Exploring Hyperbranched-Polydendron Chemistry and Architecture for Nanomedicine Applications

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by:

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Finally, I would like to thank my friends, family and Paul for their constant support and patience. My parents have always been there for me, laid no form of pressure on me, and would support me in any life choice, of which the comfort of such is priceless. I would finally like to thank my Nana, who always told me to do what made me happy.
Abstract
Exploring hyperbranched Polydendron Chemistry and Architecture for Nanomedicine Applications

The introduction of amphiphilicity into hyp-polydendrons has been achieved for the first time through the use of tertiary amine functional dendritic chain-ends and branched hydrophobic polymer chains. Explorations into the chemistry and architectural components within the hyp-polydendron structure has been carried out, offering the opportunity to control both structural and chemical behaviour. These have included: variation of the chemical composition of the primary polymer chain; utilisation of different monomers within the polymerisation; variation of dendron surface and linker chemistry; variation of primary chain architecture to produce statistical and block copolymers; variation of the degree of polymerisation; and initiation by multiple initiators (dendron and non-dendron) to result in mixed surface groups on the hyp-polydendron. The synthesis and aqueous nanoprecipitation of these branched materials is compared with their linear–dendritic polymer analogues, showing that the chemical and structural variables are all capable of influencing the ability to generate nanoparticles, the resulting nanoparticle diameter and dispersity, and subsequent response to changes in pH.

The incorporation of the monomer, 2-(diethylamino)ethyl methacrylate, and a new acid-cleavable brancher has resulted in the preparation of pH-responsive nanoparticles that undergo solubilisation upon the addition of acid. Additionally, the hydrolysis of aggregated nanoprecipitates into linear-dendritic polymer chains has been confirmed by gel permeation chromatography, and encouraging encapsulation and release studies demonstrate a promising platform for pH-responsive drug delivery vehicles.

The co-nanoprecipitation of linear-dendritic hybrids with branched copolymers has produced stable nanoparticle dispersions. Comparative nanoparticle behavioural studies, and consequent response to changes in pH have been conducted between co-nanoprecipitated nanoprecipitates in aqueous media to similarly composed hyp-polydendron nanoprecipitates containing covalently-bound dendron chain-ends.

The dye molecule fluoresceinamine has been selected as a model guest molecule for encapsulation (9 wt%) within certain nanoparticles which were stable under physiologically-relevant conditions. Cytotoxicity and transcellular permeability studies were carried out using Caco-2 cells, showing low cytotoxicity at the concentrations studied, enhanced permeation though the Caco-2 cell monolayer, and high accumulation in Caco-2 and ATHP-1 cells.

Finally, the ring opening co-polymerisation of ε-caprolactone and 4,4’-bioxepanyl-7,7’-dione using dendron initiators was explored. This resulted in stable ε-caprolactone nanoprecipitates formed in aqueous media, from biodegradable polyester hyp-polydendrons. To the best of our knowledge, this is the first example of stabilised p(CL) nanoparticles in aqueous solution, without the need for additional stabilisers.
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<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ART</td>
<td>Anti-retroviral therapy</td>
</tr>
<tr>
<td>ATHP-1</td>
<td>Activated tamm horsfall protein (monocyte derived macrophages)</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATRP</td>
<td>Atom transfer radical polymerisation</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Human epithelial colorectal adenocarcinoma cell line</td>
</tr>
<tr>
<td>CD4+</td>
<td>Cluster of differentiation 4</td>
</tr>
<tr>
<td>CAR</td>
<td>Cellular accumulation ratio</td>
</tr>
<tr>
<td>CTLs</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>CRP</td>
<td>Controlled Radical Polymerisation</td>
</tr>
<tr>
<td>CTA</td>
<td>Chain transfer agent</td>
</tr>
<tr>
<td>D</td>
<td>Dispersity (GPC)</td>
</tr>
<tr>
<td>DCR</td>
<td>Derived count rate</td>
</tr>
<tr>
<td>$D_n$</td>
<td>Number average diameter</td>
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<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>$DP_n$</td>
<td>Number average degree of polymerisation</td>
</tr>
<tr>
<td>$D_z$</td>
<td>Z-average diameter</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>ES-MS</td>
<td>Electro-spray mass spectrometry</td>
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<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>$G_x$</td>
<td>Dendron generation</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
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<tr>
<td>$Hyp$-polydendron</td>
<td>Hyperbranched polydendron</td>
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<tr>
<td>IEP</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LCST</td>
<td>Lower critical solution temperature</td>
</tr>
<tr>
<td>$M_n$</td>
<td>Number average molecular weight</td>
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<tr>
<td>$M_w$</td>
<td>Weight average molecular weight</td>
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<td>Abbreviation</td>
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<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
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<tr>
<td>pApp</td>
<td>Apparent permeability</td>
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<tr>
<td>PDI</td>
<td>Polydispersity</td>
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<td>pKa</td>
<td>Acid dissociation constant</td>
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<td>pKb</td>
<td>Base dissociation constant</td>
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<tr>
<td>PRBNs</td>
<td>pH-responsive branched copolymer nanoparticles</td>
</tr>
<tr>
<td>RAFT</td>
<td>Reversible addition-fragmentation transfer</td>
</tr>
<tr>
<td>RALS</td>
<td>Right angle light scattering (GPC)</td>
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<td>RBF</td>
<td>Round-bottomed flask</td>
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<tr>
<td>RI</td>
<td>Refractive index (GPC)</td>
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<tr>
<td>ROP</td>
<td>Ring opening Polymerisation</td>
</tr>
<tr>
<td>SCVP</td>
<td>Self-condensing vinyl polymerisation</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TBS</td>
<td>Transport Buffer Solution</td>
</tr>
<tr>
<td>UCST</td>
<td>Upper critical solution temperature</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-visible</td>
</tr>
<tr>
<td>ζ</td>
<td>Zeta potential</td>
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### Chemical Abbreviations

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<tr>
<td>3CEB</td>
<td>3-(1-chloroethyl)-ethenylbenzene</td>
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<tr>
<td>AmG&lt;sub&gt;0&lt;/sub&gt;-Br</td>
<td>Amine-functionalised generation 0 dendron ATRP initiator</td>
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<td>Amine-functionalised generation 0 dendron alcohol</td>
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<td>Amine-functionalised generation 2 dendron ATRP initiator</td>
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<td>Chloroform</td>
</tr>
<tr>
<td>CL</td>
<td>ε-Caprolactone</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>$\text{CO}_2$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Cu(I)Cl</td>
<td>Copper chloride</td>
</tr>
<tr>
<td>$\text{D}_2\text{O}$</td>
<td>Deuterated water</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DEA</td>
<td>2-Di(ethyl amino)ethyl methacrylate</td>
</tr>
<tr>
<td>DMA</td>
<td>2-Di(methyl amino)ethyl methacrylate</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethyl-aminopyridine</td>
</tr>
<tr>
<td>DMEA</td>
<td>2-(Dimethylamino) ethyl acrylate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNAA</td>
<td>Naphthalene disulfonic acid</td>
</tr>
<tr>
<td>EBiB</td>
<td>Ethyl α-bromoisobutyrate</td>
</tr>
<tr>
<td>EFV</td>
<td>Efavirenz</td>
</tr>
<tr>
<td>EGDMA</td>
<td>Ethylene glycol dimethacrylate</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>FA</td>
<td>Fluoresceinamine</td>
</tr>
<tr>
<td>GA</td>
<td>Glycolic acid</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}$</td>
<td>Water</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HOTf</td>
<td>Trifluoromethanesulfonic acid</td>
</tr>
<tr>
<td>HPMA</td>
<td>2-Hydroxypropyl methacrylate</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropanol</td>
</tr>
<tr>
<td>L</td>
<td>Ligand</td>
</tr>
<tr>
<td>LA</td>
<td>actic acid</td>
</tr>
<tr>
<td>LGA</td>
<td>Lactic-\text{-co}-glycolic acid</td>
</tr>
<tr>
<td>MMA</td>
<td>Methacrylic acid</td>
</tr>
<tr>
<td>MeOD</td>
<td>Deuterated methanol</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>M_{i}^{n}</td>
<td>Transition metal catalyst</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium hydrogen carbonate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>Na$_2$SO₄</td>
<td>Sodium sulphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>nBMA</td>
<td>n-Butyl methacrylate</td>
</tr>
<tr>
<td>OEG</td>
<td>Oligo ethyl glycol methyl ether methacrylate</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Polyamidoamine dendrimers</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEGASYS</td>
<td>Interferon α-2a PEG conjugate</td>
</tr>
<tr>
<td>PO</td>
<td>Propylene oxide</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>R-X</td>
<td>Alkyl halide</td>
</tr>
<tr>
<td>Sn(Oct₂)</td>
<td>Tin (II) 2-octanoate</td>
</tr>
<tr>
<td>tBOC</td>
<td>N-tertiary butoxycarbonyl</td>
</tr>
<tr>
<td>[BOC₂-BAPA-G₁]</td>
<td>Generation 1 3-bis(amo propyl) amine N-tertiary butoxycarbonyl</td>
</tr>
<tr>
<td>[BOC₂-BAPA-OH]</td>
<td>Generation 1 3-bis(amo propyl) amine N-tertiary butoxycarbonyl secondary alcohol</td>
</tr>
<tr>
<td>[tBOC₂-BAPA-OH’]</td>
<td>Generation 1 3-bis(amo propyl) amine N-tertiary butoxycarbonyl primary alcohol</td>
</tr>
<tr>
<td>tBuMA</td>
<td>t-Butyl methacrylate</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
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</table>
Chapter 1

General Introduction
1.1 Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) currently affects approximately 38.1 million people in the world, with sub-Saharan Africa being the most severely affected, accounting for 70% of the people living with HIV worldwide (Figure 1.1). In the same year, 1.2 million people died of acquired immunodeficiency syndrome (AIDS)-related illnesses.\(^1\)

![Number of people living with HIV worldwide](image)

**Figure 1.1** Number of people estimated to be living with HIV in 2014, *taken from ref.*\(^1\)

The HIV virus persists within cellular and tissue sanctuary sites that have poor drug permeation, such as macrophages.\(^2\) Failure to penetrate these sites is associated with discordance in viral kinetics and the evolution of resistant viruses.

1.1.1 HIV infection

HIV is a ribonucleic acid (RNA) retrovirus that exists in 2 forms – *HIV-1* and *HIV-2*. Both are responsible for human AIDS, with the latter being almost entirely confined within West Africa. HIV interacts mainly with cytotoxic T lymphocytes (CD8\(^+\) T cells) and CD4\(^+\) helper T lymphocytes. Cytotoxic T lymphocytes directly kill virally infected cells and produce and release antiviral cytokines. Once within the cell, HIV is integrated within the host deoxyribonucleic acid (DNA), undergoing transcription and generating new virions when the cell is activated. There are a large number of mutations daily at each site in the HIV genome, resulting in the virus escaping recognition by the original cytotoxic lymphocytes.\(^3\) This depletes the function of the T cells that act against all the mutations and, along with the death of the CD4\(^+\) helper cells, is what causes the immune system to fail.
1.1.2 Anti-Retroviral Therapy

HIV therapy utilises anti-retroviral therapy (ART). This is a combination of drugs that penetrate cells through diffusion or active influx and, as effective as it is, requires at least three drugs including: nucleoside/nucleotide reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, entry inhibitors and/or integrase inhibitors. ART combines several anti-retroviral drugs in order to slow the HIV virus making copies of itself (replication) in the body.

The use of three or more anti-retroviral drugs, sometimes referred to as an anti-HIV "cocktail", is the standard treatment at present for HIV infection. This treatment provides the best chance of preventing HIV from multiplying, reducing the concentration of circulating virus (viral load) until it reaches a level that is undetectable with current blood tests and allowing the immune system to stay healthy. The dose frequency, pill burden and side effects are all limitations associated with ART, generating the desire for novel nano-carrier drug delivery vehicles to target HIV-infected macrophages via endocytosis/phagocytosis, as HIV-infected macrophages show greater phagocytic activity than uninfected cells.

1.2 Nanomedicine

New drug carrier systems have been investigated thoroughly in order to overcome drug permeability issues and improve drug delivery. Important factors need to be taken into consideration when designing drug delivery vehicles such as: the capacities of the carrier to host an adequate drug payload; optimal release kinetics for each drug depending on mechanism and location(s) of release within the body; and the mode of excretion and reactivity of all side/end products that the carrier may degrade into.

The research and development of drug delivery vehicles has been comprehensive, with most attention focussed on cancer therapies and treatments. Only within the last 20 years have polymer-based medicines, which were once dismissed as interesting but impractical scientific curiosities, entered clinical practice. In recent years, research has extended from extensive manipulation of linear polymers to encompass the opportunities offered by perfectly branched macromolecules such as dendrimers.

Following the clinical approval of various polymer therapeutics, an explosion of interest in “nanotechnology” and the applications available in drug delivery has occurred. The
application of precisely engineered materials at the 10–1000 nm scale has been utilised to investigate new routes to novel therapeutics and diagnostic approaches. The vast selection of materials on offer on the nano-scale results in unequalled opportunities to modify fundamental therapy features; examples being solubility, diffusion rates, blood circulation half-life, drug release characteristics and immunogenicity; these advantages have stimulated immense interest. Within nanomedicine lies the potential to create new classes of drug delivery vehicles with a comprehensive combination of attributes.

Rational design of “nanomedicines” began nearly 50 years ago and in 2010 there were >40 products listed as completing the complex journey from laboratory to routine clinical use. The fundamental elements that are relevant to the design of practical nanomedicines and the regulatory mechanisms designed to ensure safe and timely realisation of healthcare benefits have been discussed by researchers such as Ruth Duncan and Rogerio Gasper. These reviews critically evaluate nanomedicines in clinical use, emerging nano-sized drugs and drug delivery systems. They also discuss the vital considerations undertaken in designing nano-pharmaceuticals, if they are ever to succeed in translation to clinical use.

The majority of current nanomedicine examples on the market (Table 1.1) are administered intravenously, apart from Renagel® and Welchol® which are taken orally to treat kidney disease and high blood cholesterol levels respectively. Estrasob™ used to treat hot flushes, and Bepanthen® used for skin irritation, are applied as topical emulsions. Over one third of the examples listed are used for cancer therapy and it is very apparent throughout the literature that this is where the main focus of nanomedicine research lies.

To date, there are no clinically-available nanomedicines for the treatment of HIV. Not only is the need increasing as the number of infected patients continues to rise, but there is the essential task of developing a treatment avoiding intravenous administration. HIV is a chronic disease that requires decades of dosing and patient adherence to therapy. Lack of adherence leads to drug resistance. For patients to be adherent with such long dosing regimens they need to self administer and this cannot be through a daily injection. Oral dosing is, therefore, the only clinically-relevant administration option.

<p>| Table 1.1 Examples of current nanomedicines available on the market. |
|-------------------------|-----------------|-----------------|----------------|
| Nanomaterial | Active ingredient | Brand name | Approved | Ref. |
|-------------------------|-----------------|-----------------|----------------|
| Renagel® | - | - | - | - |
| Welchol® | - | - | - | - |
| Estrasob™ | - | - | - | - |
| Bepanthen® | - | - | - | - |</p>
<table>
<thead>
<tr>
<th>Polymer-protein conjugate</th>
<th>Bovine serum albumin</th>
<th>Adagen®</th>
<th>1990 (USA)</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micelle</td>
<td>Paclitaxel</td>
<td>Taxol®</td>
<td>1992 (USA)</td>
<td>23</td>
</tr>
<tr>
<td>Polymer-protein conjugate</td>
<td>Asparaginase</td>
<td>Oncaspar®</td>
<td>1994 (USA)</td>
<td>24</td>
</tr>
<tr>
<td>Liposome</td>
<td>Amphotericin B</td>
<td>Abelcet®</td>
<td>1995 (USA)</td>
<td>25</td>
</tr>
<tr>
<td>Liposome</td>
<td>Doxorubicin</td>
<td>Caelyx®</td>
<td>1995 (USA)</td>
<td>26</td>
</tr>
<tr>
<td>Micelle</td>
<td>Docetaxel</td>
<td>Taxotere®</td>
<td>1995 (EU)</td>
<td>27</td>
</tr>
<tr>
<td>Polymeric drug</td>
<td>Glatiramer acetate</td>
<td>Copaxone®</td>
<td>1996 (USA)</td>
<td>28</td>
</tr>
<tr>
<td>Liposome</td>
<td>Daunorubicin citrate</td>
<td>DaunoXome®</td>
<td>1996 (USA)</td>
<td>29</td>
</tr>
<tr>
<td>Liposome</td>
<td>Amphoteracin B</td>
<td>Ambisome®</td>
<td>1997 (USA)</td>
<td>30</td>
</tr>
<tr>
<td>Liposome</td>
<td>Cytarabine</td>
<td>Depocyt®</td>
<td>1999 (USA)</td>
<td>31</td>
</tr>
<tr>
<td>Liposome</td>
<td>Doxorubicin</td>
<td>Myocet®</td>
<td>2000 (EU)</td>
<td>32</td>
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<tr>
<td>Polymer-protein conjugate</td>
<td>IFN-α2b</td>
<td>PEGINTRON®</td>
<td>2000 (EU)</td>
<td>33</td>
</tr>
<tr>
<td>Polymeric drug</td>
<td>Sevelamer HCl</td>
<td>Renagel®</td>
<td>2000 (USA/EU)</td>
<td>34</td>
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<tr>
<td>Liposome</td>
<td>Verteporfin</td>
<td>Visudyne®</td>
<td>2000 (USA/EU)</td>
<td>35</td>
</tr>
<tr>
<td>Polymeric drug</td>
<td>Colesevelam HCl</td>
<td>Welchol®</td>
<td>2000 (USA)</td>
<td>36</td>
</tr>
<tr>
<td>Polymer-protein conjugate</td>
<td>PEGFilgrastim</td>
<td>Neulasta®</td>
<td>2002 (USA/EU)</td>
<td>37</td>
</tr>
<tr>
<td>Polymer-protein conjugate</td>
<td>IFN-α2a</td>
<td>PEGASYS®</td>
<td>2002 (USA/EU)</td>
<td>38</td>
</tr>
<tr>
<td>Polymer-protein conjugate</td>
<td>PEGVisomant</td>
<td>Somavert®</td>
<td>2002 (EU)</td>
<td>39</td>
</tr>
<tr>
<td>Micelle</td>
<td>Estradiol</td>
<td>Estrasob™</td>
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<td>40</td>
</tr>
<tr>
<td>Liposome</td>
<td>Morphine sulphate</td>
<td>Depodur®</td>
<td>2004 (USA)</td>
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</tr>
<tr>
<td>Polymer-aptamer conjugate</td>
<td>siRNA</td>
<td>Macugen®</td>
<td>2004 (USA)</td>
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</tr>
<tr>
<td>Albumin NP</td>
<td>Paclitaxel</td>
<td>Abraxane®</td>
<td>2005 (USA)</td>
<td>43</td>
</tr>
<tr>
<td>Polymer-protein conjugate</td>
<td>Epoetin β</td>
<td>Mircera®</td>
<td>2007 (USA/EU)</td>
<td>44</td>
</tr>
<tr>
<td>Polymer-protein conjugate</td>
<td>Anti-TNF-α</td>
<td>Cimzia®</td>
<td>2008 (USA)</td>
<td>45</td>
</tr>
<tr>
<td>Liposome</td>
<td>Mifamurtide</td>
<td>Mepact®</td>
<td>2009 (EU)</td>
<td>46</td>
</tr>
<tr>
<td>Polymer-protein conjugate</td>
<td>Urate oxidase</td>
<td>Krystexxa®</td>
<td>2010 (USA)</td>
<td>47</td>
</tr>
<tr>
<td>Nanoemulsion</td>
<td>Dexpanthenol</td>
<td>Bepanthen®</td>
<td>On market</td>
<td>48</td>
</tr>
<tr>
<td>Liposome</td>
<td>Vincristine sulphate</td>
<td>Marqibo®</td>
<td>On market (2012)</td>
<td>49</td>
</tr>
</tbody>
</table>

1.2.1. Synthesis and application of solid-drug nanoparticles

Nanoparticles (NPs) are defined as solid and soft-solid colloidal particles that include both nano-spheres and nano-capsules. Over the past two decades, a progressive increase in the number of commercially available NP-based therapeutic products has been witnessed. In 2006, the European Science and Technology Observatory conducted a survey and found that >150 companies were developing nano-scale therapeutics. They also showed
that 24 nanotechnology-based therapeutic products had been approved for clinical use, with liposomal drugs and polymer–drug conjugates accounting for >80 % of the total amount at that time.

Generally, the classical methods for the preparation of NPs for medical use include: nanoprecipitation, emulsion–diffusion, double emulsification, emulsion-coacervation, polymer coating, layer-by-layer, milling and high pressure homogenisation. Solid drug NPs are produced from poorly water-soluble drugs and are stabilised by polymers and/or surfactants. These are also known as nano-suspensions and are formed by “top-down” attrition (milling) of powders and slurries, and more recently by the employment of oil-in-water emulsions (high pressure homogenisation) and emulsion template freeze drying. Emulsion processing utilises water-soluble polymers and surfactants in an aqueous phase and water-immiscible organic solvent solutions of drugs in a dispersed phase.

The association of drug to polymer NPs, either covalently attached, encapsulated or stabilised, has been studied for many different polymer classes. The potential treatment mechanisms of pathophysiological conditions by NPs includes utilising the enhanced permeability and retention (EPR) effect, resulting in favourable delivery to tumours.

### 1.2.2 Polymer therapeutics

Biologically active polymeric drugs (Figure 1.2A), polymer-protein conjugates (Figure 1.2B), polymer-drug conjugates (Figure 1.2C), block copolymeric micelles (Figure 1.2D; Section 1.2.3.1) and polyplexes (Figure 1.2E) have been designed and reported by an expansive multidisciplinary research effort, long before the term ‘nanomedicine’ became fashionable.
Ruth Duncan has termed these complex multicomponent polymer-based drugs and delivery systems as ‘polymer therapeutics’ and this terminology has become widely accepted. There have been numerous reviews summarising progress in their application and concentrating on emerging issues relating to polymer safety, the increasing use of biodegradable polymers, design of technologies for combination therapy and potential biomarkers for individualised patient treatment.

Self-assembled polymer–drug conjugates are often obtained from amphiphilic block copolymers of biodegradable or bioresorbable polymers. Polymeric-drugs are generally based on the Ringsdorf model, which describes the concept of targetable anti-cancer polymer–drug conjugates. This model lists features essential for effective design to limit cellular uptake via endocytic routes and produce long-circulating conjugates. It is important to note that although polymer-drug conjugates are nano-sized constructs (typically 2–25 nm), they are quite distinct from polymeric NPs.

Polymer-drug conjugates have proven to reduce cellular uptake via endocytic routes as well as significantly prolonging the in vivo circulation time relative to unmodified drug
compounds. This encourages passive delivery of drugs to tissues with leaky blood vessels such as solid tumours and atherosclerotic plaques.\textsuperscript{69,70} There has been a large number of polymeric materials and architectures proposed as novel drug delivery carriers, however, very few linear architectures have succeeded in full translation to clinical practice. Challenges that have arisen include: toxicity, immunogenicity, nonspecific biodistribution, \textit{in vivo} circulation stability, and low drug loading capacities with rapid drug release.\textsuperscript{71} Polyethylene glycol (PEG) was the first linear polymer accepted into clinical use in the early 1990s.\textsuperscript{72} PEG-conjugation has been shown to enhance the plasma stability and solubility of various drugs while simultaneously reducing immunogenicity. Today, there are several examples of “PEGylated” drugs in clinical practice, including PEGylated-anti-TNF Fab Cimzia\textsuperscript{20} for rheumatoid arthritis and the PEG-aptamer Macugen\textsuperscript{73} for age related macular degeneration. PEG has also been conjugated with Interferon \(\alpha\)-2a (PEGASYS), used in the treatment of chronic hepatitis C, to successfully improve the half-life and efficacy of the drug.\textsuperscript{74} Additionally, the first synthetic polymer anticancer drug conjugate went into clinical trial in 1994,\textsuperscript{75} and many conjugates have subsequently followed.

Polymer-drug conjugates are labelled as ‘new chemical entities’ rather than being considered as the drug within a delivery system or formulation that simply entraps, solubilises or controls drug release without the need for chemical conjugation. Examples include “simple” polymer–drug conjugate systems,\textsuperscript{76} including those containing doxorubicin/paclitaxel that have progressed to clinical trials (Figure 1.3A), receptor targeted polymer-drug conjugates, including recent examples containing galactosamine for liver targeting (Figure 1.3B),\textsuperscript{77,78} and combination therapies, such as those containing aminoglutethimide and doxorubicin (Figure 1.3C).\textsuperscript{79,80}
Figure 1.3 Detailed chemical and general cartoon structure of polymer-drug conjugates. The polymer is shown in red, linker region in blue, drug in green and targeting residue in purple. A) A polymer-drug conjugate containing doxorubicin that has progressed to clinical trial; B) A multivalent receptor targeted conjugate containing galactosamine to promote liver targeting; C) Polymer combination therapy containing the aromatase inhibitor aminogluthethimide (orange) and doxorubicin (green).

There has been a very large investment specifically in liposomal and antibody therapeutics; however, wider research in polymer therapeutics has progressed strongly in the past two decades through the overall interest in ‘nanomedicines’. Many challenges must be overcome in order to ensure the safe and rapid translation of polymer therapeutics into routine clinical use. There is a need for the far-sighted design of new conjugates, with cautious chemical characterisation before clinical trials, and careful safety assessment of all new polymeric carriers.

1.2.3 Self-assembling diblock copolymers for drug delivery

Diblock copolymers are amphiphilic linear polymers comprising two polymer chemistries; often a hydrophilic A block and a hydrophobic B block, (Figure 1.4A, B and C). The assembly behaviour of the diblock copolymers is governed by the ratio of the hydrophilic to hydrophobic A and B block segments. Linear amphiphilic polymers of varying monomers and block length ratios have been extensively studied for the formation
of micelles, liposomes, polymersomes, worms and cylinders.

1.2.3.1 Preparation of micelles from diblock copolymers

Micelles may be prepared from block copolymers with amphiphilic character that self-assemble in aqueous media because of the large solubility difference between the hydrophobic and hydrophilic segments (Figure 1.4).

**Figure 1.4** Diagrammatic representation of three different classes of functional micelles formed from A-B diblock copolymers containing a hydrophobic (green) and hydrophilic (blue) segment containing functionality (gold stars). The micelles are formed from i) micellisation and/or ii) cross-linking producing D) Core-crosslinked micelles formed from functionalisation of the hydrophobic block (A), E) Shell-crosslinked micelles formed from functionalisation of the hydrophilic block (B); and F) Surface functionalised micelles from functionalised chain end groups (C).

It is well-known that A-B diblock copolymers aggregate to form micelles in solvents which are selective for either the A block segment or the B block. If the block segment chain length ratio is not too asymmetric, micelles or reverse (inverted) micelles from the same block copolymer can be obtained, as long as the appropriate solvents are selected.

Armes and co-workers reported the first instance of a novel water-soluble A-B diblock copolymer, which was capable of forming both micelles (A block segment within the
core) and reverse micelles (B block segment within core) solely in aqueous media. The formation of these reverse micelles was driven by a variety of different parameters including the solution pH, electrolyte concentration, temperature and block symmetry.

Reported polymeric micelle-based drug carriers commonly comprise a biodegradable polyester core (e.g. \(p(\varepsilon\text{-caprolactone})\)) and a biocompatible and nontoxic hydrophilic shell such as PEG. Drugs can be encapsulated within the core of stable micelles and release is mediated by the rate of drug diffusion from the assembled nanostructure. Due to the pH-dependent solubility of ionic drugs, release rates are often subject to change depending on the surrounding environment of the drug carriers. This introduces a major challenge when addressing systemic absorption via oral administration where achievable plasma concentrations are affected by exposure to significant fluctuations from the large pH variation in the gastrointestinal tract.

pH-dependent release can be undesirable, especially for treatments necessitating steady plasma drug concentrations. Methods to overcome these issues have involved shell cross-linking, core cross-linking and surface functionalisation of micelles (Figure 1.4D, E and F). Recent examples include micelles with charged channels on the surface in order to reduce the difference in drug release rate upon changes in pH. Cross-linking of micelles (Figure 1.4D) provides stability at concentrations below the critical micelle concentration of the block copolymer and allows them to be isolated and re-dispersed as stable NPs. This extra stability means they are not as likely to disperse or fully dissolve in media such as the blood-stream, but rather lead to increased circulation times to allow the drugs to be released over long durations. Additionally, with shell-crosslinking (Figure 1.4E), the permeability of the corona can be controlled and fine-tuned to suit a release rate that is desirable for different drug compounds. Functionalisation of the block copolymers (Figure 1.4C) contributes to targeting specific sites of interest within the body, therefore, also adding improved distribution control throughout the whole drug delivery process.

Despite lacking the surface functionality associated with dendrimers (cf. Section 1.2.4), these colloidal carrier systems have received much attention due to their high loading capacity for drugs as well as their unique disposition characteristics in the body.
1.2.3.2 Synthesis and pharmaceutical application of liposomes

A liposome is composed of natural or synthetic amphiphilic lipid molecules (Figure 1.5A) that arrange themselves into a bi-layered membrane to create a spherical lipid vesicle (Figure 1.5B). Examples include phospholipids (Figure 1.5A) that spontaneously arrange themselves as liposomes when dispersed in aqueous media. The hydrophilic interaction of the charged head groups with water, results in the formation of multilamellar systems.

Figure 1.5 A) Detailed chemical and cartoon structure of a phospholipid showing hydrophobic fatty acid tail (green), glycerol backbone (black) and hydrophilic phosphorylated alcohol (red); B) Cartoon representation of how the phospholipid spontaneously arranges in aqueous media to form a liposome.

Liposomes have been extensively used as pharmaceutical carriers due to a number of distinctive capabilities. They are able to encapsulate hydrophobic and hydrophilic therapeutic agents, shield the encapsulated agent from the external environment and any unwanted effects that might occur in these conditions, and easily be tailored with specific ligands for targeting. Generally, their circulation half-life can be extended through coating of inert and biocompatible polymers and desired formulations can be formed depending on required properties such as composition, size and surface charge.

The first liposomal drug formulation approved by the Food and Drug Administration, USA was Doxil in 1995, for the treatment of Kaposi’s sarcoma. Doxil is a long-circulating formulation of doxorubicin, in which doxorubicin hydrochloride is encapsulated within the PEGylated liposome. The encapsulated doxorubicin within the
liposome alters its pharmacokinetic and biodistribution profile, resulting in reduced cardiotoxicity,\textsuperscript{107} however major adverse side-effects of hand-foot syndrome have also been reported compared with free doxorubicin.\textsuperscript{26} This is a distinctive and relatively frequent dermatologic toxic reaction associated with certain chemotherapeutic agents,\textsuperscript{108} and strategies investigating dose frequencies to minimise the effects are ongoing.\textsuperscript{109}

1.2.4 Dendrimers

Dendrimers (Figure 1.6B) comprise sub-units, known as dendrons (Figure 1.6A). Dendrimers are classified by generation ($G_n$), which refers to the number of repeated branching cycles that are performed during the synthesis.

Figure 1.6 Diagrammatic representation of a dendron (A), the building block of a dendrimer (B) comprised of surface groups (red), branching groups (blue) and core (green).

The first successful synthesis of dendrimers was carried out in the late 1970s,\textsuperscript{110} where the new potential of these highly branched, highly symmetrical macromolecules with maximised surface functionality began to be realised. The first synthetic example was reported by Vögtle and co-workers,\textsuperscript{111} achieved by a cascade approach via divergent growth. Following the initial development of well-defined branched macromolecules, the term “starburst polymers” was subsequently used to describe dendrimers in many of the first reports of their synthesis by Tomalia and co-workers.\textsuperscript{112} The various syntheses can offer fine control over molecular weight, levels of branching and numbers/chemistry of terminal end groups, making these perfectly branched macromolecules quite distinct from the more readily accessed, but less well-defined, hyperbranched polymers with irregular branching.

Dendrimers are generally synthesised in one of two ways, convergent and divergent growth.\textsuperscript{113}
1.2.4.1 Synthesis of Dendrimers via Divergent growth

Divergent dendrimer growth strategies start by reacting a multifunctional molecule that eventually becomes the core of the dendrimer, with reagents that exhibit the surface functionality desired (Scheme 1.1). Subsequent reactions take place at the surface of the growing dendrimer and functionality is exponentially multiplied at each generation of growth.

Scheme 1.1 Divergent growth starting with the core of the molecule (green) reacting with methyl acrylate via exhaustive Michael additions (1) followed by amidation with ethylenediamine (2) to produce amino surface groups (red) that eventually end up as branching points (blue) as the steps are repeated to produce a Generation 1 dendrimer.

Polyamidoamine (PAMAM) dendrimers (Scheme 1.1), synthesised by Tomalia et al. via divergent growth, are one of the most commercially available and widely used dendrimers in biology. They are made through a repetitive cycle of exhaustive Michael addition of amino groups with methyl acrylate, followed by amidation of the resulting esters with ethylenediamine. The broad applicability of PAMAM dendrimers has been demonstrated via modification of the surface amine groups in order to overcome various complications within living systems, including liver accumulation and toxicity seen with polycationic materials. This is often achieved by reacting the amine groups with neutral or anionic moieties.

1.2.4.2 Synthesis of Dendrimers via Convergent growth
Convergent growth begins by reacting molecules that will become the eventual dendrimer surface.\textsuperscript{119,120} A series of dendrons are formed from successive couplings to multifunctional branching molecules. Dendrons can react further to form larger generation materials or couple with a chosen core molecule at any generation of “growth”.

The first example of convergent growth was conducted by Hawker and Fréchet in 1990.\textsuperscript{121} They synthesised aryl ether dendrons up to G\textsubscript{6} which were subsequently coupled to a trivalent core to produce the equivalent \textit{p}(aryl ether) dendrimers based upon the “monomer” 3,5-dihydroxy-benzyl alcohol (Scheme 1.2). This method showed a greater control over the synthesis of dendrimers, with less imperfections arising and without the need for an excess of reagents. However, during the coupling step of the dendrons to the core molecule to form the final dendrimer, the yields declined dramatically with increasing dendrimer generation, achieving 76\% and 51\% for the G\textsubscript{5} and G\textsubscript{6} dendrimers respectively. This reduction in yield was due to steric hindrance of bulky dendron coupling with a small molecule core.

\begin{center}
\textbf{Scheme 1.2} Convergent growth starting with the aryl ether surface groups of the molecule (red) producing benzyl branching points (blue) before eventual coupling around a trivalent core (green) to produce a Generation 2 dendrimer.
\end{center}

The more globular shape of dendrimers offers several advantages over the random coil structures of most solvated linear polymers. This change in conformation assumes a lack of chain entanglements, and confers different solution and bulk properties compared to linear analogues. This topology is known to affect the interaction with biological substrates, leading to the discovery of interesting effects related to macromolecular architecture.\textsuperscript{122}

\textbf{1.2.4.3 Dendrimers for biological applications}
Dendrimers have been studied for several applications due to their controlled molecular size, distinct and tailored interior and exterior, ability to encapsulate guest molecules and micelle-like properties. In spite of such benefits, dendrimers have not currently been commercialised as nanomedicines due to their lengthy/expensive syntheses and purification, although several dendrimers are in commercial medically-related products and others are progressing through clinical trials.

Much effort has been devoted to the preparation of dendrimers in order to prepare water-soluble and highly biocompatible materials. The dendritic architecture can provide several advantages for drug delivery applications in comparison to the features of conventional linear polymers. Dendrimer drug-delivery systems include encapsulation of guest molecules in the void interior dendrimer spaces (Figure 1.7A), dendrimer-drug networks (Figure 1.7B), attachment of a therapeutic agent to the dendrimer surface either covalently (Figure 1.7C) or non-covalently (Figure 1.7D) to produce a range of new prodrug structures.

![Diagrammatic representations of dendrimer drug-delivery systems. The orange oval represents an active drug substance: A) Encapsulated guest; B) Dendrimer-drug network; C) Covalently-bound prodrug; D) Non-covalently bound prodrug.](image)

Host-guest interactions of drug with dendrimers are well established, and examples include liposomal formulations including dendrimers entrapped and subsequently slowly releasing methotrexate, and prodrug design using 5-aminolevulinic acid residues on the surface of 2nd and 3rd generation dendrimers. Advantages such as the controlled multi-
valency of dendrimers can allow several drug molecules to be attached at the molecular surface and surface groups can be tailored as targeting and solubilising groups on the periphery of the dendrimers in a controlled manner. G3-PAMAM dendrimers have been internalised and transported to endosomes and lysosomes following surface modification with lauroyl and propranolol chains.\textsuperscript{130}

A water-soluble polyester dendrimer was found to be biocompatible \textit{in vitro} and \textit{in vivo},\textsuperscript{131} therefore introducing a promising aliphatic dendrimer backbone based on 2,2-bis(hydroxymethyl) propionic acid for the development of anticancer delivery. There was an observed lack of accumulation in vital organs offering desirable behaviour; however, a longer circulatory half-life than the reported values is required to obtain passive tumour targeting \textit{via} the EPR effect – a phenomenon observed when macromolecules of a certain size accumulate more in tumours than they do in healthy tissues.\textsuperscript{132}

VivaGel® is an innovative antimicrobial developed by StarPharma and partners\textsuperscript{133} in order to combat a range of sexual health issues such as bacterial vaginosis. Surface groups imparting the most potent inhibitory activity against \textit{HIV-1} and herpes simplex virus (HSV-2) were naphthalene disulfonic acid (DNAA) and 3,5-disulfobenzoic acid exhibiting the greatest anionic charge and hydrophobicity of the seven surface groups tested. Their anti-\textit{HIV-1} activity did not appreciably increase beyond a second-generation dendrimer while dendrimers larger than two generations were required for potent anti-HSV-2 activity. Second generation (SPL7115) and fourth generation (SPL7013) (Figure 1.8) DNAA dendrimers demonstrated broad-spectrum anti-HIV activity.\textsuperscript{134}
Figure 1.8 Diagrammatic representation of a fourth generation (SPL7013) dendrimer with chemical structures for the benzhydrylamine-lysine core (green), lysine branching units (blue) and DNAA surface groups (red).

SPL7013 was more active against HSV and showed increased blocking of HIV-1 envelope mediated cell-to-cell fusion and is the active ingredient in VivaGel®. Thirty-two naphthalene disulfonate moieties, attached via amide linkages, decorate the molecule’s surface. This polyanionic structure prevents HIV infections by binding to the gp120 glycoprotein receptors on the virus surface. The interaction in turn prevents HIV from attaching to receptors on T cells in the body. Tsai and co-workers suggested a protective effect against HIV following investigations in a demanding animal model of HIV infection.135

StarPharma have also developed the drug delivery vehicle DEP™ docetaxel, a poly-L-lysine-based dendrimer-docetaxel conjugate, which is currently in Phase 1 clinical trials.136 Docetaxel is a leading chemotherapy drug that treats a range of tumours, including breast, lung and prostate cancer, and is marketed as Taxotere®. DEP™ docetaxel has demonstrated a significant enhancement of anticancer effects by eliminating neutropenia, the major dose-limiting toxicity for marketed formulations of docetaxel. It has also demonstrated higher accumulation in tumours and longer plasma half-life compared to Taxotere® alone.

The synthetic preparation of high-generation dendrimers is time consuming and the increase in hydrodynamic volume in each generation growth step is often very small as a direct result of the globular architecture. It is, however, essential in order to improve the circulation half-life as larger molecules are eliminated from the body more slowly.137 In attempts to access different sizes and different dendritic polymer properties, one route that has seen much international research interest is the combination of dendrimers and linear polymers to produce hybrids of varying architectures.

Concepts offered for the potential of dendrimer-based nanomedicines are emerging more than ever before.138 They offer substantial promise and benefits for many current nanomedicines and nano-therapies, inspired by the ‘global nanotechnology revolution’. The promise that polymers show in the development of anticancer drug delivery systems,75 encourages a greater research effort to establish applications of dendrimers in this area. The possibilities of various new polymer architectures provided by dendrimers, show great potential in relevant drug delivery needs.
1.2.5 Synthesis and application of dendronised polymers

In recent years, many reports have shown the connection of large numbers of dendritic molecules to linear polymers, resulting in dendronised polymers. These predominantly host pendant dendrons at many repeat units along the chain backbone and, at high molecular weights, assume extended rod-like conformations. This consequently leads to cylindrical core-shell architectures with biological and physical properties different to conventional dendrimers, without losing the appealing multi-valent feature of the surface functionality. Thorough investigations into the control of composition and morphologies within dendronised polymers have been explored and are ongoing. There are 3 main routes to synthesise dendronised polymers (Figure 1.9).

The “graft-from” approach involves a step-growth process progressing from the polymer backbone (Figure 1.9A), with increasing dendron generation with each synthetic step. This method was first reported by Tomalia et al., and targets a maximum degree of dendronisation, despite problems arising from structural defects. Subsequently, many examples exist within the literature including non-degradable \(p((4\text{-hydroxy})\text{styrene})\) and biodegradable substituted \(p(\text{caprolactone})\).

![Figure 1.9](image)

**Figure 1.9** Synthetic routes to dendronised polymers; A) “Graft-from” approach proceeds via a step growth process from the polymer backbone; B) Chemical and diagrammatic representation of the “graft-to” approach whereby a preformed dendron is coupled to a polymer that contains pendant groups for attachment; C) Macromonomer approach incorporates dendrons into the monomer.
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The “graft-to’ approach (Figure 1.9B) involves conjugation of preformed dendrons to pendant groups on the chosen polymer backbone. Complete coverage of the backbone has proved problematic using G3 dendrons or larger; however, successful examples include Fréchet-type dendritic azides reacting with the pendant alkynes of \( p \) (vinylacetylene) (Scheme 1.3).\(^{145}\)

![Chemical example of the “graft-to” approach](image)

**Scheme 1.3** Chemical example of the “graft-to” approach to produce a dendronised polymer *via* the reaction of dendritic azides with the pendant alkynes of \( p \) (vinylacetylene).

The macromonomer approach (Figure 1.9C) involves incorporation of dendrons into a monomer structure. This ensures a perfect pendant dendron exists at each repeat unit; however, macromonomers containing high generation dendrons have been reported to reach only low degrees of polymerisation.\(^{146,147}\)

These molecules show interesting pharmacokinetic behaviour in bio-distribution studies that differ from that displayed by linear polymers with equivalent molecular weight.\(^{148}\) They also show potential to act as drug delivery vehicles due to the long circulation half-lives and many peripheral groups, which could be utilised for drug attachment, targeting and solubilising groups.

1.2.6 Dendritic-like polymers

1.2.6.1 Linear-dendritic polymers

Dendrons, the central structural sub-units of dendrimers, have been combined with linear polymers to generate many new classes of polymer architectures.\(^ {113}\) These include the placement of dendrons at linear polymer chain ends to form linear-dendritic polymer hybrids (Figure 1.10A), linear chains with dendrons at both ends (Figure 1.10B), linear chains conjugated to the periphery of a dendron (‘bow-tie’ hybrids; Figure 1.10C) and star polymers containing dendron chain end functionality (Figure 1.10D).
1.2.6.1.1 Synthesis of linear-dendritic polymer hybrids

Linear-dendritic polymer hybrids have been synthesised using various strategies.\textsuperscript{149} The introduction of dendritic functionality to linear polymer chain ends, has led to numerous structural variations.\textsuperscript{150,151,152} The commercially available dendrons and dendrimers based on 2,2-bis(methylol)propionic acid have, overall, excelled as the leading molecule to prepare non-toxic, biocompatible materials for \textit{in vivo} applications.\textsuperscript{153,154} Examples include star branched \(p\)(3-caprolactone) chains constructed from G\(_4\) dendrons, leading to successful micelle self-assembly and loading of doxorubicin.\textsuperscript{155}

Examples of ‘bow-tie’ hybrids include polyester dendrimers and PEG, with varying architectures and molecular weights, prepared by tuning the number of PEG arms and the targeted molecular weights.\textsuperscript{156} PEG was chosen as the linear polymer segment due to its high biocompatibility and the availability of low dispersity commercial materials,\textsuperscript{157} allowing hybrids with similar dispersity to dendrimers to be created.

The demand of functionalisation and application continues to increase structural complexity, requiring more robust and versatile synthetic methods to vary the dendritic
functionality within linear-dendritic polymers. Developments in click chemistry have provided routes to prepare highly functionalised macromolecular architectures, with the consequent construction of dendritic polymers utilising Cu(I)-catalyzed azide–alkyne 1,3-dipolar cycloadditions which results in the formation of an aromatic triazole. Reported examples include A-functionalities (azides) and B-functionalities (acetylenes) for eventual repeating triazole ring groups in the final dendritic structure, for subsequent post-functionalisation. Other strategies have employed a range of commercially available acrylate monomers in multiple deprotection/functionalisation reactions with a xanthate dendron polymer chain end. The result is a range of complex polymer architectures with multiple reactive functional groups, from an efficient and facile one-pot reaction.

1.2.6.1.2 Application of linear-dendritic polymer hybrids

The conjugation of p[N-(2-hydroxypropyl)methacrylamide] to PAMAM dendrimers, followed by attachment of the anticancer drug doxorubicin to the polymer arms by a biodegradable peptide spacer, results in a star-like carrier. The star polymer has a highly dense architecture that results in slower rates of enzyme-mediated drug release, which decreases the cytotoxicity of the conjugate.

Fréchet and co-workers proceeded to synthesise a number of linear-dendritic hybrids comprising PEG with polylysine (Figure 1.11A) or polyester dendrons. These have assembled to form pH-responsive micelles whose drug release rates have the potential to be tuned and whose disintegration into unimers has been demonstrated (Figure 1.11B).
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**Figure 1.11** Example of a linear-dendritic block copolymers. A) Chemical structure of linear-dendritic comprised of poly(ethylene oxide) and a polylysine dendron to develop a stable micelle system in neutral pH (B) that disintegrates into unimers upon the addition of acid (i).

It is still a challenge to prepare dendritic polymers that circulate in the blood long enough to accumulate at target sites, and are able to be eliminated from the body at acceptable rates to avoid long-term accumulation. The tissue localisation of dendritic polymers is difficult to predict in advance and more studies are required to determine the effect of peripheral dendritic groups on these observations. The release of drugs from dendritic polymers has proved problematic due to the steric hindrance associated with the dense globular dendritic architecture, making the engineering of enzymatically cleavable linkages difficult.

### 1.2.6.2 Branched polymers producing dendrimer-like structures

The on-going development of hyperbranched polymers has led to materials with increased degrees of branching and better control over the branching, molecular weight distribution and polymer architecture, bringing hyperbranched polymers closer to perfectly branched dendrimers (Figure 1.12).

**Figure 1.12** Diagrammatic representation of the preparation of A) a hyperbranched polymer; and C) a dendrimer; from an AB₂ building block (B).

Highly branched polymer molecules have been extensively synthesised using AB₂ molecules. The first approach to the synthesis of highly branched materials via self-polymerisation was reported by Fréchet *et al.* in 1995 and concerned the polymerisation of a vinyl monomer with a pendant group, which can be transformed into an initiating moiety (an inimer) (Scheme 1.4).
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Scheme 1.4 Polymerisation of a vinyl monomer with a pendant group (an inimer) producing an initiating moiety (B) following activated monomer production (A).

This polymerisation was termed self-condensing vinyl polymerisation (SCVP) and was first demonstrated for the polymerisation of styrene and 3-(1-chloroethyl)-ethenylbenzene (3CEB). The 3CEB monomer consists of vinyl functionality and a pendant latent initiating functionality, which was activated by the addition of stannic chloride (Scheme 1.5). The synthesis of branched polymers using A-B monomers through SCVP produced architectures similar to that of dendrimers, albeit with less control. Branching points arose from propagation of the A-B monomer and the introduction of new initiating centres with each propagation step.

Scheme 1.5 Self-condensing vinyl polymerisation of 3-(1-chloroethyl)-ethenylbenzene (B) following activated monomer production (A).

Despite continuous refinements, hyperbranched polymers are notorious for being typically imperfectly branched with high dispersities, but due to the simplistic one step
syntheses, many applications have become accepting of these imperfections resulting in a variety of hyperbranched polymers becoming commercially available. These applications range from additives to coatings, advanced technologies including sensors, high loading supports in combinatorial chemistry studies and homogeneous catalysts.

Two of the most widely recognised commercially available hyperbranched polymer systems are Perstorp’s Boltorn® and DSM’s Hybrane® (Figure 1.13). Hybrane® materials have been produced at multi-tonne scale, and the modification and combination of anhydrides has led to several types of end groups and consequently a large variety of structures. Hybrane® polymers are used as strainer candidates for extractive distillation processes, and Boltorn® polymers have been employed in a succession of industrial applications in chemical engineering such as coating agents and printing inks. The distinctive dendritic properties such as lack of entanglements, large number of reactive end groups, and the possibility to interact with guest molecules has potentials to be fully exploited.

![Figure 1.13 An example of a typical structure of a hyperbranched Hybrane® macromolecule.](image)

Hyperbranched or highly branched polymers can also be synthesised via other polymerisation techniques including chain-growth polymerisation.

### 1.3 Chain-growth Polymerisation

Chain-growth polymerisation involves the successive addition of unsaturated monomers to the active site of growing polymer chains; the addition of each monomer unit regenerates the active site. Chain-growth fundamentally proceeds via three steps:
chain initiation, chain propagation and chain termination. Different types of chain-growth polymerisation include free radical polymerisation (Scheme 1.6), cationic polymerisation,\textsuperscript{185} that involves transfer of charge to a monomer from a cationic initiator, and anionic polymerisation,\textsuperscript{186} that involves a carbanion active species.

\begin{scheme}
\begin{align*}
(1) & \quad R-R \quad \rightarrow \quad R' \\
(2) & \quad R' + \quad \mathrm{R'} \quad \rightarrow \quad \mathrm{R-R} \\
(3) & \quad R' + \quad R' \quad \rightarrow \quad R-R
\end{align*}
\end{scheme}

\textbf{Scheme 1.6} Free radical chain-growth polymerisation involving 1) Chain initiation; 2) Chain propagation and 3) Chain termination.

Progressive developments over many years have led to varying degrees of control over the number average polymer chain length.\textsuperscript{187} This has predominantly been led by “living polymerisation” techniques, which essentially remove the potential for chain termination from chain growth polymerisation and controls the various rates of the key reactions within the polymerisation reaction.\textsuperscript{188} This results in the polymer chains growing at a more constant average rate until reaching the specific target degree of polymerisation as determined by the initiator to monomer ratio (Scheme 1.7).\textsuperscript{189} This attribute is desirable as it introduces precision and control over the primary polymer chain length, necessary when the properties of polymers are directed by variations in architecture and molecular weight. Examples include living anionic polymerisation,\textsuperscript{190} living cationic polymerisation,\textsuperscript{191} and free radical polymerisation (controlled radical polymerisation) (Figure 1.7). Many forms of controlled radical polymerisations (CRP) have emerged, all continually aiming to control polymerisation progression through an equilibrium of dormant/active chains.
1.3.1 Controlled radical polymerisation

CRP developments and methods have grown significantly over the past decades. All are based on establishing a rapid dynamic equilibrium between a large majority of a dormant species and a small concentration of reactive radical species. The dormant form of the radicals is controlled in a variety of ways, depending on the type of CRP technique used. Examples include reversible addition-fragmentation chain transfer polymerisation, nitroxide mediated polymerisation and degenerative transfer. The technique used within this work is atom transfer radical polymerisation (ATRP).

1.3.1.1 Atom transfer radical polymerisation

The CRP method focussed on within this study is ATRP, which produces radicals via a transition metal catalysed reaction. ATRP has been reported on widely since initial publication in 1995 by Sawamoto and co-workers and Matyjaszewski and co-workers. ATRP typically employs a transition metal complex (Mₙ/L; Scheme 1.8) as the catalyst and an alkyl halide as the initiator (R-X; Scheme 1.8). The dormant species is activated by the transition metal complex to generate the radicals, simultaneously establishing a higher oxidation state (X-Mₙ⁺/L; Scheme 1.8). This reversible process rapidly produces an equilibrium between dormant and active species, with a predominated production of low radical concentrations. The number of polymer chains is determined by the number of initiators, and each growing chain has an equal probability to propagate and produce polymers with similar molecular weights with an overall narrow molecular weight distribution.
Scheme 1.8 General ATRP mechanism involving an alkyl halide (R-X), a transition metal complex (M_{n}/L) as the catalyst, establishing a higher oxidation state (X-M_{n+1}/L) upon the generation of radicals (R') and an equilibrium between dormant and active chains.

This attractive radical polymerisation lends itself to a vast range of monomers containing substituents that can stabilise the propagating radicals; such as (meth)acrylates, \(^{199}\) styrenics \(^{200}\) and (meth)acrylamides. \(^{201}\) Equally, the effect of various synthesis parameters on the rate of polymerisation have been thoroughly examined; such as the initiator, \(^{202}\) ligand, \(^{203}\) metal catalyst \(^{204}\) and solvent choice. \(^{205}\)

1.3.1.1 Branched polymers via controlled radical polymerisation

Free radical polymerisation produces high molecular weight polymer chains that easily cross-link, \(^{206}\) resulting in gelation, even at relatively low concentrations of brancher. The ATRP branching method has been reported to rely strongly on controlling the brancher:initiator ratio in order to prevent the formation of an insoluble three dimensional network \(^{207}\) as described in the Flory-Stockmayer theory. \(^{208}\) The Flory-Stockmayer theory predicts that gelation will occur when the number of cross-links per chain is equal to one, assuming that the reactivity of each vinyl group is the same and that no intramolecular cyclisation occurs. Therefore, a ratio brancher:initiator 0.95:1 or less will prevent gelation and produce high molecular weight, soluble polymers. \(^{209}\)

In an attempt to overcome cross-linked polymer networks, Sherrington and co-workers introduced the “Strathclyde” route \(^{210,211,212,213}\) to free radical polymerisation of vinyl and divinyl monomers. This stated that gelation can be prevented by ensuring that less than one branching monomer is incorporated per primary polymer chain. The route was originally utilised in conventional free radical polymerisation and used bi-functional monomers, such as ethylene glycol dimethacrylate (EGDMA) in the presence of a chain transfer agent (CTA) to produce high levels of branching in any vinyl monomer polymerisation. The presence of a CTA avoids gelation by preventing every polymer chain from bearing a divinyl monomer. This method produced high molecular weight, soluble branched polymers via control of the primary polymer chain length.
The introduction of a low concentration of divinyl monomer (brancher) within various chain-growth polymerisation methods has led to the production of highly branched, soluble polymers with impressive molecular weights.\textsuperscript{209} Li and Armes have synthesised a number of branched copolymers using a branching agent containing a disulfide bond.\textsuperscript{214} This allowed cleavage of each branch point to degrade the high molecular weight polymers into near monodisperse primary polymer chains and confirm the mechanism of branching; controlled polymerisation of linear chains prior to combination to produce the branched copolymer. Further studies of the ATRP branching mechanism have been reported by Bannister \textit{et al}.\textsuperscript{215} uncovering a three-stage polymerisation mechanism constructed from their analysis of the evolution of the molecular weight distribution during polymerisation. Branching appeared to be negligible during the early stages of the co-polymerisation, with dispersities remaining low up to 70-80\% monomer conversion, and substantial branching occurred in the later stages of the reaction via inter-chain reaction through pendant vinyl functional groups (Figure 1.15).

\textbf{Figure 1.15} Diagrammatic formation of a branched copolymer by ATRP via: A) initiation using an ATRP initiator ( ); B) propagation and C) latent branching involving statistical linking of monomer ( ) and branching units ( ).

\subsection*{1.3.1.1.2 Synthesis of hyperbranched polydendrons \textit{via} ATRP}

In 2014, Hatton \textit{et al}. reported the introduction of simultaneous branching during the propagation of the vinyl segment of a linear-dendritic hybrid, to form a new class of macromolecular architecture: hyperbranched polydendrons (hyp-polypendrons).\textsuperscript{216} The concepts associated with the previously mentioned free-radical “Strathclyde” polymerisation, were utilised and the presence of a dendron macronitiator within the controlled radical co-polymerisation of a mixture of vinyl and divinyl monomers was used to form soluble, high molecular weight branched polymers (Scheme 1.9).
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Scheme 1.9 Co-polymerisation of 2-hydroxypropyl methacrylate (2) and ethylene glycol dimethacrylate (3) initiated by a Generation 2 1,3-dibenzylaxyloxy-2-propanol dendron ATRP initiator (1) in the presence of CuCl/bpy as the metal/catalyst to produce a hyp-polydendron.

By combining dendritic surface functionality with chain-growth polymerisation, significant sample masses, high functionality and high molecular weights (> 2,000,000 g mol\(^{-1}\)) were obtained within a relatively small number of synthetic steps. Naturally, the ideal branching concept throughout the macromolecule, compared to dendrimers, has been compromised.

1.4 Stimuli-responsive materials.

Stimuli-responsive materials are defined as materials that respond with drastic chemical or physical change after variation in external environmental conditions. They have been the subject of research for many groups over several decades due to their potential in a vast array of applications such as oil recovery,\(^{217}\) water decontamination,\(^{218}\) analytical chemistry,\(^{219}\) purification,\(^{220}\) anti-bacterial coatings\(^{221}\) and, more recently, textiles\(^{222}\) and biomedicine.\(^{223}\) Furthermore, applications in controlled release have contributed in further development of interest in stimuli-responsive polymers due to the opportunities for use in the pharmaceutical industry as new delivery systems for active agents.\(^{224}\)

The attractive characteristics affiliated with stimuli-responsive polymers, a desirable factor for drug carriers, is the ability to undergo reversible conformational changes in solution. These materials can adapt their solution behaviour in response to a range of stimuli such as temperature,\(^{225}\) pH,\(^{226}\) ionic strength,\(^{227}\) and light.\(^{228}\)

1.4.1 Temperature-responsive materials

Temperature-responsive polymers undergo a volume phase transition at well-defined
temperatures, causing an immediate change in their solvation state.\textsuperscript{229} Polymers either become insoluble or soluble upon heating possessing either a lower critical solution temperature (LCST) or upper critical solution temperature (UCST) respectively. The volume phase transition occurs because of competing hydrogen-bonding properties depending on whether the intra- and intermolecular hydrogen bonding within the polymer is more favourable than the water-polymer interaction. The solubility in water arises when the interactions between the water and polymer are preferred. Thermodynamically, the miscibility of polymer and solvent occurs when the Gibbs free-energy of the system decreases i.e. when there are no interactions between the polymers (no enthalpy ($\Delta H=0$)), there will be a preferred entropy of mixing with the solvent ($\Delta S>0$), resulting in a decrease in Gibbs free-energy. These systems are not necessarily confined to aqueous solvent environments; however, aqueous conditions are of most interest in the field of biomedical applications.

Complex multi-block responsive micelles\textsuperscript{230} have been prepared by Laschewsky and co-workers,\textsuperscript{231} Wooley and co-workers\textsuperscript{232} and Rannard and co-workers.\textsuperscript{233} These examples include temperature-responsive micelles in aqueous media that form from the self-assembly of amphiphilic A-B diblock copolymer unimers of $p$(alkylene oxide)s combined with $p$(styrene) and $p$(4-vinylpyridine).\textsuperscript{234,235}

Linear-dendritic PEG-based biocompatible copolymers have also been shown to act as thermo-responsive micelles that completely disrupt into unimers upon cooling below their LCST, with reversible self-assembly upon heating (Figure 1.16).\textsuperscript{236} These linear-dendritic polymeric micelles were investigated for temperature-induced controlled release of therapeutic agents as well as active targeting by subsequent functionalisation of the peripheral acid groups with biospecific ligands. The combination of hydrophilic, hydrophobic and charged groupings on single polymer chains, coupled with the ability to interchange these properties \textit{via} temperature or pH switching has given rise to materials with elaborate solution structures that strongly resemble biological entities. Thermo-responsive polymers are considered as one of the most widely utilised stimuli-responsive polymers, as they are easy to apply both \textit{in vitro} and \textit{in vivo}.\textsuperscript{237,238,239,240,241,242}
1.4.2 pH-responsive materials

Synthetic pH-responsive polymers are formed from monomers that are weakly basic or acidic. These moieties protonate reversibly as a function of pH, the adjustment of which alters the ionic interaction, hydrogen bonding and hydrophobic interaction with water, resulting in a reversible micro-phase separation or self-organisation phenomenon.\textsuperscript{226} As a direct result, pH-responsive polymers are fully soluble in aqueous solution at appropriate pH, as they are typically hydrated, swollen and hydrophilic in their ionic state (Figure 1.17A). As the pH varies, the polymers become dehydrated, compact and more hydrophobic in their neutral form (Figure 1.17B).\textsuperscript{243} This transition occurs at a pH that is defined as the apparent pK\textsubscript{a} or pK\textsubscript{b} and is very susceptible to change when the chemical substituents of the polymer backbone\textsuperscript{244,245} or the polymeric architectures\textsuperscript{246} are adjusted.

A change in behaviour upon a switch in pH is specifically attractive for biological
applications due to the abundant pH gradients existing in normal and pathophysiological states. A leading example is the extracellular pH of tumours\textsuperscript{247,248} and endosomal and lysosomal compartments of cells possessing a slightly more acidic pH (~4.5)\textsuperscript{249} than blood and normal tissues (~7.4).\textsuperscript{250} The synthesis of pH-responsive polymers has been extensively studied, with the influences of pH, co-solvent and electrolytes altering the solubility, volume properties and chain conformations.\textsuperscript{226} In particular, tertiary amine methacrylate-based block copolymers possess responsiveness to multiple external stimuli (such as pH, temperature and salts – largely relevant to the human body) and have received an increased amount of interest.\textsuperscript{251}

The ease of synthesis and the self-assembly of diblock copolymers to form pH-responsive micelles has extensively been investigated.\textsuperscript{252} Double hydrophilic block copolymers,\textsuperscript{253} where one hydrophilic block is susceptible to change in solution conditions such as pH, avoids the use of co-solvents. Armes and co-workers have explored this concept in detail, and confirmed that manipulating the pH of the water led to micelle formation/dissociation.\textsuperscript{254} Additionally, the construction of pH-responsive branched copolymer NPs which reversibly form “core-shell” morphologies have been studied.\textsuperscript{255}

The copolymers crafted as the “building blocks” for assembly of higher-order materials have endless possibilities.\textsuperscript{256} This has encouraged the inclusion of biocompatible polymers into pH-responsive micelles,\textsuperscript{257} combinations of different diblock copolymers to form complex micelles\textsuperscript{258} and triblock copolymers to produce multi-responsive triple-shell architectures (that are multi-responsive).\textsuperscript{259} Other approaches have included the covalent conjugation of drugs to polymers via acid degradable linkages such as hydrazone, ester and carbamate linkers.\textsuperscript{260,261,262,263} This enables control over the drug release rate, similar to smart nano-carrier systems, as opposed to the drug being physically entrapped in the hydrophobic core which is the conventional method for micelle encapsulation.

1.5 Concluding remarks

Despite the considerable on-going research into nanomedicine for drug delivery applications, there are still many biodistribution and clinical translation concerns that need to be addressed.\textsuperscript{20} Current drug delivery vehicles such as block copolymer micelles offer high loading capabilities as well as the ability to tune the chemical composition and function of the diblock copolymers.\textsuperscript{264} Micelles may be constructed to respond to triggers from external stimuli, governing self-assembly and dis-assembly and making them a
highly attractive option for drug delivery. The ability to cross-link the core and/or shell of micelles also offers increased stability in physiological media. Current research efforts are investigating functionalisation of the diblock copolymers within micelle systems,\textsuperscript{365} in order to target specific sites of interest.

Continued efforts to develop more effective syntheses, with control over molecular weight distributions, specificity of surface functionality and the ongoing understanding of \textit{in vivo} interactions of dendrimers,\textsuperscript{266} has resulted in the commercial application of PAMAM in medical use. Despite this, dendrimer syntheses continue to be expensive and time consuming. There is little detail of commercial synthesis available but the early patent by Tomalia and Dewald\textsuperscript{267} suggests the production of 51.2 g of Generation 3 polyamine dendrimer required 9 g ammonia, 978 g methanol, 1.3 kg ethylenediamine and 455 g methyl acrylate (Figure 1.18). Synthetic improvements have been vast, however, each generation of growth continues to require an exponentially increasing number of reactions in order to increase the molecular weight of the macromolecule.

![Figure 1.18](image1.png)

\textbf{Figure 1.18} Relative representation of the inefficient mass amount of reactants required: 9 g ammonia (purple), 978 g methanol (green), 1.3 kg ethylenediamine (orange) and 455 g methyl acrylate (blue) reported by Tomalia and Dewald\textsuperscript{267} to produce 51.2 g of Generation 3 polyamine dendrimer (red).

The advancement of controlled polymerisation techniques has allowed the rational design of high molecular weight materials through inexpensive and relatively straightforward procedures. In recent developments, the combined concepts of linear-dendritic hybrids and branched vinyl polymers (Figure 1.19) has resulted in the introduction of \textit{hyp}-polydendrons, incorporating the highly extensive surface functionality of dendrimers with a branched polymer core. \textit{Hyp}-polydendrons have previously been synthesised from relatively small dendrons as initiators for branched vinyl polymerisation \textit{(cf. Section}
1.3.1.2). The combination of many dendrons via a non-crosslinked, branched polymer core in one synthetic step avoids complex dendrimer synthesis. Current reports in the literature have investigated the modification of the structural components, by introducing a mixed dendron/PEG initiator system. 268

![Figure 1.19 Diagrammatic representation of the combined concepts of linear-dendritic hybrids and branched copolymers to produce hyp-polydendrons.](image)

**1.6 Project Aims**

Ultimately, the focus of hyp-polydendron NP formation is their assessment as potential nanomedicines using a series of *in vitro* pharmacological studies that includes modelling of oral administration. These novel materials are intended to offer benefits as nanomedicines through increased stability, encapsulation properties, enhanced bioavailability and decreased drug toxicity, based on the modifications made within the hyp-polydendron structure. The steps involved to produce the variety of hyp-polydendrons synthesised in order to allow such assessment is to be carried out as so:

1. Firstly, the research presented here aims to introduce amphiphilicity into hyp-polydendrons through the use of tertiary amine functional dendritic chain-ends and branched hydrophobic polymer segments, whilst allowing comparison with analogous linear-dendritic hybrids. The use of 2-hydroxypropyl methacrylate (HPMA) and the co-polymerisation of HPMA and EGDMA, will also allow comparison to similar material studies. The effects of amphiphilicity on the stability and behaviour of the polymeric NPs formed will be studied and discussed in depth.

As well as studying the effects of varying dendron generation and surface chemistry compared to the hyp-polydendrons already reported in the literature, the research presented here aims to investigate other chemical variations within the hyp-
polydendron structure. There are multiple variations available for hyp-polydendron design and synthesis, offering the opportunity to control both structural and chemical behaviour. These include: variation of the chemical composition of the primary polymer chains (Figure 1.20a), utilising different monomers within the vinyl co-polymerisation to produce primary polymer chains with different chemical properties; alteration of the chemistry of the dendron chain end (Figure 1.20b), resulting in different surface chemistry; and alternatively the linker chemistry within the molecule can be tailored to potentially generate functionality within the internal structure of the dendron.

In addition, the architecture of the primary chains is a potential site for variation with the production of statistical and block copolymers (Figure 1.20c), as well as variation of the degree of polymerisation (Figure 1.20d). The one-pot polymerisation could enable initiation by multiple initiators (dendron and non-dendron) to result in mixed surface groups on the hyp-polydendron (Figure 1.20e) and, finally, the incorporation of an alternative divinyl monomer to EGDMA can be incorporated to introduce different brancher chemistry (Figure 1.20f).

Figure 1.20 Targeted architectural and chemical variations of hyp-polydendron structure; a) primary polymer chain chemistry; b) dendron chemistry; c) primary polymer chain architecture; d) primary polymer chain length; e) mixed surface functionality and f) brancher chemistry.
2. Following the synthesis and characterisation of a range of materials with many of the structural and chemical variations outlined above, the ability of hyp-polydendrons to form stable NPs via nanoprecipitation in aqueous media (Figure 1.21B) will be compared with the linear-dendritic analogues (Figure 1.21A). The variation in chemical and structural components within the hyp-polydendron will allow investigations to be carried out to uncover the effects of chain-end dendron generation and chemistry, precipitation medium pH and polymer architecture on the ability of each material to generate NPs; the resulting NP diameter and dispersity and subsequent response to changes in pH will be studied in detail.

3. Additionally, the recently reported co-nanoprecipitation method will be applied in this work, combining linear-dendritic polymer hybrids with hydrophobic, branched copolymers prior to the nanoprecipitation process (Figure 1.21C) to produce stabilised nanoprecipitates. These studies are expected to provide insights into the synthesis variables within hyp-polydendron architectures and the effect of these modifications on NP formation and behaviour.

4. The inclusion of a pH-responsive monomer into the hyp-polydendron core is hypothesised to provide, for the first time, stabilised hyp-polydendron nanoprecipitates in neutral pH, that dis-assemble into unimolecular species upon the
addition of acid. Additionally, the introduction of an acid-cleavable brancher is expected to provide potential opportunities for a double NP degradation mechanism: first, the dissociation of the nanoprecipitate into solvated branched polymers (Figure 1.22i), and secondly the degradation of the branched polymers into their individual primary chains of relatively mono-disperse and low molecular weight (Figure 1.22ii). The size and charge of NPs used for nanomedicine applications have been shown to affect the efficient clearance of materials from the body and degradation into biologically benign components and clearance is required.270

Figure 1.22 Hypothetical diagrammatic representation of the pH-responsive hyp-polydendron containing a pH-responsive polymer core and an acid-cleavable brancher, and the proposed modes of hydrolysis resulting in initial solvated branched polymers (i), followed by linear-dendritic polymer hybrids (ii).

5. Finally, the production of biodegradable linear-dendritic hybrids and hyp-polydendrons is a key aim of the research presented. The broadening of the controlled vinyl polymerisation techniques to include ring-opening polymerisation (ROP) of cyclic and bicyclic esters will be investigated. The resulting polyesters from conventional ROP are known to undergo degradation via cleavage of the backbone ester bonds (Figure 1.23) making these, and their resulting NPs, a key target to be studied.

Figure 1.23 Diagrammatic representation of proposed degradation of biodegradable hyp-polydendrons
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via cleavage of the ester bonds.

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Synthesis and nanoprecipitation studies of 2-hydroxypropyl methacrylate linear-dendritic polymer hybrids and hyperbranched-polydendrons

Publication from this research chapter
‘Synthesis, nanoprecipitation and pH sensitivity of amphiphilic linear–dendritic hybrid polymers and hyperbranched-polydendrons containing tertiary amine functional dendrons’
_Soft Matter_, 2015, **11**, 7005-7015.
2.1 Introduction

The combination of dendron chain ends and linear polymer chains has led to numerous studies of the new class of materials, linear-dendritic polymer hybrids.\textsuperscript{1} Branching within the linear segment has recently extended this concept to the formation of hyperbranched-polydendrons (hyp-polydendrons). These were first reported by Hatton \textit{et al.},\textsuperscript{2} and produced by introducing simultaneous branching during the propagation of the vinyl segment of a linear-dendritic hybrid (Figure 2.1).

![Figure 2.1 Co-polymerisation of 2-hydroxypropyl methacrylate (HPMA) and ethylene glycol dimethacrylate (EGDMA) initiated by a Generation 2 1,3-dibenzylxy-2-propanol dendron atom transfer radical polymerisation (ATRP) initiator to produce a hyp-polydendron.](image)

The concepts associated with the free-radical “Strathclyde” polymerisation,\textsuperscript{3,4,6,7} were utilised and the presence of a dendron macroinitiator within the controlled radical co-polymerisation of a mixture of vinyl and divinyl monomers was used to form soluble, high molecular weight branched polymers.

Aqueous nanoparticles (NPs) with monomodal size distributions were prepared \textit{via} nanoprecipitation.\textsuperscript{8} These materials showed high stability and offered options for tailored functionality. Further reports have described the modification of the polymeric structural components, by introducing a library of hyp-polydendrons containing varied levels of dendron/p(ethylene glycol) (PEG) surface groups.\textsuperscript{9} These amended hyp-polydendrons
present varied biological interactions with cells that offer the potential for tuneable NP permeation through the gut epithelium.

The synthesis of pH-responsive polymers has been extensively studied, particularly the processes in which pH, co-solvent and electrolytes manipulate the solubility, volume and chain conformations.\textsuperscript{10} This has led to the construction of pH-responsive branched copolymer NPs, which reversibly form “core-shell” morphologies.\textsuperscript{11} The transition at which a pH-responsive polymer changes morphology occurs at a pH known as the apparent pK\textsubscript{a} or pK\textsubscript{b} (Chapter 1, Section 1.4.2). This is very susceptible to change when the chemical substituents on the polymer backbone\textsuperscript{12,13,14,15} or the polymeric architectures\textsuperscript{16} are adjusted.

This chapter aims to introduce amine-functionality into the dendron end groups of hyp-polydendrons to form the first amphiphilic examples of this class of material and investigate the effect of architecture, dendron generation and pH on the behaviour of the polymeric NPs formed from these complex architectures.

\textbf{2.2 Strategy for synthesising Generation 0-2 amine-functionalised dendron ATRP initiators}

In order to confer pH-responsive behaviour to the surface of dendrons, tertiary amines were introduced by using a range of dimethylamino-derived functionalities. Such functionality was targeted as these groups provide hydrophilicity under acidic conditions and allow for new amphiphilic linear-dendritic hybrids and hyp-polydendrons to be synthesised.

A series of tertiary amine functional dendron initiators was designed, ranging from the zeroth to the second generation (G\textsubscript{0}-G\textsubscript{2}), which had significant chemical similarity. The synthesis of the G\textsubscript{1} and G\textsubscript{2} dendrons required the Michael addition of 2-(dimethylamino)ethyl acrylate (DMEA) to the primary amine-containing molecules, 1-amino-2-propanol or 1-[N, N-bis(2-aminopropyl)-amino]-2-propanol (APAP) (Scheme 2.1ii). These also contained secondary hydroxyl functionality, to initially produce, AmG\textsubscript{1}-OH and AmG\textsubscript{2}-OH. AmG\textsubscript{0}-OH was commercially available as 1-dimethylamino-2-propanol.
The simple bromoesterification reaction with α-bromoisobutyryl bromide (BiB)\textsuperscript{17} was used to yield the three amine-functionalised dendritic ATRP initiators (AmG\textsubscript{0}-Br, AmG\textsubscript{1}-Br and AmG\textsubscript{2}-Br) (Scheme 2.1).

### 2.2.1 Synthesis of 1-[N, N-bis(2-aminopropyl)-amino]-2-propanol

In previous reports, an AB\textsubscript{2} monomer comprising a mixture of primary amines and secondary hydroxyls has been synthesised,\textsuperscript{18} using diethylene triamine and propylene glycol; this strategy was chosen again with slight modification (Scheme 2.2), to produce the primary amine molecule required for the AmG\textsubscript{2}-OH synthesis.
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Scheme 2.2 Synthesis of 1-[N,N-bis(2-aminopropyl)-amino]-2-propanol.

The synthesis of APAP was achieved in four synthetic steps (Scheme 2.2). $[\text{BOC}_2\text{-BAPA-G}_1]$ was firstly synthesised by reacting 1,1’-carbonyl diimidazole (CDI) with tertiary butanol to produce the imidazole carboxylic ester. Bis(3-aminopropyl)amine (BAPA) was then introduced to react selectively with the intermediate to produce $[\text{BOC}_2\text{-BAPA-G}_1]$; analysis was conducted using $^1$H (Appendix, Figure A1) and $^{13}$C nuclear magnetic resonance spectroscopy (NMR; Appendix, Figure A2) and electrospray mass-spectrometry (ES-MS; Appendix, Figure A3).

The second step of the synthesis utilised the ring opening of propylene oxide (PO) to functionalise the focal point of $[\text{BOC}_2\text{-BAPA-G}_1]$. This strategy was selected because of mild reaction conditions and reports of high yields of similar reactions. PO also exhibits a low boiling point (34°C), therefore, any excess can be easily removed by evaporation under vacuum. Similarly, $^1$H (Appendix, Figure A4) and $^{13}$C NMR (Appendix, Figure A5) and ES-MS (Appendix, Figure A6) were used to characterise the $[\text{BOC}_2\text{-BAPA-OH}]$.

Thirdly, the removal of the N-tertiary butoxycarbonyl (tBOC) protecting groups of $[\text{BOC}_2\text{-BAPA-OH}]$, using concentrated hydrochloric acid in ethyl acetate, resulted in the tris-ammonium salt APAP.3HCl. The reaction was left to stir until the generation of
CO\textsubscript{2} ceased, and was monitored using \textsuperscript{1}H NMR (D\textsubscript{2}O) until the singlet at approximately 1.50 ppm, corresponding to the tertiary butyl groups, had disappeared.

In the final step, the resulting tris-ammonium salt APAP\_3HCl was converted to the free amine AB\textsubscript{2} branching molecule APAP. The synthesis of similar molecules has used ion exchange beads to transform the salt to the free amine, and required vacuum distillation to obtain a pure product.\textsuperscript{4} After attempting to use ion exchange beads, which resulted in low yields (<20%), a strong base, 4M sodium hydroxide (NaOH) was used to deprotonate the salt. In doing so, this liberated the free-base amine APAP, which separated from solution as an oil. Extraction of the oil from the mixture, using chloroform (CHCl\textsubscript{3}), resulted in recovery of APAP as a pale yellow oil in 81% yield.

The \textsuperscript{1}H NMR spectrum (Figure 2.2) confirms complete decarboxylation due to disappearance of the singlet corresponding to the 18 protons correlating to the \textit{tBOC} protecting groups in [\textit{tBOC}\textsubscript{2}-BAPA-OH] (Appendix, Figure A4). The \textsuperscript{13}C NMR spectrum shows 6 distinct C environments (Appendix, Figure A7) and the ES-MS has a molecular ion peak of [MH]\textsuperscript{+} = 190.2 Da; calculated (C\textsubscript{9}H\textsubscript{23}N\textsubscript{3}O) = 189.3 Da (Appendix, Figure A8).

![Figure 2.2 \textsuperscript{1}H NMR spectrum (CDCl\textsubscript{3}, 400 MHz) of 1-[\textit{N,N}-bis(2-aminopropyl)-amino]-2-propanol.]
2.2.2 Synthesis of tertiary amine functional dendron ATRP initiators (AmG₀-Br, AmG₁-Br and AmG₂-Br)

As mentioned previously, the synthesis strategy aimed to employ the simple Michael addition of acrylates (in our case DMEA) to primary amines (Scheme 2.1ii). Initially, the synthesis of AmG₁-OH and AmG₂-OH was achieved by Michael addition in simple one pot reactions between either 1-amino-2-propanol or APAP with DMEA. Solvent and excess acrylate were removed \textit{in vacuo} in order to isolate the products. ES-MS, $^{1}$H and $^{13}$C NMR analysis were used to confirm the structure purity of the product. The disappearance of the two singlet signals at ~6 ppm (Figure 2.3 and 2.4), corresponding to the protons on the double bond of the DMEA, confirm all unreacted acrylate has been removed from the purified products.

![Figure 2.3](image)

Figure 2.3 $^{1}$H NMR spectrum (CDCl$_3$, 400 MHz) of AmG₁-OH.

All 22 protons neighbouring nitrogen atoms within AmG₁-OH, are collectively seen between 2.2–2.6 ppm (Figure 2.3a, b, e and f). The 4 protons neighbouring the oxygen atom on the DMEA arms are found downfield at 4.1 ppm (Figure 2.3c), due to the electronegativity of the oxygen, as expected. The methyl group (Figure 2.3h) is seen upfield and integrates to 3 protons, as expected. The $^{13}$C NMR shows a peak at 173.0 ppm, assigned as the ester carbonyl group within the DMEA arm (Appendix, Figure A9).
Molecular ion peaks at \([\text{M+H}]^+\) m/z = 362.3 and \([\text{M+Na}]^+\) m/z = 385.3 were observed in the ES-MS analysis; calculated \((\text{C}_{17}\text{H}_{35}\text{N}_3\text{O}_5) = 361.5\) Da (Appendix, Figure A10).

Similarly, for the \text{AmG}_2\text{-OH} dendron, all the protons neighbouring the nitrogen atoms (50 protons) are collectively accounted for between 2.2–2.6 ppm (Figure 2.4a, b, e, f, h and i). The protons neighbouring the oxygen atom in the DMEA arms (8 protons) are found downfield at 4.1 ppm, due to the deshielding effect of the oxygen (Figure 2.4c). The \(^{13}\text{C} \) NMR confirms a carbonyl peak at 173.0 ppm (Appendix, Figure A11). The ES-MS reveals molecular ion peaks at 762.2 m/z \([\text{M+H}]^+\) and 784.6 m/z \([\text{M+Na}]^+\); calculated \((\text{C}_{37}\text{H}_{75}\text{N}_7\text{O}_9) = 762.0\) Da (Appendix, Figure A12).

The ATRP initiators were synthesised by esterification between \text{BiB} and the different dendron alcohols (\text{AmG}_0\text{-OH}, \text{AmG}_1\text{-OH} and \text{AmG}_2\text{-OH}; Scheme 2.1i). Isolation and purification was achieved by liquid/liquid extraction (dichloromethane (DCM)/saturated sodium hydrogen carbonate (NaHCO\textsubscript{3}) solution) to remove unreacted alcohol, solvents and salts, with characterisation by NMR spectroscopy and ES-MS.

In all cases, the appearance of resonances corresponding to the two new methyl groups neighbouring the Br atom at ~ 1.9 ppm (integrating to 6 protons) (Figures 2.5d, 2.6h and 2.7k) and the shift of the lone proton in the ester focal point to ~5.1 ppm (Figures 2.5e, 2.6g and 2.7j), indicated successful synthesis and purity of the products.
The protons neighbouring the nitrogen atom in the AmG₀-Br can individually be seen at 2.29 ppm (Figure 2.5a) and separate integration patterns agree with the expected structure; 6 protons for the methyl groups neighbouring the Br atom and 3 protons in total for the CH₃ (Figure 2.5e). The ¹³C NMR contained a peak at 170.9 ppm accounting for the new carbonyl group (Appendix, Figure A13), and the ES-MS reveals a molecular ion peak at 252.0; calculated (C₉H₁₈NO₂Br) m/z = 252.15 Da (Appendix, Figure A14).

Similarly, for the AmG₁-Br and AmG₂-Br, the methyl protons neighbouring the terminal nitrogen atoms integrate to 12 and 24 protons respectively at ~2.2 ppm (Figures 2.6a and 2.7a) and agree with the integration values of 3 protons for the methyl groups in the newly formed ester group (Figure 2.6i and 2.7m). The ¹³C NMR spectra for these molecules contain a new peak at 166.6 ppm (AmG₁-Br) (Appendix, Figure A15) and 171.2 ppm (AmG₂-Br) (Appendix, Figure A16) accounting for the new carbonyl group within the ester residing in the focal point of the dendron. AmG₁-Br exhibited molecular ion peaks [M+H]⁺ at m/z = 510.2 and [M+Na]⁺ m/z = 534.2; calculated (C₂₁H₄₀N₃O₆Br) m/z = 510.5 (Appendix, Figure A17) and the AmG₂-Br at [M+H]⁺ = 912.5 m/z, [M+Na]⁺ = 934.5 m/z and [M+K]⁺ = 950.5 m/z; calculated (C₂₁H₄₀N₃O₆Br) m/z = 911.0 (Appendix, Figure A18).
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Figure 2.6 $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of AmG$_1$-Br.

Figure 2.7 $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of AmG$_2$-Br.
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This straightforward approach to low generation dendron initiator synthesis proved robust and highly reproducible and has been repeated, producing >5.37 g (99% yield) for AmG₂-OH and >3.30 g (54% yield) for AmG₂-Br.

2.3 Linear polymerisation of HPMA and co-polymerisation of HPMA and EGDMA using EBiB and AmG₀-Br, AmG₁-Br and AmG₂-Br ATRP initiators

In previous studies, an average degree of polymerisation (DPₐ) = 50 monomer units for the linear polymer segment and primary chain length has been shown to yield linear-dendritic polymers (Mₘ ~ 13,000 – 20,000 g mol⁻¹)² and hyp-polydendrons of HPMA (Mₘ > 1,000,000 g mol⁻¹)² that undergo successful nanoprecipitation into water. Therefore, this chain length was targeted for all polymers within this study to allow comparison with previous reports.

The EBiB, AmG₀-Br, AmG₁-Br and AmG₂-Br ATRP initiators were each studied in the polymerisation of HPMA. Traditionally, chain entanglement and functionality of polymer pendant groups has been shown to govern the solution and bulk properties associated with high molecular weight linear polymers. Recent examples of such tailored behaviour include dendronised polymers which exhibit worm-like morphologies due to steric hindrance between neighbouring dendrons.²¹ Alternatively, investigations into linear-dendritic polymer hybrids have unveiled the effects chain end functionality has on NP behaviour in aqueous media.²² Here, we hypothesise that the size and/or amphiphilic nature of the amine-functionalised dendron chain ends will enable NP behavioural changes in aqueous media, compared to p(HPMA₅₀) NPs previously reported and allow new opportunities.²³

2.3.1 Linear polymerisation of HPMA using EBiB and AmG₀-Br, AmG₁-Br and AmG₂-Br ATRP initiators

Linear-dendritic hybrid polymers of HPMA, targeting chain lengths of DPₐ = 50 monomer units per chain, p(HPMA₅₀), were synthesised at 30°C in methanol (MeOH) (50 w/v%) and initiated by the different dendron ATRP initiators previously described (Scheme 2.1). Copper chloride/2,2’-bipyridyl (Cu(I)Cl/bpy) was employed as the catalyst system using the following molar ratio [Initiator]:[M]:[CuCl]:[bpy] = 1:50:1:2. To allow for comparison with un-functionalised materials, the commercially available initiator
**EBiB** was also used to initiate the linear polymerisation of HPMA (Figure 2.8).

![Diagrammatic representation of the linear polymerisation of HPMA using ethyl and AmG0-G2 functional dendron ATRP initiators.](image)

**Figure 2.8** Diagrammatic representation of the linear polymerisation of HPMA using ethyl and AmG0-G2 functional dendron ATRP initiators.

Number average molecular weight ($M_n$), weight average molecular weight ($M_w$) and dispersity ($D = M_w/M_n$) of the resulting $p$(HPMA$_{50}$) polymer and dendritic polymer hybrids were determined by triple detection gel permeation chromatography (GPC) (Table 2.1).

The formation of the linear polymer and linear-dendritic hybrids using **EBiB** and the dendron initiators **AmG0-Br**, **AmG1-Br** and **AmG2-Br** achieved high monomer conversion (>94%) as determined by $^1$H NMR of the crude reaction medium samples (Table 2.1). The polymers were purified by passing through a basic alumina column to remove the catalytic system, followed by precipitation into hexane.
Table 2.1 GPC analysis of amine-functional linear-dendritic hybrids and hyp-polydendrons and materials without amine end group functionality.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Conversion&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>M&lt;sub&gt;n&lt;/sub&gt; Theory&lt;sup&gt;c&lt;/sup&gt; (g mol&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>M&lt;sub&gt;n&lt;/sub&gt; (g mol&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>M&lt;sub&gt;w&lt;/sub&gt; (g mol&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>D</th>
<th>DP&lt;sub&gt;n&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EBiB-&lt;i&gt;p&lt;/i&gt;(HPMA&lt;sub&gt;50&lt;/sub&gt;)</strong></td>
<td>99</td>
<td>7100</td>
<td>12750</td>
<td>15850</td>
<td>1.24</td>
<td>87</td>
</tr>
<tr>
<td><strong>AmG&lt;sub&gt;0&lt;/sub&gt;-&lt;i&gt;p&lt;/i&gt;(HPMA&lt;sub&gt;50&lt;/sub&gt;)</strong></td>
<td>99</td>
<td>7390</td>
<td>11250</td>
<td>16300</td>
<td>1.45</td>
<td>76</td>
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<tr>
<td><strong>AmG&lt;sub&gt;1&lt;/sub&gt;-&lt;i&gt;p&lt;/i&gt;(HPMA&lt;sub&gt;50&lt;/sub&gt;)</strong></td>
<td>96</td>
<td>7430</td>
<td>13150</td>
<td>18050</td>
<td>1.37</td>
<td>88</td>
</tr>
<tr>
<td><strong>AmG&lt;sub&gt;2&lt;/sub&gt;-&lt;i&gt;p&lt;/i&gt;(HPMA&lt;sub&gt;50&lt;/sub&gt;)</strong></td>
<td>94</td>
<td>7690</td>
<td>12900</td>
<td>16350</td>
<td>1.26</td>
<td>83</td>
</tr>
<tr>
<td><strong>EBiB-&lt;i&gt;p&lt;/i&gt;(HPMA&lt;sub&gt;50&lt;/sub&gt;-&lt;i&gt;co&lt;/i&gt;-EGDMA&lt;sub&gt;0.95&lt;/sub&gt;)</strong></td>
<td>99</td>
<td>-</td>
<td>39800</td>
<td>1019000</td>
<td>22.63</td>
<td>-</td>
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<tr>
<td><strong>AmG&lt;sub&gt;0&lt;/sub&gt;-&lt;i&gt;p&lt;/i&gt;(HPMA&lt;sub&gt;50&lt;/sub&gt;-&lt;i&gt;co&lt;/i&gt;-EGDMA&lt;sub&gt;0.9&lt;/sub&gt;)</strong></td>
<td>99</td>
<td>-</td>
<td>33600</td>
<td>728500</td>
<td>21.70</td>
<td>-</td>
</tr>
<tr>
<td><strong>AmG&lt;sub&gt;1&lt;/sub&gt;-&lt;i&gt;p&lt;/i&gt;(HPMA&lt;sub&gt;50&lt;/sub&gt;-&lt;i&gt;co&lt;/i&gt;-EGDMA&lt;sub&gt;0.9&lt;/sub&gt;)</strong></td>
<td>99</td>
<td>-</td>
<td>46800</td>
<td>595200</td>
<td>12.71</td>
<td>-</td>
</tr>
<tr>
<td><strong>AmG&lt;sub&gt;2&lt;/sub&gt;-&lt;i&gt;p&lt;/i&gt;(HPMA&lt;sub&gt;50&lt;/sub&gt;-&lt;i&gt;co&lt;/i&gt;-EGDMA&lt;sub&gt;0.9&lt;/sub&gt;)</strong></td>
<td>99</td>
<td>-</td>
<td>37500</td>
<td>216400</td>
<td>5.77</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Triple detection analysis using THF/2% triethylamine (TEA) as eluent;
<sup>b</sup> Determined by NMR;
<sup>c</sup> $M_n$ Theory = ($M_w$ monomer*$DP$ targeted)+$M_w$ initiator

The GPC refractive index (RI) and right angle light scattering (RALS) chromatograms for the linear polymer and the linear-dendritic polymer hybrids, **EBiB-<i>p</i>(HPMA<sub>50</sub>)**, **AmG<sub>0</sub>-<i>p</i>(HPMA<sub>50</sub>)**, **AmG<sub>1</sub>-<i>p</i>(HPMA<sub>50</sub>)** and **AmG<sub>2</sub>-<i>p</i>(HPMA<sub>50</sub>)**, synthesised with each initiator are shown in Figure 2.9.
Figure 2.9 GPC chromatogram overlays of A) RI chromatograms and B) RALS chromatograms for EBiB-p(HPMA<sub>50</sub>). C) RI chromatograms and D) RALS chromatograms for AmG<sub>0</sub>-p(HPMA<sub>50</sub>). E) RI chromatograms and F) RALS chromatograms for AmG<sub>1</sub>-p(HPMA<sub>50</sub>). G) RI chromatograms and H) RALS chromatograms for AmG<sub>2</sub>-p(HPMA<sub>50</sub>).

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The GPC chromatograms show that the linear and linear-dendritic polymers have a monomodal distribution, expected for a controlled polymerisation, although a slight shoulder is seen in the RALS chromatograms as previously reported for HPMA polymerisation.\textsuperscript{24} For all linear polymer samples, a higher number average molecular weight than the theoretical targeted value was obtained; either due to the inefficiency of the dendron initiators, the coupling of chain ends at high conversions, or the presence of a small amount of dimethacrylate impurity within the HPMA monomer.\textsuperscript{24} Despite this, the linear polymer series were self-consistent in their achieved \( \text{DP}_n \), all exhibiting similar \( M_n \) values ranging from 11250-13150 g mol\(^{-1} \) (Table 2.1). This allowed an accurate assessment of material behaviour with respect to differences in chain end functionality and not large differences in molecular weight or dispersity.

The \( \text{DP}_n \) could not be determined directly by \(^1\text{H} \) NMR due to the overlapping signals of the initiator chain end residues and the polymer repeat units; however, the linear materials were analysed by \(^1\text{H} \) NMR spectroscopy and the \( p(\text{HPMA}_{50}) \) spectra are shown in Figure 2.10 and Appendix, Figures A19-21.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure_2.10.png}
\caption{\(^1\text{H} \) NMR spectrum (MeOD, 400 MHz) of \textit{AmG}2-\textit{p}(\text{HPMA}_{50}).}
\end{figure}
2.3.2 Co-polymerisation of HPMA and EGDMA using EBiB and AmG₀-Br, AmG₁-Br and AmG₂-Br ATRP initiators

The EBiB, AmG₀-Br, AmG₁-Br and AmG₂-Br dendron ATRP initiators were used in the co-polymerisation of HPMA and a divinyl monomer EGDMA, in order to produce a highly branched polymer and a series of systematically varying hyp-polydendrons.

The branched co-polymerisation of HPMA and EGDMA was conducted under identical conditions to those used for linear polymer and linear-dendritic polymer synthesis, but with the addition of a low concentration of EGDMA (Figure 2.11).

When using EBiB as the initiator, an appropriate molar ratio of brancher:initiator of 0.95:1 was employed in order to avoid crosslinking and gelation (Chapter 1, Section 1.3.1.1.1); high molecular weight, soluble polymer, EBiB-p(HPMA₅₀-co-EGDMA₀.₉₅) (Table 2.1) was recovered. When using the AmG₀-Br, AmG₁-Br and AmG₂-Br
initiators, gel formation was seen within the HPMA/EGDMA co-polymerisations at the brancher:initiator ratio of 0.95:1, yet the soluble hyp-polydendrons $\text{AmG}_0 p(\text{HPMA}_{50}-\text{co-EGDMA}_{0.9})$, $\text{AmG}_1 p(\text{HPMA}_{50}-\text{co-EGDMA}_{0.9})$ and $\text{AmG}_2 p(\text{HPMA}_{50}-\text{co-EGDMA}_{0.9})$ were achieved at a slightly lower brancher:initiator ratio of 0.90:1. This further suggests a reduced initiator efficiency, as poor initiation will lead to fewer propagating polymer chains and a higher effective brancher:initiator ratio within each reaction. An identical $\text{DP}_n = 50$ monomer units was targeted for the primary polymer chain length and analysis by GPC and $^1\text{H}$ NMR spectroscopy analysis for each polymer is collated in Table 2.1. Figure 2.12 shows the GPC RI and RALS chromatogram, for each branched polymer.

The branched polymers start to elute at much lower retention volumes than their linear-polymer analogues due to the fraction of very higher molecular weight material present within the molecular weight distribution, highlighting the contrast with the linear polymers which have narrow, monomodal distributions. The broad distributions of the branched materials, including the hyp-polydendrons, is derived from both the varied number of conjoined chains within the polymer samples and a wide range of possible architectures due to the statistical incorporation of EGDMA along the primary polymer chains.

The branched materials were also analysed by $^1\text{H}$ NMR spectroscopy and the $p(\text{HPMA}_{50}-\text{co-EGDMA}_{0.9})$ spectra are shown in Figure 2.13 and Appendix, Figures A22-24. The EGDMA proton peaks are masked by the HPMA proton peaks, due to the similar chemical environment of the mono-functional monomer residues (Figure 2.13b’ and f).
Figure 2.12 GPC chromatogram overlays of A) RI chromatograms and B) RALS chromatograms for EBiB-p(HPMA$_{50}$-co-EGDMA$_{0.95}$). C) RI chromatograms and D) RALS chromatograms for AmG$_{p}$-p(HPMA$_{50}$-co-EGDMA$_{0.95}$). E) RI chromatograms and F) RALS chromatograms for AmG$_{1}$-p(HPMA$_{50}$-co-EGDMA$_{0.95}$). G) RI chromatograms and H) RALS chromatograms for AmG$_{2}$-p(HPMA$_{50}$-co-EGDMA$_{0.95}$).
Figure 2.13 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$-p (HPMA$_{50}$-co-EGDMA$_{0.9}$).

2.3.3 Kinetic studies of polymerisation of HPMA and co-polymerisation of HPMA and EGDMA using EBiB, AmG$_0$-Br, AmG$_1$-Br and AmG$_2$-Br ATRP initiators

Kinetic experiments were undertaken for each of the polymerisations to confirm first order kinetics with respect to monomer concentration, to follow the evolution of molecular weight with respect to monomer conversion and to ensure control of the polymerisation via ATRP was maintained during the reactions.

In all cases, the kinetic studies indicated first order kinetics for the linear polymerisations with linear evolution of $M_n$ with conversion (Figure 2.14).
Figure 2.14 Kinetic plots for linear DP₀₀ polymers. A) and B) EBiB₋p(HPMAₐ₀), C) and D) AmG₀₋p(HPMAₐ₀), E) and F) AmG₁₋p(HPMAₐ₀), G) and H) AmG₂₋p(HPMAₐ₀). A, C, E and G) Conversion (blue squares), ln([M]/[M]) (red circles); B, D, F and H) Mₙ (red circles) and 〈D〉 (blue lines).
Interestingly, the polymerisations initiated by AmG1-Br and AmG2-Br appeared to propagate faster than reactions using the other initiators, achieving approximately 90% conversion in 5 hours; the EBiB and AmG0-Br polymerisations showed very similar kinetics. D values were relatively consistent from 40% monomer conversion, although values tended to be 1.45 > D > 1.2.

Branched co-polymerisations initiated by either EBiB or AmG0-Br showed almost identical polymerisation rates as the respective linear polymerisations conducted in the absence of EGDMA (Figure 2.15), however, hyp-polydendron synthesis using the AmG1-Br and AmG2-Br initiators propagated noticeably slower than their analogous linear-dendritic polymer reactions. Molecular weight analyses were enabled by determining average dn/dc values of the time points analysed (Table 2.2).

**Table 2.2** Average dn/dc values of p(HPMA50) linear/linear-dendritic polymers and p(HPMA50-co-EGDMAx) copolymer/hyp-polydendrons.

<table>
<thead>
<tr>
<th>Target polymer</th>
<th>dn/dc$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBiB-p(HPMA50)</td>
<td>0.0802</td>
</tr>
<tr>
<td>AmG0-p(HPMA50)</td>
<td>0.0853</td>
</tr>
<tr>
<td>AmG1-p(HPMA50)</td>
<td>0.0716</td>
</tr>
<tr>
<td>AmG2-p(HPMA50)</td>
<td>0.0658</td>
</tr>
<tr>
<td>EBiB-p(HPMA50-co-EGDMA0.95)</td>
<td>0.0739</td>
</tr>
<tr>
<td>AmG0-p(HPMA50-co-EGDMA0.9)</td>
<td>0.0825</td>
</tr>
<tr>
<td>AmG1-p(HPMA50-co-EGDMA0.9)</td>
<td>0.0858</td>
</tr>
<tr>
<td>AmG2-p(HPMA50-co-EGDMA0.9)</td>
<td>0.0758</td>
</tr>
</tbody>
</table>

$^a$ THF eluent containing 2% TEA (v/v)
Figure 2.15 Kinetic plots for branched DP50 polymers. A) and B) EBiB\_p(HPMA50-co-EGDMA0.95), C) and D) AmG\_p(HPMA50-co-EGDMA0.9), E) and F) AmG1\_p(HPMA50-co-EGDMA0.9), G) and H) AmG2\_p(HPMA50-co-EGDMA0.9). A, C, E and G) Conversion (blue squares), ln([M]₀/[M]) (red circles); B, D, F and H) M_n (red circles) and $\bar{M}$ (blue lines), M_w (black diamonds).
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Linear semi-logarithmic plots were observed within branched polymerisations using each of the initiators, confirming first order kinetics (Figure 2.15); the development of $M_n$ and $M_w$ within the branched polymerisations showed the well reported dramatic increase at conversions $>80\%$ which was very noticeable within the relationship of $M_w$ vs. conversion, leading to multimodal GPC chromatograms (Figure 2.12).

$M_w$ values of the branched polymers up to $1.02 \times 10^6 \text{ g mol}^{-1}$ were observed, suggesting significant contributions to the physical mass of each sample from complex branched structures containing on weight average $>64$ primary chains (EBiB-$p$(HPMA$_{50}$-co-EGDMA$_{0.95}$)), $>45$ primary chains (AmG$_0$-$p$(HPMA$_{50}$-co-EGDMA$_{0.9}$)), $>33$ primary chains (AmG$_1$-$p$(HPMA$_{50}$-co-EGDMA$_{0.9}$)) and $>13$ primary chains (AmG$_2$-$p$(HPMA$_{50}$-co-EGDMA$_{0.9}$)); number average structures appear to contain at least 3 conjoined chains.

Each primary chain of the hyp-polydendrons contains a dendron initiator end group, therefore the weight average branched architectures contain approximately 45 tertiary amine (AmG$_0$-$p$(HPMA$_{50}$-co-EGDMA$_{0.9}$)), 66 tertiary amine (AmG$_1$-$p$(HPMA$_{50}$-co-EGDMA$_{0.9}$)) or 52 tertiary amine (AmG$_2$-$p$(HPMA$_{50}$-co-EGDMA$_{0.9}$)) chain end functional groups. It is notable that the size of the dendron initiator appeared to determine the extent of the branching and therefore the $M_w$ and D values obtained.

2.4 Nanoprecipitation of linear polymers, amine-functionalised linear-dendritic hybrids, branched copolymers and amine-functionalised hyp-polydendrons

Linear and branched hydrophobic polymers derived from HPMA have been previously nanoprecipitated to produce well-defined stable NPs as reported by Slater et al., with clear charge-stabilisation (negative zeta potential ($\zeta$)) as an aqueous NP dispersion. This approach has been extended to include the new linear-dendritic hybrids and hyp-polydendrons studied here to assess if stable NPs can be prepared under similar conditions.

Nanoprecipitation is a rapid, controlled assembly of hydrophobic organic molecules and has been used to form NPs of small molecule compounds and polymers. Under nanoprecipitation conditions, macromolecules are hypothesised to collapse into nonsolvated coils which aggregate to form nuclei that assemble until reaching a colloidal-stability. As the solution of solvated polymer chains in a good solvent is added to the anti-solvent, the expanded polymer coils collapse (Figure 2.16), as the water-miscible
solvent and water diffuse together and the quality of the solvent environment, for the hydrophobic polymers, diminishes.

Figure 2.16 Proposed mechanism in which the linear-dendritic polymer hybrids (A) and hyp-polydendrons (C) derived from amine-functional dendron initiators collapse and aggregate in order to achieve stabilisation; B) Representative nanoprecipitation method formed from an initial tetrahydrofuran (THF) concentration of 5 mg mL\(^{-1}\) producing an aqueous concentration of 1 mg mL\(^{-1}\) at pH = 7.8.

The aggregates must achieve colloidal stability to prevent macro-scale phase separation and this is often achieved by steric or charge repulsion. The linear-dendritic polymers and hyp-polydendrons discussed here are expected to nanoprecipitate successfully and display enhanced stability in the presence of aqueous NaCl when compared with previously reported \(p(\text{HPMA}_{50})\) and \(p(\text{HPMA}_{50-\text{co-EGDMA}_x})\) polymers, as the presence of the amine functionalised dendron chain ends provides a surface functionality capable of generating positive charge through protonation.

A range of parameters such as polymer concentration, dilution factor, primary polymer chain length, composition, and architecture have all shown to have an effect on the control of nanoprecipitation.\(^{23}\) In order to determine the impact of a varying number of parameters on NP formation for these new materials, a series of experiments were conducted including: 1) fast vs. slow addition of good solvent to a fixed volume of water, 2) variation of mass of polymer nanoprecipitated, and 3) varied polymer concentration within the good solvent. Precipitation into a fixed volume of water allowed trends to be identified.
Studies within the Rannard group have employed a rapid addition of polymer solutions to volumes of stirred water, whereas other literature examples of nanoprecipitation include the preparation of anisotropic polymer NPs via addition in a slow, drop-wise manner. In order to test the effect of the rate of nanoprecipitation, both addition methods were evaluated. Each polymer sample was dissolved in THF at a concentration of 5 mg mL\(^{-1}\) and 2 mL of each solution was subjected to a rapid solvent switch through i) rapid addition (fast; Table 2.3), and ii) drop-wise addition (slow; Table 2.3) into 10 mL of deionised water (pH = 7.8) at ambient temperature. Rapid addition involved instantaneous addition of the polymeric solution to the deionised water, whereas the slow addition was accomplished via drop-wise addition of the polymeric solution over a period of 30 seconds. Each experiment led to a final polymer concentration of 1 mg mL\(^{-1}\) in water after THF removal by evaporation overnight, as monitored by \(^1\)H NMR. The NPs that appeared stable were analysed by dynamic light scattering (DLS) to assess NP hydrodynamic diameters (\(D_z\)) and polydispersity (PDI) values.

### Table 2.3 DLS analysis of nanoprecipitated \(p(\text{HPMA}_{50}\text{-co-EGDMA}_x)\) branched copolymer and hypopolydendrons dissolved in THF (5 mg mL\(^{-1}\)) into deionised water (pH=7.8) (fast vs. slow addition) resulting in an end NP concentration of 1 mg mL\(^{-1}\).

<table>
<thead>
<tr>
<th>Addition Method</th>
<th>(D_z) (nm)</th>
<th>PDI</th>
<th>(D_z) (nm)</th>
<th>PDI</th>
<th>(D_z) (nm)</th>
<th>PDI</th>
<th>(D_z) (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast</td>
<td>99</td>
<td>0.101</td>
<td>882</td>
<td>0.420</td>
<td>79</td>
<td>0.181</td>
<td>187</td>
<td>0.231</td>
</tr>
<tr>
<td>Slow</td>
<td>178</td>
<td>0.082</td>
<td>157</td>
<td>0.269</td>
<td>165</td>
<td>0.097</td>
<td>149</td>
<td>0.099</td>
</tr>
</tbody>
</table>

The \(\text{EBiB}-, \text{AmG}_{1-}\) and \(\text{AmG}_{2-p(\text{HPMA}_{50}\text{-co-EGDMA}_x)}\) branched copolymers all formed stable NP dispersions with \(D_z\) size values similar to previous NPs formed from \(p(\text{HPMA}_{50}\text{-co-EGDMA}_x)\), regardless of the addition method. The \(\text{AmG}_{0-p(\text{HPMA}_{50}\text{-co-EGDMA}_{0.9})}\) copolymer formed much larger NPs, with a broad distribution of NP sizes, after fast administration of the polymer solution into water. This suggests a lack of controlled aggregation when this particular polymer solution is introduced rapidly into the anti-solvent. All copolymers displayed narrower particle size distributions when added to the aqueous phase via the slower, drop-wise method, implying a slightly more controlled NP formation manner when this technique is used (Figure 2.17). For all
further nanoprecipitations conducted throughout this study, the slow drop-wise method was therefore utilised.

Figure 2.17 DLS traces of fast (green) vs. slow (red) addition for A) EBiB-, B) AmG0-\textsuperscript{r}, C) AmG1-, and D) AmG2-p(HPMA\textsubscript{50-co}-EGDMA\textsubscript{x}) into deionised water (pH = 7.8) (1 mg mL\textsuperscript{-1}).

The effect of varying the mass of polymer precipitated into a fixed volume of water was studied by using differing amounts of good solvent, resulting in different end NP concentrations within the aqueous media (Table 2.4). Good solvent solutions of the \( p(\text{HPMA}\textsubscript{50-co}-\text{EGDMA}\textsubscript{0.9}) \) branched copolymer and the \( \text{hyp-} \) polydendrons were generated at concentrations of 5 mg mL\textsuperscript{-1} and 10 mg mL\textsuperscript{-1} in THF. Varying volumes of each solution, ranging from 0.5 mL of the 10 mg mL\textsuperscript{-1} solution to 5 mL of the 5 mg mL\textsuperscript{-1} solution, were nanoprecipitated into deionised water (5 mL) to achieve end concentrations of 1, 2 and 5 mg mL\textsuperscript{-1} after THF evaporation overnight; this ensured consistent fixed masses of 5 mg, 10 mg and 25 mg of each copolymer were nanoprecipitated into the fixed volume of water for each of the three experiments. Maintaining a constant mass of polymer during the different nanoprecipitations was important in order to evaluate the effect of varying solvent quality within the mixed solvent environments for each end nanoprecipitation condition.
Table 2.4 DLS analysis of \( p(\text{HPMA}_{50}\text{-co-EGDMA}_{x}) \) branched copolymer and \( \text{hyp-polydendrons} \) comparing different start and end NP concentrations in deionised water (pH=7.8).

<table>
<thead>
<tr>
<th>Concentration (mg mL(^{-1}))</th>
<th>( p(\text{HPMA}<em>{50}\text{-co-EGDMA}</em>{x}) )</th>
<th>( \text{EBiB}^- )</th>
<th>( \text{AmG}_0^- )</th>
<th>( \text{AmG}_1^- )</th>
<th>( \text{AmG}_2^- )</th>
</tr>
</thead>
</table>
| \begin{tabular}{c|c|c|c|c|c|c} \hline Start & End & \( D_z \) (nm) & PDI & \( D_z \) (nm) & PDI & \( D_z \) (nm) & PDI & \( D_z \) (nm) & PDI \\ \hline 5    & 1    & 87  & 0.082 & 108  & 0.228 & 226  & 0.086 & 245  & 0.033 \\ 2    & 1    & 118 & 0.124 & 147  & 0.392 & 258  & 0.377 & -    & -    \\ 5    & -    & -   & -     & -    & -     & -    & -     & -    & -    \\ \hline 10   & 1    & 99  & 0.080 & 110  & 0.292 & 146  & 0.280 & 170  & 0.100 \\ 2    & -    & -   & -     & -    & -     & -    & -     & -    & -    \\ 5    & -    & -   & -     & -    & -     & -    & -     & -    & -    \\ \hline \end{tabular} |}

In several instances, when larger volumes of THF solution (> 2 mL) were added to water, stable NP dispersions failed to form (Table 2.4). Only the \( \text{AmG}_1^- p(\text{HPMA}_{50}\text{-co-EGDMA}_{0.9}) \) formed stable NPs at a concentration of 5 mg mL\(^{-1}\) from the addition of 5 mL of a 5 mg mL\(^{-1}\) copolymer/THF solution. The instantaneous formation of nanoprecipitates has been demonstrated and limits of solvent mixtures have been previously considered.\(^{27}\) The addition of large volumes of good solvent to the antisolvent phase results in a mixed liquid environment capable of preventing nanoprecipitation. The large volume of polymeric solvent reduces the solvent quality and delays instant particle formation, and consequently prohibits stable NP formation in these studies. This was further confirmed when successful final NP concentrations of 2 and 5 mg mL\(^{-1}\) were achieved using a starting polymer concentration of 10 mg mL\(^{-1}\) following the addition of the same polymer mass in a reduced amount of solvent.

Overall, a general increase in the measured \( D_z \) values was observed when targeting increasing final NP concentrations. A greater difference was observed for particles prepared from the 5 mg mL\(^{-1}\) copolymer solutions, which is expected as there is a greater increase in solvent utilised during nanoprecipitation (1, 2 and 5 mL for targeted end concentrations of 1, 2 and 5 mg mL\(^{-1}\) opposed to 0.5, 1 and 2.5 mL for the nanoprecipitation prepared from 10 mg mL\(^{-1}\) copolymer solution).

When targeting final aqueous NP concentrations of 1, 2 and 5 mg mL\(^{-1}\) using \( \text{AmG}_0^- p(\text{HPMA}_{50}\text{-co-EGDMA}_{0.9}) \) and starting with 10 mg mL\(^{-1}\) copolymer solution in THF,
the NP diameters are very similar (Table 2.4); this is also seen when targeting NP concentrations of 1 and 2 mg mL\(^{-1}\) using EBiB\(\text{-p}(\text{HPMA}_{50}\text{-co-EGDMA}_{x})\), and 2 and 5 mg mL\(^{-1}\) NP dispersions using AmG\(_1\)-p(HPMA\(_{50}\text{-co-EGDMA}_{0.9}\)), also prepared from the 10 mg mL\(^{-1}\) THF solution (Table 2.4). To understand this further, the derived count rate (DCR) was taken into consideration i.e. the amount of detectable NP aggregations. The DCR displayed an increase in scattering as the final targeted NP concentration increased (87050, 92700 and 291300 kcps for the AmGo-p(HPMA\(_{50}\text{-co-EGDMA}_{0.9}\)) 1, 2 and 5 mg mL\(^{-1}\) respectively). This suggests that increasing the polymer concentration leads to an increased number of particles of similar size. Multiple nanoprecipitations have shown to be possible through direct addition of polymer solutions to aqueous NP dispersions after solvent removal, resulting in a higher particle concentration of nanoprecipitates of the same size.\(^{27}\)

Conversely, nanoprecipitation of AmG\(_1\)-p(HPMA\(_{50}\text{-co-EGDMA}_{0.9}\)) produced particles with a \(D_z = 258\) and 424 nm at a targeted final NP concentration of 2 and 5 mg mL\(^{-1}\) respectively when using an initial polymer concentration of 5 mg mL\(^{-1}\). The DCRs are similar in both these instances (400400 and 325300 kcps at 2 and 5 mg mL\(^{-1}\) respectively), suggesting that the increased polymer mass contributes to the formation of larger particles.

Stable NP dispersions were produced for all copolymers when targeting a final NP concentration of 1 mg mL\(^{-1}\), independent of the starting polymer concentration (Table 2.4). The starting polymer concentration of 5 mg mL\(^{-1}\) overall produced NPs with narrower size distributions; therefore, a starting concentration of 5 mg mL\(^{-1}\) was selected for all further nanoprecipitations, with the aim of producing aqueous polymeric NP dispersions of 1 mg mL\(^{-1}\). The volume of water for all nanoprecipitations was also standardised as 10 mL, allowing production of sufficient NP samples for further analysis and studies. The nanoprecipitation process is dependent on many different factors that influence the NPs produced and it was, therefore, paramount to fix parameters, such as concentration and dilution, in order to attribute differences in NP behaviour to the varying surface functionalities, polymer compositions and architectures investigated within this project.

Following the successful synthesis of linear polymers, linear-dendritic hybrids, branched copolymers and hyp-polydendrons, the EBiB-p(HPMA\(_{50}\)) and EBiB-p(HPMA\(_{50}\text{-co-EGDMA}_{0.95}\)) polymers were dissolved separately in THF at a concentration of 5 mg mL\(^{-1}\).
1. 2 mL of each solution was subjected to a solvent switch through drop-wise addition into 10 mL of deionised water (pH 7.8) at ambient temperature, to give a final polymer concentration of 1 mg mL$^{-1}$. Both polymers formed stable NPs in aqueous media and analysis was conducted by DLS to assess $D_z$, $\zeta$ and PDI. NPs formed from the linear EBiB-p(HPMA$_{50}$) were significantly larger and more polydisperse than their branched analogues as previously reported (Table 2.5). The same nanoprecipitation conditions were exploited in an attempt to produce stable NP dispersions from the linear-dendritic hybrids (AmG$_0$p(HPMA$_{50}$), AmG$_1$p(HPMA$_{50}$) and AmG$_2$p(HPMA$_{50}$)) and the hyp-polydendrons (AmG$_0$p(HPMA$_{50}$-co-EGDMA$_{0.9}$), AmG$_1$p(HPMA$_{50}$-co-EGDMA$_{0.9}$) and AmG$_2$p(HPMA$_{50}$-co-EGDMA$_{0.9}$)) possessing amine functionality.

**Table 2.5** DLS analysis of nanoprecipitated linear-dendritic polymer hybrids and hyp-polydendrons dissolved in THF (5 mg mL$^{-1}$) into deionised (pH=7.8) and acidic (pH=4.0) water to produce end NP concentrations of 1 mg mL$^{-1}$.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>pH = 7.8$^a$</th>
<th>pH = 4.0$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D_z$ (nm)</td>
<td>PDI</td>
</tr>
<tr>
<td>EBiB-p(HPMA$_{50}$)</td>
<td>780</td>
<td>0.230</td>
</tr>
<tr>
<td>AmG$<em>0$p(HPMA$</em>{50}$)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG$<em>1$p(HPMA$</em>{50}$)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG$<em>2$p(HPMA$</em>{50}$)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EBiB-p(HPMA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>178</td>
<td>0.082</td>
</tr>
<tr>
<td>AmG$<em>0$p(HPMA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>157</td>
<td>0.269</td>
</tr>
<tr>
<td>AmG$<em>1$p(HPMA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>165</td>
<td>0.097</td>
</tr>
<tr>
<td>AmG$<em>2$p(HPMA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>149</td>
<td>0.099</td>
</tr>
</tbody>
</table>

$^a$ Initial pH

The linear-dendritic materials failed to form stable NPs and all resulted in macrophase separation. However, the hyp-polydendrons formed stable NPs (Figure 2.18) for each generation of dendron end group (Table 2.5).
Figure 2.18 Representative SEM images of nanoprecipitated $\text{AmG}_2$-$p(\text{HPMA}_{50}-\text{co}-\text{EGDMA}_{0.9})$ formed from an initial THF concentration of 5 mg mL$^{-1}$ producing an aqueous concentration of 1 mg mL$^{-1}$ at pH = 7.8.

The difference in stability between the linear-dendritic hybrids and the equivalent hyp-polydendrons appears to result from the presence of the branching within the complex architecture, and potentially as a direct consequence from the way in which the NPs pack during the nanoprecipitation process. Regarding the obvious difference in molecular weight between these linear and branched entities, the branched hyp-polydendrons are comprised of different numbers of conjoined linear-dendritic hybrid chains and any physical mass of equivalent samples will possess the same number of primary chains and chain ends; this feature of the polymers is independent of whether the polymer under discussion is linear or branched. The GPC analysis has also shown that there is a considerable amount of linear material present within the molecular weight distributions of the hyp-polydendrons, supporting the proposition of the branched material fraction directing the behaviour of the overall distribution. It is feasible to suggest that the materials of higher molecular weight form larger nuclei under the changing solvent conditions, which would also explain the larger $D_z$ sizes for $\text{EBiB}$-$p(\text{HPMA}_{50})$ compared to the $\text{EBiB}$-$p(\text{HPMA}_{50}$-$\text{co}$-$\text{EGDMA}_{0.95}$) where rapid attainment of colloidal stability is achieved, leading to the arrest of any further aggregation processes.

The negative $\zeta$ values witnessed for the EBiB-initiated polymers are in concordance with previous studies of these materials, and with reports of the adsorption of hydroxide ions at the hydrophobic solid/water interface. The presence of amine functionality provided by the dendrons does not seem to greatly influence these observations at neutral pH and titrations of the individual dendron initiators, $\text{AmG}_0$-$\text{Br}$, $\text{AmG}_1$-$\text{Br}$ and $\text{AmG}_2$-$\text{Br}$ allowed an estimation of the $pK_a$ values of the dendrons to be $pK_a(\text{AmG}_0) \approx 6.4$, $pK_a(\text{AmG}_1) \approx 6.2$, $pK_a(\text{AmG}_2) \approx 6.2$ (Figure 2.19), confirming a relative lack of protonation at the nanoprecipitation pH of 7.8.
2.5 Impact of pH on linear-dendritic hybrids and hyp-polydendrons

Due to the amine functionality present within the linear-dendritic hybrids and hyp-polydendrons, a comparative behavioural study could be conducted upon nanoprecipitation into acidic water (pH = 4). All linear and branched polymers were insoluble at pH 4, and all but one (AmG2-p(HPMA50)) were insoluble at pH 2; AmG2-p(HPMA50) gave an aqueous, transparent solution at a concentration of 1 mg mL\(^{-1}\). The density of tertiary amine groups associated with the AmG2 dendron end group can possibly account for this solubility, however, it is notable that the equivalent hyp-polydendron did also not show solubility at this pH. Due to the hydrophobic nature of p(HPMA50), the AmG2-p(HPMA50) was not expected to be completely solvated at pH 2. DLS analysis revealed identifiable nanoprecipitats (\(D_c = 82\) nm, PDI = 0.079) within the aqueous solution after 24 hours (Appendix, Figure A25). These appear to represent an aggregated species, (further confirmed by a DCR = 20030 kcps), but the exact nature is unclear.

To investigate the impact of pH further, nanoprecipitation was carried out under identical conditions as described above, with the exception that the anti-solvent medium was aqueous HCl (1M) at pH 4 (Table 2.5). As anticipated, the formation of charge-stabilised
NPs was not achieved for either the **EBiB-p(HPMA_{50})** or **EBiB-p(HPMA_{50-co-EGDMA_{0,95}})** polymers, when nanoprecipitated into low pH and both underwent macroscale precipitation. All linear-dendritic hybrids and **hyp-polydendrons** produced stable NP dispersions, however, and once again, the **hyp-polydendrons** generated lower $D_z$ values than their linear equivalents (Table 2.5).

Highly positive $\zeta$ values were measured by DLS, even for materials bearing only a single tertiary amine chain end, which are expected for protonated amine functionality and suggests there are a large number of dendron chain ends at the surface of the NPs. It may be expected that upon assembly and formation of the NPs, dendron chain ends become entrapped within the main bulk of the nanoprecipitate, as observed in previous reports of materials containing mixtures of hydrophobic dendrons and PEG chain ends. These internalised dendrons would be predicted to also protonate after nanoprecipitation into acidic water and would, therefore, be somewhat hydrated.

In order to study the response of the linear-dendritic hybrids and **hyp-polydendron** NPs to changes in pH, the materials prepared in aqueous HCl were subjected to a titration through the slow addition of base (1M NaOH), with continuous monitoring of $\zeta$ to investigate isoelectric points (IEPs). The NPs formed from the linear-dendritic hybrids (**AmG_{0-p}(HPMA_{50}), AmG_{1-p}(HPMA_{50}) and AmG_{2-p}(HPMA_{50})**) were remarkably more robust to alterations in pH than their corresponding **hyp-polydendron** nanoprecipitates, which underwent substantial precipitation even after the initial addition of base. Data for the **hyp-polydendrons** were therefore not reliable with accurate $\zeta$ measurements being unobtainable due to phase separation during the experiment. More reliable IEP measurements for the linear-dendritic hybrids were attainable, showing a steady decrease in $\zeta$ values upon the addition of base (Figure 2.20A), and steady PDI values until approaching pH values of 5.4-5.8. A corresponding decrease in scattering intensity (DCR) was simultaneously observed as the nanoprecipitates lost charge/stabilisation and precipitation began to occur (Figure 2.20B).
Figure 2.20 Isoelectric point determination via the slow addition of base to linear-dendritic polymer NP dispersions. A) Zeta-potential and (B) and derived count rate of nanoprecipitates of AmG₄-p(HPMA₅₀) linear–dendritic hybrids (AmG₆-p(HPMA₅₀) = blue open triangles, AmG₄-p(HPMA₅₀) = red open circles and AmG₂-p(HPMA₅₀) = green open squares) formed at pH = 4.0 and undergoing base titration. All DLS measurements were at 25°C.

The lack of stability retention during slow changes in pH for all materials studied, led to an investigation of the rapid switching of pH (Table 2.6). This involved the addition of NaOH (1M; 36 μL) to each NP dispersion prepared in aqueous HCl (pH~4), producing an end pH~12 (Table 2.6), measured by a pH probe. Following this, HCl (1M; 36 μL) was added to the alkaline NP dispersions, lowering the pH (~3) once more. Neither EBiB-p(HPMA₅₀) nor EBiB-p(HPMA₅₀-co-EGDMA₀.₉₅) produced stable nanoprecipitates in acidic pH so were not included in the study.
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Table 2.6 DLS analysis of nanoprecipitated linear–dendritic polymer hybrids and *hyp*-polydendrons into acidic water (pH=4) and the effects of NP size and surface charge following the rapid switching of pH.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>pH = 4.0&lt;sup&gt;a&lt;/sup&gt; (Final pH = 3.6-4.1)</th>
<th>+ 1M NaOH (Final pH = 11.5-11.9)</th>
<th>+ 1M HCl (Final pH = 3.1-3.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D&lt;sub&gt;z&lt;/sub&gt; (nm)</td>
<td>PDI</td>
<td>ζ (mV)</td>
</tr>
<tr>
<td><strong>AmG&lt;sub&gt;0&lt;/sub&gt;-p(HPMA&lt;sub&gt;50&lt;/sub&gt;)</strong></td>
<td>581</td>
<td>0.280</td>
<td>+52</td>
</tr>
<tr>
<td><strong>AmG&lt;sub&gt;1&lt;/sub&gt;-p(HPMA&lt;sub&gt;50&lt;/sub&gt;)</strong></td>
<td>246</td>
<td>0.193</td>
<td>+40</td>
</tr>
<tr>
<td><strong>AmG&lt;sub&gt;2&lt;/sub&gt;-p(HPMA&lt;sub&gt;50&lt;/sub&gt;)</strong></td>
<td>491</td>
<td>0.165</td>
<td>+44</td>
</tr>
<tr>
<td><strong>AmG&lt;sub&gt;0&lt;/sub&gt;-p(HPMA&lt;sub&gt;50&lt;/sub&gt;-co-EGDMA&lt;sub&gt;0.9&lt;/sub&gt;)</strong></td>
<td>147</td>
<td>0.304</td>
<td>+55</td>
</tr>
<tr>
<td><strong>AmG&lt;sub&gt;1&lt;/sub&gt;-p(HPMA&lt;sub&gt;50&lt;/sub&gt;-co-EGDMA&lt;sub&gt;0.9&lt;/sub&gt;)</strong></td>
<td>102</td>
<td>0.271</td>
<td>+45</td>
</tr>
<tr>
<td><strong>AmG&lt;sub&gt;2&lt;/sub&gt;-p(HPMA&lt;sub&gt;50&lt;/sub&gt;-co-EGDMA&lt;sub&gt;0.9&lt;/sub&gt;)</strong></td>
<td>196</td>
<td>0.373</td>
<td>+61</td>
</tr>
</tbody>
</table>

<sup>a</sup> Initial pH

The linear and branched polymer nanoprecipitates prepared in acidic pH conditions and bearing **AmG<sub>0</sub>** dendron chain ends underwent macroscale precipitation upon rapid base addition. The linear-dendritic hybrids and *hyp*-polydendrons containing **AmG<sub>1</sub>** and **AmG<sub>2</sub>** dendron functionality were able to maintain stable NPs upon addition of base without any indication of phase separation; D<sub>z</sub> values decreased for all samples and a switch from highly positive ζ values to highly negative ζ values was witnessed. The decrease in D<sub>z</sub> and switch of surface charge is not unexpected as the NPs become overall more hydrophobic and deprotonated. Lack of protonation would result in the dendrons on the surface collapsing back onto the hydrophobic NP surface and the dendrons packed within the NP interior, carrying protonated amines and bound water, would also be expected to become somewhat hydrophobic resulting in the expulsion of water and an overall collapse of the entire structure.

Generally, the linear-dendritic hybrids showed a larger decrease in D<sub>z</sub> following the pH switch (the largest specifically being with the **AmG<sub>2</sub>-p(HPMA<sub>50</sub>)** material) in comparison to the branched materials. This implies a more complicated and less ordered arrangement of the linear-dendritic hybrids within the NP structures, possibly resulting
from a greater degree of freedom within each individual linear polymer chain during particle formation than their branched equivalents.

To study the reversibility of the pH switch, HCl was rapidly added to the samples that were able to avoid phase separation during base addition. This returned the NP dispersions to their original acidic environments. Within the series of linear-dendritic hybrids, only the NPs derived from AmG2-p(HPMA50) were able to maintain their colloidal stability and displayed highly positive ζ values as well as a slight increase in Dz. This small increase in hydrodynamic diameter may be due to the lack of acid penetration into the hydrophobic NP core and the protonation of dendrons only taking place at the surface, resulting in a limited amount of swelling. Interestingly, the hyp-polydendrons comprising both the AmG1- and AmG2- dendrons were able to successfully undergo the second pH switch. All final NPs exhibited similar diameters (157-168 nm) in acid following the two pH switches (Figure 2.21).

![Diagrammatic representation of amine-functional hyp-polydendron nanoprecipitates formed at pH=4 and change in solvation of the core and surface dendrons: (A) deprotonation and collapse of amine-functional dendrons on addition of base, with subsequent protonation of surface functional groups on addition of acid, (B) representation of protonated and hydrated exterior and interior dendrons with collapsed vinyl polymer chains within the hyp-polydendron structure, (C) deprotonated and collapsed structure of dendrons and vinyl polymer chains.](image)

Figure 2.21

An additional experiment was conducted where the AmG2-p(HPMA50) and AmG2-p(HPMA50-c-o-EGDMA0.9) materials were nanoprecipitated into aqueous NaOH (pH 12) in order to determine the nature of the resulting nanoprecipitates in this medium. Interestingly, nanoprecipitates were generated that exhibited very similar sizes and ζ
values compared to when the same materials were initially prepared in acidic conditions and treated with base (AmG$_2$p(HPMA$_{50}$): $D_z = 152$ nm, PDI = 0.081, $\zeta = -46.0$ mV; AmG$_2$p(HPMA$_{50}$-co-EGDMA$_{0.9}$): $D_z = 127$ nm, PDI = 0.135, $\zeta = -43.1$ mV). Once again, smaller particles were formed from hyp-polydendrons when compared to its linear analogue.

2.6 Conclusions

The synthesis of amine-functionalised dendron initiators for ATRP was achieved, with consequent controlled formation of linear-dendritic polymer hybrids and hyp-polydendrons. The nanoprecipitates derived from the materials are unique and display behaviour that appears to benefit from both the presence and generation of the dendron end groups and the branching of the methacrylate polymer chains. The linear-dendritic hybrids and hyp-polydendrons exhibit varying behaviour in aqueous media that assumes a different arrangement of dendrons and vinyl polymer segments within the nanoprecipitates. This is directed predominantly by the branched polymer architecture and the chemical dissimilarity of the charged chain-ends and the uncharged and collapsed polymer chains. These differences appear to modify the ability of the NP surfaces to protonate and deprotonate, presumably leading to rapid hydroxide adsorption in the deprotonated state.

2.7 Experimental

[$t$BOC-BAPA-G$_1$] – CDI (19.55 g, 0.12 mol, 2 eq.) was added to an oven-dried 500 mL 2-neck round-bottomed flask (RBF) fitted with a reflux condenser, magnetic stirrer and a dry N$_2$ inlet. Anhydrous toluene (200 mL) was added and the flask was purged with N$_2$ for 10 minutes. The solution was stirred at 60°C and tertiary butanol, (17.87 g, 23 mL, 0.24 mol, 4 eq.) added via a warm syringe. The mixture was left stirring at 60°C for 6 hours under a positive flow of N$_2$. BAPA (7.91 g, 8.43 mL, 0.06 mol, 1 eq.) was added drop wise, and the reaction was left stirring for a further 18 hours at 60°C under a positive flow of N$_2$. Following this, the solution was allowed to cool to room temperature, and the pale yellow solution was filtered to remove any solid imidazole, and concentrated in vacuo. The resulting viscous oil was dissolved in DCM (300 mL) washed with distilled water (2 x 200 mL) and once with brine (150 mL). The organic layer was dried with anhydrous Na$_2$SO$_4$, filtered, and concentrated in vacuo. Yield: 15.74 g, white solid, (79%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 1.45$ (s, 18H), 1.66 (m, 4H), 2.65 (t, 4H),
CHAPTER 2

3.20 (m, 4H), 5.28 (s, br, -NH). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 28.7, 30.0, 39.3, 47.8, 79.5, 156.3. Calcd: [MH]$^+$ (C$_{16}$H$_{33}$N$_3$O$_4$) m/z = 332.3 Da. Found: ES-MS: [MH]$^+$ m/z = 332.3 Da. Anal. Calcd for C$_{16}$H$_{33}$N$_3$O$_4$: C 57.98%; H 10.04%; N 12.68%. Found: C 57.84%; H 10.45%; N 12.91%.

[BOC-BAPA-OH] – [BOC-BAPA-G$_1$] (15.74 g, 0.047 mol) was added to a 500 mL 2-necked RBF fitted with a reflux condenser, magnetic stirrer and a dry N$_2$ inlet. The flask was degassed with dry N$_2$ for 10 minutes, and dissolved in dry ethanol (200 mL). Whilst stirring, and maintaining the temperature at 30°C, PO (8.27 g, 9.96 mL, 0.142 mol) was added drop wise to the solution over a period of 10 minutes. Under a positive flow of dry N$_2$, the reaction was left stirring at 30°C for 18 hours. After this time, the solvent and excess propylene oxide were removed in vacuo, and the crude product purified by liquid chromatography (silica gel, eluting with ethyl acetate (EtOAc):MeOH), 80:20). Yield: 15.7 g, pale yellow oil at ambient temperature, solidifying to an off white solid upon cooling (85%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 1.12 (d, 3H), 1.45 (s, 18H), 1.64 (m, 4H), 2.30 (d, 2H), 2.34-2.67 (d of m, 4H), 3.16 (m, 4H), 3.77 (m, 1H), 4.99 (s, br, -NH). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 27.6, 28.7, 38.8, 51.8, 60.9, 63.0, 79.5, 156.5. Calcd: [MH]$^+$ (C$_{19}$H$_{40}$N$_3$O$_5$) m/z = 390.3 Da. Found: ES-MS: [MH]$^+$ m/z = 390.3 Da. Anal. Calcd for C$_{19}$H$_{40}$N$_3$O$_5$: C 58.58%; H 10.09%; N 10.09%. Found: C 58.50%; H 10.19%; N 10.82%.

1-[N, N-bis (2-aminopropyl)-amino]-2-propanol [APAP] – To a 500 mL RBF, [BOC-BAPA-OH] (15.70 g, 0.04 mol) was dissolved in EtOAc, (160 mL) and concentrated HCl (12M, 36%) (16.33 g, 13.8 mL) was added very slowly. CO$_2$ began to rapidly evolve. The reaction vessel was heated to 55°C and stirred vigorously for 5 hours before leaving overnight at room temperature. After removal of EtOAc in vacuo, $^1$H NMR (D$_2$O) confirmed complete decarboxylation and formation of APAP·3HCl.

For the formation of APAP, the crude oil was dissolved very slowly in 4M NaOH (160 mL), and reduced by approximately half its volume on a rotary evaporator (60°C). A yellow oily substance formed on the surface of the NaOH solution. The mixture was extracted with CHCl$_3$ (2 x 160 mL), the organic layers combined, dried with anhydrous Na$_2$SO$_4$, filtered, and concentrated in vacuo. Yield: 6.2 g, pale yellow oil (81%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 1.11 (d, 3H), 1.60 (m, 4H), 2.10 (s, br, -OH), 2.32 (m, 2H), 2.39-2.67 (d of m, 4H), 2.75 (t, 4H), 3.79 (m, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 20.1, 30.7, 40.5, 52.1, 62.7, 63.9. Calcd: [MH]$^+$ (C$_9$H$_{23}$N$_3$O) m/z = 189.3 Da. Found:
CI-MS: [M+H]⁺ m/z = 190.2 Da. Anal. Calcd for C₉H₂₃N₃O: C 57.10%; H 12.25%; N 22.20%. Found: C 55.18%; H 12.15%; N 20.53%.

**AmG₀-Br** – 1-dimethylamino-2-propanol (1.12 g, 10.86 mmol, 1 eq.), TEA (1.54 g, 15.2 mmol, 1.4 eq.) and DMAP (132.7 mg, 1.086 mmol, 0.1 eq.) were added to a 250 mL 2 necked RBF containing DCM (160 mL). The flask was deoxygenated under a positive N₂ purge for 10 minutes. BiB (2.62 g, 1.4 mL, 11.4 mmol, 1.05 eq.) was added drop wise while the solution was stirring in an ice bath under a positive flow of N₂. The reaction mixture was allowed to warm to room temperature and left stirring overnight. The organic phase was washed with saturated sodium hydrogen carbonate (NaHCO₃) solution (3 x 160 mL) and the solution was subsequently dried with anhydrous sodium sulfate (Na₂SO₄). Yield: 2.38 g, yellow oil (84%). ¹H NMR (400 MHz, CDCl₃) δ = 1.27 (d, 3H), 1.89 (s, 6H), 2.17-2.55 (m, 8H), 5.07 (m, 1H). ¹³C NMR (100 MHz, CDCl₃) δ = 17.8, 31.1, 46.2, 56.1, 64.0, 70.7, 170.9. Calcd [M+H]⁺ (C₉H₁₈NO₂Br) m/z = 252.15 Da. Found: ES-MS: [M+H]⁺ m/z = 252.0 Da. Anal. Calcd for C₉H₁₈NO₂Br: C 42.86%; H 7.14%; N 5.55%. Found C 42.87%; H 7.20%; N 5.55%.

**AmG₁-OH** – DMEA (6.0 g, 42 mmol, 6 eq.) was added to a 50 mL 2 necked RBF containing IPA (12 mL). The flask was deoxygenated under a positive N₂ purge for 10 minutes. 1-amino-2-propanol, (0.53 g, 7.0 mmol, 1 eq.) dissolved in IPA (12 mL) was added drop wise while the solution was stirring in an ice bath under a positive flow of N₂. The final mixture was stirred for a further 10 minutes at 0°C before being allowed to warm to room temperature and left stirring for 48 hours. The solvent was removed and the product left to dry in vacuo overnight. Yield: 2.75 g, yellow oil (92%). ¹H NMR (400 MHz, CDCl₃) δ = 1.08 (d, 3H), 2.18-2.62 (m, 22H), 2.69 (m, 2H), 2.89 (m, 2H), 3.77 (m, 1H), 4.16 (m, 4H). ¹³C NMR (100 MHz, CDCl₃): δ = 19.7, 32.6, 45.6, 49.5, 57.8, 62.1, 62.5, 63.8, 173.0. Calcd [M+H]⁺ (C₁₇H₃₅N₃O₅) m/z = 361.5 Da. Found: ES-MS: [M+H]⁺ m/z = 362.3 and [M+Na⁺] m/z=385.3. Anal. Calcd for C₁₇H₃₅N₃O₅: C 56.43%; H 9.68%; N 11.62%. Found C 57.45%; H 9.77%; N 11.12%.

**AmG₁-Br** – **AmG₁-OH** (1.12 g, 10.86 mmol, 1 eq.), TEA (1.54 g, 15.2 mmol, 1.4 eq.) and 4-dimethyl-aminopyridine (DMAP) (132.7 mg, 1.086 mmol, 0.1 eq.) were added to a 250 mL 2 necked RBF containing DCM (160 mL). The flask was deoxygenated under a positive N₂ purge for 10 minutes. BiB (2.62 g, 1.4 mL, 11.4 mmol, 1.05 eq.) was added drop wise while the solution was stirring in an ice bath under a positive flow of N₂. The reaction mixture was allowed to warm to room temperature and left stirring overnight.
The organic phase was washed with saturated NaHCO₃ solution (3 x 160 mL) and then dried with anhydrous Na₂SO₄ and the product left to dry in vacuo overnight. Yield: 1.06 g, yellow oil (67%). ¹H NMR (400 MHz, CDCl₃) δ = 1.22 (d, 3H), 1.89 (s, 6H), 2.24-2.69 (m, 22H), 2.83 (m, 4H), 4.20 (m, 4H), 5.0 (m, 1H). ¹³C NMR (100 MHz, CDCl₃) δ = 18.5, 33.0, 44.2, 50.3, 56.4, 58.7, 59.1, 59.7, 68.3, 69.2, 166.6, 172.0. Calcd: [M+H]⁺ (C₂₁H₄₀N₃O₆Br) m/z = 510.5 Da. Found: ES-MS: [M+H]⁺ m/z = 510.2 and [M+Na]+ m/z = 534.2. Anal. Calcd for C₂₁H₄₀N₃O₆Br: C 49.41%; H 7.84%; N 8.24%. Found: C 49.41%; H 7.90%; N 8.23%.

AmG₂–OH – DMEA (6.00 g, 42 mmol, 6 eq.) was added to a 50 mL round 2 necked RBF containing IPA (12 mL). The flask was deoxygenated under a positive N₂ purge for 10 minutes. APAP (1.32 g, 6.984 mmol, 1 eq.) dissolved in IPA (12 mL) was added drop wise while the solution was stirring in an ice bath under a positive flow of N₂. The final mixture was stirred for a further 10 minutes at 0°C, allowed to warm to room temperature and left stirring for 48 hours. The solvent was removed and the product left to dry in vacuo overnight. Yield: 5.37 g, yellow oil (99%). ¹H NMR (400 MHz, CDCl₃) δ = 1.13 (d, 3H), 1.67 (m, 4H), 2.26-2.65 (m, 50H), 2.77 (m, 8H), 3.87 (m, 1H), 4.17 (m, 8H). ¹³C NMR (100 MHz, CDCl₃) δ = 20.0, 24.5, 32.2, 45.7, 48.9, 51.6, 52.2, 57.8, 62.1, 62.3, 63.6, 173.0. Calcd [M+H]⁺ (C₃₇H₇₅N₇O₉) m/z = 762.0 Da. Found: ES-MS: [M+H]⁺ m/z = 762.6 and [M+Na]+ m/z = 784.6. Anal. Calcd for C₃₇H₇₅N₇O₉: C 58.27%; H 9.84%; N 12.86%. Found C 58.32%; H 9.92%; N 12.87%.

AmG₂–Br – AmG₂–OH (5.14 g, 6.749 mmol, 1 eq.), TEA (0.96 g, 9.45 mmol, 1.4 eq.) and DMAP (82.5 mg, 0.675 mmol, 0.1 eq.) were added to a 250 mL 2 necked RBF containing DCM (160 mL). The flask was deoxygenated under a positive N₂ purge for 10 minutes. BiB (1.63 g, 0.88 mL, 7.09 mmol, 1.05 eq.) was added drop wise while the solution was stirring in an ice bath under a positive flow of N₂. The reaction mixture was allowed to warm to room temperature and left stirring overnight. The organic phase was washed with saturated NaHCO₃ solution (3 x 160 mL). The solution was dried with anhydrous Na₂SO₄ and the product left to dry in vacuo overnight. Yield: 3.30 g, yellow oil (54%). ¹H NMR (400 MHz, CDCl₃) δ = 1.26 (d, 3H), 1.56 (m, 4H), 1.91 (s, 6H), 2.22-2.67 (m, 50H), 2.76 (m, 8H), 4.19 (m, 8H), 5.04 (m, 1H). ¹³C NMR (100 MHz, CDCl₃) δ = 17.9, 24.9, 30.7, 32.6, 45.8, 48.9, 51.7, 52.7, 56.2, 57.7, 58.6, 62.0, 71.1, 171.2, 172.7. Calcd [MH]⁺ (C₄₁H₈₀N₇O₁₀Br) m/z = 911.0 Da. Found: ES-MS: [M+H]⁺ m/z = 912.5, [M+Na]+ m/z = 934.5 m/z and [M+K]+ m/z = 950.5. Anal. Calcd for C₄₁H₈₀N₇O₁₀Br: C 54.01%; H 8.78%; N 10.76%. Found C 54.05%; H 8.85%; N 10.76%.
Typical linear polymerisation of HPMA (EBiB-, AmG_0-, AmG_1- and AmG_2-\_p(HPMA_{50})) – In a typical synthesis, targeting a DP\_n = 50 monomer units \((p(\text{HPMA})_{50})\), bpy (173.3 mg, 1.1096 mmol, 2 eq.), HPMA (4.0 g, 27.7 mmol, 50 eq.) and MeOH (35% v/v based on HPMA) were placed into a 50 mL RBF. The solution was stirred and deoxygenated using a N\_2 purge for 15 minutes. Cu(I)Cl (54.9 mg, 0.5548 mmol, 1 eq.) was added to the flask and left to purge for a further 5 minutes. AmG_2-Br (0.505 g, 0.555 mmol, 1 eq.) was added to the flask under a positive flow of N\_2, and the solution was left to polymerise at 30°C. Reactions were terminated when >99 % conversion was reached, as judged by \(^1\)H NMR, by exposure to oxygen and addition of THF. The catalyst residues were removed by passing the mixture over a neutral alumina column. THF was removed under vacuum to concentrate the sample before precipitation into hexane and drying in the vacuum oven overnight.

Typical synthesis of a hyperbranched polydendron using the co-polymerisation of HPMA and EGDMA, (EBiB-, AmG_0-, AmG_1- and AmG_2-\_p(\text{HPMA}_{50-\text{co-EGDMA}_x})) – In a typical synthesis, targeting a DP\_n = 50 monomer units within the primary chain, bpy (173.3 mg, 1.11 mmol, 2 eq.), HPMA (4 g, 27.7 mmol, 50 eq.), EGDMA (99.0 mg, 0.4993 mmol, 0.9 eq.) and MeOH (35% v/v based on HPMA) were placed into a 50 mL RBF. The solution was stirred and deoxygenated using a N\_2 purge for 15 minutes. Cu(I)Cl (54.9 mg, 0.5548 mmol, 1 eq.) was added to the flask and left to purge for a further 5 minutes. AmG_2-Br (0.505 g, 0.555 mmol, 1 eq.) was added to the flask under a positive flow of N\_2, and the solution was left to polymerise at 30°C. Reactions were terminated when >99 % conversion was reached, as judged by \(^1\)H NMR, by exposure to oxygen and addition of THF. The catalyst residues were removed by passing the mixture over a neutral alumina column. THF was removed under vacuum to concentrate the sample before precipitation into hexane.

Typical procedure for aqueous nanoprecipitation of \(p(\text{HPMA}_{50})\) and \(p(\text{HPMA}_{50-\text{co-EGDMA}_x})\)] - The materials were dissolved in THF at a concentration of 5 mg mL\(^{-1}\). 2 mL of this solution was then subjected to a rapid solvent switch through drop wise addition into 10 mL of water, to give a final polymer NP concentration of 1 mg mL\(^{-1}\) in water after THF removal by evaporation overnight.

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Chapter 3

Linear polymer, linear-dendritic polymer hybrid, branched copolymer
and hyp-polydendron synthesis
3.1 Introduction

Chapter 2 demonstrated the production of amine-functionalised linear-dendritic polymer hybrids comprised of 2-hydroxypropyl methacrylate (HPMA) and corresponding hyp-polydendrons generated by the co-polymerisation of HPMA and ethylene glycol dimethacrylate (EGDMA). Recently reported hyp-polydendrons are composed of benzyl dendritic surface functionality, also containing a branched HPMA polymer core. As well as studying the effects of varying dendron surface functionality, the research presented here aims to investigate other variables within the hyp-polydendron structure (Figure 3.1).

Figure 3.1 Targeted architectural and chemical variation of hyp-polydendrons; i) dendron generation; ii) primary polymer chain chemistry; iii) dendron chemistry; iv) primary polymer chain architecture; v) primary polymer chain length; vi) brancher chemistry; vii) mixed surface functionality.

The number of options available for hyp-polydendron manipulation is considerable, offering the opportunity to control both structural and chemical behaviour. These include: variation of the chemical composition of the primary polymer chain (Figure 3.1ii), utilising different monomers within the polymerisation to produce polymers with different chemical properties; variation of dendron generation has already highlighted the ability to produce linear-dendritic polymer hybrids and hyp-polydendrons with G0-
G₂ dendron chain ends (Figure 3.1i; Chapter 2) and variation in physical behaviour; the chemistry of the dendron can be varied (Figure 3.1iii), resulting in different surface chemistry; and alternatively the linker chemistry within the molecule can be tailored to potentially generate functionality within the internal structure of the dendron. In addition, the architecture of the primary chains can be adjusted to produce statistical and block copolymers, composed of different monomers (Figure 3.1iv), and the degree of polymerisation and monomer composition can be varied. Variation in homopolymer primary chain length leads to control over the dendron:linear chain ratio, potentially analogous to hydrophilic-lipophilic balance within polymeric surfactants (Figure 3.1v) and the one-pot polymerisation allows for initiation by multiple initiators (dendron and non-dendron) to result in mixed surface groups on the hyp-polydentron (Figure 3.1vii).

This chapter aims to outline the synthesis and characterisation of a range of materials with many of the structural and chemical variations outlined above. The properties of materials will be discussed in later chapters in a series of comparative studies.

3.2 Variation of primary chain chemistry within linear polymers, linear-dendritic polymer hybrids, branched copolymers and hyp-polydentrons

Previously reported hyp-polydentrons have focussed on HPMA homopolymer primary chains (Figure 3.2i); however, atom transfer radical polymerisation (ATRP) has been utilised to polymerise a very broad range of other methacrylate monomers.² New monomers that have been taken into consideration within this work include the highly hydrophobic monomer, tertiary butyl methacrylate (tBuMA) (Figure 3.2ii) and the pH-responsive monomer, 2-(diethylamino) ethyl methacrylate (DEA) (Figure 3.2iii).

![Figure 3.2](image)

**Figure 3.2** Monomers considered for utilisation in ATRP to produce hyp-polydentrons; i) HPMA; ii) tertiary butyl methacrylate (tBuMA); iii) 2-(diethylamino) ethyl methacrylate (DEA).
CHAPTER 3

The ATRP conditions for \( \text{tBuMA} \) have been thoroughly investigated\(^3,^4\) and this monomer provides a more hydrophobic polymer to allow comparisons with HPMA. DEA was selected for this study as the presence of tertiary amines allows for the production of pH-responsive hyp-polydendrons. The use of pH-responsive materials has been widely reported in the literature for triggered release drug delivery applications in a range of different physiological conditions.\(^5\)

All ATRP polymerisations and co-polymerisations conducted in this study target a number average degree of polymerisation (\( \text{DP}_n \)) = 50 monomer units per primary polymer chain unless otherwise stated. The solvent and temperature conditions of each polymerisation have been tailored to each particular monomer following various literature procedures.\(^6\) The catalyst system for all polymerisations was copper chloride/2,2'-bipyridyl (\( \text{Cu(I)Cl/bpy} \)) using the following molar ratio [initiator]:[M]:[CuCl]:[bpy] = 1:50:1:2. In all homopolymerisation instances, the commercially available initiator, ethyl \( \alpha \)-bromoisobutyrate (\( \text{EBiB} \)), has been used as a polymerisation initiator in order to compare initiation efficiency. Additionally, comparative studies between dendritic/non-dendritic surface functionality on future nanoparticle (NP) behaviour is to be investigated (Chapter 4).

During the ATRP reactions, the monomer conversions were monitored to ensure high levels were reached in all cases; this is important to enable targeting of \( \text{DP}_n \) and for the attainment of high molecular weight branched polymers.\(^7\) Monomer conversions were determined by \(^1\)H nuclear magnetic resonance spectroscopy (NMR) of the crude reaction media. The only expected chemical difference between linear and branched polymer NMR spectra was the presence of EGDMA, but these peaks overlap with the main peaks associated with the polymer and were not observable. For most polymers, the \( \text{DP}_n \) values could not be determined by \(^1\)H NMR due to the overlapping signals of the initiators’ chain ends with the polymer repeat units.

Kinetic studies were conducted for each homopolymerisation to confirm first order kinetics with respect to monomer concentration. When new ATRP initiators were utilised, it was important to follow the evolution of molecular weight with respect to monomer conversion and to ensure no interference in the ATRP mechanism. Linear semi-logarithmic plots were used to confirm first order kinetics and the monitoring of a linear development of number average molecular weight (\( \text{M}_n \)) with respect to monomer
conversion was generally observed, leading to monomodal molecular weight distributions for all linear and linear-dendritic polymers conducted in the study.

Weight average molecular weight ($M_w$) was also closely monitored within the branched polymerisations and showed the well reported \(^8\) dramatic increase at high conversions, leading to multimodal gel permeation chromatography (GPC) chromatograms as expected. Molecular weight analyses were enabled by determining average dn/dc values for each linear/linear-dendritic/branched copolymer/hyp-polydendron. Estimations to establish the weight average number of conjoined primary linear-dendritic polymer chains within each hyp-polydendron assumed that the linear-dendritic polymer synthesised under identical conditions fully represented the primary chains within the branched polymerisation. The simple division of $M_w$ (hyp-polydendron) by $M_w$ (linear-dendritic polymer equivalent), therefore gives the weight average number of conjoined primary chains. Following this, the weight average number of surface functional groups can be estimated from the number of chain ends and the number of functional groups per dendron.

All polymer molecular weights and dispersities ($D = M_w/M_n$) were determined by triple detection GPC; for all linear polymers, $M_n$ values were greater than the theoretical values either due to initiator inefficiency and/or disproportionation leading to a small concentration of macromonomer.\(^9\) Despite the higher than targeted $M_n$ values obtained, the linear polymers and linear-dendritic polymer hybrids appeared to deviate from theoretical values in a generally consistent manner, allowing an accurate assessment of structure/chemical impact on material behaviour without specific consideration of highly variable molecular weights. All polymers are referred to using their target DP\(_n\) throughout the discussion, irrespective of the actual DP\(_n\) achieved.

Branched copolymers and hyp-polydendrons were successfully synthesised following incorporation of a low concentration of EGDMA into the polymerisation reaction, as reported in Chapter 2. When using EBiB as the initiator, a molar ratio of brancher: initiator of 0.95:1 was employed, unless otherwise stated. When using any dendron ATRP initiator ($\text{AmG}_0\text{-Br}$, $\text{AmG}_1\text{-Br}$, $\text{AmG}_2\text{-Br}$, $\text{AmG}_1^U\text{-Br}$ or $\text{BnG}_2\text{-Br}$), gel formation was seen within the co-polymerisations at this ratio but soluble hyp-polydendrons were achieved at lower brancher: initiator ratios (0.9:1). This further suggests a lower initiator efficiency, as poor initiation will lead to fewer propagating polymer chains and a higher effective brancher:chain ratio within each reaction. The
0.9:1 brancher: initiator ratio was selected for all hyp-polydendrons unless otherwise stated.

3.2.1 Linear polymerisation of DEA and co-polymerisation of DEA and EGDMA using EBiB and amine-functional dendron ATRP initiators

The controlled polymerisation conditions associated with tertiary amine methacrylates have been extensively studied and reported. The interesting behaviour of linear-dendritic HPMA polymer hybrids and hyp-polydendrons in alternating pH (Chapter 2) was investigated due to the amphiphilic behaviour of primary chains due to protonation of the amine-functionalised dendron chain ends at low pH. This, consequently, enabled the production of pH-responsive linear-dendritic and hyp-polydendron polymers.

As a weak polybase with a pKₐ of ~7.3, p(DEA) is a cationic polyelectrolyte in aqueous solution. It has been shown to effectively bind with negatively charged deoxyribonucleic acid (DNA), which has been recognised as an ideal characteristic in the design of gene delivery vehicles. Polymerisation techniques for DEA include anionic polymerisation, first reported by Nagasaki et al., group-transfer polymerisation by Armes and co-workers, and the first homopolymerisation via ATRP by Tam and co-workers.

3.2.1.1 Linear polymerisation of DEA using EBiB and amine-functional dendron ATRP initiators

Initially, linear polymers of DEA were synthesised at 40°C (56 wt% v/v) in isopropanol (IPA), initiated by EBiB (Scheme 3.1). Tertiary amine methacrylates have been reported as undergoing transesterification in methanol, rendering it an unsuitable solvent.
Scheme 3.1 Schematic representation of the linear polymerisation of DEA (2) using EBiB (1) producing the linear polymer EBiB-p(DEA₅₀).

The three dendron initiators, AmG₀-Br, AmG₁-Br and AmG₂-Br synthesised in Chapter 2, were also used to polymerise DEA in identical conditions (Figure 3.3).

Figure 3.3 Diagrammatic representation of the polymerisation of DEA using AmG₀-Br, AmG₁-Br and AmG₂-Br.

All linear and linear-dendritic polymers were purified by passing through a basic alumina column to remove the catalytic system, followed by precipitation into cold petroleum ether (40-60) before analysis by GPC. Analysis of the EBiB-p(DEA₅₀) shows higher $M_n$ values (almost double) than targeted (Table 3.1), as previously discussed.
Table 3.1 GPC analysis of EBiB and amine-functionalised DEA linear and linear-dendritic polymers and DEA-EGDMA branched copolymer and hyp-polydendrons.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Conversion (%)</th>
<th>$M_n$ Theory (g mol$^{-1}$)</th>
<th>$M_n$ (g mol$^{-1}$)</th>
<th>$M_w$ (g mol$^{-1}$)</th>
<th>$D$</th>
<th>$D_P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBiB-$p$(DEA$_{50}$)</td>
<td>98</td>
<td>9380</td>
<td>18050</td>
<td>20900</td>
<td>1.16</td>
<td>97</td>
</tr>
<tr>
<td>AmG$<em>0$-$p$(DEA$</em>{50}$)</td>
<td>99</td>
<td>9440</td>
<td>16350</td>
<td>19650</td>
<td>1.20</td>
<td>88</td>
</tr>
<tr>
<td>AmG$<em>1$-$p$(DEA$</em>{50}$)</td>
<td>97</td>
<td>9690</td>
<td>17450</td>
<td>20900</td>
<td>1.20</td>
<td>94</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(DEA$</em>{50}$)</td>
<td>97</td>
<td>10090</td>
<td>23650</td>
<td>34400</td>
<td>1.46</td>
<td>128</td>
</tr>
<tr>
<td>EBiB-$p$(DEA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>98</td>
<td>-</td>
<td>689200</td>
<td>1403000</td>
<td>2.04</td>
<td>-</td>
</tr>
<tr>
<td>AmG$<em>0$-$p$(DEA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>92</td>
<td>-</td>
<td>39500</td>
<td>173500</td>
<td>4.40</td>
<td>-</td>
</tr>
<tr>
<td>AmG$<em>1$-$p$(DEA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>99</td>
<td>-</td>
<td>21700</td>
<td>288000</td>
<td>13.28</td>
<td>-</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(DEA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>99</td>
<td>-</td>
<td>125700</td>
<td>341800</td>
<td>2.72</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* Triple detection analysis using THF/2% TEA as eluent; *b* Determined by NMR; *c* $M_n$ Theory = ($M_n$ monomer*DP targeted)+$M_w$ initiator

Despite this, the polymerisation was controlled, as confirmed by the low $D$ value and reached high monomer conversion (98%). All linear-dendritic DEA materials had similar $M_n$ values, with the AmG$_0$-$p$(DEA$_{50}$) having the lowest $M_n$ (16350 g mol$^{-1}$) and the AmG$_2$-$p$(DEA$_{50}$) having the highest (23650 g mol$^{-1}$). The AmG$_2$-$p$(DEA$_{50}$) appears to show a reduction in initiator efficiency and less control over the molecular weight distribution (Table 3.1). The linear-dendritic hybrids, AmG$_{0}$$p$(DEA$_{50}$), AmG$_{1}$$p$(DEA$_{50}$) and AmG$_{2}$$p$(DEA$_{50}$) also achieved high monomer conversion (>97%) and an example of a typical linear-dendritic DEA polymer hybrid NMR spectrum assignment is shown in Figure 3.4, and the others displayed in Appendix, Figures A26-28. The GPC refractive index (RI) and right angle light scattering (RALS) chromatograms for the linear and linear-dendritic polymers, EBiB-$p$(DEA$_{50}$), AmG$_{0}$$p$(DEA$_{50}$), AmG$_{1}$$p$(DEA$_{50}$) and AmG$_{2}$$p$(DEA$_{50}$), displayed mono-modal molecular weight distributions (Figure 3.5).
CHAPTER 3

Figure 3.4 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$-$p$(DEA$_{50}$).

Figure 3.5 GPC chromatogram overlays of A) RI chromatograms and B) RALS chromatograms for EBiB-$p$(DEA$_{50}$) (black), AmG$_0$-$p$(DEA$_{50}$) (green), AmG$_1$-$p$(DEA$_{50}$) (blue) and AmG$_2$-$p$(DEA$_{50}$) (red); C) RI chromatograms and D) RALS chromatograms for EBiB-$p$(DEA$_{50}$co-EGDMA$_{0.95}$) (black), AmG$_0$-$p$(DEA$_{50}$co-EGDMA$_{0.9}$) (green), AmG$_1$-$p$(DEA$_{50}$co-EGDMA$_{0.9}$) (blue) and AmG$_2$-$p$(DEA$_{50}$co-EGDMA$_{0.9}$) (red).
Separate kinetic studies revealed that the polymerisations initiated by AmG0-Br, AmG1-Br and AmG2-Br appeared to propagate at relatively similar rates (Figure 3.6A), with the fastest (the AmG0-Br) achieving approximately 80% conversion in 3 hours. Molecular weight analyses were enabled by determining average dn/dc values from the number of time points taken (Appendix, Table A1). The D values, for each polymerisation, decreased as the reaction progressed, although end values were as high as D = 1.46 (AmG2-p(DEA50)).

Figure 3.6 Kinetic plots for A and B) Linear-dendritic AmG0-p(DEA50), AmG1-p(DEA50) and AmG2-p(DEA50) and C) and D) AmG0-p(DEA50-co-EGDMA50). A) Conversion AmG0-p(DEA50) (green squares), ln([M]0/[M]) (green circles); AmG1-p(DEA50) (blue squares), ln([M]0/[M]) (blue circles) and AmG2-p(DEA50) (red squares), ln([M]0/[M]) (red circles). B) Mₙ AmG0-p(DEA50) (green circles); AmG1-p(DEA50) (blue circles) and AmG2-p(DEA50) (red circles); D AmG0-p(DEA50) (green lines); AmG1-p(DEA50) (blue lines) and AmG2-p(DEA50) (red lines). C) Conversion (blue squares), ln([M]0/[M]) (red circles); D) Mₙ (red circles), M_w (black diamonds) and D (blue lines).

3.2.1.2 Co-polymerisation of DEA and EGDMA using EBiB and amine-functionalised dendron ATRP initiators

Following the successful preparation of a linear DEA polymer and linear-dendritic DEA polymer hybrids containing amine functional dendron chain ends, EGDMA was
introduced into the polymerisation initiated by EBiB, to produce the branched copolymer EBiB-\textit{p}(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.95}) (Scheme 3.2).

The branched co-polymerisation of DEA with EGDMA was conducted under identical conditions to those used for linear polymer synthesis, albeit at a lower monomer concentration (38.9 wt% v/v as opposed to 56.0 wt% v/v for the linear equivalent) to avoid gelation.\textsuperscript{19,20} This resulted in a soluble, high-molecular weight polymer (M\textsubscript{n} = 689,200 g mol\textsuperscript{-1}) being recovered (Table 3.1) at high monomer conversion (98%).

Following the successful preparation of a high-molecular weight DEA branched copolymer, the amine-functionalised dendron ATRP initiators were used to co-polymerise DEA and EGDMA, to produce \textit{hyp}-polydendrons (Figure 3.7).
Figure 3.7 Diagrammatic representation of the co-polymerisation of DEA and EGDMA using AmG₀-Br, AmG₁-Br and AmG₂-Br.

The GPC RI and RALS chromatograms for the branched polymer and hyp-polydendrons (Figure 3.5C and D) confirm high levels of branching, due to broad, bimodal distributions in comparison to the narrow, mono-modal traces observed for the linear equivalent polymerisations.

The branched co-polymerisation initiated by EBiB required a longer polymerisation time (Appendix, Figure A29A and B) in contrast to the respective linear polymer reaction conducted in the absence of EGDMA (Appendix, Figure A30). The amine-functional dendron ATRP initiators required longer co-polymerisation times (Appendix, Figure A29C, D, E and F) in comparison to their respective linear-dendritic polymer reactions conducted in the absence of EGDMA (Figure 3.6); the AmG₀-p(DEA₅₀-co-EGDMA₀.₉) took > 42 hours (compared to 6 hours) to reach 80% monomer conversion (Figure 3.6C and D).

The EBiB-p(DEA₅₀-co-EGDMA₀.₉₅) reached an Mₚ of 1.4x10⁶ g mol⁻¹ suggesting a significant contribution to the physical mass of the sample from complex branched
structures containing on weight average > 67 primary polymer chains. The $M_w$ values for the amine-functional hyp-polydendrons steadily increase as the dendron generation increases (Table 3.1), although the $\text{AmG}_1$-$p$(DEA$_{50}$-co-EGDMA$_{0.9}$) suggests having more (on average) linear chains joined together (> 14 primary polymer chains) than the $\text{AmG}_0$- or $\text{AmG}_2$-$p$(DEA$_{50}$-co-EGDMA$_{0.9}$) (> 9 and 10 primary polymer chains respectively) (Table 3.1). All hyp-polydendrons show similar levels of primary chain contribution per branched polymer. Therefore, as each primary chain of the hyp-polydendrons contains a dendron initiator end group, the weight average branched architectures contain approximately 14 tertiary amine ($\text{AmG}_0$-$p$(DEA$_{50}$-co-EGDMA$_{0.9}$)), 28 tertiary amine ($\text{AmG}_1$-$p$(DEA$_{50}$-co-EGDMA$_{0.9}$)) and 40 tertiary amine ($\text{AmG}_2$-$p$(DEA$_{50}$-co-EGDMA$_{0.9}$)) functional chain end groups. An example of a typical $p$(DEA$_{50}$-co-EGDMA$_x$) $^1$H NMR spectrum is shown (Figure 3.8) with major peaks assigned, with the others displayed in Appendix, Figures A31-33.

Figure 3.8 $^1$H NMR spectrum (MeOD, 400 MHz) of $\text{AmG}_2$-$p$(DEA$_{50}$-co-EGDMA$_{0.9}$)
3.2.2 Linear polymerisation of tBuMA and co-polymerisation of tBuMA and EGDMA using EBiB and AmG2-Br ATRP initiators

Both ATRP initiators, **EBiB** (Scheme 3.3 1) and **AmG2-Br** (Scheme 3.3 3) were used to polymerise tBuMA (Scheme 3.3 2) and copolymerise tBuMA and EGDMA (Scheme 3.4 3) in IPA/H2O at 20°C. Rannard and co-workers first reported the ambient temperature waterborne ATRP of a hydrophobic monomer (nBuMA) in homogenous alcoholic media in 2001. A ratio of 92.5:7.5% IPA:H2O proved to be ideal solvent conditions, allowing short reaction times without compromising the control of the polymerisation. All materials were purified by passing through a basic alumina column to remove the catalytic system, followed by precipitation into cold hexane before analysis by GPC (Table 3.2).

Scheme 3.3 Schematic representation of the linear polymerisation of tBuMA (2) using EBiB (1) and AmG2-Br (3) as ATRP initiators producing the linear polymer EBiB-p(tBuMA50) and linear-dendritic polymer hybrid AmG2-p(tBuMA50).
Scheme 3.4 Schematic representation of the branched co-polymerisation of tBuMA (2) and EGDMA (3) using EBiB (1) and AmG2-Br (4) as ATRP initiators producing the branched copolymer EBiB-p(tBuMA$_{50}$-co-EGDMA$_{0.95}$) and hyp-polydendron AmG2-p(tBuMA$_{50}$-co-EGDMA$_{0.9}$).

The linear polymer, EBiB-p(tBuMA$_{50}$), and linear-dendritic polymer hybrid, AmG2-p(tBuMA$_{50}$) both reached high monomer conversion (>94 %); however, the AmG2-p(tBuMA$_{50}$) exhibited a more controlled polymerisation, resulting in a lower dispersity value (Đ = 1.11). The linear-dendritic polymer hybrid was also recovered with an $M_n = 6900$ g mol$^{-1}$ and $D_P$ close to the targeted theoretical value of 7940 g mol$^{-1}$. The branched copolymer (EBiB-p(tBuMA$_{50}$-co-EGDMA$_{0.95}$)) and hyp-polydendron (AmG2-p(tBuMA$_{50}$-co-EGDMA$_{0.9}$)) were recovered at high monomer conversion (>96 %) and analysis was conducted by GPC as previously stated (Table 3.2). The hyp-polydendron was recovered with a higher $M_n$ and $M_w$ value (273300 g mol$^{-1}$) than the EBiB-initiated branched copolymer ($M_w = 150400$ g mol$^{-1}$), which could be due to the former reaching 99% conversion. The AmG2-p(tBuMA$_{50}$-co-EGDMA$_{0.9}$) contained on average > 36 primary chains in comparison to > 5 primary chains for the EBiB-p(tBuMA$_{50}$-co-EGDMA$_{0.95}$) due to the hyp-polydendron consisting of a higher molecular weight. Each primary chain of the hyp-polydendron contains an AmG2 dendron chain end, therefore the weight average branched architecture contains approximately 144 tertiary amine chain end functional groups. The $^1$H NMR spectra of AmG2-p(tBuMA$_{50}$) and AmG2-p(tBuMA$_{50}$-co-EGDMA$_{0.9}$) with major peaks assigned is presented in Figures 3.9 and 3.10 respectively, with the EBiB-p(tBuMA$_{50}$) and EBiB-p(tBuMA$_{50}$-co-EGDMA$_{0.95}$) shown in Appendix, Figures A34 and A35.
### Table 3.2 GPC analysis of EBiB and amine-functionalised tBuMA linear polymer, linear-dendritic polymer hybrid, copolymer and hyp-polydendron.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Conversion (%)</th>
<th>$M_n$ Theory (g mol$^{-1}$)</th>
<th>$M_n$ (g mol$^{-1}$)</th>
<th>$M_w$ (g mol$^{-1}$)</th>
<th>$D$</th>
<th>$D_P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBiB-$p$(tBuMA$_{50}$)</td>
<td>95</td>
<td>7230</td>
<td>19750</td>
<td>27450</td>
<td>1.38</td>
<td>138</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(tBuMA$</em>{50}$)</td>
<td>94</td>
<td>7940</td>
<td>6900</td>
<td>7650</td>
<td>1.11</td>
<td>48</td>
</tr>
<tr>
<td>EBiB-$p$(tBuMA$<em>{50}$-$co$-EGDMA$</em>{0.95}$)</td>
<td>96</td>
<td>-</td>
<td>63800</td>
<td>150400</td>
<td>2.36</td>
<td>-</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(tBuMA$</em>{50}$-$co$-EGDMA$_{0.95}$)</td>
<td>99</td>
<td>-</td>
<td>88200</td>
<td>273300</td>
<td>3.10</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ Triple detection analysis using THF/2% TEA as eluent; $^b$ Determined by NMR; $^c$ $M_n$ Theory = (M$_n$ monomer$^a$DP targeted) + M$_n$ initiator

**Figure 3.9** $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$-$p$(tBuMA$_{50}$).
Figure 3.10 $^1$H NMR spectrum (MeOD, 400 MHz) of $\text{AmG}_2\text{p}(\text{tBuMA}_{50} \text{co-EGDMA}_{0.9})$.

The linear polymers $\text{EBiB}\text{-p}(\text{tBuMA}_{50})$ and $\text{AmG}_2\text{-p}(\text{tBuMA}_{50})$ exhibited mono-modal molecular weight distributions upon inspection of the GPC RI and RALS chromatograms (Figure 3.11). Figure 3.11 also shows the GPC RI and RALS chromatograms for the branched copolymer and $\text{hyp}$-polydendron, showing broader size distributions in comparison to their linear analogues.
Figure 3.11 GPC chromatogram overlays of A) RI chromatograms and B) RALS chromatograms for EBiB-\(p\)(BuMA\(_{50}\)) (black) and AmG\(_2\)-\(p\)(BuMA\(_{50}\)) (red); C) RI chromatograms and D) RALS chromatograms for EBiB-\(p\)(BuMA\(_{50}\)-co-EGDMA\(_{0.95}\)) (black) and AmG\(_2\)-\(p\)(BuMA\(_{50}\)-co-EGDMA\(_{0.9}\)).

The linear polymerisation initiated by EBiB appeared to propagate faster (Appendix, Figure A36A and B) than the reaction using AmG\(_2\)-Br, achieving approximately 60% conversion in 5 hours; in comparison, the AmG\(_2\)-Br polymerisation required ~16 hours to reach a similar conversion (Figure 3.12A). Dispersity values decreased gradually as the polymerisations proceeded and the polymers reached high conversion. Molecular weight analyses were enabled by determining average dn/dc values from the number of time points taken (Appendix, Table A2).
Figure 3.12 Kinetic plots for linear AmG2-p(tBuMA50) and branched AmG2-p(tBuMA50-co-EGDMA0.9). A and C) Conversion (blue squares), ln([M]/[M]) (red circles); B and D) M_n (red circles), D (blue lines) and M_w (black diamonds).

Kinetic studies suggest that the EBiB-initiated branched co-polymerisation has an almost identical polymerisation rate to the respective linear polymerisation conducted in the absence of EGDMA (Appendix, Figure A36C and D), reaching 95% conversion in 8 hours. The hyp-polydendron synthesis using AmG2-Br initiator propagated noticeably slower than the analogous linear-dendritic polymer reaction, taking 24 hours to reach 94% conversion (Figure 3.12C).

3.3 Variation of primary chain architecture to produce statistical and block linear-dendritic polymer hybrids and hyp-polydendrons

DEA has been co-polymerised with a wide variety of monomers via facile controlled polymerisation techniques in order to synthesise materials that easily produce pH-responsive NPs,22 with huge potential in the drug delivery field.23 The incorporation of DEA into highly branched polymers allows for subsequent quaternisation of the tertiary amine groups leading to water-soluble cationic polyelectrolytes.24 Monomers included in...
the co-polymerisation with DEA include tBuMA and methacrylic acid (MAA) to investigate the micellar behaviour under varying pH and the effects of NaCl addition.\textsuperscript{25} Triple-shell architectures, consisting of DEA, vinlycaprolactam and 2-(dimethylamino)ethyl methacrylate (DMA) triblock terpolymers have even been obtained by sequential reversible addition–fragmentation chain transfer polymerisations to produce a double responsive system.\textsuperscript{26} The application of ATRP to design and synthesise a variety of novel polymeric architectures, has also included reports of shell-cross linked particles comprising DEA and $\rho$(ethylene glycol) (PEG) that swell upon a change in pH.\textsuperscript{27}

These ideologies encourage modification of the primary chain architecture to produce statistical and block linear-dendritic copolymers and hyp-polydendrons, composed of different monomers. The amounts of each monomer composition and block length can, also, be modified.

### 3.3.1 Statistical co-polymerisations using AmG\textsubscript{2}-Br ATRP initiator to produce linear-dendritic copolymer hybrids and hyp-polydendrons

Following the successful homopolymerisations of HPMA (Chapter 2), DEA and tBuMA to produce a variety of linear-dendritic polymer hybrids and hyp-polydendrons, the incorporation of different monomer units to produce statistical linear-dendritic polymer hybrids and hyp-polydendrons was investigated (Figure 3.13).

![Figure 3.13](image.png)

**Figure 3.13** Diagrammatic representation of the statistical linear-dendritic polymer hybrids and hyp-polydendrons synthesised incorporating HPMA (blue), DEA (green) and tBuMA (red).
DEA was incorporated into both the HPMA and tBuMA polymerisations in order to introduce a pH-response into both hyp-polydendron systems. Previous homopolymerisations with HPMA (Chapter 2) and DEA have utilised varying generations of dendrons as ATRP initiators, producing a variety of dendron generation as a chain end/surface group for the linear-dendritic hybrids/hyp-polydendrons. In this study, only the AmG2-Br ATRP initiator was selected for polymerisation initiation, in order to focus investigations on varying compositions and architectural differences of the polymers produced. The statistical and block linear-dendritic polymer hybrids and hyp-polydendrons all targeted a DPn = 50 monomer units for the primary chain, in ratios of 33:66, 50:50 and 66:33 mol%, DEA: HPMA/tBuMA unless otherwise stated.

3.3.1.1 Statistical co-polymerisation of DEA and HPMA using AmG2-Br ATRP initiator

AmG2-Br (Scheme 3.5 1) was used to co-polymerise DEA (Scheme 3.5 2) and HPMA (Scheme 3.5 3) at 40°C in IPA in a one-pot statistical polymerisation to produce three linear-dendritic polymers, AmG2-p(DEA17-co-HPMA33), AmG2-p(DEA25-co-HPMA25) and AmG2-p(DEA33-co-HPMA17).

The linear-dendritic hybrids were collected at high monomer conversion (>99 %) with the p(DEA_x-co-HPMA_y) 1H NMR spectra shown in Figure 3.14 and Appendix, Figures A37 and 38, with major peaks assigned. In all cases, the polymers were purified by
passing through a basic alumina column to remove the catalytic system and precipitating into cold petroleum ether (40-60). The linear-dendritic polymer hybrids all possessed similar $M_n$ values that were closer to targeted theoretical values than any of the previously synthesised linear-dendritic DEA and HPMA homopolymer hybrids. $M_w$ values were also similar and $D$ values were low (Table 3.3), confirmed further by the monomodal, narrow RI and RALS chromatograms (Figure 3.15).

Table 3.3 GPC analysis of AmG2-initiated amine-functional DEA-HPMA and DEA-tBuMA statistical linear-dendritic hybrids and hyp-polydendrons.

<table>
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<tr>
<th>Polymer</th>
<th>Conversion $^b$ (%)</th>
<th>$M_n$ Theory (g mol$^{-1}$)</th>
<th>$M_n$ (g mol$^{-1}$)</th>
<th>$M_w$ (g mol$^{-1}$)</th>
<th>$D$</th>
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<td>AmG2-p(DEA33-co-HPMA17)</td>
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<td>9400$^c$</td>
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<td>1971000</td>
<td>7.33</td>
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<td>4510000</td>
<td>66.15</td>
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<td>9360$^c$</td>
<td>9000</td>
<td>10350</td>
<td>1.15</td>
</tr>
<tr>
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<td>9020$^c$</td>
<td>9800</td>
<td>11350</td>
<td>1.16</td>
</tr>
<tr>
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<td>140900</td>
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<tr>
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<td>-</td>
<td>149200</td>
<td>531100</td>
<td>3.56</td>
</tr>
</tbody>
</table>

$^a$ Triple detection analysis using THF/2% TEA eluent; $^b$ Determined by 1H NMR; $^c$ $M_n$ Theory = ($M_n$ HPMA monomer*DP HPMA targeted)+($M_n$ DEA monomer*DP DEA targeted)+$M_n$ AmG2; $^d$ $M_n$ Theory = ($M_n$ tBuMA monomer*DP tBuMA targeted)+($M_n$ DEA monomer*DP DEA targeted)+$M_n$ AmG2
Figure 3.14 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$p(DEA$_{33}$-co-HPMA$_{17}$).
3.3.1.2 Statistical co-polymerisation of DEA, HPMA and EGDMA using AmG2-Br ATRP initiator

Following the successful preparation of linear-dendritic DEA-HPMA polymer hybrids containing amine dendron chain end functionality, EGDMA (Scheme 3.6 4) was introduced into the polymerisation initiated by AmG2-Br, to produce the hypopolydendrons $\text{AmG}_2\text{-}p(\text{DEA}_{33}\text{-co-HPMA}_{17}\text{-co-EGDMA}_{0.9})$, $\text{AmG}_2\text{-}p(\text{DEA}_{25}\text{-co-HPMA}_{25}\text{-co-EGDMA}_{0.9})$ and $\text{AmG}_2\text{-}p(\text{DEA}_{17}\text{-co-HPMA}_{33}\text{-co-EGDMA}_{0.9})$. The $\text{p}(\text{DEA}_x\text{-co-HPMA}_y\text{-co-EGDMA}_{0.9})^1$H NMR spectra is shown in Figure 3.16 and Appendix, Figures A39 and 40 with major peaks assigned.
Scheme 3.6 Schematic representation of the statistical branched co-polymerisations of DEA (2), HPMA (3) and EGDMA (4) using AmG2-Br (1) as the ATRP initiator producing the hyp-polydendrons AmG2-p(DEA33-co-HPMA17-co-EGDMA0.9), AmG2-p(DEA25-co-HPMA25-co-EGDMA0.9) and AmG2-p(DEA17-co-HPMA33-co-EGDMA0.9).

Figure 3.16 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG2-p(DEA25-co-HPMA25-co-EGDMA0.9).
The statistical hyp-polydendrons formed materials of noticeably high molecular weights in comparison to previously synthesised DEA and HPMA hyp-polydendrons, following high monomer conversions for all, with M_w values up to 4.51x10^6 g mol^-1 (Table 3.3). There is a noticeable increase in M_n and M_w (and Đ) as the HPMA content within the polymer is increased, with the AmG2-p(DEA17-co-HPMA33-co-EGDMA0.9) containing the most linear chains joined together, on weight average > 282 primary chains (1,128 tertiary amine surface groups) (> 27 primary chains (108 tertiary amine surface groups) (AmG2-p(DEA33-co-HPMA17-co-EGDMA0.9)) and > 119 primary chains (476 tertiary amine surface groups) (AmG2-p(DEA25-co-HPMA25-co-EGDMA0.9)). The hyp-polydendrons show a significant level of primary chain contribution per branched polymer, also displayed by the broad RI and RALS molecular weight distributions (Figure 3.15).

3.3.1.3 Statistical co-polymerisation of DEA and tBuMA using AmG2-Br ATRP initiator

AmG2-Br (Scheme 3.7 1) was additionally used to co-polymerise DEA (Scheme 3.7 2) and tBuMA (Scheme 3.7 3) at 40°C in IPA/H_2O_28 in a one-pot statistical polymerisation to produce three linear-dendritic polymers, AmG2-p(DEA17-co-tBuMA33), AmG2-p(DEA25-co-tBuMA25) and AmG2-p(DEA33-co-tBuMA17).

Scheme 3.7 Schematic representation of the statistical linear co-polymerisations of DEA (2) and tBuMA (3) using AmG2-Br (1) as the ATRP initiator producing the linear-dendritic polymer hybrids AmG2-p(DEA33-co-tBuMA17), AmG2-p(DEA25-co-tBuMA25) and AmG2-p(DEA17-co-tBuMA33).
The statistical linear-dendritic hybrids were collected at high monomer conversion (>99%), as determined by $^1$H NMR of the crude polymerisation media. Polymers were purified by passing through a basic alumina column and precipitating into cold petroleum ether (40-60). The $^1$H NMR spectra for the AmG$_2$p(DEA$_x$-co-tBuMA$_y$) statistical linear-dendritic hybrids are shown in Figure 3.17 and Appendix, Figures A41 and 42.

![Figure 3.17 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$p(DEA$_{17}$-co-tBuMA$_{33}$).](image)

The linear-dendritic polymer hybrid series were consistent in their achieved DP$_n$, as well as exhibiting high levels of control confirmed by the low dispersity values ($D=1.12-1.16$) (Table 3.3) and narrow monomodal weight distributions (Figure 3.18). Interestingly, the linear-dendritic polymer containing the highest content of tBuMA, AmG$_2$p(DEA$_{17}$-co-tBuMA$_{33}$) demonstrated the highest level of control. The AmG$_2$p(tBuMA$_{50}$) previously displayed greater levels of polymerisation control ($D=1.11$) in comparison to the AmG$_2$p(DEA$_{50}$) ($D=1.46$).
Figure 3.18 GPC chromatogram overlays of A) RI chromatograms and B) RALS chromatograms for $\text{AmG}_2$p(DEA$_{33}$-co-tBuMA$_{17}$) (green), $\text{AmG}_2$p(DEA$_{25}$-co-tBuMA$_{25}$) (orange) and $\text{AmG}_2$p(DEA$_{17}$-co-tBuMA$_{33}$) (red); C) RI chromatograms and D) RALS chromatograms for $\text{AmG}_2$p(DMA$_{33}$-co-tBuMA$_{17}$-co-EGDMA$_{0.9}$) (green), $\text{AmG}_2$p(DMA$_{25}$-co-tBuMA$_{25}$-co-EGDMA$_{0.9}$) (orange) and $\text{AmG}_2$p(DMA$_{17}$-co-tBuMA$_{33}$-co-EGDMA$_{0.9}$) (red).

3.3.1.4 Statistical co-polymerisation of DEA, tBuMA and EGDMA using AmG$_2$-Br ATRP initiator

EGDMA (Scheme 3.8 4) was subsequently introduced to the co-polymerisations in order to produce the tBuMA-containing statistical hyp-polydendrons, $\text{AmG}_2$p(DMA$_{33}$-co-tBuMA$_{17}$-co-EGDMA$_{0.9}$), $\text{AmG}_2$p(DMA$_{25}$-co-tBuMA$_{25}$-co-EGDMA$_{0.9}$) and $\text{AmG}_2$p(DMA$_{17}$-co-tBuMA$_{33}$-co-EGDMA$_{0.9}$) (Scheme 3.8).
Scheme 3.8 Schematic representation of the statistical branched co-polymerisations of DEA (2), tBuMA (3) and EGDMA (4) using AmG2-Br (1) as the ATRP initiator producing the hyp-polydendrons AmG2-p(DEA33-co-tBuMA17-co-EGDMA0.9), AmG2-p(DEA25-co-tBuMA25-co-EGDMA0.9) and AmG2-p(DEA17-co-tBuMA33-co-EGDMA0.9).

Highly branched materials with broad molecular weight distributions (Figure 3.18) were recovered with molecular weights ranging from 140900 g mol\(^{-1}\) (AmG2-p(DEA33-co-tBuMA17-co-EGDMA0.9)) to 531100 g mol\(^{-1}\) (AmG2-p(DEA33-co-tBuMA17-co-EGDMA0.9)) (Table 3.3). The branched structures contained on weight average >38 primary chains (AmG2-p(DEA33-co-tBuMA17-co-EGDMA0.9)), >12 primary chains (AmG2-p(DEA25-co-tBuMA25-co-EGDMA0.9)) and >48 primary chains (AmG2-p(DEA17-co-tBuMA33-co-EGDMA0.9)). Each primary chain of the hyp-polydendrons contains an AmG2 dendron initiator end group, therefore the weight average branched architectures contain approximately 152 tertiary amine (AmG2-p(DEA33-co-tBuMA17-co-EGDMA0.9)), 48 tertiary amine (AmG2-p(DEA25-co-tBuMA25-co-EGDMA0.9)) or 192 tertiary amine (AmG2-p(DEA17-co-tBuMA33-co-EGDMA0.9)) chain end functional groups. The \(^1\)H NMR spectra of p(DEA\(_x\)-co-tBuMA\(_y\)-co-EGDMA\(_0.9\)) statistical hyp-polydendrons are shown in Figure 3.19 and Appendix, Figures A43 and 44.
As well as a range of statistical linear-dendritic polymer hybrids and hyp-polydendrons, dendritic block copolymer hybrids and block hyp-polydendrons of varying branched tBuMA core were also synthesised.

3.3.2 Block co-polymerisations using AmG₂-Br ATRP initiator to produce linear-dendritic copolymer hybrids and hyp-polydendrons

Rannard and co-workers have previously documented the synthesis of complex polymer nanostructures using a one-pot ATRP approach, avoiding separate self-assembly and chemical-fixation steps.²⁹ Well-defined branched block terpolymer NPs were synthesised by the initial production of simple linear hydrophilic \( p(\text{o}l\text{igo(ethylene \text{glycol}) \text{methacrylate}_x}) \) (OEG\(_x\)), followed by subsequent addition of the hydrophobic \( n\text{BuMA} \) and EGDMA to the growing OEG chain. This allowed branching to occur and chemical bond formation between other growing \( p(n\text{BuMA}_x) \) blocks, hence building a structure composed of covalently linked copolymer chains (Scheme 3.9). This chemical synthesis strategy allowed design and manipulation of NP size by variation of the block lengths, whereby a systematic increase in particle size was observed. This simplistic one-pot method allows for the facile production of block terpolymers, where the block length can easily be altered.
This work utilised the same concepts as just described, albeit with the initial fabrication of linear-dendritic polymer hybrids from which to grow the branched hydrophobic polymer core from. Initially, diblock copolymers with dendron chain ends were produced in order to confer control. Succeeding this, EGDMA was introduced in the second feed of monomer.

Much attention has been invested into block copolymers containing MAA segments, where the properties of such weak polyelectrolytes govern the self-assembled structures by changes in pH and salt concentrations. The route to such MAA functionality is introduced by the incorporation of protected monomers with masked acid groups into the polymer chains. Essential prerequisites for the protected monomer are good ‘livingness’ under each polymerisation condition and selective deprotection under mild conditions.

The controlled polymerisation of the block copolymer \( p(\text{DEA}_x-b-\text{tBuMA}_y) \) has previously been reported, using both DEA-Cl and tBuMA-Cl as the macroinitiators. Armes and co-workers have already considered the opportunities in combining a protected monomer (methacrylic acid) with a pH-responsive monomer (DMA) and uncovered difficulties upon hydrolysis. The research described claimed that conditions required to remove the tertiary butyl groups were likely to cause intermolecular cross-
linking between the DMA blocks. Fortunately, in this study, the rBuMA polymer is merely integrated into the dendritic materials to provide a hydrophobic domain for drug encapsulation.

The design of these branched block hyp-polydendrons also provides a robust hydrophobic core for drug encapsulation, surrounded by dendritic pH-responsive chain ends to provide stability in aqueous media. The growth of linear DEA arms surrounding a branched hydrophobic core (Figure 3.20), presents a wholly different architecture platform in which nanoprecipitates collapse and self-assemble from.

**Figure 3.20** Diagrammatic representation of: i) Block linear-dendritic DEA-rBuMA polymer hybrid (AmG2-p(DEAα-b-rBuMAβ)) and ii) Block DEA-rBuMA-EGDMA hyp-polydendron (AmG2-p(DEAα-b-(rBuMAβ-co-EGDMAαβ))).

AmG2-Br (Scheme 3.10 1) was used to polymerise DEA (Scheme 3.10 2) at 40°C in IPA using the following molar ratio [Initiator]:[M]:[CuCl]:[bpy]=1:33/25/17:1:2. Upon reaching 73-92% monomer conversion, the second feed of rBuMA monomer (Scheme 3.10 3) of varying compositions (33, 50, 66 mol%) (DPα=17, 25, 33 respectively) was introduced, with Cu(I)Cl/bpy ([M]:[CuCl]:[bpy]=17/25/33:1:2) in IPA/H2O (92.75:7.5%) to produce the three linear-dendritic block polymer hybrids, AmG2-(DEA33-b-rBuMA17), AmG2-(DEA25-b-rBuMA25) and AmG2-(DEA17-b-rBuMA33). The addition of EGDMA (Scheme 3.10 4) into the second feed to rBuMA monomer feed to encourage branching to occur between the growing rBuMA polymer chains, resulted in the production of 3 block hyp-polydendrons, AmG2-(DEA33-b-(rBuMA17-co-EGDMAαβ)), AmG2-(DEA25-b-(rBuMA25-co-EGDMAαβ)) and AmG2-(DEA17-b-(rBuMA33-co-EGDMAαβ)).
Scheme 3.10 Schematic representation of the initial polymerisation of DEA (2) initiated by $\text{AmG}_2\text{-Br}$ (1), followed by a second monomer feed of $t\text{BuMA}$ (3) or $r\text{BuMA}$ and EGDMA (4) to produce block linear-dendritic polymer hybrids ($\text{AmG}_2$-(DEA$_{33}$-$b$-$t\text{BuMA}_{17}$), $\text{AmG}_2$-(DEA$_{25}$-$b$-$r\text{BuMA}_{25}$) and $\text{AmG}_2$-(DEA$_{17}$-$b$-$r\text{BuMA}_{33}$)) and hyp-polydendrons ($\text{AmG}_2$-(DEA$_{33}$-$b$-($t\text{BuMA}_{17}$-$co$-EGDMA$_{0.9}$)), $\text{AmG}_2$-(DEA$_{25}$-$b$-($r\text{BuMA}_{25}$-$co$-EGDMA$_{0.9}$)) and $\text{AmG}_2$-(DEA$_{17}$-$b$-($r\text{BuMA}_{33}$-$co$-EGDMA$_{0.9}$))).

Conversions of both the linear DEA chain and the final linear-dendritic polymer and hyp-polydendron were monitored via $^1$H NMR spectroscopy of the crude polymerisation media. Purification to remove the catalytic system was carried out by passage through a basic alumina column, followed by precipitation into cold petroleum ether (40-60). The $^1$H NMR spectra for the linear-dendritic block DEA-$t\text{BuMA}$ polymers and block DEA-$r\text{BuMA}$-EGDMA hyp-polydendron are shown in Figure 3.21, and Appendix A45-48.
The linear-dendritic block polymer hybrids displayed considerably higher $M_n$ values compared to the theoretical values predicted (Table 3.4). However, a high level of control was still present due to equally high $M_w$ values, resulting in significantly low $Đ$ values (1.16-1.21). The RI and RALS chromatograms also displayed monomodal
CHAPTER 3

distributions (Figure 3.22). Block hyp-polydendrons were collected at high monomer conversions and GPC confirms the presence of highly branched materials (Table 3.4 and Figure 3.22). The AmG$_2$-p(DEA$_{25}$-b-(tBuMA$_{25}$-co-EGDMA$_{0.9}$)) possessed the highest molecular weight (528900 g mol$^{-1}$), which was > twice the molecular weight of the other block hyp-polydendrons.

Table 3.4 GPC analysis of AmG$_2$-initiated amine-functional block DEA-tBuMA linear-dendritic polymer hybrids and block DEA-tBuMA-EGDMA hyp-polydendrons.

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<th>tBuMA (DP$_n$)</th>
<th>Conversion (%)$^b$</th>
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</table>

---

$^a$ Triple detection analysis using THF/2% TEA as eluent, $^b$ Determined by NMR, $^c$ $M_w$ Theory = ($M_w$ tBuMA monomer*DP tBuMA targeted) + ($M_w$ DEA monomer*DP DEA targeted) + $M_w$ AmG$_2$ dendron chain end
The complex branched structures contained on weight average > 5 primary chains (20 tertiary amine surface groups) \( (\text{AmG}_{2-p}(\text{DEA}_{33-b-\text{BuMA}_{17}})) \), > 11 primary chains (44 tertiary amine surface groups) \( (\text{AmG}_{2-p}(\text{DEA}_{25-b-\text{BuMA}_{25}})) \), > 6 primary chains (24 tertiary amine surface groups) \( (\text{AmG}_{2-p}(\text{DEA}_{17-b-\text{BuMA}_{33}})) \).

### 3.4 Variation of primary polymer chain length

Slater et al. have previously investigated architecture-driven aqueous stability of hydrophobic linear and branched polymers.\(^3\) Linear vs. branched dendritic polymeric HPMA NPs have similarly showed behavioural differences in aqueous media (Chapter 2). The report continues to study differences of varying polymer chain lengths within...
branched polymers, ultimately presenting a clear trend of decreasing solubility with increasing chain length. The hydrophobic branched polymers form NPs between 60-800 nm with the lowest DP_n primary chains forming the largest NPs, driven by the polymer chain length. Such subtle variations in the branched structure led to eventual macroscale precipitation in some cases, confirming that the origins of long term stability are architecture-dependent. In lieu of these reported observations, the primary polymer chain length within the block DEA-tBuMA hyp-polydendron was increased.

Following the successful synthesis of the hyp-polydendrons targeting a DP_n=50 monomer units per primary polymer chain, a branched block hyp-polydendron with increased lengths of DEA and tBuMA was designed AmG_2-p(DEA_{50-b}-(tBuMA_{65-co-EGDMA_{0.9}})) (Figure 3.23). The preparation of AmG_2-p(DEA_{50-b}-(tBuMA_{65-co-EGDMA_{0.9}})) would allow for behavioural comparisons to be made to the DP_n=50 analogues, containing similar polymer compositions but with different block lengths.

AmG_2-Br was used to initiate DEA (DP_n=50) using the following molar ratio [Initiator]:[M]:[CuCl]:[bpy] = 1:50:1:2. Following this, a second feed of tBuMA (DP_n=65) and EGDMA monomer was introduced with the molar ratio Cu(I)Cl/bpy ([M]:[CuCl]:[bpy] = 65:1:2. Identical temperature, solvents and purification techniques for the DP_n=50 hyp-polydendrons were applied. Analysis by ^1^H NMR (Appendix, Figure A49) and GPC confirmed the production of the hyp-polydendron, AmG_2-p(DEA_{50-b}-(tBuMA_{65-co-EGDMA_{0.9}})) (Table 3.4 and Figure 3.24). Despite the longer polymer blocks, the AmG_2-p(DEA_{50-b}-(tBuMA_{65-co-EGDMA_{0.9}})) was recovered with a lower
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$M_w$ and $M_n$ values compared to the block DEA-$t$BuMA-EGDMA hyp-polydendrons containing a DP$_n$=50 monomer units.

![Figure 3.24](image)

Figure 3.24 GPC chromatograms of $\text{AmG}_2$-$p(\text{DEA}_{50}-co-(t\text{BuMA}_{65}-co-\text{EGDMA}_{0.9}))$; A) RI chromatogram and B) RALS chromatogram.

3.5 Variation of dendron chemistry via introduction of benzyl surface functionality and urethane linker chemistry

Chapter 2 introduced the new synthesis of tertiary amine-functionalised Generation 0-2 dendron ATRP initiators, $\text{AmG}_0$-Br, $\text{AmG}_1$-Br and $\text{AmG}_2$-Br (Figure 3.25).

![Figure 3.25](image)

Figure 3.25 Amine-functionalised dendron ATRP initiators of varying generation synthesised in Chapter 2; i) $\text{AmG}_2$-Br; ii) $\text{AmG}_1$-Br and iii) $\text{AmG}_0$-Br represented by red, blue and green wedges (iv) respectively.
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These were utilised in the polymerisation of HPMA to produce linear-dendritic hybrids and hyp-polydendrons. Further to this, the amine-functionalised dendrons have been utilised as ATRP initiators to successfully polymerise DEA and tBuMA, demonstrating examples of variable polymer cores on offer for incorporation within the hyp-polydendron. Following the chemistry variation of the polymer primary chains, the chemistry within the dendron chain end was considered.

The alternatives for dendron surface chemistry are very broad and tertiary amines offer one option, due to their pH responsive nature. Comparisons between hyp-polydendrons, comprising different dendritic surface groups, present options for unique behaviour and applications. Amine vs. benzyl-functionality is an interesting comparison due to the former bearing pH-responsive groups, and the latter being hydrophobic (Figure 3.26i). Further to this, the surface chemistry is not the only structural variable within the dendron, the linker chemistry also has the potential to be modified. A tertiary amine-functionalised Generation 1 dendron ATRP initiator was synthesised via 1,1'-carbonyldiimidazole (CDI) coupling chemistry, introducing a urethane group into the dendron molecule (Figure 3.26ii).

3.5.1 Synthesis of tertiary amine functional G1 dendron ATRP initiator containing urethane linker chemistry (AmG1U-Br)

The amine-functionalised dendrons previously utilised as ATRP initiators within this work were initially synthesised via Michael addition to produce dendrons bearing a secondary alcohol as the focal point. A similar approach was taken, through initial
CHAPTER 3

dendron precursor production (AmG₁⁻OH), albeit via CDI chemistry. The second step utilised the same bromoesterification reaction witnessed in Chapter 2, to produce the ATRP initiator (AmG₁⁻Br) from the alcohol bearing dendron (Scheme 3.11).

Scheme 3.11 Synthesis of AmG₁⁻Br by initial production of AmG₁⁻OH via CDI chemistry followed by bromoesterification.

The synthesis of AmG₁⁻OH was achieved by a simple one pot reaction beginning with the reaction of 1-dimethylamino-2-propanol (Scheme 3.11 1) with CDI (Scheme 3.11 2) to give the imidazole carboxylic ester (Scheme 3.11 3). The imidazole carboxylic ester underwent selective coupling to the primary amines of 1-[N, N-bis(2-aminopropyl)-amino-2-propanol (APAP) (Scheme 3.11 4), previously synthesised in Chapter 2. AmG₁⁻OH was analysed by ¹H and ¹³C NMR and electro-spray mass spectrometry (ES-MS) (Figure 3.27 and Appendix, Figures A50 and 51). All protons neighbouring tertiary or secondary amine groups collectively integrated to 22 (Figure 3.27a, b, g, h). The methine proton situated within the initial APAP molecule, integrates to 1 proton (Figure 3.27i) and is located in a different environment to the 2 methine protons (Figure 3.27c) donated by the imidazole carboxylic ester intermediate (Figure 3.27c). The ¹³C NMR shows a peak at 156.7 ppm, attributing to the carbonyl residing in the urethane group. Molecular ion peaks are seen at 448.3 m/z ([M+H]+) and 470.3 m/z ([M+Na]+) in the ES-MS, (theoretical [M+H]⁺ (C₂₁H₄₅N₅O₅) m/z = 447.6).
The synthesis of the ATRP initiator, AmG\textsubscript{1}\textsuperscript{U}-Br, was achieved by esterification of AmG\textsubscript{1}\textsuperscript{U}-OH using α-bromoisobutyryl bromide (BiB) (Scheme 3.11 5) in a one pot reaction. The product was isolated by a dichloromethane/saturated sodium hydrogen carbonate solution (DCM/aq. NaHCO\textsubscript{3}) wash to remove any unreacted alcohol and characterised by \textsuperscript{1}H and \textsuperscript{13}C NMR and ES-MS (Figure 3.28 and Appendix, Figures A52 and 53).

A singlet peak appears at 1.92 ppm (Figure 3.28k), corresponding to the 2 methyl groups neighbouring the Br atom, integrating to 6 protons. The downfield shift of the methine proton, neighbouring the new ester bond, from 3.78 ppm to 5.06 ppm (Figure 3.28i), suggests the successful production of AmG\textsubscript{1}\textsuperscript{U}-Br. The \textsuperscript{13}C NMR spectrum witnesses a peak at 171.5 and 30.9 ppm, accounting for the new carbonyl group in the ester, and the new methyl groups respectively. Molecular ion peaks are obtained at 598.3 m/z [M+H]+, 620.3 m/z [M+Na]+ and 636.3 m/z [M+K]+ (calculated [M+H]+ (C\textsubscript{25}H\textsubscript{30}N\textsubscript{5}O\textsubscript{6}Br) m/z = 596.6).
3.5.2 Synthesis of benzyl ester functional G₂ dendron ATRP initiator (BnG₂-Br)

The AmG₂-Br was previously synthesised via the Michael addition \(^3^4\) of 2-(dimethylamino) ethyl acrylate to APAP. The same strategy was employed to produce the new Generation 2 hydrophobic benzyl ester dendron ATRP initiator, BnG₂-Br, by initially reacting benzyl acrylate (Scheme 3.12 1) with APAP (Scheme 3.12 2) to produce BnG₂-OH.
Scheme 3.12 Synthesis of BnG₂-Br by initial production of BnG₂-OH via Michael addition followed by bromoesterification.

BnG₂-OH was isolated following concentration in vacuo to remove solvent and any excess acrylate. ES-MS, ^1^H and ^13^C NMR analysis were used to confirm the structure of the product (Figure 3.29 and Appendix, Figures A54 and 55). The absence of the 2 singlet signals at ~6 ppm, corresponding to the benzyl acrylate protons, confirm all acrylate has been removed. All 20 aromatic protons are collectively seen between 7.27-7.41 ppm (Figure 3.29a, b, c). The 8 methylene protons neighbouring the oxygen atom on the benzyl acrylate arm are found downfield at 5.09 ppm (Figure 3.29d), due to the electronegativity of the oxygen. The methyl protons, situated on the focal point of the dendron, are seen upfield and integrate to three protons (Figure 3.29l). The aromatic protons are clearly seen in the ^13^C NMR at 128.6 ppm and the carbonyls in the ester groups are seen at 172.7 ppm. The molecular ion peak is situated at 838.5 m/z (theoretical [M+H]^+ (C₄₉H₆₃N₃O₉) m/z = 838.0).
BiB (Scheme 3.12 3) was then used in an esterification reaction overnight\textsuperscript{35} with the dendron alcohol, BnG\textsubscript{2}-OH, to prepare the ATRP initiator, BnG\textsubscript{2}-Br (Scheme 3.12). The product was isolated by liquid/liquid extraction using DCM/aq. NaHCO\textsubscript{3}, in order to remove any unreacted alcohol and characterised by \textsuperscript{1}H and \textsuperscript{13}C NMR spectroscopy (Figure 3.30 and Appendix, Figure A56) and ES-MS (Appendix, Figure A57).
The appearance of the signal at 1.90 ppm (Figure 3.30m) correspond to the 2 methyl groups neighbouring the Br. The shift of the methine proton neighbouring the ester bond from 3.71 ppm (Figure 3.29k) to 5.00 ppm (Figure 3.30k), confirms the esterification reaction has taken place. The aromatic protons integrate to 20 protons (Figure 3.30a, b, c), likewise the protons for the methyl group neighbouring the new ester group (Figure 3.30l) resumes to integrate to 3. The $^{13}$C NMR shows the appearance of a peak at 170.8 ppm accounting for the introduction of a second carbonyl group into the molecule. An m/z = 988.4 was obtained which was in concordance with the predicted m/z = 987.0 for C$_{53}$H$_{68}$N$_3$O$_{10}$Br. Once again, this straightforward approach to low generation dendron initiator synthesis proved robust, and their ability to initiate polymerisations was subsequently investigated.

### 3.5.3 Linear polymerisation of DEA and co-polymerisation of DEA and EGDMA using AmG$_1^U$-Br and BnG$_2$-Br dendron ATRP initiators

AmG$_1^U$-Br and BnG$_2$-Br dendron ATRP initiators were used to polymerise DEA in identical conditions previously utilised to produce the amine-functionalised linear-dendritic DEA polymer hybrids (Figure 3.31). The AmG$_1^U$-Br ATRP initiator was, also,
utilised in the co-polymerisation of DEA and EGDMA to produce the hyp-polydendron AmG1 U-p(DEA50-co-EGDMA0.9).

Both the AmG1 U- and BnG2-p(DEA50) linear-dendritic polymer hybrids were collected at high monomer conversion (>95 %). As previously witnessed, the M_n values were > twice as high as the targeted M_n (Table 3.5), with DP_n values > twice the targeted theoretical value estimated by GPC. ¹H NMR analysis of Bn-p(DEA50) also allowed assessment of the DP_n, by comparison of the aromatic signal attributing to the benzyl chain end group (Figure 3.32), with resonances assigned to p(DEA50) proton peaks residing in the polymer backbone, following subtraction of the integration accounting for the methyl protons neighbouring the Br atom (Figure 3.32q). The AmG1 U-p(DEA50) ¹H NMR spectrum is displayed in Appendix, Figure A58.

Figure 3.31 Diagrammatic representation of the polymerisation of DEA using AmG1 U-Br and BnG2-Br, and co-polymerisation of DEA and EGDMA using AmG1 U-Br.
Table 3.5 GPC analysis of amine-functionalised and benzyl-functionalised DEA linear-dendritic polymer hybrids and hyp-polydendron.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Conversion (%)</th>
<th>$M_n$ Theory (g mol$^{-1}$)</th>
<th>$M_n$ (g mol$^{-1}$)</th>
<th>$M_w$ (g mol$^{-1}$)</th>
<th>$D$</th>
<th>$DP_n$</th>
<th>$DP_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{AmG}<em>1^{U-p}(\text{DEA}</em>{50})$</td>
<td>99</td>
<td>9780</td>
<td>20300</td>
<td>22850</td>
<td>1.13</td>
<td>109</td>
<td>-</td>
</tr>
<tr>
<td>$\text{BnG}<em>2-p(\text{DEA}</em>{50})$</td>
<td>95</td>
<td>10220</td>
<td>20750</td>
<td>25850</td>
<td>1.25</td>
<td>107</td>
<td>97</td>
</tr>
<tr>
<td>$\text{AmG}<em>1^{U-p}(\text{DEA}</em>{50}-co-\text{EGDMA}_{0.9})$</td>
<td>99</td>
<td>-</td>
<td>125000</td>
<td>327000</td>
<td>2.62</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ Triple detection analysis using THF/2%TEA as eluent; $^b$ Determined by NMR; $^c$ $M_n$ Theory = ($M_w$ monomer*DP targeted) + $M_w$ initiator; $^d$ Determined by GPC; $^e$ Determined by $^1$H NMR.

Both linear-dendritic hybrids demonstrated high levels of control, observed by the narrow dispersity values and the monomodal, narrow molecular weight distributions displayed in the GPC chromatograms (Figure 3.33) (particularly the $\text{AmG}_1^{U-p}(\text{DEA}_{50})$ ($D = 1.13$)).
A soluble, high molecular weight hyp-polydendron was collected succeeding high monomer conversion (99%) (Table 3.5). The $\text{AmG}_1^\text{U} - \text{p(DEA}_{50}\text{-co-EGDMA}_{0.9})$ was analysed by $^1$H NMR (Appendix, Figure A59) and GPC, possessing an $M_w$ (327000 g mol$^{-1}$) that was less than the $\text{AmG}_2^\text{P-DEA}_{50}-\text{co-EGDMA}_{0.9}$ (341800 g mol$^{-1}$) but greater than the $\text{AmG}_1^\text{P-DEA}_{50}-\text{co-EGDMA}_{0.9}$ (288000 g mol$^{-1}$), suggesting an increase in dendron generation encourages the production of more highly branched polymers, when producing DEA-EGDMA hyp-polydendrons. The broad GPC RI and RALS chromatograms are shown in Figure 3.33.

Kinetic experiments were undertaken for the $\text{AmG}_1^\text{U}$-initiated linear polymerisation, confirming first order kinetics with respect to monomer concentration. The molecular weight, with respect to monomer conversion, appeared to evolve slower (Figure 3.34A) than the reactions initiated by $\text{AmG}_0^\text{Br}$ and $\text{AmG}_1^\text{Br}$. The reaction required 24 hours
to reach high conversion, similarly to the AmG1-Br polymerisation. The kinetics of the branched polymerisation were first order in respect to $M_n$ and $M_w$ development, showing a dramatic increase at high conversions (Figure 3.34D), which was very noticeable within the relationship of $M_w$ and conversion. Similarly, to the AmG2-p(DEA50-co-EGDMA0.9), a high $M_n$ was recovered, suggesting a large number of linear-dendritic polymer chains have joined together; > 14 primary chains, suggesting 28 tertiary amine chain end functional groups contribute to the hyp-polydendron surface functionality on average.

![Kinetic plots](image)

Figure 3.34 Kinetic plots for linear AmG1-p(DEA0) (A and B) and branched AmG1-p(DEA50-co-EGDMA0.9) (C and D). A and C) Conversion (blue squares), $\ln([M]_0/[M])$ (red circles); B and D) $M_n$ (red circles), $M_w$ (blue lines) and $M_w$ (black diamonds).

3.6 Variation of initiators in a one-pot $t$BuMA and EGDMA co-polymerisation

The introduction of multi-functional initiators has led to the formation of dumbbell and clover-leaf NPs. Rannard and co-workers have subsequently utilised mixed initiator systems, involving a Generation 2 dendron initiator and a PEG macroinitiator.
new synthetic strategy enabled the formation of complex spherical NPs with mixed surface functionality. The synthesis of hyp-polypolydendrons offers a unique opportunity for systematic incorporation of mixed functionalities through the mixing of ATRP initiators.

A tBuMA hyp-polypolydendron was designed to contain a minimal amount of amine-functional dendritic surface functionality (Figure 3.35). Hyp-polypolydendrons comprising of mixed surface functionality have uncovered behavioural differences when existing as nanoprecipitates. A molar ratio EBiB:AmG2-Br 0.9:0.1 was used to initiate tBuMA in the presence of EGDMA. Analysis was conducted via $^1$H NMR (Appendix, Figure A60) and GPC, confirming a high molecular weight hyp-polypolydendron, EBiB$_{0.9}$-(AmG$_2$)$_{0.1}$-$p$(tBuMA$_{50}$-co-EGDMA$_{0.95}$) ($M_w = 137100$ g mol$^{-1}$, $M_n = 78800$ g mol$^{-1}$ and $D=1.74$; Figure 3.36).

Figure 3.35 Diagrammatic representation of $p$(tBuMA$_{50}$-co-EGDMA$_{0.95}$) containing 90 mol% EBiB chain ends and 10% AmG$_2$ chain ends.

Figure 3.36 GPC chromatograms of A) RI chromatogram and B) RALS chromatogram for EBiB$_{0.9}$-(AmG$_2$)$_{0.1}$-$p$(tBuMA$_{50}$-co-EGDMA$_{0.95}$).
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3.7 Variation of brancher within the hyp-polydendron structure

Previous reports have described the synthesis of a bifunctional methacrylate monomer, ethylene glycol di(1-methacryloyloxy)ethyl ether via an addition reaction between ethylene glycol divinyl ether and MAA.\(^{38}\) This bifunctional methacrylate monomer was used as a cross-linker in the preparation of several star-shaped polymers, where the cross-linked points could easily be cleaved by hydrolysis following acid addition. Here, the synthesis of a new pH-responsive brancher, 1,4-butanediol di(methacryloyloxy)-ethyl ether (BDME), (Figure 3.37ii) is reported. The use of such a cross-linker at low concentrations relative to initiators would be able to make pH-cleavable hyp-polydendrons.

Figure 3.37 Branchers utilised in hyp-polydendron synthesis. i) ethylene glycol dimethacrylate (EGDMA), ii) 1,4-butanediol di(methacryloyloxy)-ethyl ether (BDME).

3.7.1 Synthesis of new acid-cleavable brancher

The pH-responsive branching unit BDME was successfully prepared through an addition reaction between MAA (Scheme 3.13 1) and 1,4-butanediol divinyl ether (BDVE) (Scheme 3.13 2), utilising a trace amount of 4-tert-butylcatechol acting as a radical inhibitor. The product was confirmed by \(^1\)H NMR (Figure 3.38), \(^{13}\)C NMR and ES-MS (Appendix, Figures A61 and 62).
Scheme 3.13 Schematic representation and mechanism of addition reaction between 1,4-butanediol divinyl ether (BDVE) (2) and methacrylic acid (1) to produce 1,4-butanediol di(methacryloyloxy)ethyl ether (BDME).

Figure 3.38 $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of BDME with important integrations assigned.

The methine proton signals at 5.97 ppm integrating to 2 protons (Figure 3.38c) and the 2 methyl groups neighbouring the methine proton integrate to 6 protons at 1.95 ppm (Figure 3.38d) suggests the successful bond formation between the divinyl ether and the carboxylic acid on the MAA. The methyl groups neighbouring the carbon double bond integrate to 6 protons (Figure 3.38a) and the methylene protons in the backbone of the
molecule integrate collectively to 4 protons in each environment (Figure 3.38e and f). The $^{13}$C NMR shows peaks at 166.9 and 136.5 ppm, which can be assigned to the carbonyl groups and the vinyl carbons respectively. Molecular ion peaks of 337.2 and 353.1 m/z were observed, accounting for the [M+Na]$^+$ and [M+K]$^+$ respectively (Calculated m/z = 314.4).

3.7.2 Utilisation of BDME brancher in ATRP to produce a series of hyp-polydendrons

Following successful production of a range of branched copolymers and hyp-polydendrons using EGDMA, the newly synthesised BDME was incorporated into three hyp-polydendron procedures, all initiated by AmG$_2$-Br (Scheme 3.14 1). Initially, BDME (Scheme 3.14 3) was copolymerised with DEA (Scheme 3.14 2) to produce AmG$_2$p(DEA$_{50}$-co-BDME$_{2.0}$). To ensure incorporation of the divinyl monomer into the polymer backbone, the molar concentration of BDME was increased (2 molar eq.). This molar ratio was used for all BDME-containing hyp-polydendrons, and the polymerisation conditions were identical to each EGDMA equivalent procedure.

Scheme 3.14 Schematic representation of the polymerisation of DEA (2) and BDME (3) initiated by AmG$_2$-Br (1) to produce AmG$_2$p(DEA$_{50}$-co-BDME$_{2.0}$).

The incorporation of BDME into the polymerisation of DEA resulted in a highly branched polymer (Table 3.6). The $M_n$ and $M_w$ calculated by GPC were similar values to the AmG$_2$-Br initiated DEA and EGDMA co-polymerisation, insinuating consistency with initiation efficiency. The RI and RALS traces display broad, bimodal molecular weight distributions (Figure 3.39). The AmG$_2$p(DEA$_{50}$-co-BDME$_{2.0}$) contains on
average 9 primary polymer chains, and consequently 37 tertiary amine chain ends per hyp-polydendron. The $^1$H NMR spectrum is shown in Figure 3.40.

### Table 3.6 GPC analysis of BDME-containing and their EGDMA-equivalent hyp-polydendrons.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Conversion (%)</th>
<th>$M_n$ (g mol$^{-1}$)</th>
<th>$M_w$ (g mol$^{-1}$)</th>
<th>$D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmG2-p(DEA50-co-EGDMA0.9)</td>
<td>99</td>
<td>125700</td>
<td>341800</td>
<td>2.72</td>
</tr>
<tr>
<td>AmG2-p(DEA50-co-BDME2.0)</td>
<td>99</td>
<td>157300</td>
<td>321100</td>
<td>2.04</td>
</tr>
<tr>
<td>AmG2-p(DEA33-co-HPMA17-co-EGDMA0.9)</td>
<td>99</td>
<td>247500</td>
<td>398300</td>
<td>1.61</td>
</tr>
<tr>
<td>AmG2-p(DEA33-co-HPMA17-co-BDME2.0)</td>
<td>99</td>
<td>305300</td>
<td>733400</td>
<td>2.40</td>
</tr>
<tr>
<td>AmG2-p(DEA17-b-(tBuMA33-co-EGDMA0.9))</td>
<td>99</td>
<td>92300</td>
<td>251800</td>
<td>2.73</td>
</tr>
<tr>
<td>AmG2-p(DEA17-b-(tBuMA33-co-BDME2.0))</td>
<td>99</td>
<td>444100</td>
<td>594800</td>
<td>1.34</td>
</tr>
</tbody>
</table>

$^a$Triple detection analysis using THF/2% TEA eluent; $^b$Determined by NMR

### Figure 3.39 GPC chromatogram overlays of A) RI chromatograms and B) RALS chromatograms for AmG2-p(DEA50-co-BDME2.0) (green), AmG2-p(DEA33-co-HPMA17-co-BDME2.0) (purple) and AmG2-p(DEA17-b-(tBuMA33-co-BDME2.0)) (orange).
Following the successful synthesis of a branched polymer via inclusion of BDME, the brancher was incorporated into the statistical co-polymerisation of DEA and HPMA and the block co-polymerisation of DEA and tBuMA to produce $\text{AmG}_2-p(\text{DEA}_{33}-co-\text{HPMA}_{17}-co-\text{BDME}_{2.0})$ (Figure 3.41i) and $\text{AmG}_2-p(\text{DEA}_{17}-b-(\text{tBuMA}_{33}-co-\text{BDME}_{2.0}))$ (Figure 3.41ii) respectively.
Figure 3.41 Diagrammatic representation of the statistical co-polymerisation of DEA, HPMA and BDME to produce i) AmG$_2$$p$(DEA$_{33}$$-$$co$$-$$HPMA$_{17}$$-$$co$$-$$BDME$_{2.0}$); and the block polymerisation of DEA, tBuMA and BDME to produce ii) AmG$_2$$p$(DEA$_{17}$$-$$b$$-$(tBuMA$_{33}$$-$$co$$-$$BDME$_{2.0}$)).

Both BDME-containing hyp-polydendrons contained higher $M_n$ and $M_w$ values (≈ twice as large) as their EGDMA equivalents (Table 3.6). This may be due to the longer BDME molecule allowing easier access for the linear polymer chains to branch within the reaction media. AmG$_2$$p$(DEA$_{33}$$-$$co$$-$$HPMA$_{17}$$-$$co$$-$$BDME$_{2.0}$) and AmG$_2$$p$(DEA$_{17}$$-$$b$$-$(tBuMA$_{33}$$-$$co$$-$$BDME$_{2.0}$)) $^1$H NMR spectra are shown in Appendix, Figures A63 and 64 respectively. Highly branched materials were also confirmed from the multimodal weight distributions observed during GPC analysis (Figure 3.39). The AmG$_2$$p$(DEA$_{17}$$-$$co$$-$$HPMA$_{33}$$-$$co$$-$$BDME$_{2.0}$) contained > 50 primary chains suggesting ≈ twice as many primary polymer chains in comparison to the AmG$_2$$p$(DEA$_{33}$$-$$co$$-$$HPMA$_{17}$$-$$co$$-$$EGDMA$_{0.9}$), with 200 tertiary amine chain end functional groups. The $M_n$ value obtained for AmG$_2$$p$(DEA$_{17}$$-$$b$$-$(tBuMA$_{33}$$-$$co$$-$$BDME$_{2.0}$)) was noticeably large, with the hyp-polydendron comprised of > 13 primary chains, with 52 tertiary amine chain end functional groups.

3.8 Conclusions

The homopolymerisations of DEA using dendron ATRP initiators produced linear-dendritic hybrids with $M_n$ values > twice the targeted theoretical value. The hyp-
polydendrons proceeded to high monomer conversions and produced materials with molecular weights > 300,000 g mol\(^{-1}\). The linear-dendritic hybrid AmG\(_2\)-p(tBuMA\(_{50}\)) displayed more polymerisation control compared to the EBiB-p(tBuMA\(_{50}\)); and the hyp-polydendron AmG\(_2\)-p(tBuMA\(_{50}\)-co-EGDMA\(_{0.9}\)) contained a higher molecular weight than the EBiB-containing branched copolymer. Generally, the polymerisations and co-polymerisations monitored proceeded via first order kinetics, as expected, and produced monomodal distributions of polymer chains when a linear polymer was targeted and a much broader disperse polymer species when the divinyl monomer was incorporated and a branched polymer was targeted.

The statistical linear-dendritic DEA-HPMA hybrids conveyed high levels of control, and the statistical DEA-HPMA hyp-polydendrons had considerably high molecular weights compared to the DEA and HPMA hyp-polydendrons (Chapter 2). tBuMA was successfully incorporated into the linear-dendritic polymer chain and hyp-polydendron core, and subsequently utilised to produce block DEA-tBuMA hybrids and a branched tBuMA core produced from growing linear DEA chains in one-pot block co-polymerisations.

A tBuMA-EGDMA hyp-polydendron containing both dendron and non-dendron surface groups was successfully synthesised via incorporation of both EBiB and AmG\(_2\)-Br ATRP initiators within the reaction. New dendron ATRP initiators were successfully synthesised containing different surface and linker chemistry. The linear polymerisations of DEA proceeded in a similar manner to the amine-functionalised DEA hybrids. Finally, DEA, statistical DEA-HPMA and block DEA-tBuMA hyp-polydendrons were successfully produced containing a new acid-cleavable brancher.

Overall, the research presents synthetic explorations within a new polymeric material class. The versatility observed in hyp-polydendron synthesis has been successfully demonstrated. The hyp-polydendron structure has been altered by variation of multiple components, including the monomer chemistry, the hyp-polydendron architecture, the surface functionality, the use of multiple initiators and the incorporation of a newly synthesised brancher.
3.9 Experimental

Typical linear polymerisation of DEA (EBiB-, AmG₀-, AmG₁-, AmG₂-, AmG₁⁻ and BnG₂⁻p(DEA₅₀)) – In a typical synthesis, targeting a DPₙ = 50 monomer units (p(DEA₅₀)), bpy (134.9 mg, 0.8637 mmol, 2 eq.), DEA (4 g, 21.59 mmol, 50 eq.) and IPA (56% v/v based on DEA) were placed into a 25 mL round-bottomed flask (RBF). The solution was stirred and deoxygenated using a N₂ purge for 15 minutes. Cu(Ι)Cl (42.8 mg, 0.4318 mmol, 1 eq.) was added to the flask under a positive flow of N₂ and the solution was left to polymerise at 40°C. Reactions were terminated when >99 % conversion was reached, as judged by ¹H NMR, by exposure to oxygen and addition of acetone. The catalyst residues were removed by passing the mixture over a basic alumina column. Acetone was removed under vacuum to concentrate the sample before precipitation into cold petroleum ether (40-60).

Typical synthesis of branched copolymer and hyperbranched polydendrons using the co-polymerisation of DEA and EGDMA, (EBiB-, AmG₀-, AmG₁-, AmG₂- and AmG₁⁻-p(DEA₅₀-co-EGDMAₙ)) – In a typical synthesis, targeting a DPₙ = 50 monomer units (p(DEA₅₀-co-EGDMAₙ)), bpy (134.9 mg, 0.8637 mmol, 2 eq.), DEA (4 g, 21.59 mmol, 50 eq.), EGDMA (77.0 mg, 0.3886 mmol, 0.9 eq.) and IPA (38.9% v/v based on DEA) were placed into a 25 mL RBF. The solution was stirred and deoxygenated using a N₂ purge for 15 minutes. Cu(Ι)Cl (42.8 mg, 0.4318 mmol, 1 eq.) was added to the flask under a positive flow of N₂ and the solution was left to polymerise at 40°C. Reactions were terminated when >99 % conversion was reached, as judged by ¹H NMR, by exposure to oxygen and addition of acetone. The catalyst residues were removed by passing the mixture over a basic alumina column. Acetone was removed under vacuum to concentrate the sample before precipitation into cold petroleum ether (40-60).

Typical linear polymerisation of tBuMA (EBiB- and AmG₂⁻p(tBuMA₅₀)) – In a typical synthesis, targeting a DPₙ = 50 monomer units (p(tBuMA₅₀)), bpy (134.9 mg, 0.8637 mmol, 2 eq.), tBuMA (3.07 g, 21.59 mmol, 50 eq.) and IPA/H₂O (92.5:7.5 %) (24.2% v/v based on tBuMA) were placed into a 25 mL RBF. The solution was stirred and deoxygenated using a N₂ purge for 15 minutes. Cu(Ι)Cl (42.8 mg, 0.4318 mmol, 1 eq.) was added to the flask under a positive flow of N₂ and the solution was left to polymerise at 40°C. Reactions were terminated when >99 % conversion was reached, as judged by ¹H NMR, by exposure to oxygen and addition of acetone. The catalyst residues were removed by passing the mixture over a basic alumina column. Acetone was removed under vacuum to concentrate the sample before precipitation into cold petroleum ether (40-60).
g, 0.4318 mmol, 1 eq.) was added to the flask under a positive flow of N₂ and the solution was left to polymerise at 20°C. Reactions were terminated when >99 % conversion was reached, as judged by ¹H NMR, by exposure to oxygen and addition of THF. The catalyst residues were removed by passing the mixture over a basic alumina column. THF was removed under vacuum to concentrate the sample before precipitation into cold hexane.

Typical synthesis of branched copolymer and hyperbranched polydendrons using the co-polymerisation of tBuMA and EGDMA, (EBiB- and AmG₂-p(tBuMA₅₀-co-EGDMAₓ)) – In a typical synthesis, targeting a DPₙ = 50 monomer units (p(tBuMA₅₀)), bpy (134.9 mg, 0.8637 mmol, 2 eq.), tBuMA (3.07 g, 21.59 mmol, 50 eq.), EGDMA (77 mg, 0.3886 mmol, 0.9 eq.) and IPA/H₂O (92.5:7.5 %) (32.4% v/v based on tBuMA) were placed into a 25 mL RBF. The solution was stirred and deoxygenated using a N₂ purge for 15 minutes. Cu(I)Cl (42.8 mg, 0.4318 mmol, 1 eq.) was added to the flask and left to purge for a further 5 minutes. AmG₂-Br (0.3934 g, 0.4318 mmol, 1 eq.) was added to the flask under a positive flow of N₂ and the solution was left to polymerise at 20°C. Reactions were terminated when >99 % conversion was reached, as judged by ¹H NMR, by exposure to oxygen and addition of THF. The catalyst residues were removed by passing the mixture over a basic alumina column. THF was removed under vacuum to concentrate the sample before precipitation into cold hexane.

Typical synthesis of statistical hyperbranched polydendrons using the co-polymerisation of DEA, HPMA and EGDMA, (AmG₂-p(DEAₓ-co-HPMAₓ-co-EGDMAₓ)) – In a typical synthesis, targeting a DPₙ = 50 monomer units (p(DEA₂₅-co-HPMA₂₅)), bpy (134.9 mg, 0.8637 mmol, 2 eq.), DEA (2 g, 10.80 mmol, 25 eq.), HPMA (1.5567 g, 10.80 mmol, 25 eq.) and IPA (25.9% v/v based on monomer) were placed into a 25 mL RBF. The solution was stirred and deoxygenated using a N₂ purge for 15 minutes. Cu(I)Cl (42.8 mg, 0.4318 mmol, 1 eq.) was added to the flask and left to purge for a further 5 minutes. AmG₂-Br (0.3934 g, 0.4318 mmol, 1 eq.) was added to the flask under a positive flow of N₂, and the solution was left to polymerise at 40°C. Reactions were terminated when >99 % conversion was reached, as judged by ¹H NMR, by exposure to oxygen and addition of acetone. The catalyst residues were removed by passing the mixture over a basic alumina column. Acetone was removed under vacuum to concentrate the sample before precipitation into cold petroleum ether (40–60).

Typical synthesis of statistical hyperbranched polydendrons using the co-polymerisation of DEA, HPMA and EGDMA, (AmG₂-p(DEAₓ-co-HPMAₓ-co-EGDMAₓ)) – In a typical synthesis, targeting a DPₙ = 50 monomer units (p(DEA₂₅-co-HPMA₂₅)), bpy (134.9 mg, 0.8637 mmol, 2 eq.), DEA (2 g, 10.80 mmol, 25 eq.), HPMA (1.5567 g, 10.80 mmol, 25 eq.) and IPA (25.9% v/v based on monomer) were placed into a 25 mL RBF. The solution was stirred and deoxygenated using a N₂ purge for 15 minutes. Cu(I)Cl (42.8 mg, 0.4318 mmol, 1 eq.) was added to the flask and left to purge for a further 5 minutes. AmG₂-Br (0.3934 g, 0.4318 mmol, 1 eq.) was added to the flask under a positive flow of N₂, and the solution was left to polymerise at 40°C. Reactions were terminated when >99 % conversion was reached, as judged by ¹H NMR, by exposure to oxygen and addition of acetone. The catalyst residues were removed by passing the mixture over a basic alumina column. Acetone was removed under vacuum to concentrate the sample before precipitation into cold petroleum ether (40–60).
EGDMA0.9)) – In a typical synthesis, targeting a DP$_n$ = 50 monomer units ($p$(DEA$_{25}$-co-HPMA$_{25}$-co-EGDMA$_{0.9}$)), bpy (134.9 mg, 0.8637 mmol, 2 eq.), DEA (2 g, 10.80 mmol, 25 eq.), HPMA (1.5567 g, 10.80 mmol, 25 eq.), EGDMA (77.0 mg, 0.3886 mmol, 0.9 eq.) and IPA (25.9% v/v based on monomer) were placed into a 25 mL RBF. The solution was stirred and deoxygenated using a N$_2$ purge for 15 minutes. Cu(I)Cl (42.8 mg, 0.4318 mmol, 1 eq.) was added to the flask and left to purge for a further 5 minutes. AmG$_2$-Br (0.3934 g, 0.4318 mmol, 1 eq.) was added to the flask under a positive flow of N$_2$, and the solution was left to polymerise at 40°C. Reactions were terminated when >99% conversion was reached, as judged by $^1$H NMR, by exposure to oxygen and addition of acetone. The catalyst residues were removed by passing the mixture over a basic alumina column. Acetone was removed under vacuum to concentrate the sample before precipitation into cold petroleum ether (40–60).

Typical statistical linear polymerisation of DEA and tBuMA (AmG$_2$-p(DEA$_x$-co-tBuMA$_y$)) – In a typical synthesis, targeting a DP$_n$ = 50 monomer units ($p$(DEA$_{25}$-co-tBuMA$_{25}$)), bpy (134.9 mg, 0.8637 mmol, 2 eq.), DEA (2 g, 10.80 mmol, 25 eq.), tBuMA (1.5352 g, 10.80 mmol, 25 eq.) and IPA/H$_2$O (92.5:7.5%) (30.6% v/v based on monomer) were placed into a 25 mL RBF. The solution was stirred and deoxygenated using a N$_2$ purge for 15 minutes. Cu(I)Cl (42.8 mg, 0.4318 mmol, 1 eq.) was added to the flask and left to purge for a further 5 minutes. AmG$_2$-Br (0.3934 g, 0.4318 mmol, 1 eq.) was added to the flask under a positive flow of N$_2$, and the solution was left to polymerise at 40°C. Reactions were terminated when >99% conversion was reached, as judged by $^1$H NMR, by exposure to oxygen and addition of acetone. The catalyst residues were removed by passing the mixture over a basic alumina column. Acetone was removed under vacuum to concentrate the sample before precipitation into cold petroleum ether (40–60).

Typical synthesis of statistical hyperbranched polydendrons using the co-polymerisation of DEA, tBuMA and EGDMA, (AmG$_2$-p(DEA$_x$-co-tBuMA$_y$-co-EGDMA$_{0.9}$)) – In a typical synthesis, targeting a DP$_n$ = 50 monomer units ($p$(DEA$_{25}$-co-tBuMA$_{25}$-co-EGDMA$_{0.9}$)), bpy (134.9 mg, 0.8637 mmol, 2 eq.), DEA (2 g, 10.80 mmol, 25 eq.), tBuMA (1.5352 g, 10.80 mmol, 25 eq.), EGDMA (77.0 mg, 0.3886 mmol, 0.9 eq.) and IPA/H$_2$O (92.5:7.5%) (30.6% v/v based on monomer) were placed into a 25 mL RBF. The solution was stirred and deoxygenated using a N$_2$ purge for 15 minutes. Cu(I)Cl (42.8 mg, 0.4318 mmol, 1 eq.) was added to the flask and left to purge for a further 5 minutes. AmG$_2$-Br (0.3934 g, 0.4318 mmol, 1 eq.) was added to the flask.
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under a positive flow of N₂, and the solution was left to polymerise at 40°C. Reactions were terminated when >99% conversion was reached, as judged by ¹H NMR, by exposure to oxygen and addition of acetone. The catalyst residues were removed by passing the mixture over a basic alumina column. Acetone was removed under vacuum to concentrate the sample before precipitation into cold petroleum ether (40–60).

**Typical block linear polymerisation of DEA and tBuMA** (AmG₂₋p(DEAₓ-b-tBuMAₙ)) – In a typical synthesis, targeting an overall DPₙ = 50 monomer units (p(DEA₂₅-b-tBuMA₂₅)), bpy (134.9 mg, 0.8637 mmol, 2 eq.), DEA (2.00 g, 10.80 mmol, 25 eq.) and IPA (29.6-45.6% v/v based on monomer) were placed into a 25 mL RBF. The solution was stirred and deoxygenated using a N₂ purge for 15 minutes. Cu(I)Cl (42.8 mg, 0.4318 mmol, 1 eq.) was added to the flask and left to purge for a further 5 minutes. Cu(I)Cl/bpy was added to the reaction flask in the molar ratio ([M]:[CuCl]:[bpy]=25:1:2). AmG₂-Br (0.3934 g, 0.4318 mmol, 1 eq.) was added to the flask under a positive flow of N₂, and the solution was left to polymerise at 40°C. Upon 73-92% monomer conversion, the second feed of tBuMA (1.54 g, 10.80 mmol, 25 eq.) was introduced, with Cu(I)Cl/bpy ([M]:[CuCl]:[bpy]=25:1:2) in IPA/H₂O (92.75:7.5%) (31.6-32.9 w/v%). Reactions were terminated when >99% conversion was reached, as judged by ¹H NMR, by exposure to oxygen and addition of acetone. The catalyst residues were removed by passing the mixture over a basic alumina column. Acetone was removed under vacuum to concentrate the sample before precipitation into cold petroleum ether (40–60).

**Typical synthesis of a block hyperbranched polydendron using the copolymerisation of DEA, tBuMA and EGDMA** (AmG₂₋p(DEAₓ-b-(tBuMAₓ-co-EGDMA₀₉))) – In a typical synthesis, targeting an overall DPₙ = 50 monomer units (p(DEA₂₅-b-(tBuMA₂₅-co-EGDMA₀₉)), bpy (134.9 mg, 0.8637 mmol, 2 eq.), DEA (2.00 g, 10.80 mmol, 25 eq.) and IPA (29.6-45.6% v/v based on monomer) were placed into a 25 mL RBF. The solution was stirred and deoxygenated using a N₂ purge for 15 minutes. Cu(I)Cl (42.8 mg, 0.4318 mmol, 1 eq.) was added to the flask and left to purge for a further 5 minutes. Cu(I)Cl/bpy was added to the reaction flask in the molar ratio ([M]:[CuCl]:[bpy]=25:1:2). AmG₂-Br (0.3934 g, 0.4318 mmol, 1 eq.) was added to the flask under a positive flow of N₂, and the solution was left to polymerise at 40°C. Upon 73-92% monomer conversion, the second feed of tBuMA (1.54 g, 10.80 mmol, 25 eq.) and EGDMA (77 mg, 0.389 mol, 0.9 eq.) was introduced, with Cu(I)Cl/bpy
([M]:[CuCl]:[bpy]=25:1:2) in IPA/H₂O (92.75:7.5%) (31.6-32.9 w/v%). Reactions were terminated when >99 % conversion was reached, as judged by ¹H NMR, by exposure to oxygen and addition of acetone. The catalyst residues were removed by passing the mixture over a basic alumina column. Acetone was removed under vacuum to concentrate the sample before precipitation into cold petroleum ether (40–60).

**AmG₁⁻OH** – CDI (1.9460 g, 12 mmol, 2 eq.) was added to an oven-dried 100 mL 2-neck RBF fitted with a reflux condenser, magnetic stirrer and a dry N₂ inlet. Anhydrous toluene (20 mL) was added and the flask purged with N₂ for 10 minutes. The solution was stirred at 60°C and dimethylamino propan-2-ol (2.4758 g, 24 mmol, 4 eq.) added via a syringe. The mixture was left stirring at 60°C for 6 hours under a positive flow of N₂. **APAP** (1.1338 g, 6 mmol, 1 eq.) was added drop wise, and the reaction was left stirring for a further 18 hours at 60°C under a positive flow of N₂. Following this, the solution was allowed to cool to room temperature, and the pale yellow solution was filtered to remove any solid imidazole, and concentrated in vacuo. The resulting viscous oil was dissolved in DCM (20 mL) washed with 1M NaHCO₃ aq. (3 x 30 mL). The organic layer was dried with anhydrous sodium sulfate (Na₂SO₄), filtered, and concentrated in vacuo. Yield: 1.5424 g, yellow oil, (58%). ¹H NMR (400 MHz, CDCl₃) δ 1.11 (m, 3H), 1.22 (m, 3H), 1.29 (d, 3H), 1.65 (m, 4H), 2.04 – 2.68 (m, 22H), 3.22 (m, 4H), 3.80 (m, 1H), 4.89 (m, 2H), 5.59 (s, br, -NH), 5.73 (s, br, -NH), 5.98 (s, br, -OH). ¹³C NMR (100 MHz, CDCl₃) δ 18.9, 20.8, 27.2, 39.7, 46.0, 52.6, 62.6, 64.4, 68.4, 72.5, 156.7. Calcd [M+H]⁺ (C₂₁H₄₅N₅O₅) m/z = 447.6. Found: ESI-MS: [M+H]⁺ m/z = 447.2 and [M+Na]+ m/z = 470.3. Anal. Calcd for C₂₁H₄₅N₅O₅: C, 56.38; H, 10.07; N, 15.66. Found C, 56.06; H, 10.33; N, 14.78.

**AmG₁⁻Br** – **AmG₁⁻OH** (1.5424 g, 3.4 mmol, 1 eq.), triethylamine (TEA) (0.4886 g, 4.8 mmol, 1.4 eq.) and 4-dimethyl-aminopyridine (DMAP; 42.1 mg, 0.34 mmol, 0.1 eq.) were added to a 250 mL 2 necked RBF containing DCM (110 mL). The flask was deoxygenated under a positive N₂ purge for 10 minutes. **BiB** (0.8326 g, 0.45 mL, 3.62 mmol, 1.05 eq.) was added drop wise while the solution was stirring in an ice bath under a positive flow of N₂. The reaction mixture was allowed to warm to room temperature and left stirring overnight. The organic phase was washed with saturated NaHCO₃ solution (3 x 110 mL). The solution was dried with anhydrous Na₂SO₄ and the product left to dry in vacuo overnight. Yield: 0.6937 g, yellow oil, (37%). ¹H NMR (400 MHz, CDCl₃) δ 1.22 (d, 3H), 1.25 (d, 3H), 1.29 (d, 3H) 1.64 (m, 4H), 1.92 (s, 6H), 2.15-2.87 (m, 24H), 3.19 (m, 4H), 4.92 (m, 2H), 5.06 (m, 1H), 5.22 (s, br, -NH), 5.50 (s, br, -NH), 5.50 (s, br, -NH),
5.64 (s, br, -OH). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 18.3, 19.2, 27.4, 30.9, 39.5, 46.0, 52.3, 56.3, 59.4, 64.4, 68.4, 72.4, 156.4, 171.5. Calcd [M+H]$^+$ (C$_{25}$H$_{50}$N$_5$O$_6$Br) m/z = 596.6. Found: ESI-MS: [M+H]$^+$ m/z = 598.3, [M+Na]$^+$ m/z = 620.3 and [M+K]$^+$ m/z = 636.3

Anal. Calcd for C$_{25}$H$_{50}$N$_5$O$_6$Br: C, 50.28; H, 8.38; N, 11.73. Found C, 50.39; H, 8.58; N, 11.51.

**BnG$_2$-OH** – BA (3.00 g, 18 mmol, 6 eq.) was added to a 50 mL round 2 necked RBF containing IPA (12 mL). The flask was deoxygenated under a positive N$_2$ purge for 10 minutes. APAP (1.32 g, 6.984 mmol, 1 eq.) dissolved in IPA (12 mL) was added drop wise while the solution was stirring in an ice bath under a positive flow of N$_2$. The final mixture was stirred for a further 10 minutes at 0°C, allowed to warm to room temperature and left stirring for 48 hours. The solvent was removed and the product left to dry in vacuo overnight. Yield: 1.6262 g, yellow oil, (63%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.07 (d, 3H), 1.53 (m, 4H), 2.19-2.56 (m, 18H), 2.77 (m, 8H), 3.69 (m, 1H), 5.08 (m, 8H), 7.33 (m, 20H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ = 20.0, 24.5, 32.2, 45.7, 48.9, 51.6, 52.2, 57.8, 62.1, 62.3, 63.6, 173.0. Calcd [M+H]$^+$ (C$_{49}$H$_{63}$N$_3$O$_9$) m/z = 838.0. Found: ESI-MS: [M+H]$^+$ m/z = 838.5. Anal. Calcd for C$_{49}$H$_{63}$N$_3$O$_9$: C, 70.17; H, 7.52; N, 5.01%. Found C, 69.56; H, 7.41; N, 4.57%.

**BnG$_2$-Br** – BnG$_2$-OH (1.6262 g, 1.9 mmol, 1 eq.), TEA (0.2739 g, 2.6 mmol, 1.4 eq.) and DMAP (23.7 mg, 0.19 mmol, 0.1 eq.) were added to a 250 mL 2 necked RBF containing DCM (60 mL). The flask was deoxygenated under a positive N$_2$ purge for 10 minutes. BiB (0.5577 g, 0.30 mL, 2.3 mmol, 1.25 eq.) was added drop wise while the solution was stirring in an ice bath under a positive flow of N$_2$. The reaction mixture was allowed to warm to room temperature and left stirring overnight. The organic phase was washed with saturated NaHCO$_3$ solution (3 x 60 mL). The solution was dried with anhydrous Na$_2$SO$_4$ and the product left to dry in vacuo overnight. Yield: 1.19 g, yellow oil (62%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.22 (d, 3H), 1.54 (m, 4H), 1.90 (s, 6H), 2.24-2.65 (m, 18H), 2.77 (m, 8H), 5.00 (m, 1H), 5.09 (s, 8H), 7.33 (m, 20H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ = 17.0, 23.9, 29.7, 31.6, 48.3, 50.8, 51.6, 55.2, 57.7, 65.2, 70.0, 127.15, 127.2, 127.5, 135.0, 171.4. Calcd [M+H]$^+$ (C$_{53}$H$_{68}$N$_3$O$_{10}$Br) m/z = 987.0. Found: ESI-MS: [M+H]$^+$ m/z = 988.4. Anal. Calcd for C$_{53}$H$_{68}$N$_3$O$_{10}$Br: C, 70.17; H, 6.89; N, 4.26%. Found C, 63.40; H, 6.96; N, 4.18%.

**BDME** – BDVE (5.03 g, 5.6 mL, 35.36 mmol, 1 eq.) was added to a two-necked 250 mL RBF equipped with a condenser, a magnetic stirrer and a positive flow of N$_2$. A
small amount of radical inhibitor 4-<i>tert</i>-butylcatechol (end of a spatula) was added and the mixture deoxygenated using a N₂ purge for 15 minutes. Once dissolved, the temperature was raised to 70°C. MAA (15.22 g, 15.1 mL, 0.1769 mol, 5 eq.) was added drop wise over 10 minutes through a septa. The reaction was allowed to proceed at 70°C for a further 6 hours with stirring. After this time, the reaction was stopped by cooling and exposing to the air. The crude product was dissolved in chloroform (100 mL) and washed with basic H₂O (~pH 12, 3 x 100 mL). The combined washings were collected and dried over Na₂SO₄ and the solvent removed by rotary evaporation. Yield: 8.73 g, yellow oil, (79%). ¹H NMR (400 MHz, CDCl₃) δ 1.43 (d, 6H), 1.65 (m, 4H), 1.95 (s, 6H), 3.51 (m, 2H), 3.68 (m, 2H), 5.60 (s, 2H), 5.97 (m, 2H), 6.15 (s, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 18.4, 20.6, 26.0, 30.9, 68.9, 97.0, 126.0, 136.5, 166.9. Calcd [M+H]+ (C₁₆H₂₆O₆) m/z = 314.4. Found: ESI-MS: [M+Na]+ m/z = 337.2 and [M+K]+ m/z = 353.1. Anal. Calcd for C₁₆H₂₆O₆: C, 61.07; H, 8.27%. Found C, 61.33; H, 8.45%.

Chapter 4

A comparison of the nanoprecipitation of linear polymers, linear-dendritic hybrids, branched copolymers, terpolymers and hyp-polydendrons
CHAPTER 4

4.1 Introduction

Polymeric nanoparticles (NPs) have been extensively studied as drug carriers in the pharmaceutical field\textsuperscript{1,2,3,4,5} and reviews of the mechanism of NP formation,\textsuperscript{6,7} the classification of NP systems and preparation techniques\textsuperscript{8,9} have been published by a number of research collaborations. The interest in nanoprecipitation is on a continuous rise due to its versatility and compatibility with a range of polymeric materials, as well as the rapid formation of well-defined NPs in water.\textsuperscript{10,11,12}

The nanoprecipitation method, also known as solvent displacement or interfacial deposition, was first reported in 1989 by Fessi \textit{et al.}\textsuperscript{13} Accordingly, the preparation of NPs was described to require both solvent and anti-solvent phases in order to produce a rapid and controlled assembly of stable hydrophobic organic macromolecules. The aggregation of macromolecules has been shown to continue until a colloidally stable dispersion is formed, with stability derived from steric or electrostatic repulsion. Nanoprecipitation is believed to form NPs in three distinct stages: nucleation, growth and aggregation.\textsuperscript{14} The rate of each step governs the particle size and the driving force of these phenomena is super-saturation, which is the ratio of polymer concentration and polymer solubility in the solvent mixture.

During a typical aqueous nanoprecipitation experiment a solution of hydrophobic polymer in a good, water-miscible organic solvent, is added to water which acts as the solvent-miscible anti-solvent (Figure 4.1A). This leads to NP formation \textit{via} the suggested nucleation/growth mechanism.\textsuperscript{14} During diffusion of the solvent and anti-solvent phases, the solvated and expanded polymer chains collapse and consequently associate to form colloidally-stable NPs.

Successful literature examples of nanoprecipitation include the large-scale generation of polymeric NPs under clinically relevant conditions, highlighted in recent reports of positive phase II human clinical trial results from nanoprecipitates derived from linear A-B block copolymers of $p$(ethylene glycol) (PEG) and either $p$(lactic acid)$\textsuperscript{15,16,17}$ or $p$(alkylcyanoacrylate)$\textsuperscript{18}$. Nanoprecipitation provides a facile, less complex and widely applicable technique compared to dialysis.$\textsuperscript{19}$ It also allows for polymer and guest molecules to be co-dissolved in a common solvent to generate early indications of potential encapsulation before undertaking further, more complicated studies.
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Figure 4.1 General nanoprecipitation method. A) Drop-wise addition of polymer dissolved in water miscible good solvent to water, leading to mixing and formation of a solvent/anti-solvent environment (i); B) Evaporation of water miscible solvent from water causing aggregation of NPs (ii); forming (C) a stable aqueous NP dispersion.

4.2 Nanoprecipitation of linear polymers, linear-dendritic hybrids, branched copolymers, terpolymers and hyp-polydendrons

All linear polymers, linear-dendritic polymer hybrids, branched copolymers, terpolymers and hyp-polydendrons described in Chapter 3 were evaluated for their ability to nanoprecipitate. Figure 4.2 shows the suggested collapse and aggregation behaviour of linear-dendritic polymer hybrids and hyp-polydendrons following nanoprecipitation.

Figure 4.2 Diagrammatic representation of the nanoprecipitation of A) linear-dendritic hybrids, and B) hyperbranched-polydendrons derived from dendron initiators. A proposed stable NP dispersion is achieved following: i) initial collapse of hydrophobic polymers and; ii) association and aggregation to produce stabilised nanoprecipitates.
A variety of nanoprecipitation parameters were previously investigated in order to optimise the conditions for NP formation and identify common parameters for use with the materials synthesised in Chapter 2; this included comparisons of slow addition (Figure 4.3A) and fast addition (Figure 4.3B) of the polymer solution to the aqueous anti-solvent media. The majority of these \( p(2\text{-hydroxypropyl methacrylate-co-ethylene glycol dimethacrylate}) \) \( (p(\text{HPMA}_{50}\text{-co-EGDMA}_x)) \) materials displayed similar NP sizes with narrower particle size distributions when added to the aqueous phase via the slower, drop-wise method. This implied a slightly more controlled NP formation manner when this technique was used.

Figure 4.3 Diagrammatic representation of the nanoprecipitation parameters investigated in Chapter 2 including fast (A) vs. slow (B) addition of polymer solution to aqueous media; and targeting varying polymer end concentrations in aqueous media, where (C) represents a lower polymer concentration and (D) represents a higher polymer concentration following evaporation of good solvent overnight.

Additionally, the initial polymer concentration in solvent and the targeted final NP concentration in water was varied (Figure 4.3C and D). Stable NP dispersions were generally produced for all copolymers in Chapter 2, when targeting a final NP concentration of 1 mg mL\(^{-1}\). This was independent of the starting polymer concentration, and a starting polymer concentration of 5 mg mL\(^{-1}\) overall produced NPs with narrower size distributions.

Following the studies outlined in Chapter 2, and to allow direct comparisons for the range of polymer variants synthesised in Chapter 3, all polymers reported here were dissolved at a standardised initial concentration of 5 mg mL\(^{-1}\) to produce a targeted 1 mg mL\(^{-1}\) aqueous NP dispersion, after drop-wise addition and subsequent solvent removal by evaporation overnight at ambient temperature. The tertiary butyl
methacrylate polymers (\(p(t\text{BuMA}_{50})\) and \(p(t\text{BuMA}_{50-c-o-EGDMA}_{3})\)) were dissolved in tetrahydrofuran (THF) as the good solvent phase, whilst all other linear, linear-dendritic and branched polymers containing 2-(diethylamino)ethyl methacrylate (DEA) were dissolved in acetone, following the failure of THF to act as a good solvent in these cases.

Analysis of the stable NP dispersions was conducted by dynamic light scattering (DLS) to assess NP hydrodynamic diameters (\(D_z\)), zeta-potentials (\(\zeta\)) and polydispersities (PDI). For all materials containing DEA, nanoprecipitation studies utilising acidic water as the anti-solvent were conducted and final pH values after nanoprecipitation were measured. Due to protonation of these materials at low pH (or upon the addition of acid following pH studies), number average diameters (\(D_n\)) and derived count rates (DCR) were interpreted with caution as unreliable data may be readily generated. The reasoning for this behaviour is described below.

According to Rayleigh Theory,\(^{20}\) the light scattering intensity produced by particles is proportional to \(r^6\) where \(r\) is the particle radius. Light scattered by a particle of diameter, \(2r\), and refractive index, \(n\), from a beam of unpolarised light of wavelength \(\lambda\) and intensity \(I_0\) is given in Equation 4.1, where \(R\) is the distance to the particle and \(\theta\) is the scattering angle.

\[
l = I_0 \frac{1 + \cos^2 \theta}{2R^2} \left(\frac{2\pi}{\lambda}\right)^2 \left(\frac{n^2 - 1}{n^2 + 2}\right) r^6
\]

**Equation 4.1** Rayleigh Theory.

This infers that a theoretical particle diameter of 50 nm scatters light up to 1,000,000 times more than a particle diameter of 5 nm (i.e. factor of the 10 in radius), meaning the presence of any large particles, even a very small number, in a NP dispersion will misrepresent the size distribution to generate an inaccurate weighting of the particle sizes present when studied under scattering intensity considerations only. It is, therefore, vital to take into consideration the number distribution of the dispersion; number distributions are calculated from scattering data using Mie Theory,\(^{21}\) which calculates the particle size distribution assuming a volume equivalent sphere model. This model is an exact description of how spherical particles of all sizes and optical properties scatter light. The DLS instrument directly measures light scattering and utilises Mie theory to extrapolate to a number distribution, therefore a number of assumptions, such as the
perfect spherical nature of the particles, are utilised which may not accurately represent
the sample under investigation and lead to additional errors within the calculated number
distribution.

Figure 4.4 shows a hypothetical example of a population comprising two distinct near-
mono-disperse particle populations with diameters of 5 and 50 nm in a 1:1 number ratio. The
number distribution (Figure 4.4A) shows two peaks centred at 5 and 50 nm, with a
1:1 number ratio. The volume distribution (Figure 4.4B), shows the two peaks in a
1:1,000 ratio, as the volume of a sphere is equal to \( 4/3\pi r^3 \). The intensity distribution
shows a 1:1,000,000 ratio between the two distributions, as the intensity of scattering is
proportional to \( r^6 \) from Rayleigh's approximation. The DCR was also monitored to
confirm either nanoprecipitate aggregation, or the existence of solubilised polymers in
low pH.

![Diagrammatic representation of DLS analysis through A) number; B) volume and C) intensity.](image)

Figure 4.4 Diagrammatic representation of DLS analysis through A) number; B) volume and C) intensity.

4.2.1 Nanoprecipitation studies of linear, linear-dendritic DEA polymers and
DEA-EGDMA branched copolymers and hyp-polydendrons

Polymers derived from DEA are well known for solubilising in acidic media and
existing as a cationic polyelectrolyte due to protonation of the tertiary amines.\(^{22}\) Reports
of \( \rho \)(DEA) also describe quarternisation of the tertiary amine groups using reagents such
as methyl iodide,\(^{23}\) in some cases forming highly branched water-soluble
polyelectrolytes after polymerisation using atom transfer radical polymerisation
(ATRP)-based self-condensing vinyl polymerisation techniques.\textsuperscript{24} DEA-based NPs have also been widely exploited due to the advantageous applications that polycations have to offer.\textsuperscript{25} Cross-linked polymer NPs containing a pH-responsive core and hydrophilic charged shell have been designed to disrupt endosomes and mediate drug/cell binding.\textsuperscript{26} These developments were designed in consideration of the polymer’s ability to absorb protons in response to acidification within endosomes, theoretically disrupting vesicles. Double hydrophilic graft copolymers containing p(DEA) have also been prepared to introduce colloidal stability to gold NPs with controllable size in aqueous media without any external reducing agent.\textsuperscript{27} Weaver et al. have polymerised DEA and EGDMA from a PEG macroinitiator under conventional free radical polymerisation conditions to produce pH-responsive branched NPs.\textsuperscript{28} Further investigations have led to cross-linking the hydrophilic shell domains, rendering the micellar aggregates as single nanoparticulates that do not display critical micelle concentrations and are stable to very high dilution.\textsuperscript{29}

Over recent decades, the self-assembly behaviour of tertiary amine methacrylate-based copolymers in alternating pH environments has been extensively explored.\textsuperscript{30} The design and synthesis of such copolymers has provided a means to control the pK\textsubscript{a} values of varying copolymers \textit{via} changes in the chemical structure and/or the composition of the pH-responsive polymer to meet the requirements of particular target applications. By amending the solubility/insolubility balance, pK\textsubscript{a} values have existed between plasma physiological pH (~7.4) and abnormal pathologic tissue\textsuperscript{31} (~7.2-6.5) or intracellular micro-environmental pH.\textsuperscript{32,33}

Solution properties and micellisation behaviour of double hydrophilic diblock copolymers, \(p(\text{hexaethylene glycol methacrylate}_{x-co-\text{DEA}y})\) comprising an ionisable \(p(\text{DEA})\) domain, in aqueous media have been investigated through DLS and \(^1\text{H} \) nuclear magnetic resonance (NMR) spectroscopy over a range of different pH values.\textsuperscript{34} These findings revealed a copolymer in its unimer state at low pH due to the hydrophilicity of the protonated tertiary amine units, while an increase of the solution pH resulted in the deprotonation of the amine residues, which subsequently become hydrophobic and lead to the formation of micelles consisting of a DEA core and a \(p(\text{hexaethylene glycol methacrylate})\) corona. These investigations have demonstrated the potential for drug delivery vehicle platforms to be generated.
Within this study, nanoprecipitation studies were conducted initially for the range of p(DEA)-containing materials described in Chapter 3; namely linear EBiB-p(DEA\textsubscript{50}), linear-dendritic AmG\textsubscript{0}-p(DEA\textsubscript{50}), AmG\textsubscript{1}-p(DEA\textsubscript{50}), AmG\textsubscript{2}-p(DEA\textsubscript{50}), AmG\textsubscript{1}-U-p(DEA\textsubscript{50}), branched EBiB-p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.95}) and the hyp-polydendrons AmG\textsubscript{0}-p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}), AmG\textsubscript{1}-p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}), AmG\textsubscript{2}-p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) and AmG\textsubscript{1}-U-p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}), Table 4.1.

NPs formed from EBiB-p(DEA\textsubscript{50}) were significantly more polydisperse than dispersions generated from the branched analogue, suggesting a much more controlled aggregation of highly branched polymers and the rapid attainment of stability during the nanoprecipitation of these materials. This architectural effect is also seen across all of the linear-dendritic and hyp-polydendron materials in Table 4.1, strongly suggesting the influence of branching being a critical parameter within the behaviour of these materials. EBiB-p(DEA\textsubscript{50}) formed smaller NPs than EBiB-p(HPMA\textsubscript{50}) under neutral aqueous conditions, however, the size distribution was bimodal when using tertiary-amine methacrylates. Despite the somewhat broad and multi-modal distributions within the linear-dendritic polymer series, both these materials and their hyp-polydendron equivalents, across all generations of dendron end group, appeared to form stable NPs (Table 4.1 and Figure 4.5) following the same trend as seen with EBiB-initiated materials; linear dendritic polymer hybrids much more polydisperse than their branched equivalents. The linear polymer and linear-dendritic polymer hybrids produced bimodal size distributions in neutral water upon inspection by DLS (Figure 4.5A), resulting in their large PDI values, and existed as two populations showing lack of control in NP formation.

![Figure 4.5 DLS size distribution analysis of A) AmG\textsubscript{2}-p(DEA\textsubscript{50}) and B) AmG\textsubscript{0}-p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) (green), AmG\textsubscript{1}-p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) (blue) and AmG\textsubscript{2}-p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) (red) NP dispersions in pH=7.8.](image-url)
All HPMA-containing linear-dendritic polymer hybrids failed to form stabilised nanoprecipitates in neutral pH aqueous conditions (Chapter 2). The marked difference in NP formation between the linear-dendritic hybrids and the hyp-polydendrons appears to result from branching, which may influence how the initial nuclei form, aggregate and assemble during the nanoprecipitation process. The formation of nuclei from hyp-polydendrons will benefit from the fraction of the overall molecular weight distribution comprising large numbers of conjoined chains as, in principle, the collapse of a single branched molecule containing, for example, > 50 primary chains will form a nucleus that would otherwise require 50 linear-dendritic hybrid chains to coalesce into a single structure (Figure 4.6). Formation of such nuclei allow the linear fraction of the hyp-polydendron molecular weight distribution to assemble quickly, rather than form small structures containing low numbers of chains that slowly build into nuclei themselves.

Figure 4.6 Proposed aggregation in aqueous media of A) Linear-dendritic polymer hybrids; and B) hyp-polydendrons.
Table 4.1 DLS analysis of nanoprecipitated particles from linear, linear-dendritic and branched polymers and hyp-polypendrons prepared in neutral water (pH 7.8) at a concentration of 1 mg mL\(^{-1}\).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>(D_z) (nm)</th>
<th>PDI</th>
<th>(D_n) (nm)</th>
<th>(\zeta) (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBiB-p(HPMA(_{50}))</td>
<td>780</td>
<td>0.230</td>
<td>-</td>
<td>-34</td>
</tr>
<tr>
<td>AmG(<em>{0})-p(HPMA(</em>{50}))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG(<em>{1})-p(HPMA(</em>{50}))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG(<em>{2})-p(HPMA(</em>{50}))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EBiB-p(HPMA(<em>{50})-co-EGDMA(</em>{0.95}))</td>
<td>178</td>
<td>0.082</td>
<td>145</td>
<td>-20</td>
</tr>
<tr>
<td>AmG(<em>{0})-p(HPMA(</em>{50})-co-EGDMA(_{0}))</td>
<td>157</td>
<td>0.269</td>
<td>75</td>
<td>-12</td>
</tr>
<tr>
<td>AmG(<em>{1})-p(HPMA(</em>{50})-co-EGDMA(_{0}))</td>
<td>165</td>
<td>0.097</td>
<td>124</td>
<td>-31</td>
</tr>
<tr>
<td>AmG(<em>{2})-p(HPMA(</em>{50})-co-EGDMA(_{0}))</td>
<td>149</td>
<td>0.099</td>
<td>108</td>
<td>-22</td>
</tr>
<tr>
<td>EBiB-p(DEA(_{50}))</td>
<td>47</td>
<td>0.249</td>
<td>24</td>
<td>+23</td>
</tr>
<tr>
<td>AmG(<em>{0})-p(DEA(</em>{50}))</td>
<td>51</td>
<td>0.413</td>
<td>21</td>
<td>+21</td>
</tr>
<tr>
<td>AmG(<em>{1})-p(DEA(</em>{50}))</td>
<td>148</td>
<td>0.249</td>
<td>13</td>
<td>+16</td>
</tr>
<tr>
<td>AmG(<em>{2})-p(DEA(</em>{50}))</td>
<td>65</td>
<td>0.381</td>
<td>12</td>
<td>+19</td>
</tr>
<tr>
<td>AmG(<em>{1})-U-p(DEA(</em>{50}))</td>
<td>82</td>
<td>0.408</td>
<td>33</td>
<td>+45</td>
</tr>
<tr>
<td>EBiB-p(DEA(<em>{50})-co-EGDMA(</em>{0.95}))</td>
<td>139</td>
<td>0.088</td>
<td>102</td>
<td>+34</td>
</tr>
<tr>
<td>AmG(<em>{0})-p(DEA(</em>{50})-co-EGDMA(_{0}))</td>
<td>168</td>
<td>0.086</td>
<td>123</td>
<td>+47</td>
</tr>
<tr>
<td>AmG(<em>{1})-p(DEA(</em>{50})-co-EGDMA(_{0}))</td>
<td>74</td>
<td>0.086</td>
<td>54</td>
<td>+31</td>
</tr>
<tr>
<td>AmG(<em>{2})-p(DEA(</em>{50})-co-EGDMA(_{0}))</td>
<td>58</td>
<td>0.065</td>
<td>43</td>
<td>+22</td>
</tr>
<tr>
<td>AmG(<em>{1})-U-p(DEA(</em>{50})-co-EGDMA(_{0}))</td>
<td>68</td>
<td>0.172</td>
<td>39</td>
<td>+26</td>
</tr>
<tr>
<td>AmG(<em>{2})-p(DEA(</em>{33})-co-HPMA(_{17}))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG(<em>{2})-p(DEA(</em>{33})-co-HPMA(_{17}))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG(<em>{2})-p(DEA(</em>{33})-co-HPMA(_{17}))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG(<em>{2})-p(DEA(</em>{33})-co-HPMA(<em>{17})-co-EGDMA(</em>{0}))</td>
<td>144</td>
<td>0.113</td>
<td>106</td>
<td>+16</td>
</tr>
<tr>
<td>AmG(<em>{2})-p(DEA(</em>{25})-co-HPMA(<em>{25})-co-EGDMA(</em>{0}))</td>
<td>183</td>
<td>0.166</td>
<td>140</td>
<td>+16</td>
</tr>
<tr>
<td>AmG(<em>{2})-p(DEA(</em>{17})-co-HPMA(<em>{33})-co-EGDMA(</em>{0}))</td>
<td>113</td>
<td>0.099</td>
<td>82</td>
<td>+13</td>
</tr>
</tbody>
</table>

\(^{1}\) Italics indicate a bimodal size distribution measured by DLS.

\(^{2}\) – indicates failure to form stabilised NPs without observable macroscale precipitation.
CHAPTER 4

The hyp-polydendrons experience a decrease in PDI as the amount of tertiary amine increases on the dendron (Figure 4.5B), suggesting the NPs arrange and pack themselves in a slightly more controlled manner and achieve colloidal stability earlier in the growth/aggregation stage of nanoprecipitation. This is also observed in the decrease in $D_z$ and $D_n$ values as the tertiary amine density at the polymer chain ends increases (see Chapter 2) from AmG$_0^-$ through to AmG$_2^-$. This does also imply that a decreasing amount of polymeric material is required to achieve colloidally stable NPs; these clear trends are not seen in the linear equivalent materials. The HPMA-containing series of hyp-polydendrons (Chapter 2), also show a decrease in PDI with increasing dendron generation, albeit to a single value, with broader size distributions (PDI = 0.269) generated when using the AmG$_0^-$ end group; comparison to nanoprecipitations of DEA-branched materials containing the AmG$_0^-$ end group (PDI = 0.086) suggesting the importance of primary chain chemistry.

The EBiB-, AmG$_0$, AmG$_1^-$, AmG$_2^-$ and AmG$_1^U^-$ hyp-polydendrons exhibited positive $\zeta$ values, with the presence of extra amine functionality provided by the dendrons not significantly influencing these observations; although a higher zeta potential is seen for all branched EBiB-, AmG$_0$, AmG$_1^-$ and AmG$_2^-$ materials in comparison to their linear equivalents and the branched materials do show a weak trend towards higher potentials with decreasing dendron generation. The highly positive zeta potentials observed for the EBiB-p(DEA$_{50}$) and EBiB-p(DEA$_{50^{0.95}}$) indicate the values are highly governed by the primary polymer chains. It is probable that, upon assembly and formation of these NPs, dendron chain ends would also become entrapped within the main bulk of the nanoprecipitates, as well as arranging themselves on the surface, hence further suggesting the zeta potential appears to be dominated by the amine functionality of the primary p(DEA$_{50}$) chains.

Both hyp-polydendrons bearing AmG$_1$ dendron chain ends with differing dendron chemistry (AmG$_1^-p$(DEA$_{50^{0.9}}$-co-EGDMA$_{0.95}$) and AmG$_1^U^-p$(DEA$_{50^{0.9}}$-co-EGDMA$_{0.9}$)) have almost identical $D_z$ values of 74 and 68 nm respectively (Table 4.1). This is a strong indication that the chemistry of the dendron end group is not as important as the number of terminal groups; however, the PDI does vary with the AmG$_1^U$ end group generating a much higher PDI.
4.2.2 Nanoprecipitation studies of linear, linear-dendritic, branched copolymers and hyp-polydendrons comprising tBuMA

Chapter 2 has interestingly shown stable NPs are produced by amine-functionalised linear-dendritic polymer hybrids in pH 4, however EBiB initiated materials, equivalent to those initiated by amine-functional dendrons, were not stable. This was determined to be due to the increased charge stabilisation available by the tertiary amine groups at the periphery of the dendron chain ends. The instability of EBiB-\(p(\text{HPMA}_{50}-\text{co-EGDMA}_{0.95})\) has already been reported in aqueous NaCl, and salt stability is essential for many biological applications.\(^1\)

Chapter 2 also explored the production of NPs by precipitation into aqueous HCl from amine-functionalised linear-dendritic \(p(\text{HPMA}_{50})\) and amine-functionalised \(p(\text{HPMA}_{50}-\text{co-EGDMA}_{0.9})\), following the failure of the former to form stable nanoprecipitates in water at near-neutral pH. This clearly indicated that the dendron chain ends contribute to the stabilisation of the nanoprecipitates, as the EBiB-initiated linear and branched analogues underwent macro-scale precipitation in acidic pH.

The nanoprecipitation of a range of materials containing \(p(\text{tBuMA}_{50})\) and \(p(\text{tBuMA}_{50}-\text{co-EGDMA})\) was therefore investigated within this study, as these highly-hydrophobic primary chains may behave very differently to the similar structures containing \(p(\text{HPMA}_{50})\) and \(p(\text{HPMA}_{50}-\text{co-EGDMA})\), discussed in Chapter 2.

Upon nanoprecipitation of the EBiB-\(p(\text{tBuMA}_{50})\), AmG2-\(p(\text{tBuMA}_{50})\), EBiB-\(p(\text{BuMA}_{50}-\text{co-EGDMA}_{0.95})\) and AmG2-\(p(\text{BuMA}_{50}-\text{co-EGDMA}_{0.9})\) polymers into neutral water (pH = 7.8), precipitation was observed with large polymer masses forming rapidly. Upon investigation by scanning electron microscopy (SEM), the macroscale precipitates were seen to be comprised of nanoparticles, strongly suggesting a successful nanoprecipitation process but a high level of instability of the NPs leading to uncontrolled aggregation (Figure 4.7). The instability of these polymers in pH 7.8, compared to the linear and branched HPMA materials, demonstrates the difference that polymer composition has on stable NP aggregation. The AmG2-\(p(\text{tBuMA}_{50})\), similarly resulted in macroscale precipitation compared to the AmG2-\(p(\text{HPMA}_{50})\), however, the latter was able to produce stable NPs in acidic water. This concept is applied to the AmG2-\(p(\text{tBuMA}_{50})\) later on in this study, in an attempt to produce stable NPs (cf. Section 4.2.4.3).
4.2.3 Nanoprecipitation studies of statistical linear-dendritic DEA-HPMA polymers and DEA-HPMA-EGDMA hyp-polydendrons

As previously observed, the AmG2 dendron chain end has provided excellent nanoprecipitation behaviour to a range of materials, possibly through playing a key role in determining the packing and arrangement of the nanoprecipitates produced from linear-dendritic polymer hybrids and hyp-polydendrons derived from HPMA. The range of statistical copolymers synthesised in Chapter 3, vary systematically in the degree of amphiphilicity in the primary polymer chains comprising the branched structure of the hyp-polydendrons, and this is expected to also modify the behaviour and stability of the NPs formed.

The linear-dendritic hybrid $\text{AmG}_{21} - p(\text{HPMA}_{50})$ failed to produce stable NPs upon nanoprecipitation into neutral (pH 7.8) water; however, $\text{AmG}_{21} - p(\text{HPMA}_{50} - co - \text{EGDMA}_{0.9})$ was conversely able to form NPs exhibiting control during nanoprecipitation into neutral water with polydispersity values $< 0.1$. This may be due to, as already mentioned, the instantaneous collapse of a large number of conjoined primary polymer chains within the hyp-polydendron on addition to water, compared to the collapse of single linear-dendritic hybrid polymer chains and subsequent assembly. The $\text{AmG}_{21} - p(\text{DEA}_{50})$ equally failed to demonstrate any level of control in neutral pH, confirmed by the bimodal distribution measured by DLS. The $\text{AmG}_{21} - p(\text{DEA}_{50} - co - \text{EGDMA}_{0.9})$ succeeded in producing stable NPs with a narrow size distribution (PDI = 0.065).
The DEA-HPMA statistical copolymer linear-dendritic hybrids, $\text{AmG}_2-p(\text{DEA}_{33}-\text{co}-\text{HPMA}_{17})$, $\text{AmG}_2-p(\text{DEA}_{25}-\text{co}-\text{HPMA}_{25})$, $\text{AmG}_2-p(\text{DEA}_{17}-\text{co}-\text{HPMA}_{33})$ and statistical hyp-polydendrons ($\text{AmG}_2-p(\text{DEA}_{33}-\text{co}-\text{HPMA}_{17}-\text{co}-\text{EGDMA}_{0.9})$, $\text{AmG}_2-p(\text{DEA}_{25}-\text{co}-\text{HPMA}_{25}-\text{co}-\text{EGDMA}_{0.9})$ and $\text{AmG}_2-p(\text{DEA}_{17}-\text{co}-\text{HPMA}_{33}-\text{co}-\text{EGDMA}_{0.9})$ were also evaluated under nanoprecipitation conditions in neutral water. All linear-dendritic polymers, once again, failed to form stable NPs and resulted in large aggregates precipitating out of solution. The hyp-polydendrons, however, successfully produced stable NP dispersions, supported by monomodal size distributions measured by DLS (Table 4.1 and Figure 4.8).

The inability of the linear-dendritic hybrids to form particles and the contrasting ready formation of nanoprecipitates by hyp-polydendrons, again, appears to confirm the clear benefit from having the complex architecture. As previously described, this is hypothesised to be due to the rate and mechanism of nucleation (cf. Figure 4.6), the efficiency of aggregation and attainment of charge stabilisation.

The positive $\zeta$ values measured for these statistical terpolymer hyp-polydendron nanoprecipitates were slightly lower (+13 to +16 mV) than the values of the NPs produced from $\text{AmG}_2-p(\text{DEA}_{50}-\text{co}-\text{EGDMA}_{0.9})$ (+22 mV); this may result directly from the minor decrease in DEA content within the primary chains. The nanoprecipitates formed from the $\text{AmG}_2-p(\text{DEA}_{17}-\text{co}-\text{HPMA}_{33}-\text{co}-\text{EGDMA}_{0.9})$ contained the narrowest size distribution and lowest $\zeta$ value, suggesting an increased level of control when using
a higher content of HPMA co-monomer in the NP formation, compared to the other statistical terpolymer hyp-polydendrons, and also the impact of the lower DEA content on the eventual ζ values.

4.2.4 Nanoprecipitation studies of linear-dendritic hybrid polymers and hyp-polydendrons containing DEA and tBuMA, and the effect of primary chain architecture

4.2.4.1 Nanoprecipitation studies of statistical linear-dendritic copolymers and hyp-polydendrons comprising DEA and tBuMA

Upon the nanoprecipitation of the statistical linear-dendritic hybrids and hyp-polydendrons containing tBuMA and DEA: namely AmG2-p(DEA33-co-tBuMA17), AmG2-p(DEA25-co-tBuMA25), AmG2-p(DEA17-co-tBuMA33), AmG2-p(DEA33-co-tBuMA17-co-EGDMA0.9), AmG2-p(DEA25-co-tBuMA25-co-EGDMA0.9) and AmG2-p(DEA17-co-tBuMA33-co-EGDMA0.9); macroscale precipitation was observed in all cases following evaporation of acetone. This is in marked contrast to the hyp-polydendrons comprising statistical primary chains of DEA and HPMA, and the incorporation of the highly hydrophobic monomer, tBuMA, appears to completely dominate the solution behaviour.

4.2.4.2 Nanoprecipitation studies of block linear-dendritic DEA-tBuMA polymers and DEA-tBuMA-EGDMA hyp-polydendrons

The polymer architecture of the primary chain copolymers could also be modified to form a block copolymer (or gradient copolymer), where the hydrophobic tBuMA monomers that dominated the statistical copolymer behaviour were predominantly isolated into one block segment. It was hypothesised that this architectural difference would potentially allow the formation of stable NPs comprised of DEA and tBuMA. The synthesis of linear-dendritic block polymer hybrids (Figure 4.9i) and block hyp-polydendrons (Figure 4.9ii) was therefore undertaken, using the same molar ratios of tBuMA: DEA utilised within the analogous statistical copolymer equivalents (66: 33, 50: 50 and 33: 66). This also allows a direct behavioural comparison based on the polymer architecture of the primary chains within the hyp-polydendron and the linear-dendritic hybrids.
Figure 4.9 Architectural variation of linear-dendritic hybrids and hyp-polydendrons comprising DEA and tBuMA to establish stable NPs in neutral water. i) Statistical and linear-dendritic block copolymers and; ii) statistical and block copolymer hyp-polydendrons.

Amphiphilic block copolymers conventionally contain connected hydrophilic and hydrophobic segments, providing an array of morphologies in the solid state and selective solvents.\textsuperscript{35,36} The nature of each polymer fragment has been extensively manipulated using many controlled polymerisation techniques,\textsuperscript{37} allowing for control over the chemical and physical properties desired for the block copolymer, and resulting in highly ordered structures during the self-assembly process.

Linear-dendritic block copolymer hybrids in low pH, allow for a bespoke opportunity for new micelle species through self-assembly. Previous studies bear witness to changes in self-assembly behaviour upon incorporation of stimuli-responsive polymers within the linear polymer chain.\textsuperscript{38} Examples include PEG-based biocompatible linear-dendritic copolymers that form thermo-responsive micelles that completely disrupt into unimers upon cooling below their lower critical solution temperature and then reform again upon heating,\textsuperscript{39} and assembled pH-responsive linear-dendritic micelles with tuneable drug release rates.\textsuperscript{40} The curious amphiphilic characteristic associated with the linear-dendritic hybrid architectures pinpoint evolving application potential,\textsuperscript{41} particularly the construction of nano-sized particles in environmentally friendly media.

The nanoprecipitation of all of the block linear-dendritic hybrids and block hyp-polydendrons into neutral water (pH=7.8), namely: \textit{AmG}_2-p(\textit{DEA}_{33}-b-t\textit{BuMA}_{17}), \textit{AmG}_2-p(\textit{DEA}_{25}-b-t\textit{BuMA}_{25}), \textit{AmG}_2-p(\textit{DEA}_{17}-b-t\textit{BuMA}_{33}), \textit{AmG}_2-p(\textit{DEA}_{33}-b-(t\textit{BuMA}_{17}-co-\textit{EGDMA}_{0.9})), \textit{AmG}_2-p(\textit{DEA}_{25}-b-(t\textit{BuMA}_{25}-co-\textit{EGDMA}_{0.9})) and \textit{AmG}_2-p(\textit{DEA}_{17}-b-(t\textit{BuMA}_{33}-co-\textit{EGDMA}_{0.9})); equally led to macroscale precipitation. This
CHAPTER 4

suggests the architecture of the primary chain copolymers is not critical to successful nanoprecipitation under these conditions.

4.2.4.3 The production of stable nanoprecipitates from DEA-\(t\)BuMA linear-dendritic polymer hybrids and and DEA-\(t\)BuMA-EGDMA \(hyp\)-polydendrons

Following the failed production of NPs from statistical and block DEA-\(t\)BuMA materials, two additional strategies to overcome this inability to controllably self-assemble were considered. Initially, the overall primary polymer chain length was increased within the block \(hyp\)-polydendron to produce chains with a similar DEA to \(t\)BuMA ratio to the shorter chain-containing block polymers but with an overall larger DEA component relative to dendron chain end. This may lead to a higher contribution to charge stabilisation from the \(p\)(DEA) block segment (Figure 4.10B); \textbf{AmG}_{2}\textbf{-}p(\text{DEA}_{50}\textbf{-}b-\text{(tBuMA}_{65}\textbf{-}co\text{-}EGDMA}_{0.9})) was subsequently synthesised to evaluate this hypothesis. This subtle variation in the branched polymer structure was observed to generate successful NP formation and prevent macroscale precipitation in neutral water; offering additional flexibility to the \(hyp\)-polydendron approach.

![Figure 4.10 Proposed solutions for producing stable \(p\)(DEA,\(t\)BuMA)-derived NPs: A) Nanoprecipitation into aqueous HCl for increased charge stabilisation; B) increased primary polymer length within the block \(hyp\)-polydendron.](image_url)

Secondly, the anti-solvent was replaced with acidic water (Figure 4.10A) due to the successful nanoprecipitation of \textbf{AmG}_{2}\textbf{-}p(\text{HPMA}_{50}\textbf{-}co\text{-}EGDMA}_{0.9}) NPs under these...
conditions, presumably due to the increased protonation of the dendron chain ends (Chapter 2). In the current case, charge-stabilisation was expected via dendron protonation with an additional contribution from primary polymer chain protonation.

The use of the acidic anti-solvent medium (aqueous HCl; pH = 4) was studied for all the statistical and block linear-dendritic polymer hybrids and hyp-polydendron materials comprising DEA and tBuMA (Table 4.2), to evaluate the ability of increased protonation to enable successful nanoprecipitation; nanoprecipitates were successfully formed for all materials without observable macrophase separation. In addition, the AmG2-p(DEA50) and AmG2-p(DEA50-co-EGDMA0.9) were nanoprecipitated under these conditions for comparison.

All statistical and block copolymer linear-dendritic hybrids and hyp-polydendrons containing all ratios of DEA and tBuMA formed stable NPs when nanoprecipitating into acidic water (Table 4.2). For these particular materials, the hyp-polydendrons were more polydisperse than the relevant linear equivalents, and often multi-modal within their size distributions.

The $D_n$ values for the statistical linear-dendritic polymers containing DEA and tBuMA were >17 nm (Table 4.2), confirming the lack of dissolution at the final solution pH, which was much higher than the initial pH = 4, similarly witnessed for the linear-dendritic DEA polymer and DEA-EGDMA hyp-polydendron. The narrow size distributions (Figure 4.11A) may have resulted from arrangement of the highly dense tertiary amine dendron to the NP surface in order to protect the hydrophobic tBuMA polymer components, within the nanoprecipitate, from the aqueous media. Upon DLS analysis of the corresponding hyp-polydendron samples, broader size distributions were clearly evident, suggesting that the complex architectures may be restricted in their mobility and unable to rearrange under these conditions to form clearly defined structures (Figure 4.11B).
Table 4.2 DLS analysis of nanoprecipitated particles (1 mg mL\(^{-1}\)) prepared from linear, linear-dendritic, branched polymer and hyp-polydendron solutions (5 mg mL\(^{-1}\)) derived from DEA and tBuMA into neutral (pH=7.8) and acidic (pH=4) water.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>pH=7.8*</th>
<th>pH=4.0(^{\dagger}) (Final pH=5.9-7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(D_z) (nm)</td>
<td>PDI</td>
</tr>
<tr>
<td>AmG2-p(DEA50)</td>
<td>65</td>
<td>0.381</td>
</tr>
<tr>
<td>AmG2-p(DEA50-co-EGDMA0.9)</td>
<td>58</td>
<td>0.065</td>
</tr>
<tr>
<td>AmG2-p(DEA33-co-tBuMA17)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(DEA25-co-tBuMA25)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(DEA17-co-tBuMA33)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(DEA33-co-tBuMA17-co-EGDMA0.9)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(DEA25-co-tBuMA25-co-EGDMA0.9)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(DEA17-co-tBuMA33-co-EGDMA0.9)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(DEA33-b-tBuMA17)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(DEA25-b-tBuMA25)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(DEA17-b-tBuMA33)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(DEA33-b-(tBuMA17-co-EGDMA0.9))</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(DEA25-b-(tBuMA25-co-EGDMA0.9))</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(DEA17-b-(tBuMA33-co-EGDMA0.9))</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(DEA50-b-(tBuMA65-co-EGDMA0.9))</td>
<td>163</td>
<td>0.082</td>
</tr>
<tr>
<td>AmG2-p(tBuMA50)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG2-p(tBuMA50-co-EGDMA0.9)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Initial pH of water.
\(^{\dagger}\) Italics indicates a bimodal size distribution measured by DLS.
\(^{\ddagger}\) – indicates failure to form stabilised NPs with observable macroscale precipitation.
Figure 4.11 DLS size distribution analysis for A) $\text{AmG}_2\text{-}p(\text{DEA}_{33}\text{-}co\text{-}t\text{BuMA}_{17})$ (green), $\text{AmG}_2\text{-}p(\text{DEA}_{25}\text{-}co\text{-}t\text{BuMA}_{25})$ (orange) and $\text{AmG}_2\text{-}p(\text{DEA}_{17}\text{-}co\text{-}t\text{BuMA}_{33})$ (red); B) $\text{AmG}_2\text{-}p(\text{DEA}_{33}\text{-}co\text{-}t\text{BuMA}_{17}\text{-}co\text{-}\text{EGDMA}_{0.9})$ (green), $\text{AmG}_2\text{-}p(\text{DEA}_{25}\text{-}co\text{-}t\text{BuMA}_{25}\text{-}co\text{-}\text{EGDMA}_{0.9})$ (orange) and $\text{AmG}_2\text{-}p(\text{DEA}_{17}\text{-}co\text{-}t\text{BuMA}_{33}\text{-}co\text{-}\text{EGDMA}_{0.9})$ (red) NP dispersions in pH=4.

The observed $D_n$ values within the statistical hyp-polydendron nanoprecipitates appear to increase as the DEA content decreases and the hyp-polydendrons become less soluble in low pH (Table 4.2); this is a general trend witnessed within each series of these materials studied.

Small NP diameters were observed for all linear-dendritic block copolymer hybrids ($D_n$ = 19-25 nm), all with monomodal size distributions (Figure 4.12A) and behaving very similarly to the equivalent statistical copolymer hybrid analogues. None of the materials were soluble in acidic water, and the $D_n$ confirms nanoprecipitate formation. A slight increase in $D_n$ values was witnessed as the level of hydrophobic $t$BuMA primary polymer chain was increased. The dispersity of the linear-dendritic block copolymer hybrid nanoprecipitate samples improved as the hydrophobic content increased, suggesting an increase in the driving forces for self-assembly and aggregation. In spite of the variation in DEA segment lengths, within the self-assembled linear-dendritic block copolymer nanoparticles, the $\zeta$ values obtained were virtually identical (+37, +38 and +37 mV) suggesting there is an equal tertiary amine density surrounding the nanoprecipitates, created by a combination of the DEA polymer and $\text{AmG}_2$ dendrons that are protonated in acidic pH.
Evidently, the low pH plays a vital role in the generation of stabilised NPs, again, suggesting a predominantly charge stabilised mechanism for such nanoprecipitates. In comparison to the statistical linear-dendritic equivalents, no significant difference in particle size ($D_z$ or $D_n$) was witnessed. The only note-worthy observation could be the high DCR obtained for $\text{AmG}_2p(\text{DEA17}-b-\text{tBuMA33})$, the polymer containing the highest level of hydrophobicity across the range of these co-polymeric dendritic hybrids.

Upon nanoprecipitation of the block copolymer hyp-polydendrons, $\text{AmG}_2p(\text{DEA33}-b-(\text{tBuMA}_{17}-\text{co-EGDMA}_{0.9}))$, $\text{AmG}_2p(\text{DEA25}-b-(\text{tBuMA}_{25}-\text{co-EGDMA}_{0.9}))$ and $\text{AmG}_2p(\text{DEA17}-b-(\text{tBuMA}_{33}-\text{co-EGDMA}_{0.9}))$ all formed stable nanoprecipitates at low pH (Figure 4.12B). $\text{AmG}_2p(\text{DEA25}-b-(\text{tBuMA}_{25}-\text{co-EGDMA}_{0.9}))$ produced the only monomodal size distribution (Figure 4.12B) and NPs with the largest $D_z$ and $D_n$ size values. The $\text{AmG}_2p(\text{DEA33}-b-(\text{tBuMA}_{17}-\text{co-EGDMA}_{0.9}))$ and $\text{AmG}_2p(\text{DEA17}-b-(\text{tBuMA}_{33}-\text{co-EGDMA}_{0.9}))$ produced bimodal size distributions accounting for the existence of a variety of nanoprecipitate species. For these two materials, almost identical values for particle sizes, count rates and surface charge were measured by DLS (Table 4.2).

As mentioned above, all block copolymer hyp-polydendrons exhibited broader size distributions in comparison to the linear-dendritic block copolymer hybrid equivalents. This trend was also observed when DEA-tBuMA statistical hyp-polydendrons were nanoprecipitated, suggesting the linear-dendritic polymer hybrids have a higher degree of freedom to form nanoprecipitates. This, however, was not witnessed within the DEA
series of linear-dendritic hybrids and hyp-polydendrons, where branching seemed to encourage more stabilised aggregations, having lower polydispersity values.

In all cases of statistical and block dendritic polymer materials comprising DEA and tBuMA, stable NPs were not generated via nanoprecipitation into neutral water. The block copolymer hyp-polydendron with increased primary chain length, \text{AmG}_2-p(\text{DEA}_{50}-b-(\text{tBuMA}_{65}-co-\text{EGDMA}_{0.9})), (Chapter 3, Section 3.4) was, however, able to successfully undergo nanoprecipitation into both neutral and acidic water (Figure 4.13), unlike any of the previous statistical and block hyp-polydendrons consisting of primary polymer chain lengths containing average degrees of polymerisation (DP\text{av}) = 50 monomer units (Table 4.2). The nanoprecipitates formed from the \text{AmG}_2-p(\text{DEA}_{50}-b-(\text{tBuMA}_{65}-co-\text{EGDMA}_{0.9})) hyp-polydendron exhibited a \(D_z = 163\) nm with a very low PDI = 0.082 in neutral water; a size within the previously reported range as successful for various nanocarrier candidates within the literature.\(^4\)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{dls_size_analysis.png}
\caption{DLS size analysis by intensity (A) for \text{AmG}_2-p(\text{DEA}_{50}-b-(\text{tBuMA}_{65}-co-\text{EGDMA}_{0.9})) in neutral (green) and acidic (red) water; and by number (B) in neutral (green) and acidic (red) water at a concentration of 1 mg mL\(^{-1}\).}
\end{figure}

The stable nanoprecipitates of \text{AmG}_2-p(\text{DEA}_{50}-b-(\text{tBuMA}_{65}-co-\text{EGDMA}_{0.9})) prepared in low pH = 4 water showed noticeably smaller \(D_z\) values than those formed in neutral water, suggesting a decreased amount of packing within the growth stages of the nanoprecipitation. This observation again suggests that the protonated \(p(\text{DEA})\) block segments contribute strongly to the stabilisation, but may also indicate that the longer chains may be providing steric stabilisation as solvated chains at the surface of the NP. It is also possible that the increased hydrophobicity from the longer \(p(\text{tBuMA})\) block segment may also play a role in the successful nanoprecipitation.
Comparatively, the nanoprecipitation of $\text{AmG}_2\text{p(DEA}_{50}\text{)}$ and $\text{AmG}_2\text{p(DEA}_{50'}\text{co-EGDMA}_{0.9})$ into aqueous HCl produced stable NPs, supported by the monomodal size distributions (Table 4.2 and Figure 4.14).

![Figure 4.14 DLS size distribution analysis of $\text{AmG}_2\text{p(DEA}_{50}\text{)}$ (red) and $\text{AmG}_2\text{p(DEA}_{50'}\text{co-EGDMA}_{0.9})$ (green) prepared in acidic water (pH=4) at a concentration of 1 mg mL$^{-1}$.

The $\text{AmG}_2\text{p(DEA}_{50}\text{)}$ produced NPs with more narrow PDI values within aqueous HCl than at neutral pH, most likely due to the more rapid and controlled establishment of charge stabilisation via efficient protonation. The $D_z$ values obtained for $\text{AmG}_2\text{p(DEA}_{50}\text{)}$ was very similar to the DEA/tBuMA-containing linear-dendritic block polymer hybrid NPs prepared in aqueous HCl than, for example, the large sizes measured for the linear amphiphilic hybrids in Chapter 2. The $\text{AmG}_2\text{p(DEA}_{50'}\text{co-EGDMA}_{0.9})$ produced stable, slightly smaller NPs in acidic media compared to those prepared in neutral water ($D_z = 49$ and 58 nm respectively) and the $D_n$ value of 32 nm confirms a lack of complete solubilisation at lower pH.

In a similar study, a comparison of the nanoprecipitation of $\text{EBiB}\text{p(tBuMA}_{50}\text{)}$, $\text{AmG}_2\text{p(tBuMA}_{50}\text{)}$, $\text{EBiB}\text{p(tBuMA}_{50'}\text{co-EGDMA}_{0.95})$ and $\text{AmG}_2\text{p(tBuMA}_{50'}\text{co-EGDMA}_{0.9})$ using an acidic water anti-solvent was conducted. Chapter 2 witnessed stable NP formation due to the amine functionality present in the linear-dendritic polymer hybrids and hyp-polydendrons of materials comprising $p$(HPMA) chains and the benefits of the amine functional dendron were also expected to be seen with $p$(tBuMA)-containing materials. As anticipated, the NPs were not successfully achieved for either the $\text{EBiB}\text{p(tBuMA}_{50}\text{)}$ polymer or $\text{EBiB}\text{p(tBuMA}_{50'}\text{co-EGDMA}_{0.95})$ copolymer under these conditions and macroscale precipitation was observed. In contrast, the linear-dendritic hybrid, $\text{AmG}_2\text{p(tBuMA}_{50}\text{)}$ and hyp-polydendron, $\text{AmG}_2\text{p(tBuMA}_{50'}\text{co-EGDMA}_{0.9})$
samples produced stable NP dispersions during addition to water at a low pH (Table 4.2) following no previous success at neutral pH (cf. Section 4.2.2). NPs formed from both the linear-dendritic polymer hybrid and hyp-polydendron exhibited highly positive ζ values as expected. Both NP distributions are relatively broad compared to the \( p(\text{HPMA}) \)-derived NPs; particularly the linear-dendritic polymer hybrid in comparison to \( \text{AmG}_2-p(\text{HPMA}_{50}) \) (PDI = 0.165), suggesting a less controlled aggregation; this may be due to the increased hydrophobicity of the \( \text{tBuMA} \) primary chains. Unlike the \( p(\text{HPMA}) \)-derived materials, the branched \( \text{AmG}_2-p(\text{tBuMA}_{50-co}-\text{EGDMA}_{0.9}) \) produced nanoprecipitates with a \( D_\zeta \) value greater than the linear equivalent. This may be due to the high molecular weight hyp-polydendron containing an increased amount of hydrophobic polymer compared to the linear-dendritic equivalent, and undergoing increased aggregation to achieve stabilisation. It is also important to note that the glass transition temperature of \( p(\text{tBuMA}) \) homopolymer is well known to be 118°C and as such, the rapid nanoprecipitation may lead to the “locking in” of polymer conformations and a reduced ability to relax after addition to the anti-solvent.

4.3 pH-responsive studies of linear polymers, linear-dendritic hybrids, branched copolymers and hyp-polydendrons

The materials that produced monomodal, stable NP dispersions in neutral water were subjected to the rapid addition of acid (1M HCl) to establish any pH-responsive behaviour or ability to withstand environmental changes. Following acid addition, the changes in nanoprecipitate structure and assembly were investigated by DLS analysis and compared. The materials which were stable when generated using aqueous anti-solvent at \( \text{pH} = 4 \), and those that produced bimodal distributions, were not included in the study. If the materials were to be considered as nanomedicine drug delivery vehicles, a large number of drug molecules are acidic or basic and the requirements of anti-solvent pH may be highly restrictive. For example, encapsulation of a basic drug in acidic aqueous conditions would be impractical, therefore, NPs prepared in neutral water are more widely applicable and less likely to cause difficulties in drug encapsulation.

Chapter 2 demonstrated interesting behaviour upon the rapid-switching of pH, for nanoprecipitates formed from linear-dendritic polymer hybrids and hyp-polydendrons comprised of HPMA. Successful NP formation has been observed for the majority of linear polymers, linear-dendritic hybrids, copolymers, terpolymer and hyp-polydendrons.
synthesised in Chapter 3, following nanoprecipitation into neutral water. The majority of these materials contain pH-responsive compositions with varying DEA content and primary polymer architecture, therefore the behaviour of the different materials upon a switch in pH is of interest.

pH-responsive polymers have gathered much attention due to their emerging \textit{in vitro} studies, \textit{in vivo} biomedical applications and the relevance of modulating pH in living organisms.\textsuperscript{43} This attractiveness has extended to the exploration of terpolymers, the majority of which exhibit pH-responsiveness \textit{via} pH-cleavable chemical linkages.\textsuperscript{44} Tertiary amine methacrylate-based polymers, such as \textit{p}(DEA\textsubscript{50}-\textit{co}-EGDMA\textsubscript{x}), have the advantage of possessing pH-responsive properties without the production of any byproducts upon hydrolysis or acidolysis.

Also included in this study, was the preparation of NPs from \textit{hyp}-polydendrons containing the acid-cleavable brancher, 1,4-butanediol di(methacryloxy)-ethyl ether (BDME; Chapter 3, Section 3.7), such as AmG\textsubscript{2}-\textit{p}(DEA\textsubscript{50}-\textit{co}-BDME\textsubscript{2.0}), AmG\textsubscript{2}-\textit{p}(DEA\textsubscript{33}-\textit{co}-HPMA\textsubscript{17}-\textit{co}-BDME\textsubscript{2.0}) and AmG\textsubscript{2}-\textit{p}(DEA\textsubscript{17}-b-(\textit{t}BuMA\textsubscript{33}-\textit{co}-BDME\textsubscript{2.0})), which all successfully produced stable NPs in neutral water.

In general, the size and charge of the majority of NPs used for nanomedicine applications can have an effect on efficient clearance from the body as intact NPs and degradation into biologically benign components and clearance is required.\textsuperscript{45} This process still creates concerns for many clinical applications as, without efficient clearance or degradation, toxicity may arise from the nanocarrier material which is a potential long term health issue. For globular proteins, a $D_z$ of approximately 5–6 nm appears to allow rapid clearance from the body \textit{via} renal filtration and urinary excretion.\textsuperscript{46}

The BDME-containing \textit{hyp}-polydendrons synthesised here provide potential opportunities for two modes of NP degradation: first, the dissociation of the nanoprecipitate into solvated branched polymers, and secondly the degradation of the branched polymers into their individual primary chains of relatively mono-disperse and low molecular weight (Figure 4.15).
4.3.1 pH-responsive studies of linear, linear-dendritic DEA polymers and DEA-EGDMA branched copolymers and hyp-polydendrons

Synthetic pH-responsive polymers are formed from monomers that are weakly basic or acidic. These moieties protonate/deprotonate reversibly as a function of pH, the adjustment of which alters the ionic interaction, hydrogen bonding and hydrophobic interaction and results in a reversible microphase separation or self-organisation phenomenon. Consequently, acid-responsive polymers are fully soluble in aqueous solution at low pH, being largely hydrated, swollen and hydrophilic in their ionic state. At high pH, the polymers become dehydrated, compact and more hydrophobic in their neutral form. This transition occurs at a pH that is defined as the apparent pKₐ or pKₖ and is very susceptible to change when the chemical substituents on the polymer backbone or the polymeric architectures are adjusted. A change in behaviour upon a switch in pH is specifically attractive for biological applications due to the abundant pH gradients existing in normal and pathophysiological states.

All NPs generated from DEA-containing polymers were subjected to the rapid switching of pH to observe and understand their pH-responsive behaviour (Table 4.3). Only the AmG₂₋₇p(DEA₅₀₋₇₀-BDME₂₀₀) from the branched DEA hyp-polydendron series was included for comparative NP behaviour.
Table 4.3 DLS analysis following the addition of acid on nanoprecipitated particles prepared from branched DEA, DEA-HPMA and DEA-\(t\)BuMA \(hyp\)-polydendron NP dispersions prepared in neutral water pH=7.8.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>(D_z) (nm)</th>
<th>PDI</th>
<th>(D_n) (nm)</th>
<th>DCR (kcps)</th>
<th>(D_z) (nm)</th>
<th>PDI</th>
<th>(D_n) (nm)</th>
<th>DCR (kcps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(AmG_2-p(\text{DEA}<em>{50}-co-\text{EGDMA}</em>{0.9}))</td>
<td>58</td>
<td>0.065</td>
<td>43</td>
<td>97000</td>
<td>47</td>
<td>0.364</td>
<td>6</td>
<td>850</td>
</tr>
<tr>
<td>(AmG_2-p(\text{DEA}<em>{33}-co-\text{HPMA}</em>{17}-co-\text{EGDMA}_{0.9}))</td>
<td>144</td>
<td>0.113</td>
<td>106</td>
<td>391100</td>
<td>37</td>
<td>0.515</td>
<td>6</td>
<td>300</td>
</tr>
<tr>
<td>(AmG_2-p(\text{DEA}<em>{25}-co-\text{HPMA}</em>{25}-co-\text{EGDMA}_{0.9}))</td>
<td>183</td>
<td>0.166</td>
<td>140</td>
<td>533400</td>
<td>63</td>
<td>0.462</td>
<td>7</td>
<td>700</td>
</tr>
<tr>
<td>(AmG_2-p(\text{DEA}<em>{17}-co-\text{HPMA}</em>{33}-co-\text{EGDMA}_{0.9}))</td>
<td>113</td>
<td>0.099</td>
<td>82</td>
<td>95100</td>
<td>164</td>
<td>0.363</td>
<td>16</td>
<td>1500</td>
</tr>
<tr>
<td>(AmG_2-p(\text{DEA}<em>{50}-b-(\text{tBuMA}</em>{65}-co-\text{EGDMA}_{0.9})))</td>
<td>163</td>
<td>0.082</td>
<td>123</td>
<td>451800</td>
<td>192</td>
<td>0.079</td>
<td>156</td>
<td>232960</td>
</tr>
<tr>
<td>(AmG_2-p(\text{DEA}<em>{50}-co-\text{BDME}</em>{2.0}))</td>
<td>61</td>
<td>0.241</td>
<td>32</td>
<td>38700</td>
<td>457</td>
<td>0.535</td>
<td>5</td>
<td>266</td>
</tr>
<tr>
<td>(AmG_2-p(\text{DEA}<em>{33}-co-\text{HPMA}</em>{17}-co-\text{BDME}_{2.0}))</td>
<td>410</td>
<td>0.204</td>
<td>310</td>
<td>46400</td>
<td>131</td>
<td>0.418</td>
<td>5</td>
<td>925</td>
</tr>
<tr>
<td>(AmG_2-p(\text{DEA}<em>{17}-b-(\text{tBuMA}</em>{33}-co-\text{BDME}_{2.0})))</td>
<td>107</td>
<td>0.086</td>
<td>76</td>
<td>286300</td>
<td>134</td>
<td>0.073</td>
<td>103</td>
<td>284200</td>
</tr>
</tbody>
</table>

\(a\) Initial pH

\(i\) Italics indicates a bimodal size distribution measured by DLS.

Upon rapid acid addition, \(AmG_2-p(\text{DEA}_{50}-co-\text{EGDMA}_{0.9})\) NPs resulted in a colourless solution (final pH = 2.2-3.2) (Figure 4.16B), confirming complete solubilisation of the materials at this low pH. The DCR value also dropped significantly, clearly indicating that this \(hyp\)-polydendron nanoprecipitate existed as dissolved unimolecular species under these conditions (Figure 4.16A). This confirms that the \(D_z\) and broad PDI values measured following acid addition were not true indications of the main population of particles existing in this acidic environment. A review of the \(D_n\) value following the pH change showed a diameter of 6 nm, typical of a fully solvated high molecular weight branched polymer (Figure 4.17A), as opposed to the measured \(D_z\) value of 43 nm measured under neutral conditions.
4.3.2 pH-responsive studies of statistical linear-dendritic DEA-HPMA polymers and DEA-HPMA-EGDMA hyp-polydendrons

Following the observation of the fully soluble AmG$_2$-$p$(DEA$_{50}$-co-EGDMA$_{0.9}$) hyp-polydendron in aqueous solution at low pH, the response of the range of statistical AmG$_2$-$p$(DEA$_{33}$-co-HPMA$_{17}$-co-EGDMA$_{0.9}$), AmG$_2$-$p$(DEA$_{25}$-co-HPMA$_{25}$-co-EGDMA$_{0.9}$) and AmG$_2$-$p$(DEA$_{17}$-co-HPMA$_{33}$-co-EGDMA$_{0.9}$) hyp-polydendron NPs was investigated. As previously observed, the measured $D_z$ values suggested the presence of large, stable nanoprecipitates still remaining within the dispersion (Figure 4.18A).
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However, as suggested by the large PDI values implying a broad distribution of species, upon evaluation of the $D_n$ values (Figure 4.18B) and the DCR, it is quite clear that the dispersion contains merely dissolved material.

![Figure 4.18 DLS analysis of AmG2-p(DEA25-co-HPMA25-co-EGDMA0.9). A) Size distribution analysis and; B) Number distribution analysis before (purple) and after addition (ret-dotted).](image)

The $D_n$ values increase slightly with increasing HPMA within the hyp-polydendron copolymers, with the largest diameter of 16 nm observed for the $\text{AmG}_2-p(\text{DEA}_{17}-\text{co-HPMA}_{33}-\text{co-EGDMA}_{0.9})$ hyp-polydendron. The fraction of protonatable monomer residues within the polymers at low pH decreases across the series of materials studied which may result in a low level of residual polymer-polymer interaction at higher HPMA content. The sizes may also be an effect of the molecular weight values of the statistical hyp-polydendrons, with the $\text{AmG}_2-p(\text{DEA}_{17}-\text{co-HPMA}_{33}-\text{co-EGDMA}_{0.9})$ possessing a noticeably larger molecular weight (4,510,000 g mol$^{-1}$) in comparison to the $\text{AmG}_2-p(\text{DEA}_{33}-\text{co-HPMA}_{17}-\text{co-EGDMA}_{0.9})$ and $\text{AmG}_2-p(\text{DEA}_{25}-\text{co-HPMA}_{25}-\text{co-EGDMA}_{0.9})$ (398,300 and 1,971,000 g mol$^{-1}$ respectively).

4.3.3 pH-responsive studies of AmG2-p(DEA50-b-(tBuMA65-co-EGDMA0.9))

Upon the addition of acid to $\text{AmG}_2-p(\text{DEA}_{50}-b-(\text{tBuMA}_{65}-\text{co-EGDMA}_{0.9}))$ NPs, a surprising phenomenon was observed. The NP dispersion did not solubilise, despite the addition of a large excess of acid (360 mg to 10 mL water). The polymeric NPs, instead, swelled slightly upon protonation (Figure 4.19), suggesting the potential extension of surface $\text{AmG}_2-p(\text{DEA}_{50})$ block segments extending into the surrounding aqueous environment and acting to provide steric stabilisation. It is also possible that the increased hydrophobicity within the core of the NP was able to hold together the assembled structure whilst some of the internal $p(\text{DEA})$ became protonated at such low
pH. This would lead to NP core swelling as well as the formation of extended surface polymer chains as the internal chains became hydrated.

\[ \text{Figure 4.19 DLS size distribution analysis of AmG}_2\text{-}(DEA}_{50}\text{-b-}(tBuMA}_{65}\text{-co-EGDMA}_{0.9}) \] before (red) and after (ret-dotted) acid addition.

4.3.4 pH-responsive studies of BDME-containing hyp-polydendrons

The AmG\(_2\)-p(DEA\(_{50}\)-co-BDME\(_{2.0}\)) nanoprecipitates (\(D_z = 61 \text{ nm}; D_n = 32 \text{ nm}\)) prepared in neutral water (pH=7.8) displayed a broader polydispersity than the corresponding AmG\(_2\)-p(DEA\(_{50}\)-co-EGDMA\(_{0.9}\)) (Figure 4.20), despite both hyp-polydendrons possessing similar molecular weights (Chapter 3).

\[ \text{Figure 4.20 DLS size distribution analysis of AmG}_2\text{-p(DEA}_{50}\text{-co-BDME}_{2.0}) \] (green) and AmG\(_2\)-p(DEA\(_{50}\)-co-EGDMA\(_{0.9}\)) (red).

Upon rapid acid addition, the stabilised AmG\(_2\)-p(DEA\(_{50}\)-co-BDME\(_{2.0}\)) NPs became soluble as expected, also confirmed by the observed colourless solution replacing a
slightly turbid NP dispersion. As previously observed, the $D_z$ values suggest an increase in size but the considerable decrease in count rate and $D_n$ values (Table 4.3 and Figure 4.21) confirmed the existence of individual species.

![Figure 4.21](image)

**Figure 4.21** DLS number distribution analysis of $\text{AmG}_2\text{-}p(\text{DEA}_{35}\text{-}co\text{-}\text{BDME}_{2.0})$ before (green) and after acid addition (red-dotted).

The nanoprecipitation and pH-responsive nature of $\text{AmG}_2\text{-}p(\text{DEA}_{33}\text{-}co\text{-}\text{HPMA}_{17}\text{-}co\text{-}\text{BDME}_{2.0})$ was studied as a direct comparison. The statistical incorporation of hydrophobic monomers within the hyp-polydendron produces control over the chemistry of the primary polymer chains and offers modification of the internal NP environment.

The $\text{AmG}_2\text{-}p(\text{DEA}_{33}\text{-}co\text{-}\text{HPMA}_{17}\text{-}co\text{-}\text{BDME}_{0.9})$ produced nanoprecipitates in neutral water that were more than double the size of the EGDMA equivalent $\text{AmG}_2\text{-}p(\text{DEA}_{35}\text{-}co\text{-}\text{HPMA}_{17}\text{-}co\text{-}\text{EGDMA}_{0.9})$ hyp-polydendron NPs (Figure 4.22). The NP dispersion displayed a broader size distribution, suggesting a less controlled aggregation process. Upon the addition of acid, the nanoprecipitates became soluble as expected, with a dramatic decrease in the $D_n$ value from 310 to 5 nm.
Figure 4.22 DLS size by intensity (A) and number (B) analysis of AmG2-p(DEA33-co-HPMA17-co-BDME2.0) (pink) and AmG2-p(DEA33-co-HPMA17-co-EGDMA0.9) (green) prepared in neutral water (pH 7.8) at a concentration of 1 mg mL\(^{-1}\). DLS size by intensity (C) and number (D) analysis of AmG2-p(DEA33-co-HPMA17-co-BDME2.0) (pink) and AmG2-p(DEA33-co-HPMA17-co-EGDMA0.9) (green) following the addition of acid.

Nanoprecipitation of AmG2-p(DEA17-b-(tBuMA33-co-BDME2.0)) produced stabilised aggregated nanoprecipitates in neutral water, exhibiting high levels of control during the aggregation process confirmed by the narrow PDI values. The AmG2-p(DEA17-b-(tBuMA33-co-BDME2.0)) possesses a very high weight average molecular weight of 594800 g mol\(^{-1}\) which is greater than twice the equivalent EGDMA-analogue with an \(M_w = 251800\) g mol\(^{-1}\). This would imply instantaneous formation of much larger nuclei on addition to water; however, these speculations are yet to be investigated. Upon the addition of acid to the NPs, the nanoprecipitates failed to become soluble and continued to exist as nanoprecipitates (Figure 4.23), as previously observed for the AmG2-p(DEA60-b-(tBuMA65-co-EGDMA0.9)). An increase in \(D_z\) and \(D_n\) values of ~ 30 nm in both cases was again seen (Table 4.3), suggesting polymer swelling during protonation of dendron and primary polymer chains.
Figure 4.23 DLS trace of AmG$_2$-p(DEA$_{17}$-b-(tBuMA$_{33}$-co-BDME$_{2.0}$)) before (green) and after (red) acid addition.

The DLS measurements performed on the BDME-containing nanoprecipitates following the addition of acid, despite confirming soluble materials, are not sufficient to verify the cleavage of the pH-responsive brancher, nor the existence of primary linear-dendritic polymer chains. Hydrolysis of the hyp-polydendrons (AmG$_2$-p(DEA$_{50}$-co-BDME$_{2.0}$), AmG$_2$-p(DEA$_{33}$-co-HPMA$_{17}$-co-BDME$_{2.0}$), and AmG$_2$-p(DEA$_{17}$-b-(tBuMA$_{33}$-co-BDME$_{2.0}$))) was carried out in acetone at room temperature in the presence of a small amount of aqueous HCl. The hyp-polydendrons became water-soluble upon protonation following acid addition. The existence of a solid precipitate, accounting for the cloudy solution, cleared immediately upon the addition of deionised water as the precipitate dissolved. Cleavage of the brancher upon addition of acid was readily observed from the monomodal distributions in the post-hydrolysis gel permeation chromatography (GPC) refractive index (RI; Figure 4.24) chromatograms of the BDME-containing branched copolymers.
Figure 4.24 GPC RI chromatograms of A) $\text{AmG}_2-p(\text{DEA}_{50})$ (red) and $\text{AmG}_2-p(\text{DEA}_{50}-\text{co}-\text{BDME}_{2.0})$ before (green) and after (red-dotted) hydrolysis; B) $\text{AmG}_2-p(\text{DEA}_{33}-\text{co}-\text{HPMA}_{17})$ (blue and $\text{AmG}_2-p(\text{DEA}_{33}-\text{co}-\text{HPMA}_{17}-\text{co}-\text{BDME}_{2.0})$ before (green) and after (red-dotted) hydrolysis; C) $\text{AmG}_2-p(\text{DEA}_{17}-b-\text{tBuMA}_{33})$ (red) and $\text{AmG}_2-p(\text{DEA}_{17}-b-(\text{tBuMA}_{33}-\text{co}-\text{BDME}_{2.0}))$ before (green) and after hydrolysis (red-dotted).
The hydrolysis of the BDME-containing hyp-polydendrons result in polymers with lower molecular weights and narrower molecular weight distributions than the precursor branched hyp-polydendron. These narrow, monomodal molecular weight distributions are indicative of the primary linear-dendritic hybrid polymer chains and the change in distribution is readily seen when compared to the highly branched polymer. These hydrolysed products are most likely statistical copolymers consisting of \( p(\text{DEA}) \) and \( p(\text{methacrylic acid}) \). The hydrolysed molecular weight distributions of each hydrolysed hyp-polydendron is highly comparable to the GPC analysis of each previously synthesised linear-dendritic equivalent polymer. Overlays of the molecular weight distributions of \( \text{AmG}_2-p(\text{DEA}_{50}) \) (Figure 4.24A), \( \text{AmG}_2-p(\text{DEA}_{33}-\text{co}-\text{HPMA}_{17}) \) (Figure 4.24B) and \( \text{AmG}_2-p(\text{DEA}_{17}-b-\text{BuMA}_{33}) \) (Figure 4.24C) were highly indicative of degradation to a distribution of primary chains.

### 4.4 Encapsulation and release studies

The association of drug molecules to NPs, either covalently attached or encapsulated, has been reported for many different polymer classes.\(^{52}\) Drugs can also be encapsulated within stable micelles with release mediated by the rate of drug diffusion from the core of the micelle.\(^{53}\) Liposomes have also been reported to encapsulate hydrophobic and hydrophilic therapeutic agents;\(^{54}\) they can shield the encapsulated agent from the external environment and required properties can be tailored through composition, size and surface charge to improve circulation half-life.\(^{55}\)

The hydrophobic dye molecule, fluoresceinamine (FA), was chosen as a model drug and dissolved in acetone at a concentration of 1 mg mL\(^{-1}\). FA was selected due to it being partly soluble in water, which was an essential characteristic for the release studies. 1 mL (1 mg) of the FA solution was combined with 2 mL of a 5 mg mL\(^{-1}\) solution (10 mg) of each \( \text{AmG}_2-p(\text{DEA}_{50}-\text{co}-\text{EGDMA}_{0.9}) \), \( \text{AmG}_1^{U}-p(\text{DEA}_{50}-\text{co}-\text{EGDMA}_{0.9}) \) and \( \text{AmG}_2-p(\text{DEA}_{50}-\text{co}-\text{BDME}_{2.0}) \) polymer solution (3 mL total; 9 wt% FA). The solutions were then subjected to a rapid solvent switch through drop-wise addition into 10 mL of deionised water (pH 7.8) at ambient temperature, to give a final polymer concentration of 1 mg mL\(^{-1}\) in water after acetone removal by evaporation overnight. All combinations formed stable uniform NPs in aqueous media and were analysed by DLS (Figure 4.25 and Table 4.4).
Figure 4.25 DLS size distribution analysis of encapsulated FA (9 wt%) $\text{AmG}_1\text{U}-p(\text{DEA}_{50}-\text{co-EGDMA}_{0.9})$ (orange), $\text{AmG}_2\text{p}(\text{DEA}_{50}-\text{co-EGDMA}_{0.9})$ (red) and $\text{AmG}_2\text{p}(\text{DEA}_{50}-\text{co-BDME}_{2.0})$ (green) NP dispersions.

Table 4.4 DLS analysis of DEA hyp-polydendrons (blank) and containing encapsulated FA (9 wt%) prepared in neutral water.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$D_0$ (nm)</th>
<th>PDI</th>
<th>$D_z$ (nm)</th>
<th>$D_0$ (nm)</th>
<th>PDI</th>
<th>$D_z$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{AmG}<em>2\text{p}(\text{DEA}</em>{50}-\text{co-EGDMA}_{0.9})$</td>
<td>58</td>
<td>0.065</td>
<td>43</td>
<td>45</td>
<td>0.197</td>
<td>28</td>
</tr>
<tr>
<td>$\text{AmG}<em>1\text{U}-p(\text{DEA}</em>{50}-\text{co-EGDMA}_{0.9})$</td>
<td>68</td>
<td>0.172</td>
<td>39</td>
<td>59</td>
<td>0.243</td>
<td>28</td>
</tr>
<tr>
<td>$\text{AmG}<em>2\text{p}(\text{DEA}</em>{50}-\text{co-BDME}_{2.0})$</td>
<td>61</td>
<td>0.241</td>
<td>32</td>
<td>38</td>
<td>0.190</td>
<td>20</td>
</tr>
</tbody>
</table>

$^a$ Blank DEA hyp-polydendrons; $^b$ DEA hyp-polydendrons nanoprecipitated with FA.

Due to the pH-responsive nature of the DEA, a visual release study of the encapsulated dye was conducted. The 9 wt % dye-loaded NP dispersions (0.1 mg FA encapsulated in 1 mg hyp-polydendron) were transferred independently into a dialysis membrane (molecular weight cut-off: 2000 g mol$^{-1}$) and submerged into a body of deionised water at pH 7.8, (Figure 4.26i).
The dialysis membrane within the water was left stirring for 48 hours to allow for any free or weakly-adsorbed FA to pass through the membrane. After 48 hours, the water had a slight yellow colour that was collected and analysed for the calculation of un-encapsulated FA (Figure 4.27), and subsequently the amount of encapsulated FA. In order to calculate the amount of FA released, a calibration curve was produced (Appendix, Figure A65), measuring the absorbance of varying concentrations of FA in acidic water using ultraviolet-visible (UV-Vis) spectroscopy. This allowed samples to be analysed via UV-Vis, measuring the absorbance at different time points, to determine the amount released.
The water was replaced with fresh, deionised water and left for a further 24 hours to ensure only encapsulated FA remained within the dialysis membrane. After this 24 hour period, the water remained clear. Acid (HCl, 140 mg) was added to the water until the pH reached ~2, and samples of water were taken over a period of time. The majority of FA was released within 8 hours for all samples (Figure 4.28). After 24 hours the water was replaced with fresh, aqueous HCl (pH ~2) due to equilibration of FA, after which the remaining fraction of FA was released.

A difference in the release of FA within the different hyp-polydendron NP dispersions was observed. The AmG$_2$-$p$(DEA$_{50}$-co-BDME$_{2.0}$) appeared to release the encapsulated FA faster, potentially due to additional hydrolysis of the brancher, resulting in a more rapid dissolution of the branched polymer core. Following the release study, the remaining AmG$_2$-$p$(DEA$_{50}$-co-BDME$_{2.0}$) NP dispersion within the dialysis membrane, was collected and analysed by GPC. A lower molecular weight and a narrow molecular weight distribution (Table 4.5) can be seen from the RI (Figure 4.29) GPC chromatogram, confirming hydrolysis of the BDME-containing hyp-polydendron once again.
**Figure 4.29** GPC RI chromatogram overlays of $\text{AmG}_2\text{-}p(\text{DEA}_{50})$ (red); $\text{AmG}_2\text{-}p(\text{DEA}_{50}\text{-}co\text{-}BDME_{2.0})$ (green) and $\text{AmG}_2\text{-}p(\text{DEA}_{50}\text{-}co\text{-}BDME_{2.0})$ after release study (orange).

**Table 4.5** GPC analysis of $\text{AmG}_2\text{-}p(\text{DEA}_{50})$ and $\text{AmG}_2\text{-}p(\text{DEA}_{50}\text{-}co\text{-}EGDMA_{0.9})$ and $\text{AmG}_2\text{-}p(\text{DEA}_{50}\text{-}co\text{-}BDME_{2.0})$ before and after the release study.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$M_n$ (g mol$^{-1}$)</th>
<th>$M_w$ (g mol$^{-1}$)</th>
<th>$D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{AmG}<em>2\text{-}p(\text{DEA}</em>{50})$</td>
<td>23650</td>
<td>34400</td>
<td>1.46</td>
</tr>
<tr>
<td>$\text{AmG}<em>2\text{-}p(\text{DEA}</em>{50}\text{-}co\text{-}BDME_{2.0})$</td>
<td>157300</td>
<td>39950</td>
<td>68800</td>
</tr>
<tr>
<td>- After hydrolysis</td>
<td>39950</td>
<td>51150</td>
<td>1.28</td>
</tr>
<tr>
<td>- After release study</td>
<td>68800</td>
<td>99500</td>
<td>1.45</td>
</tr>
<tr>
<td>$\text{AmG}<em>2\text{-}p(\text{DEA}</em>{50}\text{-}co\text{-}EGDMA_{0.9})$</td>
<td>200645</td>
<td>395261</td>
<td>1.97</td>
</tr>
<tr>
<td>- After release study</td>
<td>1513000</td>
<td>3938000</td>
<td>2.60</td>
</tr>
</tbody>
</table>

*Triple detection analysis using THF/2% TEA eluent*

Additionally, the remaining $\text{AmG}_2\text{-}p(\text{DEA}_{50}\text{-}co\text{-}EGDMA_{0.9})$ NP dispersion in the dialysis bag following the release study, was collected and analysed by GPC. Figure 4.30 shows a highly branched polymer remaining in the dialysis bag, compared to the narrower, monomodal peak witnessed for the $\text{AmG}_2\text{-}p(\text{DEA}_{50}\text{-}co\text{-}BDME_{2.0})$, due to the EGDMA brancher not undergoing hydrolysis. Distinguishable peaks in the RI overlay exactly with distinct peaks observed for the $\text{AmG}_2\text{-}p(\text{DEA}_{50}\text{-}co\text{-}EGDMA_{0.9})$ before the release study.
4.5 Conclusions

Overall, the results demonstrate the effects that linear-dendritic polymer and hyp-polydendron chemistry and architecture, play on the ability to form stable NPs in aqueous media. Generally, the hyp-polydendrons produced stable nanoprecipitates with narrow size distributions, compared to the linear-dendritic hybrids that typically resulted in broad and bimodal size distributions. The proposed explanation stems from how the initial nuclei form, with a higher molecular weight hyp-polydendron collapsing to form particles already containing a number of conjoined chains, as opposed to the linear materials slowly producing nuclei from small structures. Statistical linear-dendritic polymer hybrids and hyp-polydendrons containing tBuMA failed to produce stable NPs in neutral water (pH=7.8). The change in architecture to produce block hyp-polydendrons, also failed to produce stable NP dispersions, however, nanoprecipitation into acidic water, and increasing the primary polymer chain length, yielded stable nanoprecipitates. Again, the ability to tailor the hyp-polydendron architecture to form stable NPs is demonstrated.

All DEA-containing NPs underwent a pH-responsive study, with most existing as soluble, hydrated polymers at low pH, from protonation of the tertiary amines residing in the pendant polymer groups. These propositions were confirmed by observable clear
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solutions, and noticeable decreases in particle sizes ($D_n$) and derived count rates measured by DLS. All BDME-containing hyp-polydendrons succeeded in producing stable NP dispersions. Hydrolysis to linear-dendritic polymer chains was confirmed by GPC, displaying monomodal molecular weight distributions. Interestingly, the $\text{AmG}_2$-$p(\text{DEA}_{17}-b-(t\text{BuMA}_{33}-co-\text{BDME}_{2.0}))$ failed to solubilise upon the addition of acid, proposing a NP packing arrangement that prevents acid entry. The successful encapsulation of a model dye guest molecule was achieved, with release studies revealing changes in the release rate based on minor modifications within the hyp-polydendron, i.e. the acid-cleavable brancher.

Overall, the ability to form stable nanoprecipitates and the procedure to release a model drug has been demonstrated to depend on a variety of different chemical and architectural properties within the hyp-polydendron, allowing modifications within the structure for tuning properties that have not been previously demonstrated.

4.6 Experimental

Typical procedure for aqueous nanoprecipitation – The materials were dissolved in acetone/THF at a concentration of 5 mg mL$^{-1}$. 2 mL of this solution was then subjected to a rapid solvent switch through drop wise addition into 10 mL of water, to give a final polymer nanoparticle concentration of 1 mg mL$^{-1}$ in water after acetone/THF removal by evaporation overnight.

Typical procedure for fluoresceinamine encapsulation – FA was dissolved in acetone at a concentration of 1 mg mL$^{-1}$. 1 mL of this solution, along with 2 mL of the $p(\text{DEA}_{50}-co-\text{EGDMA}_{x})$ solution (5 mg mL$^{-1}$), was then subjected to a rapid solvent switch through drop wise addition into 10 mL of water, to give a final polymer nanoparticle concentration of 1 mg mL$^{-1}$ in water after acetone removal by evaporation overnight.

Typical procedure for hydrolysis of BDME-containing hyp-polydendrons – $\text{AmG}_2$-$p(\text{DEA}_{50}-co-\text{BDME}_{2.0})$ was dissolved in acetone at a concentration of 40 mg mL$^{-1}$ (9 mL). HCl (5M, 400 $\mu$L) was added drop wise to the solution and stirred vigorously at room temperature for 20 minutes, resulting in a cloudy solution with solid precipitate. Deionised water (9 mL) was added to the acidic polymer solution and left to stir overnight in a sealed vial. The hydrolysed polymer solution was frozen in liquid nitrogen and lyophilized for 72 hours.
Co-nanoprecipitation studies of linear-dendritic polymer hybrids and branched, hydrophobic copolymers
5.1 Introduction

In 2015, Ford et al. described the formation of stable polymeric nanoparticles (NPs) using mixed polymer feeds and through a process known as co-nanoprecipitation. Co-nanoprecipitation differs from the established nanoprecipitation method as two polymers are dissolved in the same organic good solvent prior to precipitation into water, which acts as an anti-solvent. This published report described the stabilisation of a branched, hydrophobic, vinyl copolymer, $p$(2-hydroxypropyl methacrylate-$co$-ethylene glycol dimethacrylate) initiated by ethyl $\alpha$-bromoisobutyrate ($\text{EBiB}$-$p$(HPMA$_{50}$-$co$-EGDMA$_{0.9}$)) (Scheme 5.1i), with an amphiphilic A-B block copolymer $p$(ethylene glycol-$co$-HPMA) (PEG$_{45}$-$co$-HPMA$_{120}$) (Scheme 5.1ii) to generate NP dispersions with narrow particle size distributions.

![Scheme 5.1](image)

Scheme 5.1 Synthesis of branched, hydrophobic, vinyl copolymer, $\text{EBiB}$-$p$(HPMA$_{50}$-$co$-EGDMA$_{0.9}$) (i), and amphiphilic A-B block copolymer, (PEG$_{45}$-$co$-HPMA$_{120}$) (ii) via atom transfer radical polymerisation (ATRP).

By varying the ratios of the two polymers within the good solvent solution, differences in NP behaviour were observed, ultimately resulting in materials with optimised salt stability. The formation of sterically stabilised NPs via the co-nanoprecipitation approach is presented schematically in Figure 5.1. Thorough investigations were carried...
out to show the range of parameters that effect and control the NP stabilisation process: dilution factor, primary polymer composition and architecture. The mechanism of NP formation was investigated and it was apparent that NPs are formed immediately after addition of the polymer solution (good solvent) to the anti-solvent, the addition of large volumes of good solvent can prevent nanoprecipitation and repeated nanoprecipitations into the same anti-solvent sample led to increased numbers of particles, supporting the nucleation and growth mechanism.\(^2\)

![Figure 5.1 Co-nanoprecipitation of branched HPMA copolymer, EBiB-\(p\)(HPMA\(_{50}\)-co-EGDMA\(_{0.9}\)), and linear A-B block copolymer (PEG\(_{45}\)-co-HPMA\(_{120}\)), to produce stabilised NPs.](image)

Overall, the co-nanoprecipitation concept proved a relatively facile technique to introduce steric stability to branched polymer nanoprecipitates, particularly in aqueous salt conditions, which is essential for *in vitro* pharmacological studies. Previous research reports have described dendrons and conjugated PEG chains covalently-bound to the *hyp*-polydendron surface, where PEG was also confirmed to be present inside the NPs. This may present issues, that co-nanoprecipitation may overcome.

The co-nanoprecipitation method allows for investigations of NPs formed from the self-assembly from different materials. In this current study, linear polymers, copolymers and linear-dendritic polymer hybrids will be evaluated in co-nanoprecipitation with hydrophobic, branched copolymers (Figure 5.2). A clear advantage of this approach is the ability to relatively quickly change the chemical composition of different NP structural components, and the ease of tailoring the surface chemistry of the resulting NPs.
5.2 Co-nanoprecipitation studies of linear polymers and linear-dendritic hybrids with branched hydrophobic copolymers

Co-nanoprecipitation studies of a series of selected linear polymers, linear-dendritic polymer hybrids and branched copolymers synthesised in Chapter 3, were conducted to evaluate their suitability for this NP fabrication approach.

For all co-nanoprecipitation experiments, polymers were dissolved in the water-miscible good solvent at a concentration of 5 mg mL\(^{-1}\). Solutions of linear polymers and linear-dendritic polymer hybrids were combined with branched copolymer solutions at a ratio of 10:90 wt% (linear:branched) (cf. Section 5.2.2). An aqueous NP dispersion with an end concentration of 1 mg mL\(^{-1}\) was targeted, following solvent removal by evaporation overnight at ambient temperature. All polymers were dissolved in acetone, apart from the \(t\)-butyl methacrylate containing polymers, \(p(tBuMA_{50})\) and \(EBiB-p(tBuMA_{50}-co-EGDMA_{0.05})\) samples, which were solubilised in tetrahydrofuran (THF).

![Diagrammatic representation of the co-nanoprecipitation method](image)

**Figure 5.2** Diagrammatic representation of the proposed co-nanoprecipitation method utilising linear-dendritic and branched polymers; A) addition of combined polymer solutions; B) collapse and aggregation of polymers to produce; C) stabilised nanoprecipitates.

Analysis of the stable NP dispersions was conducted by scanning electron microscopy (SEM) and dynamic light scattering (DLS) to assess number average diameters (\(D_n\)), hydrodynamic diameters (\(D_z\)), zeta-potentials (\(\zeta\)) and polydispersities (PDI).
5.2.1 Co-nanoprecipitation studies of linear polymers and linear-dendritic hybrids with EBiB-\textit{p}(DEA_{50}-co-EGDMA_{0.95}) branched copolymer

Initially, the co-nanoprecipitation strategy was studied in combination with the pH-responsive branched copolymer EBiB-\textit{p}(DEA_{50}-co-EGDMA_{0.95}) using the linear polymer EBiB-\textit{p}(DEA_{50}) and linear-dendritic polymer hybrid, AmG_{2-\textit{p}}(DEA_{50}) (Figure 5.3). Each linear polymer component was co-nanoprecipitated with the EBiB-\textit{p}(DEA_{50}-co-EGDMA_{0.95}) into 10 mL of deionised water at pH = 7.8. Both combinations formed stable uniform NPs in aqueous media and were analysed by DLS (Table 5.1). Upon inspection by DLS, both co-nanoprecipitations formed monomodal, stable NP dispersions (Figure 5.4).

\textbf{Figure 5.3} Diagrammatic representation of the co-nanoprecipitation of AmG_{2-\textit{p}}(DEA_{50}) (i) and EBiB-\textit{p}(DEA_{50}-co-EGDMA_{0.95}) (ii) at 10:90 wt\% to produce stable NPs in neutral water at a concentration of 1 mg mL\textsuperscript{-1}. 
### Table 5.1 DLS analysis for co-nanoprecipitated particles formed from linear and linear-dendritic polymers and branched copolymers at 90:10 wt% (linear:branched) in aqueous media pH=7.8.

<table>
<thead>
<tr>
<th>Branched Polymer</th>
<th>Linear Polymer</th>
<th>$D_z$ (nm)</th>
<th>PDI</th>
<th>$D_n$ (nm)</th>
<th>$\zeta$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-EBiB-$p$(DEA$_{50}$)</td>
<td>-</td>
<td>47</td>
<td>0.249</td>
<td>24</td>
<td>+23</td>
</tr>
<tr>
<td>-AmG$<em>2$-$p$(DEA$</em>{50}$)</td>
<td>-</td>
<td>65</td>
<td>0.381</td>
<td>12</td>
<td>+19</td>
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<tr>
<td>-BnG$<em>2$-$p$(DEA$</em>{50}$)</td>
<td>-</td>
<td>48</td>
<td>0.348</td>
<td>14</td>
<td>+45</td>
</tr>
<tr>
<td>-EBiB-$p$(HPMA$_{50}$)</td>
<td>-</td>
<td>780</td>
<td>0.230</td>
<td>654</td>
<td>-34</td>
</tr>
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<td>-AmG$<em>2$-$p$(HPMA$</em>{50}$)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EBiB-$p$(DEA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>-</td>
<td>139</td>
<td>0.087</td>
<td>102</td>
<td>+34</td>
</tr>
<tr>
<td>EBiB-$p$(HPMA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>-</td>
<td>178</td>
<td>0.082</td>
<td>145</td>
<td>-20</td>
</tr>
<tr>
<td>EBiB-$p$(tBuMA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EBiB-$p$(DEA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>EBiB-$p$(DEA$_{50}$)</td>
<td>38</td>
<td>0.227</td>
<td>20</td>
<td>+38</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(DEA$</em>{50}$)</td>
<td>AmG$<em>2$-$p$(DEA$</em>{50}$)</td>
<td>43</td>
<td>0.171</td>
<td>24</td>
<td>+28</td>
</tr>
<tr>
<td>EBiB-$p$(HPMA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>EBiB-$p$(HPMA$_{50}$)</td>
<td>161</td>
<td>0.086</td>
<td>124</td>
<td>-34</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(HPMA$</em>{50}$)</td>
<td>AmG$<em>2$-$p$(HPMA$</em>{50}$)</td>
<td>210</td>
<td>0.072</td>
<td>187</td>
<td>-21</td>
</tr>
<tr>
<td>EBiB-$p$(DEA$_{50}$)</td>
<td>EBiB-$p$(DEA$_{50}$)</td>
<td>136</td>
<td>0.094</td>
<td>99</td>
<td>+48</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(DEA$</em>{50}$)</td>
<td>AmG$<em>2$-$p$(DEA$</em>{50}$)</td>
<td>144</td>
<td>0.102</td>
<td>106</td>
<td>+52</td>
</tr>
<tr>
<td>EBiB-$p$(tBuMA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>EBiB-$p$(DEA$_{50}$)</td>
<td>192</td>
<td>0.109</td>
<td>131</td>
<td>+40</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(DEA$</em>{50}$)</td>
<td>AmG$<em>2$-$p$(DEA$</em>{50}$)</td>
<td>234</td>
<td>0.051</td>
<td>216</td>
<td>+40</td>
</tr>
<tr>
<td>BnG$<em>2$-$p$(DEA$</em>{50}$)</td>
<td>BnG$<em>2$-$p$(DEA$</em>{50}$)</td>
<td>168</td>
<td>0.131</td>
<td>116</td>
<td>+38</td>
</tr>
</tbody>
</table>

1 Italics indicates a bimodal size distribution measured by DLS.

2 – indicates failure to form stabilised NPs with observable macroscale precipitation.

---

**Figure 5.4** DLS size distribution analysis of co-nanoprecipitated particles comprised of EBiB-$p$(DEA$_{50}$-co-EGDMA$_{0.95}$) with EBiB-$p$(DEA$_{50}$) (green) and AmG$_2$-$p$(DEA$_{50}$) (red) in neutral water at a concentration of 1 mg mL$^{-1}$. 

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The formation of monomodal size distributions suggests that the co-nanoprecipitation occurs via a controlled self-assembly. Comparatively, neither the EBiB-\textit{p}(DEA\textsubscript{50}) nor AmG\textsubscript{2}-\textit{p}(DEA\textsubscript{50}) produced stable NP dispersions independently, confirmed by bimodal and broad size distributions (Table 5.1), suggesting a clear and active contribution to the co-nanoprecipitation approach by the branched polymer component. In contrast, the nanoprecipitation of both EBiB-\textit{p}(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.95}) and AmG\textsubscript{2}-\textit{p}(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) produced larger, well-defined nanoprecipitates with narrow size distributions which indicates that the linear and linear-dendritic polymers are also key to the process. In this instance, the preliminary studies suggest a covalently-bound dendron chain end produces NPs via a more controlled aggregation than the co-nanoprecipitation approach. The presence of independent linear and branched DEA-containing polymers may allow for a variety of outcomes and consequently broad polydispersities.

5.2.2 Co-nanoprecipitation studies of linear polymers and linear-dendritic hybrids with EBiB-\textit{p}(HPMA\textsubscript{50}-co-EGDMA\textsubscript{0.95}) hydrophobic copolymer

Following the different behaviour and NP size outcomes from nanoprecipitations of covalently dendron-bound AmG\textsubscript{2}-\textit{p}(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) and co-nanoprecipitation of AmG\textsubscript{2}-\textit{p}(DEA\textsubscript{50}) with EBiB-\textit{p}(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.95}), co-nanoprecipitation of linear-dendritic hybrid polymers and branched copolymers containing HPMA was investigated. This allowed comparison of the behaviour of other polymer compositions when the chain end was covalently-bound or co-nanoprecipitated with the branched copolymer. Initially, co-nanoprecipitation was carried out through combination of the branched, hydrophobic copolymer EBiB-\textit{p}(HPMA\textsubscript{50}-co-EGDMA\textsubscript{0.95}) with EBiB-\textit{p}(HPMA\textsubscript{50}) and AmG\textsubscript{2}-\textit{p}(HPMA\textsubscript{50}), previously reported in Chapter 2, at two different linear:branched polymer ratios; 50:50 and 10:90 wt% (Figure 5.5). The 50:50 wt% combination failed to produce stable NPs, with considerable macroscale precipitation witnessed. The combination carried out at 10:90 wt% formed stable uniform NPs in aqueous media (Table 5.1 and Figure 5.6).
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Figure 5.5 Diagrammatic representation of the co-nanoprecipitation of AmG2-p(HPMA50) (i) and EBiB-p(HPMA50-co-EGDMA0.95) (ii) at 10:90 wt% to produce stable NPs in neutral water at a concentration of 1 mg mL\(^{-1}\).

Figure 5.6 DLS size distribution analysis of co-nanoprecipitated EBiB-p(HPMA-co-EGDMA0.95) with EBiB-p(HPMA50) (blue) and AmG2-p(HPMA50) (red) in neutral water at a concentration of 1 mg mL\(^{-1}\).

The notably narrow PDI values measured by DLS suggest that the EBiB-initiated linear material has solely formed uniform mixed NPs with the branched, hydrophobic copolymer, as this material generates very large and unstable NPs when nanoprecipitated alone (Chapter 2). The monomodal size distributions also confirms the presence of one main size distribution, again supporting the co-nanoprecipitation mechanism as the EBiB-p(HPMA50) will independently form nanoprecipitates with \(D_z = 780\) nm and PDI = 0.230; no evidence of very large nanoprecipitates of this size was seen (Figure 5.6). The AmG2-p(HPMA50) failed to form NPs in pH=7.8 when nanoprecipitated alone; however, when combined with the EBiB-p(HPMA50-co-EGDMA0.95) no macroscale precipitation
was observed. The NPs containing $\text{AmG}_2\text{p}(\text{HPMA}_{50})$ produced larger NPs than those co-nanoprecipitated particles containing $\text{EBiB}_x\text{p}(\text{HPMA}_{50})$; compared to the opposing trend observed with the individual nanoprecipitation of the branched $\text{EBiB}_x\text{p}(\text{HPMA}_{50-co-EGDMA}_{0.95})$ and hyp-polydendron $\text{AmG}_2\text{p}(\text{HPMA}_{50-co-EGDMA}_{0.9})$ material (NP $D_z=178$ and 149 respectively).

To investigate the observed effects and rule out the potential for the varying polymer masses directing the nanoprecipitation results, control experiments were conducted using two individual THF solutions of $\text{EBiB}_x\text{p}(\text{HPMA}_{50-co-EGDMA}_{0.95})$ and $\text{AmG}_2\text{p}(\text{HPMA}_{50})$ resembling the ratios of branched and linear-dendritic hybrid polymer mixtures used in the co-nanoprecipitation experiments. Volumes of branched copolymer and linear-dendritic hybrid polymer solutions (5 mg mL$^{-1}$) of 1.8 mL and 0.2 mL were used to mimic the 90:10 wt% ratios of the co-nanoprecipitation experiments; these were added dropwise to deionised water in separate vials. Stable NPs (0.9 mg mL$^{-1}$) were formed from $\text{EBiB}_x\text{p}(\text{HPMA}_{50-co-EGDMA}_{0.95})$ as expected, with $D_z$ and PDI values similar to NPs formed at a concentration of 1 mg mL$^{-1}$ ($D_z = 165$ nm, PDI = 0.086). The $\text{AmG}_2\text{p}(\text{HPMA}_{50})$ linear-dendritic hybrid, however, failed to form stable NPs at 0.1 mg mL$^{-1}$ and resulted in macroscale precipitation. As already discussed, the linear-dendritic hybrids tend to form smaller nuclei in the changing solvent system and, therefore, aggregate to larger sizes. $\text{AmG}_2\text{p}(\text{HPMA}_{50})$ also previously failed to produce stable NPs in neutral water in previous studies (Chapter 2), suggesting that the combination of $\text{AmG}_2\text{p}(\text{HPMA}_{50})$ with the branched copolymer allows formation of stabilised NPs and a direct interaction between the two materials is seen.

Negative $\zeta$ values were once again obtained for all NPs formed, in concordance with previous reports of the adsorption of hydroxide ions at the hydrophobic/water interface and lack of protonation of the amine functionalities at pH = 7.8.

In addition, the combination of two branched polymers within a co-nanoprecipitation process was studied. $\text{EBiB}_x\text{p}(\text{HPMA}_{50-co-EGDMA}_{0.95})$ and $\text{AmG}_2\text{p}(\text{HPMA}_{50-co-EGDMA}_{0.9})$ were co-nanoprecipitated into deionised water at pH = 7.8 at a branched copolymer: hyp-polydendron ratio of 90:10 wt% to match the previous experiments. Stable NPs ($D_z=595$ nm, PDI=0.213 and $\zeta=-17$ mV) were formed. This is not unexpected as both branched polymers previously formed stable NPs independently (Chapter 2); however, the larger size suggested an indication that the mixing of the polymer structures
during nanoprecipitation was more hindered than the relatively simple incorporation of the linear-dendritic hybrids.

Following the preparation of stable NPs across the range of statistical hyp-polydendrons comprising varying DEA and HPMA molar ratios (Chapter 4), co-nanoprecipitation was carried out between combinations of EBiB-\(p(\text{HPMA}_{50}-\text{co}-\text{EGDMA}_{0.95})\) and either EBiB-\(p(\text{DEA}_{50})\) or AmG\(_2\)-\(p(\text{DEA}_{50})\) (Figure 5.7).

Figure 5.7 Diagrammatic representation of the co-nanoprecipitation of AmG\(_2\)-\(p(\text{DEA}_{50})\) (i) and EBiB-\(p(\text{HPMA}_{50}-\text{co}-\text{EGDMA}_{0.95})\) (ii) at 10:90 wt% to produce stable NPs in neutral water at a concentration of 1 mg mL\(^{-1}\).

This allowed for the formation of NPs containing similar chemical compositions to the statistical DEA/HPMA-containing hyp-polydendrons, where comparisons could be drawn between covalently-bound and combination materials and their self-assembled particles. Additionally, it was hypothesised that a pH-response could be introduced into the co-nanoprecipitated particles. EBiB-\(p(\text{DEA}_{50})\) and AmG\(_2\)-\(p(\text{DEA}_{50})\) were independently co-nanoprecipitated with EBiB-\(p(\text{HPMA}_{50}-\text{co}-\text{EGDMA}_{0.95})\) into deionised water (pH = 7.8) and stable NPs indeed formed for both combinations (Figure 5.8 and Table 5.1).
The NPs formed from the co-nanoprecipitation of either EBiB\(-p(\text{DEA}_{50})\) or AmG\(_2\)-\(p(\text{DEA}_{50})\) with EBiB\(-p(\text{HPMA}_{50}\text{-co-EGDMA}_{0.95})\) formed stable NPs with narrow polydispersities and highly positive $\zeta$ values; both $D_z$ and $D_n$ size values were essentially identical ($D_z=136$ and 144 nm respectively). The positive $\zeta$ values and the near identical size distributions indicates the influence of the $p(\text{DEA})$ chains, as seen in other nanoprecipitations of DEA-containing materials, and not observed when the amine-functional dendron alone is present; these values were greater than those observed for the nanoprecipitats of statistical $\text{hyp}$-polydendrons, suggesting a high density of the linear DEA polymer chains at the surface of the particles.

5.2.3 Co-nanoprecipitation studies of linear and linear-dendritic polymers with EBiB\(-p(\text{BuMA}_{50}\text{-co-EGDMA}_{0.95})\)

The successful co-nanoprecipitation of various combinations of linear and linear-dendritic polymers with branched copolymers suggested the potential to produce stable NPs of $\text{BuMA}$-containing polymers in neutral water; these had failed to do so previously as reported in Chapter 4. Initially, either the linear polymer EBiB\(-p(\text{BuMA}_{50})\) or the linear-dendritic polymer AmG\(_2\)-\(p(\text{BuMA}_{50})\) was co-nanoprecipitated with EBiB\(-p(\text{BuMA}_{50}\text{-co-EGDMA}_{0.95})\) in aqueous media pH=7.8 (Figure 5.9).
A stable NP dispersion failed to form for either, resulting in macrophase separation. This was, perhaps, not surprising as all polymers failed to produce stable NPs during previous nanoprecipitation studies (Chapter 4).

The positive impact of \( p(\text{DEA}) \) chains in previous studies of co-nanoprecipitation suggested that the production of stable NPs in aqueous media from hydrophobic EBiB-\( p(\text{BuMA}_{50}-\text{co-EGDMA}_{0.95}) \) may be improved by inclusion of EBiB-\( p(\text{DEA}_{50}) \) or the linear-dendritic polymer hybrid \( \text{AmG}_2-\text{p(DEA}_{50}) \). It was hypothesised that a fraction of the \( p(\text{DEA}) \) chains may ultimately reside close to the surface of the NP and provide charge stabilisation as seen in studies of \( p(\text{HPMA}) \)-containing materials (Figure 5.10).
Both experiments were successful, generating NPs with similar $D_z$ values of 192 nm and 234 nm from co-nanoprecipitates containing $\text{EBiB-}p(\text{BuMA}_{50}^{\text{-co-EGDMA}_{0.95}})$ or $\text{AmG}_2-p(\text{DEA}_{50})$ respectively. The combination containing the linear-dendritic polymer hybrid was slightly larger than the non-dendritic material, possibly due to arrangement of dendrons on the NP surface or packing within the NP; however, the NP comprising $\text{AmG}_2-p(\text{DEA}_{50})$ displayed a narrower size distribution (Figure 5.11A). Both NPs exhibited highly positive $\zeta$ values, as seen in previous experiments, but interestingly the linear DEA materials and $\text{EBiB-}p(\text{BuMA}_{50}^{\text{-co-EGDMA}_{0.95}})$ have all previously failed to generate uniform nanoprecipitates.
Figure 5.11 A) DLS size distribution analysis of EBiB-$p$($t$BuMA$_{50}$-co-EGDMA$_{0.95}$) with EBiB-$p$(DEA$_{50}$) (green) and AmG$_2$-$p$(DEA$_{50}$) (red); and B) SEM image of co-nanoprecipitated AmG$_2$-$p$(DEA$_{50}$) with EBiB-$p$($t$BuMA$_{50}$-co-EGDMA$_{0.95}$) in neutral water at a concentration of 1 mg mL$^{-1}$.

Comparatively, the statistical and block DEA and $t$BuMA hyp-polydendrons with an average degree of polymerisation=$50$ primary polymer chains for all varying DEA:$t$BuMA compositions failed to create stable NP dispersions in pH=7.8 and the requirement of additional charge by nanoprecipitating into aqueous HCl was essential to eventually produce stable NPs (Figure 5.12i). To combat this, a hyp-polydendron with an increased primary polymer chain length, AmG$_2$-$p$(DEA$_{50}$-$b$($t$BuMA$_{65}$-co-EGDMA$_{0.9}$)), was synthesised and the resulting NPs were stable in neutral water (Figure 5.12ii). The co-nanoprecipitation of AmG$_2$-$p$(DEA$_{50}$) with EBiB-$p$($t$BuMA$_{50}$-co-EGDMA$_{0.95}$) produced nanoprecipitates, that are possibly not too dissimilar to the AmG$_2$-$p$(DEA$_{50}$-$b$($t$BuMA$_{65}$-co-EGDMA$_{0.9}$)) as the combination of the two polymers of significant block length successfully produced NPs, albeit, of larger sizes (Figure 5.12iii).
The impact of the presence of the dendron chemistry within the linear-dendritic hybrid on co-nanoprecipitation was studied by utilising a combination of \textit{BnG}$_2$-\textit{p}(DEA$_{50}$) (Chapter 3) with \textit{EBiB}-\textit{p}((BuMA$_{50}$-co-EGDMA$_{0.95}$)). Experiments were conducted using deionised water (pH=7.8) and aqueous HCl (pH=4) and stable NPs were produced in both aqueous environments. The \textit{BnG}$_2$-\textit{p}(DEA$_{50}$) exhibited a bimodal size distribution when nanoprecipitated independently (Table 5.1).

In a comparative study, the \textit{EBiB}-\textit{p}((BuMA$_{50}$-co-EGDMA$_{0.95}$) failed to form stable co-nanoprecipitates with either \textit{EBiB}-\textit{p}(DEA$_{50}$) or \textit{AmG}$_2$-\textit{p}(DEA$_{50}$) in acidic water, due to the solubilisation of the linear DEA polymer and linear-dendritic DEA hybrid at low pH. This indicates the importance of the collapse of the linear polymer chains and co-nanoprecipitation, and not just the simple surface adsorption of polymer chains to the growing NPs during the precipitation process. In comparison, the large hydrophobic benzyl-functional dendritic chain end on the \textit{BnG}$_2$-\textit{p}(DEA$_{50}$) prevents solubilisation of the polymer in aqueous HCl, therefore, offering the potential to yield stable nanoprecipitates within different pH environments. Indeed, the co-nanoprecipitation of \textit{BnG}$_2$-\textit{p}(DEA$_{50}$) with \textit{EBiB}-\textit{p}((BuMA$_{50}$-co-EGDMA$_{0.95}$) led to self-assembled NPs in water at both pH = 4 ($D_c$=165 nm, PDI=0.140, $D_n$=120 nm, $\zeta$=+68 mV) and 7.8 (Table 5.1). The co-nanoprecipitates were nearly identical in size with similar size distributions ($D_c$=168 nm at pH = 7.8; 165 nm at pH = 4). Both NP dispersions had highly positive $\zeta$ values suggesting the presence of protonated linear DEA chains at the nanoprecipitate surface, and potentially entrapped hydrophobic chain ends within the nanoprecipitate.
bulk. The NPs in acidic water exhibited a more positive $\zeta$ value due to the increased abundance of H$^+$ ions in this media, offering increased levels of protonation of the linear DEA polymer chains.

### 5.3 Co-nanoprecipitation studies of linear and linear-dendritic polymers containing tBuMA with EBiB-$p$(tBuMA$_{50}$-co-EGDMA$_{0.95}$) in acidic water

Following the unsuccessful production of NPs via co-nanoprecipitation of EBiB-$p$(tBuMA$_{50}$-co-EGDMA$_{0.95}$) using deionised water (Section 5.2.3), the branched copolymer was co-nanoprecipitated with EBiB-$p$(tBuMA$_{50}$) and AmG$_2$-$p$(tBuMA$_{50}$) into acidic water. Only the co-nanoprecipitation containing AmG$_2$-$p$(tBuMA$_{50}$) successfully produced NPs (Table 5.2 and Figure 5.13), due to the ability of the dendron chain end to protonate at low pH.

#### Table 5.2 DLS analysis for co-nanoprecipitated particles formed from linear/linear-dendritic DEA polymers and EBiB-$p$(BuMA-co-EGDMA$_{0.95}$) in acidic water.

<table>
<thead>
<tr>
<th>Branched Polymer</th>
<th>Polymer</th>
<th>$D_z$ (nm)</th>
<th>PDI</th>
<th>$D_n$ (nm)</th>
<th>$\zeta$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBiB-$p$(tBuMA$_{50}$)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(tBuMA$</em>{50}$)</td>
<td>104</td>
<td>0.250</td>
<td>43</td>
<td>+46</td>
<td></td>
</tr>
<tr>
<td>EBiB-$p$-(BuMA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>EBiB-$p$-(BuMA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(tBuMA$</em>{50}$)</td>
<td>161</td>
<td>0.114</td>
<td>114</td>
<td>+46</td>
<td></td>
</tr>
</tbody>
</table>

$^*$ - indicates failure to form stabilised NPs with observable macroscale precipitation.

The failure to form stabilised NPs from the co-nanoprecipitation of EBiB-$p$(tBuMA$_{50}$-co-EGDMA$_{0.95}$) and AmG$_2$-$p$(tBuMA$_{50}$) in neutral water confirms the necessity of charge stabilisation, which is delivered by the protonated dendron when utilising an acidic anti-solvent environment and has also been observed for the AmG$_2$-$p$(tBuMA$_{50}$-co-EGDMA$_{0.9}$) hyp-polydendron ($D_z$=233 nm, PDI=0.295) and AmG$_2$-$p$(tBuMA$_{50}$) linear-dendritic polymer forming stable NPs in acidic water ($D_z$=104 nm, PDI=0.250). A narrow size distribution was obtained by DLS, compared to the broad size distributions measured for the amine-functionalised materials nanoprecipitated independently.
Figure 5.13 DLS size distribution analysis (A) and SEM image (B) of co-nanoprecipitated AmG2-p(tBuMA50) and EBiB-p(tBuMA50-co-EGDMA0.95) in aqueous HCl (1 mg mL⁻¹).

In previous reports of hyp-polydendron synthesis, mixed initiators have been used to tailor the chain-end functionality and provide systematically varying behaviour from nanoprecipitated materials. The stabilisation of EBiB-p(tBuMA50-co-EGDMA0.95) nanoprecipitates at low pH using co-nanoprecipitation with a linear-dendritic polymer hybrid, suggested that a branched tBuMA-containing copolymer, synthesised using a mixed EBiB and AmG2-Br initiation (Chapter 3) may also be able to form stable NPs using a conventional single component nanoprecipitation into acidic media. The resultant polymer, EBiB0.9-(AmG2)0.1-p(tBuMA50-co-EGDMA0.95) underwent nanoprecipitation into deionised water and aqueous HCl and stable NPs failed to form in either pH environment.

Nanoprecipitation was then conducted in highly acidic water (pH = 2), and stable NPs ($D_z = 204$ nm; PDI=0.202) were produced. The lower pH requirement may indicate that very few dendron chain ends were available at the nanoprecipitate surface. A large
amount of chain-ends are situated within the main bulk of the nanoprecipitate after the assembly of nuclei and unimers and a significant number if dendrons would be lost and incapable of contributing to the charge stabilisation. This would also suggest a more efficient dendron chain end arrangement on the nanoprecipitate surface from the linear-dendritic hybrid upon co-nanoprecipitation. The NPs exhibited a $\zeta = +58$ mV, further suggesting nanoprecipitate stabilisation from surface charge but the increased acidity may be required to fully protonate the available dendrons.

### 5.4 pH-responsive studies of co-nanoprecipitated NPs prepared in neutral water

Following the successful incorporation of a pH-responsive linear or linear-dendritic polymer into a variety of co-nanoprecipitated particles, the effect of acid addition to the resultant NPs was investigated. Initially, EBiB-$p$(DEA$_{50}$) or AmG$_2$-$p$(DEA$_{50}$) co-nanoprecipitated with EBiB-$p$(DEA$_{50}$-co-EGDMA$_{0.95}$) was studied and comparisons were made to similarly composed polymeric NPs formed in Chapter 4, where all components were covalently-bound.

The rapid addition of acid (1M HCl) to the NPs prepared from either EBiB-$p$(DEA$_{50}$) or AmG$_2$-$p$(DEA$_{50}$) co-nanoprecipitated with EBiB-$p$(DEA$_{50}$-co-EGDMA$_{0.95}$) (Table 5.3) led to complete solubilisation of both the linear and branched DEA component, confirmed by the decrease in $D_n$ and derived count rate (DCR) values (Table 5.3). This behaviour was equally observed for the nanoprecipitates comprised solely of EBiB-$p$(DEA$_{50}$-co-EGDMA$_{0.95}$) and AmG$_2$-$p$(DEA$_{50}$-co-EGDMA$_{0.9}$).

<table>
<thead>
<tr>
<th>Branched Polymer</th>
<th>$p$(DEA$_{50}$)</th>
<th>pH = 7.8$^a$</th>
<th>+ acid$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$D_z$ (nm)</td>
<td>PDI</td>
</tr>
<tr>
<td>EBiB-$p$(DEA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>EBiB-</td>
<td>38</td>
<td>0.227</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(DEA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>AmG$_2$-</td>
<td>43</td>
<td>0.171</td>
</tr>
</tbody>
</table>

$^a$ Initial pH  
$^b$ Addition of 35 $\mu$L HCl (aq.) (1M)  
$^c$ Italics indicate a bimodal size distribution measured by DLS.
The pH-response of the \textit{EBiB}-\textit{p}(DEA_{50}) and \textit{AmG2}-\textit{p}(DEA_{50}) co-nanoprecipitated with \textit{EBiB}-\textit{p}(HPMA_{50}-co-EGDMA_{0.95}) was also investigated via rapid addition of acid (1M HCl). The statistical \textit{p}(DEA_{x}-co-HPMA_{y}-co-EGDMA_{0.9}) \textit{hyp}-polydendron NPs were fully soluble in aqueous solution at low pH confirmed by increased PDI values and decreased $D_n$ size values (Chapter 4). Upon the addition of acid to the co-nanoprecipitated NP dispersions (Figure 5.14A) large aggregates were formed resulting in macroscale precipitation (Figure 5.14B).

![Figure 5.14](image)

\textbf{Figure 5.14} Photographs of co-nanoprecipitated \textit{AmG2}-\textit{p}(DEA_{50}) with \textit{EBiB}-\textit{p}(HPMA_{50}-co-EGDMA_{0.95}) NP dispersion in neutral water at a concentration of 1 mg mL$^{-1}$. A) Stable, cloudy NP dispersion before acid addition. B) Large, polymeric aggregates precipitating out of solution upon acid addition.

Upon acid addition, the linear-dendritic polymer of the co-nanoprecipitated system was solubilised and, therefore, unable to stabilise the \textit{EBiB}-\textit{p}(HPMA_{50}-co-EGDMA_{0.95}), which as previously demonstrated in Chapter 2, is not stable in acidic conditions. The \textit{EBiB}-\textit{p}(HPMA_{50}-co-EGDMA_{0.95}), therefore, aggregates to such a large size that it precipitates out of solution, which is due to a lack of stability. The co-nanoprecipitation of \textit{EBiB}-\textit{p}(HPMA_{50}-co-EGDMA_{0.95}) with linear DEA, provides a facile route to produce pH-triggered NPs with a hydrophobic branched core, however, the large polymeric aggregates produced upon acid addition are potentially problematic in the area of drug delivery, with concerns of effective clearance from the body. The potential to form wholly soluble molecules upon changes in pH is more attractive when considering excretion routes.$^5$ NPs are filtered out through either the glomerular capillary or remain within the vasculature, depending on their properties.$^6$ During extraction, they pass through several layers of cells which can filter materials up to sizes of 43 nm, however,
the functional or physiologic pore size is only around 5 nm.\textsuperscript{7}

Following this, the pH-response of the co-nanoprecipitated NPs consisting of \textit{EBiB-}p(tBuMA\textsubscript{50}-co-EGDMA\textsubscript{0.95}) stabilised by \textit{EBiB-}, \textit{AmG\textsubscript{2}}- and \textit{BnG\textsubscript{2}}-p(DEA\textsubscript{50}) prepared in neutral water were subjected to the rapid addition of acid (1M HCl) (Table 5.4). Upon the addition of acid to nanoprecipitates formed from \textit{AmG\textsubscript{2}}-p(DEA\textsubscript{50}-b-(tBuMA\textsubscript{65}-co-EGDMA\textsubscript{0.9}))), the NPs remained stable. As previously observed, the DEA/HPMA-containing co-nanoprecipitates underwent observable precipitation following dissolution of the linear-dendritic DEA polymer hybrid, compared to the covalently-bound statistical \textit{AmG\textsubscript{2}}-p(DEA\textsubscript{x}-co-HPMA\textsubscript{y}-co-EGDMA\textsubscript{0.9}) NPs becoming soluble in low pH.

Initially, the addition of acid (36 \textmu L 1M HCl aq.) was administered to the \textit{EBiB-}p(tBuMA\textsubscript{50}-co-EGDMA\textsubscript{0.95}) co-nanoprecipitated with the \textit{EBiB-}p(DEA\textsubscript{50}) and \textit{AmG\textsubscript{2}}-p(DEA\textsubscript{50}). Both NP dispersions continued to exist as self-assembled nanoprecipitates. The $D_z$ size values obtained for \textit{AmG\textsubscript{2}}-p(DEA\textsubscript{50}):\textit{EBiB-}p(tBuMA\textsubscript{50}-co-EGDMA\textsubscript{0.95}) NPs remained completely unchanged (Table 5.4), and the \textit{EBiB-}p(DEA\textsubscript{50}):\textit{EBiB-}p(tBuMA\textsubscript{50}-co-EGDMA\textsubscript{0.95}) NPs swelled slightly from DEA protonation on the nanoprecipitate surface, as previously witnessed in Chapter 2.

Table 5.4 DLS analysis for co-nanoprecipitated particles formed from linear DEA polymer and linear-dendritic DEA hybrids with \textit{EBiB-}p(tBuMA-co-EGDMA\textsubscript{0.95}) following the addition of acid.

<table>
<thead>
<tr>
<th>Branched polymer</th>
<th>pH = 7.8\textsuperscript{a}+1M HCl\textsuperscript{b} (Final pH 3.0-3.2)</th>
<th>+1M HCl\textsuperscript{c} (Final pH 3.0-3.2)</th>
<th>pH = 1.7-1.9 (24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{EBiB-}p(tBuMA-co-EGDMA\textsubscript{0.95})</td>
<td>$D_z$ (nm)</td>
<td>PDI</td>
<td>$D_z$ (nm)</td>
</tr>
<tr>
<td>\textit{EBiB-}</td>
<td>192</td>
<td>0.109</td>
<td>214</td>
</tr>
<tr>
<td>\textit{AmG\textsubscript{2}}-</td>
<td>234</td>
<td>0.051</td>
<td>233</td>
</tr>
<tr>
<td>\textit{BnG\textsubscript{2}}-</td>
<td>168</td>
<td>0.131</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Initial pH, \textsuperscript{b} 35 \textmu L, \textsuperscript{c} 350 \textmu L.

\textsuperscript{1} Italic indicates a bimodal size distribution measured by DLS.

\textsuperscript{2} – indicates no measurement.

In order to encourage hydrolysis of these NPs, more acid was administered (350 \textmu L, 1M HCl (aq.)). This was enough to fully solubilise the \textit{EBiB-}p(DEA\textsubscript{50}), causing macroscale precipitation of the \textit{EBiB-}p(tBuMA\textsubscript{50}-co-EGDMA\textsubscript{0.95}) (Figure 5.15A) due to \textit{EBiB-}p(tBuMA\textsubscript{50}-co-EGDMA\textsubscript{0.95}) previously demonstrating poor stability in any form of
aqueous media (Chapter 4). The **AmG2-p(DEA50):EBiB-p(tBuMA50-co-EGDMA0.95)** nanoprecipitates, however, retained stability and experienced an increase in particle size due to swelling of the NPs (Table 5.4 and Figure 5.15B).

![Figure 5.15](image)

**Figure 5.15** DLS size distribution analysis for co-nanoprecipitated particles formed from **EBiB-p(tBuMA50-co-EGDMA0.95)** with linear and linear-dendritic DEA polymers. A) **EBiB-p(DEA50)** and **EBiB-p(tBuMA50-co-EGDMA0.95)** before (green) and after addition of 35 μL (red) and 350 μL (red-dotted) 1M HCl (aq.); B) **AmG2-p(DEA50)** and **EBiB-p(tBuMA50-co-EGDMA0.95)** before (green) and after addition of 350 μL HCl (1M, aq.) (red) and after 24 hours (red-dotted); C) **BnG2-p(DEA50)** and **EBiB-p(tBuMA50-co-EGDMA0.95)** before (purple) and after 24 hours following the addition of acid 350 μL 1M HCl (aq.) (red).

The highly acidic NP dispersion was left over 24 hours and particle size was analysed, confirming the eventual solubilisation of the linear-dendritic polymer hybrid and the existence of large **EBiB-p(tBuMA50-co-EGDMA0.95)** precipitates (Figure 5.15B). These observations suggest higher levels of protonation are required to solubilise the increased amount of tertiary amine groups within the **AmG2-p(DEA50)**. The prolonged exposure to acid also suggests inaccessible dendron chain ends within the main bulk of the
nanoprecipitate and suggest that it is the protonation of the amine functional groups which finally achieves solubilisation.

Upon the addition of acid to co-nanoprecipitated \textit{BnG}_{2}\textit{p}(\textit{DEA}_{50})::\textit{EBiB}-\textit{p}(\textit{tBuMA}_{50}-\textit{co}-\textit{EGDMA}_{0.95}), the NPs experienced swelling due to protonation of the DEA chain on the nanoprecipitate surface (Table 5.4 and Figure 5.15C). After 24 hours, the NPs remained intact, suggesting continued stabilisation of the nanoprecipitates from the \textit{BnG}_{2}-\textit{p}(\textit{DEA}_{50}) from a lack of protonation and a stabilised combination of polymers.

5.5 Conclusions

The co-nanoprecipitation concept provides a relatively facile technique to produce stabilised polymer nanoprecipitates. Overall, the co-nanoprecipitated particles prepared with linear-dendritic polymers produced nanoprecipitates that were noticeably different compared to the nanoprecipitates prepared from similarly composed hyp-polydendrons. Independently, the linear-dendritic polymers generally failed to produce stable NP dispersions in neutral water when nanoprecipitated alone. The facile construction of NPs via co-nanoprecipitation produces a hydrophobic domain, with the opportunity to introduce specific characteristics and easily tailored surface functionality, to provide eventual enhanced stability. The stabilisation of \textit{EBiB}-\textit{p}(\textit{tBuMA}_{50}-\textit{co}-\textit{EGDMA}_{0.95}), achieved by co-nanoprecipitation with \textit{p}(\textit{DEA}_{50}), provides the first example within this project of stabilised \textit{p}(\textit{tBuMA}_{50}-\textit{co}-\textit{EGDMA}_{0.95}) NPs in neutral water. Additionally, a pH-response was introduced into the NP system, where a slower solubilisation of the pH-responsive \textit{AmG}_{2}-\textit{p}(\textit{DEA}_{50}) was observed compared to the particles containing \textit{EBiB}-\textit{p}(\textit{DEA}_{50}). The lack of solubilisation upon the addition of acid to particles co-nanoprecipitated from \textit{EBiB}-\textit{p}(\textit{tBuMA}_{50}-\textit{co}-\textit{EGDMA}_{0.95}) with \textit{BnG}_{2}-\textit{p}(\textit{DEA}_{50}), provides an insight into the close packed arrangement of NPs, and suggests presence of the dendron chain end within the main bulk of the nanoprecipitate.

The endurance of these co-nanoprecipitated NPs within different environments is essential should drug delivery application be considered. These studies provide an initial insight into how NPs behave differently depending on whether surface functionality is covalently bound or not.
5.6 Experimental

**Typical procedure for aqueous nanoprecipitation** – The materials were dissolved in acetone/THF at a concentration of 5 mg mL\(^{-1}\). 2 mL of this solution was then subjected to a rapid solvent switch through drop wise addition into 10 mL of water, to give a final polymer NP concentration of 1 mg mL\(^{-1}\) in water after acetone/THF removal by evaporation overnight.

**Typical procedure for aqueous co-nanoprecipitation** – The linear/linear-dendritic polymer solution (5 mg mL\(^{-1}\), 0.2 mL) and branched copolymer/hyp-polydendron solution (5 mg mL\(^{-1}\), 1.8 mL) were combined (2 mL) prior to a rapid solvent switch through drop wise addition into 10 mL of water, to give a final polymer NP concentration of 1 mg mL\(^{-1}\) in water after acetone/THF removal by evaporation overnight.

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Chapter 6

Pharmacological analysis of hyperbranched-polydendrons and co-nanoprecipitated systems
6.1 Introduction

The human immunodeficiency virus (HIV) is transmitted via contact with infected blood\(^1\) or through unprotected sex with an infected person.\(^2,3\) It initially infects dendritic cells, that consequently transport the virus into the lymphatic system to infect CD4+ T cells (a cytotoxic T lymphocyte that directly kill virally infected cells and produce and release antiviral cytokines) (Chapter 1, Section 1.1.1).\(^4\) The virus can reside in reservoirs, such as monocytes and macrophages,\(^5\) or in cells and tissues (known as sanctuary sites) that are inaccessible to drugs within the systemic circulation;\(^6\) current anti-retroviral therapies (ART) aim to reduce the viral levels in the blood. Ideally, a more effective treatment method would allow penetration of the sanctuary sites.

ART\(^7\) is a combination of drugs that penetrate cells through diffusion or active influx.\(^8\) Many of the drugs are poorly water-soluble,\(^9\) resulting in a lack of bioavailability. This equates to the need for large doses of administered drug in order to achieve therapeutic concentrations in the bloodstream. These drugs lead to a variety of dose-dependent side effects\(^10,11\) and, additionally, the large pill burden generates a high risk of treatment failure, due to poor patient adherence with the subsequent development of viral resistance.\(^12\) Numerous research efforts into the development of simplified and improved dosing regimens have been reported using nanocarrier strategies, particularly to overcome drug permeability issues\(^13\) and/or target specific cells or tissues.\(^14\)

The important factors that are considered during the design of drug delivery vehicles include: the capacity of the carrier to host an adequate drug payload; optimal release kinetics for each drug depending on mechanism and location of release within the body; mode of excretion and reactivity of all carrier metabolites.\(^15\) Specifically, the association of drug to a nanoparticle (NP) may be achieved either covalently or via encapsulation and several examples have been produced using many different polymer classes; encapsulation is featured in this work.\(^16\)

NPs possess high surface area to mass ratios, and drug NPs may result in more rapid and complete dissolution in the gut lumen and offer an enhanced delivery of an effective therapeutic dosage into the systemic system.\(^17,18\) In many cases, the modification of drug distribution and tissue penetration requires the circulating drug to be present as a NP rather than a dissolved drug molecule, and the benefits of nanocarriers are derived from the presentation of the drug in a NP format. In general, nanocarriers are injected directly
CHAPTER 6

into the bloodstream but orally-dosed NPs that can enter the bloodstream through the
gut, are a key nanomedicine target globally. This will allow simple oral dosing and
remove the need for daily injection within a clinical setting.

There are five main routes a NP can utilise to cross the gut epithelium and enter into the
bloodstream (Figure 6.1). In the case of solid drug NPs, surface adhesion followed by
subsequent dissolution results in high local concentrations that saturate drug transporters
(Figure 6.1.1). Solid drug NPs may also become trapped in the mucous resulting in rapid
dissolution close to the intestinal barrier; again, high local concentrations saturate efflux
systems (Figure 6.1.2). NPs of various types may avoid transport systems by
paracellular movement across the intestinal barrier (i.e. between cells); tight junctions
only allow permeation of small (<100 Da.) sized materials,19 so nanomaterials can be
functionalised to reversible opening tight junctions (Figure 6.1.3). The particulate nature
of the NPs may result in endocytosis/phagocytosis by immune cells or enterocytes; this
leaves potential for entry into the systemic circulation via Peyer’s patches20 (Figure
6.1.4). Finally NPs may enter the lymphatic system prior to entering the systemic
circulation21 (Figure 6.1.5), either releasing drug within during this process or after
prolonged circulation and accumulation.

Figure 6.1 The five main routes in which a NP can enter the systematic circulation: 1) Surface
interaction and subsequent dissolution; 2) Saturation of efflux systems: 3) Paracellular movement
across the intestinal barrier; 4) Peyer’s patches and; 5) Lymphatic system.

Stable NPs have been produced for the majority of the materials synthesised within this
project (Chapters 4 and 5), with particle sizes, surface charge and varying behaviour in
aqueous media investigated. A selection of these NP dispersions were taken forward for
pharmacological testing. A range of pharmacological evaluations were conducted
including, modelling the NPs crossing the gut epithelium, predicting the behaviour after oral administration, studying cellular uptake and cytotoxicity. These were carried out in order to evaluate whether these materials possessed any potential drug delivery benefits. The pharmacological experiments were carried out by Dr. Lee Tatham and Prof. Andrew Owen in the Department of Molecular and Clinical Pharmacology at the University of Liverpool.

Initially, the materials’ stability in the presence of a buffering vehicle media (Transport Buffer Solution; TBS) was assessed (Figure 6.2A). The stability in such is essential, as pharmacological tests were carried out in this media. TBS contains Hanks Buffer Saline Solution (25 mM), Bovine Serum Albumin (0.1% w/v) and is adjusted to pH = 7.4. Following this, materials were selected to investigate their loading capabilities with a hydrophobic traceable marker, fluoresceinamine (FA), as a surrogate for anti-retroviral drugs (Figure 6.2B). The successfully loaded materials were then progressed to in vitro pharmacological testing as previously mentioned (Figure 6.2C). The loading capabilities of the lead candidate exhibiting the most encouraging behaviour as an orally administered NP, was also loaded with an ART drug, Efavirenz (EFV) for future evaluations (Figure 6.2D) (cf. Section 6.3.5).

![Figure 6.2](image_url)

**Figure 6.2** The progression of selection and assessment for NP dispersions to undergo pharmacological testing. A) Addition of transport buffer solution to NP dispersion; B) Investigation of loading capabilities of the hydrophobic traceable marker, fluoresceinamine; C) In vitro pharmacological testing modelling the gut epithelium and; D) Investigation of loading capability of the anti-retroviral drug, Efavirenz.
6.2 Stability and encapsulation studies of linear and linear-dendritic polymers, branched copolymers and hyp-polypolydendrons

6.2.1 Stability studies in buffering vehicle media

A majority of the materials that produced monomodal, stable NP dispersions in neutral water (Chapters 2, 4 and 5) were subjected to the addition of buffering vehicle media (1 mL to 10 mL NP dispersion), in order to establish the ability of the nanoprecipitates to retain stability in a physiologically-relevant media. This included: all \( p(\text{HPMA}_{50}-\text{co-EGDMA}_{x}) \), \( p(\text{DEA}_{50}-\text{co-EGDMA}_{x}) \), \( p(\text{DEA}_{x}-\text{co-HPMA}_{y}-\text{co-EGDMA}_{0.9}) \), \( \text{AmG}_2-p(\text{DEA}_{50}-\text{co-EGDMA}_{0.95}) \); and the co-nanoprecipitated \( \text{EBiB}-p(\text{HPMA}_{50}-\text{co-EGDMA}_{0.95})\). Dispersions were adjusted to the physiological pH of blood, with TBS acting as a buffering vehicle. The buffering vehicle has no detectable impact on the various cells studied, allowing for accurate assessment of the nanocarrier effects in vitro. Following TBS addition, the changes in nanoprecipitate structure and assembly were investigated by dynamic light scattering (DLS) analysis and compared. In cases in which the NPs were unable to retain stability, observable macroscale precipitation was witnessed, without the need for DLS measurement, and only those that retained stability are shown in Table 6.1.

### Table 6.1 DLS analysis of nanoprecipitated and co-nanoprecipitated NP dispersions (10 mL, 1 mg mL\(^{-1}\)) that remained stable following the addition of transport buffer solution (1 mL).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>( D_z ) (nm)</th>
<th>PDI</th>
<th>( D_n ) (nm)</th>
<th>+ TBS (aq.)</th>
<th>( D_z ) (nm)</th>
<th>PDI</th>
<th>( D_n ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{AmG}<em>2-p(\text{HPMA}</em>{50}):\text{EBiB}-p(\text{HPMA}<em>{50}-\text{co-EGDMA}</em>{0.95}) )</td>
<td>210</td>
<td>0.072</td>
<td>187</td>
<td>356</td>
<td>0.126</td>
<td>290</td>
<td></td>
</tr>
<tr>
<td>( \text{AmG}<em>2-p(\text{DEA}</em>{50}-\text{co-EGDMA}_{0.9}) )</td>
<td>295</td>
<td>0.159</td>
<td>244</td>
<td>346</td>
<td>0.190</td>
<td>308</td>
<td></td>
</tr>
<tr>
<td>( \text{AmG}<em>1-p(\text{DEA}</em>{50}-\text{co-EGDMA}_{0.9}) )</td>
<td>134</td>
<td>0.148</td>
<td>82</td>
<td>39</td>
<td>0.228</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>( \text{AmG}<em>2-p(\text{DEA}</em>{50}-\text{b-(tBuMA}<em>{65}-\text{co-EGDMA}</em>{0.9}) )</td>
<td>163</td>
<td>0.082</td>
<td>123</td>
<td>163</td>
<td>0.100</td>
<td>123</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) Initial pH, \( ^b \) TBS (aq.) (1 mL) added to 10 mL (1 mg mL\(^{-1}\)) NP dispersion

The only NP dispersion containing HPMA monomer residues that was stable in TBS was the co-nanoprecipitated \( \text{AmG}_2-p(\text{HPMA}_{50}):\text{EBiB}-p(\text{HPMA}_{50}-\text{co-EGDMA}_{0.95}) \). This suggests the presence of the dendron chain end on the nanoprecipitate surface.
contributes a stabilising effect due to the amine functionality, potentially through the positive surface charge and possible small steric repulsion. This demonstrates that the dendritic functionality is advantageous, allowing stable NPs to exist in physiologically relevant media. The $\text{AmG}_2 \cdot p(\text{HPMA}_{50}) : E\text{Bib} \cdot p(\text{HPMA}_{50} \cdot co \cdot \text{EGDMA}_{0.95})$ NPs experienced an increase in particle size (210 nm to 356 nm) and size distribution (0.072 to 0.126), suggesting a slight aggregation under these conditions, possibly as a result of charge screening (Figure 6.3A).

![Figure 6.3](image)

**Figure 6.3** DLS size distribution analysis before and after TBS addition. A) $\text{AmG}_2 \cdot p(\text{HPMA}_{50}) : E\text{Bib} \cdot p(\text{HPMA}_{50} \cdot co \cdot \text{EGDMA}_{0.95})$ before (blue) and after (red-dotted) TBS addition; B) $\text{AmG}_2 \cdot p(\text{DEA}_{50} \cdot co \cdot \text{EGDMA}_{0.9})$ before (green) and after (red-dotted) TBS addition; C) $\text{AmG}_1 \cdot U \cdot p(\text{DEA}_{50} \cdot co \cdot \text{EGDMA}_{0.9})$ before (orange) and after (red-dotted) TBS addition; and D) $\text{AmG}_2 \cdot p(\text{DEA}_{50} \cdot b \cdot (\text{tBuMA}_{65} \cdot co \cdot \text{EGDMA}_{0.9}))$ before (red) and after (red-dotted) TBS addition.

The NP dispersions containing DEA monomer residues required either $\text{AmG}_1 \cdot U$- or $\text{AmG}_2$- dendritic surface functionality to retain stabilisation on addition of TBS. The $\text{AmG}_2 \cdot p(\text{DEA}_{50} \cdot co \cdot \text{EGDMA}_{0.9})$ NPs increased in size ($D_z = 346$ nm) and broadened in size distribution (PDI=0.190) (Figure 6.3B) whilst the $\text{AmG}_1 \cdot U \cdot p(\text{DEA}_{50} \cdot co \cdot \text{EGDMA}_{0.9})$ underwent a decrease in $D_z$ to 45 nm (Figure 6.3C). None of the $p(\text{DEA}_{x} \cdot co \cdot \text{HPMA}_{y} \cdot co$-
EGDMA0.9) NPs retained stability upon the addition of TBS and resulted in large polymeric aggregates from uncontrollable precipitation. All these materials contain a less positive surface charge (Chapter 4) compared to the \( p(\text{DEA}_{50}\text{-co-EGDMA}_x) \) NPs, suggesting a reduced level of charge stabilisation unable to withstand variation of electrolytes in aqueous media. The \( \text{AmG}_2-p(\text{DEA}_{50}-b-(\text{tBuMA}_{65}\text{-co-EGDMA}_{0.9})) \) proved stable suggesting that the increased primary polymer chain length, and architecture of linear-dendritic DEA polymer block arms contribute in some manner to the NP stabilisation process (Figure 6.3D). Following these simple stability studies, these materials underwent pharmacological testing.

### 6.2.2 Encapsulation studies of a hydrophobic fluorescent marker

Many polymeric drug carriers are designed to target two main administration routes: intravenous\(^{22}\) (IV) and oral.\(^{23}\) For the treatment of infectious diseases such as HIV, oral administration is preferable due to long-term daily dosing, despite the advantageous immediate introduction to the systemic circulation with IV administration. The ability of the polymeric particles to be loaded with a hydrophobic fluorescent marker acting as a drug model, was assessed. FA was selected for encapsulation due to its hydrophobic nature, thereby modelling poorly soluble drugs such as many anti-retroviral drugs.

The NPs were prepared as described in Chapter 4 with dissolution of FA (10 mg) in the good solvent (10 mL, producing a 1 mg mL\(^{-1}\) FA solution) before combination with the polymeric solutions, and subsequent nanoprecipitation into water; the NP sizes containing encapsulated FA are shown in Table 6.2 and the monomodal size distributions measured by DLS in Figure 6.4.
Table 6.2 DLS analysis of nanoparticles containing encapsulated FA.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>pH=7.8</th>
<th>+ FA (9 wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D_z$</td>
<td>PDI</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(HPMA$</em>{50}$):EBiB-$p$(HPMA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>210</td>
<td>0.072</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(DEA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>295</td>
<td>0.159</td>
</tr>
<tr>
<td>AmG$<em>1$-$p$(DEA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>134</td>
<td>0.148</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(DEA$</em>{50}$-b-(tBuMA$<em>{65}$-co-EGDMA$</em>{0.9}$))</td>
<td>163</td>
<td>0.082</td>
</tr>
</tbody>
</table>

$^a$ Initial pH, $^b$ 0.9 wt% FA loading, $^c$ 9 wt% FA loading

Figure 6.4 DLS size by intensity distributions of nanoprecipitated and co-nanoprecipitated particles with and without encapsulated FA: A) Blank AmG$_2$-$p$(HPMA$_{50}$):EBiB-$p$(HPMA$_{50}$-co-EGDMA$_{0.95}$) (blue), and encapsulated FA (0.9 wt%; orange-dotted); B) Blank AmG$_2$-$p$(DEA$_{50}$-co-EGDMA$_{0.9}$) (green), and encapsulated FA (9 wt%; orange-dotted); C) Blank AmG$_1$-$p$(DEA$_{50}$-co-EGDMA$_{0.9}$) (orange), and encapsulated FA (9 wt%; orange-dotted); and D) Blank AmG$_2$-$p$(DEA$_{50}$-b-(tBuMA$_{65}$-co-EGDMA$_{0.9}$)) (red), and encapsulated FA (9 wt%; orange-dotted).
The FA encapsulated NPs for pharmacological studies were prepared at 9 wt% (final polymer concentration 1 mg mL$^{-1}$ and theoretical FA amount 0.1 mg mL$^{-1}$), apart from the co-nanoprecipitated AmG$_2$p(HPMA$_{50}$):EBiB$\cdot$p(HPMA$_{50}$-co-EGDMA$_{0.95}$), which only managed to encapsulate 0.9 wt% FA following several attempts at higher loading. This may suggest a high proportion of dendron is residing within the main bulk of the nanoprecipitate aggregations. The polymeric NPs for this study included: a co-nanoprecipitated NP dispersion comprising of HPMA, two pH-responsive DEA hyp-polydendrons with varying generation of dendritic amine-functionality and dendron linker chemistry, and a block DEA-$\tau$BuMA-EGDMA hyp-polydendron.

These collectively produce a combined variation of branched polymer core, polymer properties, hyp-polydendron NPs and NPs arranged from a separate linear-dendritic and branched polymer, different dendron surface and linker chemistry and different polymer architecture, to be assessed in vitro using various pharmacological assays.

6.3 Pharmacological studies of hyp-polydendron and co-nanoprecipitated NPs containing encapsulated FA

6.3.1 Caco-2 Transcellular Permeation Assays

The oral delivery of polymeric nanocarriers via permeation through the gut epithelium into the systemic circulation was investigated, initially to determine whether the nanocarrier could permeate the monolayer. Following this, cellular accumulation studies were undertaken, and experiments carried out to define what mechanisms were responsible for the permeation.

The pharmacological assays performed were selected depending on the potential route of administration of the drug delivery vehicle. In the case of orally administered therapeutics, the Caco-2 (human epithelial colorectal adenocarcinoma) transcellular permeability assay is typically used to predict the permeability of materials across a model intestinal monolayer. This assay was adopted for this project in order to evaluate the potential permeation of the selected nanoprecipitated and co-nanoprecipitated NPs.

The Caco-2 cell line can be cultured to differentiate and become polarised to model the intestinal epithelial cells. It is important to consider, however, that Caco-2 cells do not form as tight monolayers as those found in vivo, with paracellular (between cells)
permeation reported for several NP systems. Additionally, the expression levels of the cell enzymes are highly unlikely to identically mimic those in the human body. Despite this, the movement of drug across the monolayer is predominantly restricted to permeation or active transport through the cells, and subsequent supporting cellular accumulation studies are carried out to help determine the mechanisms responsible.

A graphical representation of the transwell experiment is shown in Figure 6.5, with the apical (A) and basolateral (B) chambers shown either side of the epithelial monolayer and supporting membrane. The (A) chamber represents the gut side of the epithelium, whilst the (B) chamber represents the blood side of the epithelium. The experiment was conducted through incubation of the monolayer with the sample added to the (A) compartment, measuring the amount of movement across the monolayer over a 4 hour period from the A to B compartment (A>B); subsequent measurements were made by adding sample to the B compartment and monitoring the movement of sample from the B to A compartment (B>A).

![Figure 6.5](image)

The movement of sample across the membrane is typically reported as apparent permeability (pApp), which describes the flux at which the material traverses per unit area of the cell barrier. pApp is estimated using equation 6.1: where pApp is apparent permeability (cm s⁻¹); dQ/dt is the rate of transport (nM min⁻¹); v is the volume of the receiver compartment; A is the surface area of the membrane (cm²); and C₀ is the initial donor concentration.
\[ P_{\text{app}} = \frac{(dQ/dt) \times v}{A \times C_0} \]

**Equation 6.1** Calculation of apparent permeability where \( P_{\text{app}} \) is apparent permeability (cm s\(^{-1}\)); \( dQ/dt \) is the rate of transport (nM min\(^{-1}\)); \( v \) is the volume of the receiver compartment; \( A \) is the surface area of the membrane (cm\(^2\)); and \( C_0 \) is the initial donor concentration.

Several assumptions are made for an accurate measure of permeability.\(^{30}\) These include assumptions that the drug accumulated in the receiver compartment is proportional to time; “sink conditions” are compliant; and that cellular accumulation, metabolism and nonspecific binding to plastic-ware are absent. “Sink conditions” implies that once the material has traversed the monolayer it will not pass back across, however, this is not always the case with highly permeable materials.

The behaviour of the materials was investigated with respect to the impact of variation within their structural components. First, permeation across the Caco-2 *in vitro* model of human gut epithelium. This would allow a proof-of-concept to be developed for the synthesis and potential application of these novel systems.

### 6.3.1.1 Monolayer Integrity Assessment

The first experiment conducted was to assess the potential of the FA loaded NPs to compromise the cell monolayer as damage to the epithelium model will lead to a false assessment of permeation. To allow for comparisons, an “aqueous solution” of FA was prepared to mimic the permeation of un-encapsulated FA as a control; due to the insolubility of FA in water, FA was dissolved in dimethyl sulfoxide (DMSO) and subsequently used to spike TBS, so that < 0.5 vol% DMSO was present within the total aqueous volume. 10 \( \mu \)M of FA loaded co-nanoprecipitated, nanoprecipitated materials or 10 \( \mu \)M aqueous FA were added to the A or B chamber of the wells to quantify transport in both the A>B and B>A directions, and the plates were sampled 4 hours after incubation.

To assess the monolayer integrity following incubation, 100 \( \mu \)L of TBS containing 2 \( \mu \)L/mL \(^{14}\)C mannitol (0.2 \( \mu \)Ci/100 \( \mu \)L) was added to the A compartment and incubated for 1 hour. Scintillation fluid (4 mL) was added to 100 \( \mu \)L of both A and B sampled contents and quantified on the scintillation counter (Packard Tri-Carb 3100 Liquid Scintillation Analyser). The monolayer was considered compromised if the \(^{14}\)C-mannitol
pApp was $> 0.953 \times 10^{-6}$ cm s$^{-1}$. The apparent permeability results for the encapsulated FA samples are presented in Figure 6.6.

![Figure 6.6](image)

**Figure 6.6** pApp of $^{14}$C mannitol following 1 hour incubation, after monolayer was exposed to each FA sample (red-dotted line represents threshold value).

The co-nanoprecipitated $\text{AmG}_2\text{p}(\text{HPMA}_{50}:\text{EBiB}\text{-p}(\text{HPMA}_{50}\text{-co-EGDMA}_{0.95})$ appeared to compromise the monolayer over the 4 hour incubation period (pApp=1.23 $\times 10^{-6}$ cm s$^{-1}$) and was removed from further study. $\text{AmG}_1\text{U}\text{-p}(\text{DEA}_{50}\text{-co-EGDMA}_{0.9})$, $\text{AmG}_2\text{-p}(\text{DEA}_{50}\text{-co-EGDMA}_{0.9})$ and $\text{AmG}_2\text{-p}(\text{DEA}_{50}\text{-b-(tBuMA}_{65}\text{-co-EGDMA}_{0.9}))$ were taken forward for further testing.

### 6.3.1.2 Transcellular permeation studies

The transcellular permeation of FA across the Caco-2 cell monolayer following 4 hours incubation was measured through fluorescence monitoring (*via* high performance liquid chromatography; HPLC) to determine hyp-polydendron passage from the A compartment (A>B). Additionally, the movement of NPs from the B to A compartment (B>A) was measured to estimate the permeation from blood>gut, resulting from the presence of active transport proteins that have been known to limit oral bioavailability. Comparisons were made with aqueous A>B and B>A permeation.
The pApp results for the encapsulated FA materials are shown in Figure 6.7. The A>B movements are shown in blue and the B>A are shown in red.

**Figure 6.7** pApp of aqueous FA and NPs containing encapsulated FA (9 wt%) across the Caco-2 cell monolayer (A>B) (blue) and (B>A) (red) following a 4 hour incubation period. Data shown as +/- standard deviation.

**AmG1**-p(DEA50-co-EGDMA0.9) showed the greatest increase in the A>B movement of FA in the series (5 x 10^{-6} cm s^{-1}), although not statistically significant (P > 0.5) compared to the aqueous (1.44 x 10^{-6} cm s^{-1}). All encapsulated FA NPs showed an increased (or equal) B>A movement, although also not statistically significant (P > 0.5). The A>B movement provides a more informative measurement compared to B>A movement, and is overall an underestimation of drug permeation. The drug would not accumulate in the B compartment *in vivo*, but would move with the systematic circulation, therefore quenching any possible equilibrium of drug concentration. Equally, the B>A is an overestimation compared to *in vivo*, as the flow of the systematic circulation would prevent the nano-carriers being in contact with the cells for such a prolonged period of time. Despite this, **AmG1**-p(DEA50-co-EGDMA0.9) showed improved transcellular permeability properties compared to the equivalent aqueous preparation.

In order to provide a relative indication of apparent oral administration, the pApp ratio (A>B)/(B>A) was calculated (Figure 6.8). The Caco-2 cell monolayer was ensured to be
intact throughout. The pApp ratio (A>B/B>A) (Figure 6.8A) and efflux ratio (B>A/A>B) (Figure 6.8B), display the relative amount of encapsulated FA NPs that is transported in and out of the cell respectively, compared to aqueous FA.

Figure 6.8 A) pApp ratio A>B/B>A and B) efflux ratio B>A/A>B for FA encapsulated materials compared to the aqueous preparation of FA. Data shown as +/- standard deviation.

The pApp ratio of encapsulated FA $\text{AmG}_1^{U^{-}p(\text{DEA}_{50^{-}}\text{co-EGDMA}_{0.9})}$ NPs (>0.34) indicates considerable A>B movement, relative to B>A movement (more movement from gut to blood); and the efflux ratio of encapsulated FA $\text{AmG}_1^{U^{-}p(\text{DEA}_{50^{-}}\text{co-EGDMA}_{0.9})}$ NPs, indicates lessened B>A movement (>2.94) relative to A>B movement, suggesting less movement from blood to gut.

### 6.3.2 Cytotoxicity assays

Cytotoxicity assays were deemed appropriate to ensure the nanomaterials were not toxic to cells. Typical cytotoxicity assays include the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay and adenosine triphosphate (ATP) assay. Both of these assays measure the mitochondrial function of the cells, which may result in an underestimated level of cytotoxicity, as cytotoxic effects experienced by the entire cell may go unnoticed.

The MTT assay is based on the conversion of MTT to formazan crystals by mitochondria present in cells to determine mitochondrial activity. The mitochondrial activity is related to the number of viable living cells, therefore this assay can be used to assess a drug or material’s cytotoxic effects. The ATP assay method starts with the extraction of cells from the surface of cell culture flasks, in order to suspend in culture
media. The assay uses polypropylene plates to prevent the growth of non-neoplastic cells over a 5-day incubation period followed by detergent based extraction of cellular ATP. The cell production of ATP is a measure of the cell viability as viable cells produce ATP whereas dead cells do not. All NPs considered to not compromise the integrity of the monolayer were utilised in both cytotoxicity assays (Figures 6.9 and 6.10).

Cytotoxicity of the 4 aqueous nanoprecipitates was evaluated against the Caco-2 cell line and no appreciable cytotoxicity was observed at achievable concentrations in assays assessing either ATP (Figure 6.9) or MTT turnover (Figure 6.10) as such the determination of an IC$_{50}$ value for each polymer was not possible, indicating very low
cytotoxicity towards Caco-2 cells over the concentration range investigated.

Figure 6.10 Caco-2 cell MTT assay, 5 day incubation: A) Aqueous FA B) DMSO C) AmG2-p(DEA₅₀-b-(BuMA₆₅-co-EGDMA₀.₉)) D) AmG₂-p(DEA₅₀-co-EGDMA₀.₉) E) AmG₁-U-p(DEA₅₀-co-EGDMA₀.₉). Data shown as +/- standard deviation.

Additional cytotoxicity testing was carried out on the lead nanoprecipitated nanomaterial, AmG₁-U-p(DEA₅₀-co-EGDMA₀.₉) using Activated Tamm Horsfall Protein (ATHP-1) cells. ATHP-1 cells are monocyte-derived macrophage cells so it was important to ascertain the cytotoxicity of the materials with these cells, and ensure the NPs do not have any overt cytotoxicity to the host cell. The FA loaded sample showed increased cytotoxicity to ATHP-1 cells (35% (ATP) and 32% (MTT) cell viability) when compared with the aqueous sample of FA (76% (ATP) and 97% (MTT) cell
viability) during both assays over the 5 day incubation period (Figure 6.11 and 6.12). This may have been due to enhanced accumulation of FA in the ATHP-1 cells compared to the aqueous FA (cf. Caco-2 and macrophage cell accumulation studies), however, a cytotoxic effect or assay interference from the NPs cannot be ruled out.38

Figure 6.11 ATHP-1 cell ATP assay, 5 day incubation: A) Aqueous FA B) AmG1 U-p(DEA50-co-EGDMA0.9). Data shown as +/- standard deviation.

Figure 6.12 ATHP-1 cell MTT assay, 5 day incubation: A) Aqueous FA B) AmG1 U-p(DEA50-co-EGDMA0.9). Data shown as +/- standard deviation.

6.3.3 Caco-2 and ATHP-1 cell accumulation studies

AmG1 U-p(DEA50-co-EGDMA0.9) containing encapsulated FA was utilised in experiments to measure the cellular accumulation ratio (CAR) in Caco-2 and ATHP-1 cells (Figure 6.13). It is important to note, that the CAR results may also include NPs associated with the cell membrane, without definite confirmation of cell entry. ATHP-1 can model accumulation of molecules in macrophages.39
Figure 6.13 Cellular accumulation ratio for aqueous FA and AmG_1^U-p(DEA_{50-co}-EGDMA_{0.9}) containing encapsulated FA (9 wt%) in A) Caco-2 cells; and B) ATHP-1 cells. Data shown as +/- standard deviation; **, P < 0.01; and ***, P < 0.001 (ANOVA) (n=3).

Figure 6.13A shows that AmG_1^U-p(DEA_{50-co}-EGDMA_{0.9}) provides an increased accumulation of FA in Caco-2 cells (>0.018) compared to the aqueous (>0.007). Figure 6.13B also shows AmG_1^U-p(DEA_{50-co}-EGDMA_{0.9}) has an increased accumulation of FA in ATHP-1 cells (>0.024) compared to the aqueous (>0.005). The increased accumulation in Caco-2 cells suggests that the earlier observed permeation occurs via a transcellular, as opposed to paracellular, pathway. If this is the case, studies can be performed to understand whether it is an active or passive process through the cell.

6.3.4 Endocytosis inhibition

Polymeric complexes can enter mammalian cells through different endocytic pathways. For efficient optimisation of the nanocarrier it is important to profile its cellular uptake, because this largely determines its intracellular processing and subsequent permeation efficiency. Most of the current information on uptake of gene-delivery vehicles is based on data following the use of chemical inhibitors of endocytic pathways. Here, a detailed characterisation was performed using 5 commonly used endocytosis inhibitors: Chlorpromazine hydrochloride (Chl), Dansylcadaverine (Dan), Indomethacin (Ind), Genistein (Gen) and Dynasore hydrate (Dyn) on transcellular permeation and CAR in Caco-2 cells.

Initially, the % luminescence was measured with different levels of ATP depletion in Caco-2 cells, relative to an untreated control, using 2-Deoxyglucose and Rotenone. This was the control experiment to confirm that treating Caco-2 cells with 2-deoxyglucose
and rotenone depletes ATP, and was used to decide what concentrations to proceed with (Figure 6.14). In this instance, the measurement of ATP was performed using a luciferin-luciferase assay in a luminometer, which measures the production of ATP.42 Following this, cells were incubated with 2-Deoxyglucose/Rotenone for 20 min at 37°C, and encapsulated FA AmG1 U-p(DEA50-co-EGDMA0.9) NPs were added to the well in order to gain an understanding of whether transport was potentially active (ATP-dependent) or passive (non ATP-dependent). Figure 6.15 shows the Caco-2 CAR % of AmG1 U-p(DEA50-co-EGDMA0.9) encapsulated with FA (9 wt%), indicating a reduced, but not significant, CAR (48%) upon ATP depletion.

Figure 6.14 % Luminescence relative to untreated control following treatment of varying concentrations of 2-deoxyglucose/Rotenone. Data shown as +/- standard deviation.
Figure 6.15 Caco-2 CAR % of AmG1\textsuperscript{U}-p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) containing encapsulated FA (9 wt%) with and without ATP depletion. Data shown as +/- standard deviation.

Following this, the effect on transcellular permeation (pApp) in the presence of each inhibitor was considered (Figure 6.16). Transport across the endocytosis pathway is an active process\textsuperscript{43} and when these processes were inhibited, there was no significant drop in pApp (P > 0.5) for AmG1\textsuperscript{U}-p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) containing encapsulated FA. This suggests that the movement is not dependant on ATP.

Figure 6.16 pApp of aqueous FA (blue) and AmG1\textsuperscript{U}-p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) (9 wt% FA) (red) with various endocytosis inhibitors across the Caco-2 cell monolayer following a 4 hour incubation period. Data shown as +/- standard deviation.
Following this, the $^{14}$C-mannitol control experiment was repeated to investigate the integrity of the monolayer in the presence of the inhibitors (i.e. that the passage of FA is transcellular and not due to a compromised monolayer). Both experiments, aqueous FA (Figure 6.17A) and $\text{AmG}_1^{U-p(\text{DEA}_{50}^{co}-\text{EGDMA}_{0.9})}$ containing encapsulated FA (Figure 6.17B), suggest the monolayer remains intact throughout the incubation with aqueous FA and $\text{AmG}_1^{U-p(\text{DEA}_{50}^{co}-\text{EGDMA}_{0.9})}$ with the selected inhibitors.

**Figure 6.17** pApp of $^{14}$C mannitol following 1 hour incubation, after monolayer was exposed to aqueous FA (A) and $\text{AmG}_1^{U-p(\text{DEA}_{50}^{co}-\text{EGDMA}_{0.9})}$ containing encapsulated FA (9 wt%; B) with various endocytosis inhibitors (red-dotted line represents threshold value). Data shown as +/- standard deviation.
6.3.5 Encapsulation of an anti-retroviral therapy drug

\( \text{AmG}_1 \text{U}-p(\text{DEA}_{50}-co-\text{EGDMA}_{0.9}) \) was loaded with EFV using identical nanoprecipitation conditions for FA encapsulation. EFV is a non-nucleoside reverse transcriptase inhibitor which prevents the conversion of viral ribonucleic acid strands to deoxyribonucleic acid by the reverse transcriptase enzyme. EFV is considered a first-line global therapy for new cases of HIV infection and is well-tolerated by patients. Side-effects of EFV administration include neuropsychiatric disturbances (which may be persistent), low incidence of hepatotoxicity and metabolic alterations.\(^{44,45,46,47,48} \)

EFV was successfully encapsulated within the \( \text{AmG}_1 \text{U}-p(\text{DEA}_{50}-co-\text{EGDMA}_{0.9}) \) hypopolydendron NP dispersion at 9 wt%. This was confirmed by no observable macroscale precipitation of nanomaterial or drug, and a stable NP dispersion producing a monomodal size distribution (Figure 6.18).

![Figure 6.18](image)

**Figure 6.18** DLS size by intensity trace of blank \( \text{AmG}_1 \text{U}-p(\text{DEA}_{50}-co-\text{EGDMA}_{0.9}) \) (green), encapsulated FA (9 wt%; orange) and encapsulated EFV (9 wt%; blue).

**Table 6.3** DLS analysis of \( \text{AmG}_1 \text{U}-p(\text{DEA}_{50}-co-\text{EGDMA}_{0.9}) \) (blank) and containing encapsulated FA (9 wt%) or encapsulated EFV (9 wt%).

<table>
<thead>
<tr>
<th>pH=7.8</th>
<th>+ FA (9 wt%)</th>
<th>+ EFV (9 wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( D_c ) (nm)</td>
<td>( D_n ) (nm)</td>
<td>( D_c ) (nm)</td>
</tr>
<tr>
<td>134</td>
<td>0.148</td>
<td>59</td>
</tr>
<tr>
<td>82</td>
<td></td>
<td>28</td>
</tr>
</tbody>
</table>

The size of the encapsulated EFV NPs (\( D_c \) and \( D_n \)) was very similar to the encapsulated FA \( \text{AmG}_1 \text{U}-p(\text{DEA}_{50}-co-\text{EGDMA}_{0.9}) \) NPs, which were both noticeably smaller than the
un-encapsulated NPs (Table 6.3). This may suggest the dye or drug acts as a nucleation point and directs the aggregation process. Future studies for AmG1\textsuperscript{U-}p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) containing encapsulated EFV should include assessing pApp, cytotoxicity assessment, accumulation, and the efficacy of the nanocarrier against HIV, compared to aqueous EFV efficacy. Furthermore, in order to reduce the likelihood of HIV becoming resistant to a class of anti-retroviral drugs, healthcare providers recommend that people infected with HIV take a combination of anti-retroviral drugs in previously mentioned ART. Future studies may include the encapsulation of other drugs such as the protease inhibitors, Lopinavir and Ritonovir.\textsuperscript{49,50}

6.4 Conclusions

Overall, the research presented represents the study of previously synthesised materials within a pharmacological relevant manner. It is important to note, and issues have been addressed throughout, that there are limitations associated with these in vitro studies and how further investigations are essential to understand the behaviour of hyp-polydendron NPs. These results primarily suggest that the AmG1\textsuperscript{U-}p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) in particular, showed negligible cytotoxicity with a marked increase in transcellular pApp (A>B) without damage to the monolayer (membrane). The increased accumulation of encapsulated FA compared to the aqueous FA in Caco-2 and ATHP-1 cells could suggest uptake of the nano-carrier into primary lymphocytes and consequently, macrophages. This presents great advances for future developments that may allow oral dosing leading to circulating polymeric NPs. This may prove clinically desirable to many nonterminal or chronic diseases that utilise nanomedicines, but wish to avoid regular or repeated intravenous administration.

6.5 Experimental

Transcellular permeation studies: Cells were seeded at a density of 3.5 x 10\textsuperscript{4} per well into 24 well Costar→HTS transwell plates, with 0.33 cm\textsuperscript{2} surface area, and 3 \textmu M pore size, and propagated to a monolayer over a 21 day period. Only wells with transepithelial electrical resistance (TEER) values >800\textOmega were used. 10 \textmu M of FA hyp-polydendron or 10 \textmu M aqueous FA (transport buffer spiked with DMSO dissolved FA, DMSO final volume <0.5% of total volume), was added to the apical or basolateral compartment of the wells to quantify transport in both A>B and B>A directions. Plates
CHAPTER 6

were sampled following 4 hours incubation, A and B contents were stored at -30°C prior to analysis.

**Monolayer integrity studies:** To assess monolayer integrity following incubation, 100 µL of transport buffer containing 2 µL mL⁻¹ ¹⁴C mannitol was added to the apical compartment and incubated for 1 hour. 4 mL of scintillation fluid was added to 100 µL of the sampled contents and quantified on the scintillation counter.

**HPLC quantification of FA:** 150 µL of sample and prepared calibration for each hypopolypendron material, were extracted using 9 volumes of acetone, sonicated for 6 min and centrifuged for 3 min prior to drying at 30°C on a vacuum centrifuge. Each sample was reconstituted using 150 µL of 25% DMSO. Samples were run on a Dionex HPLC using a Fortis C18 column (100 mm x 4.6 mm i.d., 3 µm). The mobile phase consisted of: (A) 95% H₂O; 5% ACN; 5 mM NH₄FA (B) 95% ACN; 5% H₂O; 5 mM NH₄FA. Elution peaks were monitored with a fluorescence detector at; 490 (ex), 530 (em) (Thermo Spectrasystem FL3000) and subsequently analysed using Chromeleon v.6.8. software.

**Caco-2 and macrophage cell accumulation studies:** Caco-2 cells were seeded into 6 well plates (Nuncлон™) at a density of 4 x 10⁶ per well and incubated at 37°C 5% CO₂ for 24 hours. THP-1 cells were seeded at a density of 4 x 10⁶ cells per well in a 6 well plate and allowed to differentiate to ATHP-1 cells for 7 days in 10 nM PMA supplemented RPMI-1640 10% FBS prior to use. Following incubation, the media was aspirated and cells washed twice with HBSS (37°C) and subsequently replaced with prewarmed (37°C) Transport Buffer containing either 10 µM (final concentration) aqueous FA or 10 µM (final concentration) hypopolypendron formulated FA. Following 24 hours incubation at 37°C 5% CO₂, 150 µL of the extracellular media was sampled. The remaining media was aspirated and cells were washed twice with ice cold HBSS. The ice cold HBSS was aspirated and replaced with 500 µL of a 50% acetone 50% water solution and incubated for 24 hours at -20°C, 150 µL of the lysate was subsequently sampled. Finally, 9 volumes of acetone was added to each intracellular and extracellular sample to extract FA for quantification on the HPLC as previously described. Average cell volumes were previously determined using a Scepter 2.0 Automated Cell Counter (Millipore) and used to calculate Cellular Accumulation Ratios (CAR); (Intracellular concentration/Volume)/(Extracellular concentration/Volume).
Cytotoxicity assays: Caco-2 cells were seeded into 96 well plates (Nunclon™) at a density of $1 \times 10^4$ per well and incubated at 37°C 5% CO₂ for 24 hours. THP-1 cells were seeded at a density of $1 \times 10^4$ cells per well in 96 well plates and allowed to differentiate to ATHP-1 cells for 7 days in 10 nM PMA supplemented RPMI-1640 10% FBS at 37°C 5% CO₂ prior to use. Following incubation, the media was aspirated and replaced with media containing either varying concentrations (0.1 – 15 µM) of aqueous or hyp-polydendron formulated FA as determined by the molarity and mass of FA contained in each formulation. The treated cells were then incubated for either 24 or 120 hours at 37°C 5% CO₂. All cytotoxicity analyses were determined using both ATP and MTT cell viability assays.

ATP assays: ATP assays were conducted using CellTiter-Glo→ Reagent (Promega) in accordance with manufacturer’s instructions. Briefly, the plates and contents were initially equilibrated to room temperature. Subsequently, all but 20 µL of media was removed from each well and 20 µL CellTiter-Glo→ reagent was added to produce a 1:1 ratio. The contents were then mixed for 10 minutes on an orbital shaker to induce lysis and allow for stabilisation of luminescence signal prior to reading on a TECAN GENios plate reader. MTP assay: For MTT assays, 20 µL of a 5 mg mL⁻¹ MTT reagent was added to each well and incubated for 2 hours. Subsequently, 100 µL MTT lysis buffer (50% N-N-Dimethylformamide in water containing 20% SDS, 2.5% glacial acetic acid and 2.5% hydrochloric acid, pH 4.7) was added to each well and lysed overnight at 37°C 5% CO₂. Following incubation, the absorbance of each well was read using a TECAN GENios plate reader (560 nm).

ATP depletion of Caco-2 cells using varying concentrations of 2-Deoxyglucose and rotenone: Caco-2 cells were incubated with varying concentrations of 2-Deoxyglucose/rotenone (0/0, 1/50, 2/100 and 3/150 nM) as outlined for 20 min at 37°C 5% CO₂. Following incubation, ATP assays were carried out using CellTiter-GLO® kit as previously described to determine relative depletion of ATP compared to an untreated control.

Apparent permeability (Papp) of fluoresceinamine encapsulated in polymeric nanocarrier materials: Caco-2 cell monolayers were pre-incubated for 30 min in transport buffer containing either: 30 µM chlorpromazine (Chl); 200 µM dansylcadaverine (Dan); 10 µM genistein (Gen); 150 µM dynasore (Dyn); or 150 µM Indomethacin (Ind) prior to washing three times with pre-warmed (37°C) HBSS.
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Following washing, the Caco-2 monolayers were co-incubated with the various endocytosis inhibitors as outlined, and either aqueous (DMSO spiked; <0.1% v/v) or nanocarrier formulated fluoresceinamine for 4 h at 37°C 5% CO₂.

**Statistical analysis:** Data was statistically analysed using Prism 5 (GraphPad) and StatsDirect statistical software (version 2.7.9).

Chapter 6

Pharmacological analysis of hyperbranched-polydendrons and co-nanoprecipitated systems
6.1 Introduction

The human immunodeficiency virus (HIV) is transmitted via contact with infected blood\textsuperscript{1} or through unprotected sex with an infected person.\textsuperscript{2,3} It initially infects dendritic cells, that consequently transport the virus into the lymphatic system to infect CD4+ T cells (a cytotoxic T lymphocyte that directly kill virally infected cells and produce and release antiviral cytokines) (Chapter 1, Section 1.1.1).\textsuperscript{4} The virus can reside in reservoirs, such as monocytes and macrophages,\textsuperscript{5} or in cells and tissues (known as sanctuary sites) that are inaccessible to drugs within the systemic circulation;\textsuperscript{6} current anti-retroviral therapies (ART) aim to reduce the viral levels in the blood. Ideally, a more effective treatment method would allow penetration of the sanctuary sites.

ART\textsuperscript{7} is a combination of drugs that penetrate cells through diffusion or active influx.\textsuperscript{8} Many of the drugs are poorly water-soluble,\textsuperscript{9} resulting in a lack of bioavailability. This equates to the need for large doses of administered drug in order to achieve therapeutic concentrations in the bloodstream. These drugs lead to a variety of dose-dependent side effects\textsuperscript{10,11} and, additionally, the large pill burden generates a high risk of treatment failure, due to poor patient adherence with the subsequent development of viral resistance.\textsuperscript{12} Numerous research efforts into the development of simplified and improved dosing regimens have been reported using nanocarrier strategies, particularly to overcome drug permeability issues\textsuperscript{13} and/or target specific cells or tissues.\textsuperscript{14}

The important factors that are considered during the design of drug delivery vehicles include: the capacity of the carrier to host an adequate drug payload; optimal release kinetics for each drug depending on mechanism and location of release within the body; mode of excretion and reactivity of all carrier metabolites.\textsuperscript{15} Specifically, the association of drug to a nanoparticle (NP) may be achieved either covalently or via encapsulation and several examples have been produced using many different polymer classes; encapsulation is featured in this work.\textsuperscript{16}

NPs possess high surface area to mass ratios, and drug NPs may result in more rapid and complete dissolution in the gut lumen and offer an enhanced delivery of an effective therapeutic dosage into the systemic system.\textsuperscript{17,18} In many cases, the modification of drug distribution and tissue penetration requires the circulating drug to be present as a NP rather than a dissolved drug molecule, and the benefits of nanocarriers are derived from the presentation of the drug in a NP format. In general, nanocarriers are injected directly
into the bloodstream but orally-dosed NPs that can enter the bloodstream through the gut, are a key nanomedicine target globally. This will allow simple oral dosing and remove the need for daily injection within a clinical setting.

There are five main routes a NP can utilise to cross the gut epithelium and enter into the bloodstream (Figure 6.1). In the case of solid drug NPs, surface adhesion followed by subsequent dissolution results in high local concentrations that saturate drug transporters (Figure 6.1.1). Solid drug NPs may also become trapped in the mucous resulting in rapid dissolution close to the intestinal barrier; again, high local concentrations saturate efflux systems (Figure 6.1.2). NPs of various types may avoid transport systems by paracellular movement across the intestinal barrier (i.e. between cells); tight junctions only allow permeation of small (<100 Da.) sized materials, so nanomaterials can be functionalised to reversible opening tight junctions (Figure 6.1.3). The particulate nature of the NPs may result in endocytosis/phagocytosis by immune cells or enterocytes; this leaves potential for entry into the systemic circulation via Peyer’s patches (Figure 6.1.4). Finally NPs may enter the lymphatic system prior to entering the systemic circulation (Figure 6.1.5), either releasing drug within during this process or after prolonged circulation and accumulation.

Stable NPs have been produced for the majority of the materials synthesised within this project (Chapters 4 and 5), with particle sizes, surface charge and varying behaviour in aqueous media investigated. A selection of these NP dispersions were taken forward for pharmacological testing. A range of pharmacological evaluations were conducted.
including, modelling the NPs crossing the gut epithelium, predicting the behaviour after oral administration, studying cellular uptake and cytotoxicity. These were carried out in order to evaluate whether these materials possessed any potential drug delivery benefits. The pharmacological experiments were carried out by Dr. Lee Tatham and Prof. Andrew Owen in the Department of Molecular and Clinical Pharmacology at the University of Liverpool.

Initially, the materials’ stability in the presence of a buffering vehicle media (Transport Buffer Solution; TBS) was assessed (Figure 6.2A). The stability in such is essential, as pharmacological tests were carried out in this media. TBS contains Hanks Buffer Saline Solution (25 mM), Bovine Serum Albumin (0.1% w/v) and is adjusted to pH = 7.4. Following this, materials were selected to investigate their loading capabilities with a hydrophobic traceable marker, fluoresceinamine (FA), as a surrogate for anti-retroviral drugs (Figure 6.2B). The successfully loaded materials were then progressed to in vitro pharmacological testing as previously mentioned (Figure 6.2C). The loading capabilities of the lead candidate exhibiting the most encouraging behaviour as an orally administered NP, was also loaded with an ART drug, Efavirenz (EFV) for future evaluations (Figure 6.2D) (cf. Section 6.3.5).

Figure 6.2 The progression of selection and assessment for NP dispersions to undergo pharmacological testing. A) Addition of transport buffer solution to NP dispersion; B) Investigation of loading capabilities of the hydrophobic traceable marker, fluoresceinamine; C) In vitro pharmacological testing modelling the gut epithelium and; D) Investigation of loading capability of the anti-retroviral drug, Efavirenz.
6.2 Stability and encapsulation studies of linear and linear-dendritic polymers, branched copolymers and hyp-polydendrons

6.2.1 Stability studies in buffering vehicle media

A majority of the materials that produced monomodal, stable NP dispersions in neutral water (Chapters 2, 4 and 5) were subjected to the addition of buffering vehicle media (1 mL to 10 mL NP dispersion), in order to establish the ability of the nanoprecipitates to retain stability in a physiologically-relevant media. This included: all $p$(HPMA$_{50}$-co-EGDMA$_x$), $p$(DEA$_{50}$-co-EGDMA$_x$), $p$(DEA$_x$-co-HPMA$_x$-co-EGDMA$_{0.9}$), $\text{AmG}_2$-$p$(DEA$_{50}$-co-(tBuMA$_{65}$-co-EGDMA$_{0.9}$)); and the co-nanoprecipitated $\text{EBiB}$-$p$(HPMA$_{50}$-co-EGDMA$_{0.95}$):$p$(HPMA$_{50}$). Dispersions were adjusted to the physiological pH of blood, with TBS acting as a buffering vehicle. The buffering vehicle has no detectable impact on the various cells studied, allowing for accurate assessment of the nanocarrier effects in vitro. Following TBS addition, the changes in nanoprecipitate structure and assembly were investigated by dynamic light scattering (DLS) analysis and compared. In cases in which the NPs were unable to retain stability, observable macroscale precipitation was witnessed, without the need for DLS measurement, and only those that retained stability are shown in Table 6.1.

**Table 6.1** DLS analysis of nanoprecipitated and co-nanoprecipitated NP dispersions (10 mL, 1 mg mL$^{-1}$) that remained stable following the addition of transport buffer solution (1 mL).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$D_z$ (nm)</th>
<th>PDI</th>
<th>$D_n$ (nm)</th>
<th>$D_z$ (nm)</th>
<th>PDI</th>
<th>$D_n$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{AmG}<em>2$-$p$(HPMA$</em>{50}$):$\text{EBiB}$-$p$(HPMA$<em>{50}$-co-EGDMA$</em>{0.95}$)</td>
<td>210</td>
<td>0.072</td>
<td>187</td>
<td>356</td>
<td>0.126</td>
<td>290</td>
</tr>
<tr>
<td>$\text{AmG}<em>2$-$p$(DEA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>295</td>
<td>0.159</td>
<td>244</td>
<td>346</td>
<td>0.190</td>
<td>308</td>
</tr>
<tr>
<td>$\text{AmG}<em>1$-$p$(DEA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>134</td>
<td>0.148</td>
<td>82</td>
<td>39</td>
<td>0.228</td>
<td>18</td>
</tr>
<tr>
<td>$\text{AmG}<em>2$-$p$(DEA$</em>{50}$-$b$-(tBuMA$<em>{65}$-co-EGDMA$</em>{0.9}$))</td>
<td>163</td>
<td>0.082</td>
<td>123</td>
<td>163</td>
<td>0.100</td>
<td>123</td>
</tr>
</tbody>
</table>

$^a$ Initial pH, $^b$ TBS (aq.) (1 mL) added to 10 mL (1 mg mL$^{-1}$) NP dispersion

The only NP dispersion containing HPMA monomer residues that was stable in TBS was the co-nanoprecipitated $\text{AmG}_2$-$p$(HPMA$_{50}$):$\text{EBiB}$-$p$(HPMA$_{50}$-co-EGDMA$_{0.95}$). This suggests the presence of the dendron chain end on the nanoprecipitate surface
contributes a stabilising effect due to the amine functionality, potentially through the positive surface charge and possible small steric repulsion. This demonstrates that the dendritic functionality is advantageous, allowing stable NPs to exist in physiologically relevant media. The $\text{AmG}_2\cdot p(\text{HPMA}_{50}):\text{EBib}-p(\text{HPMA}_{50}-\text{co}-\text{EGDMA}_{0.95})$ NPs experienced an increase in particle size (210 nm to 356 nm) and size distribution (0.072 to 0.126), suggesting a slight aggregation under these conditions, possibly as a result of charge screening (Figure 6.3A).

![DLS size distribution analysis before and after TBS addition. A) $\text{AmG}_2\cdot p(\text{HPMA}_{50}):\text{EBib}-p(\text{HPMA}_{50}-\text{co}-\text{EGDMA}_{0.95})$ before (blue) and after (red-dotted) TBS addition; B) $\text{AmG}_2\cdot p(\text{DEA}_{50}-\text{co}-\text{EGDMA}_{0.9})$ before (green) and after (red-dotted) TBS addition; C) $\text{AmG}_1\cdot u^{-} p(\text{DEA}_{50}-\text{co}-\text{EGDMA}_{0.9})$ before (orange) and after (red-dotted) TBS addition; and D) $\text{AmG}_2\cdot p(\text{DEA}_{50}\cdot b^{-} (\text{tBuMA}_{65}-\text{co}-\text{EGDMA}_{0.9}))$ before (red) and after (red-dotted) TBS addition.](image)

The NP dispersions containing DEA monomer residues required either $\text{AmG}_1\cdot u^{-}$ or $\text{AmG}_2\cdot$ dendritic surface functionality to retain stabilisation on addition of TBS. The $\text{AmG}_2\cdot p(\text{DEA}_{50}-\text{co}-\text{EGDMA}_{0.9})$ NPs increased in size ($D_z = 346$ nm) and broadened in size distribution (PDI=0.190) (Figure 6.3B) whilst the $\text{AmG}_1\cdot u^{-} p(\text{DEA}_{50}-\text{co}-\text{EGDMA}_{0.9})$ underwent a decrease in $D_z$ to 45 nm (Figure 6.3C). None of the $p(\text{DEA}_{x}-\text{co}-\text{HPMA}_{y}-\text{co}-\text{EGDMA}_{0.9})$ NPs experienced aggregation under these conditions.
EGDMA_{0.9} NPs retained stability upon the addition of TBS and resulted in large polymeric aggregates from uncontrollable precipitation. All these materials contain a less positive surface charge (Chapter 4) compared to the \( p(\text{DEA}_{50}\text{-co-EGDMA}_x) \) NPs, suggesting a reduced level of charge stabilisation unable to withstand variation of electrolytes in aqueous media. The \( \text{AmG}_2\text{-p(DEA}_{50}\text{-b-(tBuMA}_{65}\text{-co-EGDMA}_{0.9})} \) proved stable suggesting that the increased primary polymer chain length, and architecture of linear-dendritic DEA polymer block arms contribute in some manner to the NP stabilisation process (Figure 6.3D). Following these simple stability studies, these materials underwent pharmacological testing.

### 6.2.2 Encapsulation studies of a hydrophobic fluorescent marker

Many polymeric drug carriers are designed to target two main administration routes: intravenous\(^2\) (IV) and oral.\(^3\) For the treatment of infectious diseases such as HIV, oral administration is preferable due to long-term daily dosing, despite the advantageous immediate introduction to the systemic circulation with IV administration. The ability of the polymeric particles to be loaded with a hydrophobic fluorescent marker acting as a drug model, was assessed. FA was selected for encapsulation due to its hydrophobic nature, thereby modelling poorly soluble drugs such as many anti-retroviral drugs.

The NPs were prepared as described in Chapter 4 with dissolution of FA (10 mg) in the good solvent (10 mL, producing a 1 mg mL\(^{-1}\) FA solution) before combination with the polymeric solutions, and subsequent nanoprecipitation into water; the NP sizes containing encapsulated FA are shown in Table 6.2 and the monomodal size distributions measured by DLS in Figure 6.4.
### Table 6.2 DLS analysis of nanoparticles containing encapsulated FA.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$D_1$ (nm)</th>
<th>PDI</th>
<th>$D_6$ (nm)</th>
<th>$D_1$ (nm)</th>
<th>PDI</th>
<th>$D_6$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmG$<em>2$p(HPMA$</em>{50}$)-EBiB$<em>p$(HPMA$</em>{50}$-co-EGDMA$_{0.95}$)</td>
<td>210</td>
<td>0.072</td>
<td>187</td>
<td>441$^b$</td>
<td>0.265</td>
<td>247</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>295</td>
<td>0.159</td>
<td>244</td>
<td>45$^c$</td>
<td>0.197</td>
<td>28</td>
</tr>
<tr>
<td>AmG$<em>1^U$p(DEA$</em>{50}$-co-EGDMA$_{0.9}$)</td>
<td>134</td>
<td>0.148</td>
<td>82</td>
<td>59$^c$</td>
<td>0.243</td>
<td>28</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{50}$-b-(tBuMA$<em>{65}$-co-EGDMA$</em>{0.9}$))</td>
<td>163</td>
<td>0.082</td>
<td>26</td>
<td>41$^c$</td>
<td>0.256</td>
<td>26</td>
</tr>
</tbody>
</table>

$^a$ Initial pH, $^b$ 0.9 wt% FA loading, $^c$ 9 wt% FA loading

**Figure 6.4** DLS size by intensity distributions of nanoprecipitated and co-nanoprecipitated particles with and without encapsulated FA: A) Blank AmG$_2$p(HPMA$_{50}$)-EBiB$_p$(HPMA$_{50}$-co-EGDMA$_{0.95}$) (blue), and encapsulated FA (0.9 wt%; orange-dotted); B) Blank AmG$_2$p(DEA$_{50}$-co-EGDMA$_{0.9}$) (green), and encapsulated FA (9 wt%; orange-dotted); C) Blank AmG$_1^U$p(DEA$_{50}$-co-EGDMA$_{0.9}$) (orange), and encapsulated FA (9 wt%; orange-dotted); and D) Blank AmG$_2$p(DEA$_{50}$-b-(tBuMA$_{65}$-co-EGDMA$_{0.9}$)) (red), and encapsulated FA (9 wt%; orange-dotted).
The FA encapsulated NPs for pharmacological studies were prepared at 9 wt% (final polymer concentration 1 mg mL\(^{-1}\) and theoretical FA amount 0.1 mg mL\(^{-1}\)), apart from the co-nanoprecipitated \(\text{AmG}_2-p(\text{HPMA}_{50}) : \text{EBiB}-p(\text{HPMA}_{50} - \text{co-EGDMA}_{0.95})\), which only managed to encapsulate 0.9 wt% FA following several attempts at higher loading. This may suggest a high proportion of dendron is residing within the main bulk of the nanoprecipitate aggregations. The polymeric NPs for this study included: a co-nanoprecipitated NP dispersion comprising of HPMA, two pH-responsive DEA hyp-polydendrons with varying generation of dendritic amine-functionality and dendron linker chemistry, and a block DEA-\(\text{rBuMA-EGDMA}\) hyp-polydendron.

These collectively produce a combined variation of branched polymer core, polymer properties, hyp-polydendron NPs and NPs arranged from a separate linear-dendritic and branched polymer, different dendron surface and linker chemistry and different polymer architecture, to be assessed \textit{in vitro} using various pharmacological assays.

### 6.3 Pharmacological studies of hyp-polydendron and co-nanoprecipitated NPs containing encapsulated FA

#### 6.3.1 Caco-2 Transcellular Permeation Assays

The oral delivery of polymeric nanocarriers \textit{via} permeation through the gut epithelium into the systemic circulation was investigated, initially to determine whether the nanocarrier could permeate the monolayer. Following this, cellular accumulation studies were undertaken, and experiments carried out to define what mechanisms were responsible for the permeation.

The pharmacological assays performed were selected depending on the potential route of administration of the drug delivery vehicle. In the case of orally administered therapeutics, the Caco-2 (human epithelial colorectal adenocarcinoma) transcellular permeability assay is typically used to predict the permeability of materials across a model intestinal monolayer\(^{24}\). This assay was adopted for this project in order to evaluate the potential permeation of the selected nanoprecipitated and co-nanoprecipitated NPs.

The Caco-2 cell line can be cultured to differentiate and become polarised to model the intestinal epithelial cells\(^{25}\). It is important to consider, however, that Caco-2 cells do not form as tight monolayers as those found \textit{in vivo}, with paracellular (between cells)
permeation reported for several NP systems. Additionally, the expression levels of the cell enzymes are highly unlikely to identically mimic those in the human body. Despite this, the movement of drug across the monolayer is predominantly restricted to permeation or active transport through the cells, and subsequent supporting cellular accumulation studies are carried out to help determine the mechanisms responsible.

A graphical representation of the transwell experiment is shown in Figure 6.5, with the apical (A) and basolateral (B) chambers shown either side of the epithelial monolayer and supporting membrane. The (A) chamber represents the gut side of the epithelium, whilst the (B) chamber represents the blood side of the epithelium. The experiment was conducted through incubation of the monolayer with the sample added to the (A) compartment, measuring the amount of movement across the monolayer over a 4 hour period from the A to B compartment (A>B); subsequent measurements were made by adding sample to the B compartment and monitoring the movement of sample from the B to A compartment (B>A).

![Figure 6.5 Transwell experiment setup showing apical and basolateral chambers separated by Caco-2 cell monolayer.](image)

The movement of sample across the membrane is typically reported as apparent permeability (pApp), which describes the flux at which the material traverses per unit area of the cell barrier. pApp is estimated using equation 6.1: where pApp is apparent permeability (cm s⁻¹); dQ/dt is the rate of transport (nM min⁻¹); v is the volume of the receiver compartment; A is the surface area of the membrane (cm²); and C₀ is the initial donor concentration.
Equation 6.1 Calculation of apparent permeability where \( p_{\text{App}} \) is apparent permeability (cm s\(^{-1}\)); \( \frac{dQ}{dt} \) is the rate of transport (nM min\(^{-1}\)); \( \nu \) is the volume of the receiver compartment; \( A \) is the surface area of the membrane (cm\(^2\)); and \( C_0 \) is the initial donor concentration.

Several assumptions are made for an accurate measure of permeability. These include assumptions that the drug accumulated in the receiver compartment is proportional to time; “sink conditions” are compliant; and that cellular accumulation, metabolism and nonspecific binding to plastic-ware are absent. “Sink conditions” implies that once the material has traversed the monolayer it will not pass back across, however, this is not always the case with highly permeable materials.

The behaviour of the materials was investigated with respect to the impact of variation within their structural components. First, permeation across the Caco-2 \textit{in vitro} model of human gut epithelium. This would allow a proof-of-concept to be developed for the synthesis and potential application of these novel systems.

### 6.3.1.1 Monolayer Integrity Assessment

The first experiment conducted was to assess the potential of the FA loaded NPs to compromise the cell monolayer as damage to the epithelium model will lead to a false assessment of permeation. To allow for comparisons, an “aqueous solution” of FA was prepared to mimic the permeation of un-encapsulated FA as a control; due to the insolubility of FA in water, FA was dissolved in dimethyl sulfoxide (DMSO) and subsequently used to spike TBS, so that < 0.5 vol\% DMSO was present within the total aqueous volume. 10 \( \mu \)M of FA loaded co-nanoprecipitated, nanoprecipitated materials or 10 \( \mu \)M aqueous FA were added to the A or B chamber of the wells to quantify transport in both the A>B and B>A directions, and the plates were sampled 4 hours after incubation.

To assess the monolayer integrity following incubation, 100 \( \mu \)L of TBS containing 2 \( \mu \)L/mL \(^{14}\)C mannitol (0.2 \( \mu \)Ci/100 \( \mu \)L) was added to the A compartment and incubated for 1 hour. Scintillation fluid (4 mL) was added to 100 \( \mu \)L of both A and B sampled contents and quantified on the scintillation counter (Packard Tri-Carb 3100 Liquid Scintillation Analyser). The monolayer was considered compromised if the \(^{14}\)C-mannitol
pApp was > 0.953 x 10^{-6} \text{ cm s}^{-1}. The apparent permeability results for the encapsulated FA samples are presented in Figure 6.6.

![Figure 6.6](image)

**Figure 6.6** pApp of ^{14}\text{C} mannitol following 1 hour incubation, after monolayer was exposed to each FA sample (red-dotted line represents threshold value).

The co-nanoprecipitated AmG_2-p(HPMA_{50}):EBiB-p(HPMA_{50}-co-EGDMA_{0.95}) appeared to compromise the monolayer over the 4 hour incubation period (pApp=1.23 x 10^{-6} \text{ cm s}^{-1}) and was removed from further study. AmG_1^{U}-p(DEA_{50}-co-EGDMA_{0.9}), AmG_2-p(DEA_{50}-co-EGDMA_{0.9}) and AmG_2-p(DEA_{50}-b-(tBuMA_{65}-co-EGDMA_{0.9})) were taken forward for further testing.

### 6.3.1.2 Transcellular permeation studies

The transcellular permeation of FA across the Caco-2 cell monolayer following 4 hours incubation was measured through fluorescence monitoring (*via* high performance liquid chromatography; HPLC) to determine hyp-polypendron passage from the A compartment (A>B). Additionally, the movement of NPs from the B to A compartment (B>A) was measured to estimate the permeation from blood>gut, resulting from the presence of active transport proteins that have been known to limit oral bioavailability. Comparisons were made with aqueous A>B and B>A permeation.
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The pApp results for the encapsulated FA materials are shown in Figure 6.7. The A>B movements are shown in blue and the B>A are shown in red.

Figure 6.7 pApp of aqueous FA and NPs containing encapsulated FA (9 wt%) across the Caco-2 cell monolayer (A>B) (blue) and (B>A) (red) following a 4 hour incubation period. Data shown as +/- standard deviation.

AmG1U-p(DEA50-co-EGDMA0.9) showed the greatest increase in the A>B movement of FA in the series (5 x 10^-6 cm s^-1), although not statistically significant (P > 0.5) compared to the aqueous (1.44 x 10^-6 cm s^-1). All encapsulated FA NPs showed an increased (or equal) B>A movement, although also not statistically significant (P > 0.5). The A>B movement provides a more informative measurement compared to B>A movement, and is overall an underestimation of drug permeation. The drug would not accumulate in the B compartment in vivo, but would move with the systematic circulation, therefore quenching any possible equilibrium of drug concentration. Equally, the B>A is an overestimation compared to in vivo, as the flow of the systematic circulation would prevent the nano-carriers being in contact with the cells for such a prolonged period of time. Despite this, AmG1U-p(DEA50-co-EGDMA0.9) showed improved transcellular permeability properties compared to the equivalent aqueous preparation.

In order to provide a relative indication of apparent oral administration, the pApp ratio (A>B)/(B>A) was calculated (Figure 6.8). The Caco-2 cell monolayer was ensured to be
intact throughout. The pApp ratio (A>B/B>A) (Figure 6.8A) and efflux ratio (B>A/A>B) (Figure 6.8B), display the relative amount of encapsulated FA NPs that is transported in and out of the cell respectively, compared to aqueous FA.

The pApp ratio of encapsulated FA AmG1_U-p(DEA50-co-EGDMA0.9) NPs (>0.34) indicates considerable A>B movement, relative to B>A movement (more movement from gut to blood); and the efflux ratio of encapsulated FA AmG1_U-p(DEA50-co-EGDMA0.9) NPs, indicates lessened B>A movement (>2.94) relative to A>B movement, suggesting less movement from blood to gut.

6.3.2 Cytotoxicity assays

Cytotoxicity assays were deemed appropriate to ensure the nanomaterials were not toxic to cells. Typical cytotoxicity assays include the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay and adenosine triphosphate (ATP) assay. Both of these assays measure the mitochondrial function of the cells, which may result in an underestimated level of cytotoxicity, as cytotoxic effects experienced by the entire cell may go unnoticed.

The MTT assay is based on the conversion of MTT to formazan crystals by mitochondria present in cells to determine mitochondrial activity. The mitochondrial activity is related to the number of viable living cells, therefore this assay can be used to assess a drug or material’s cytotoxic effects. The ATP assay method starts with the extraction of cells from the surface of cell culture flasks, in order to suspend in culture.
media. The assay uses polypropylene plates to prevent the growth of non-neoplastic cells over a 5-day incubation period followed by detergent based extraction of cellular ATP. The cell production of ATP is a measure of the cell viability as viable cells produce ATP whereas dead cells do not.\(^{37}\) All NPs considered to not compromise the integrity of the monolayer were utilised in both cytotoxicity assays (Figures 6.9 and 6.10).

**Figure 6.9** Caco-2 cell ATP assay, 5 day incubation: A) Aqueous FA B) DMSO C) AmG\(_2\)-p(DEA\(_{50}\)-b-(BuMA\(_{65}\)-co-EGDMA\(_{0.9}\))) D) AmG\(_2\)-p(DEA\(_{50}\)-co-EGDMA\(_{0.9}\)) E) AmG\(_1\)U-p(DEA\(_{50}\)-co-EGDMA\(_{0.9}\)). Data shown as +/- standard deviation.

Cytotoxicity of the 4 aqueous nanoprecipitates was evaluated against the Caco-2 cell line and no appreciable cytotoxicity was observed at achievable concentrations in assays assessing either ATP (Figure 6.9) or MTT turnover (Figure 6.10) as such the determination of an IC\(_{50}\) value for each polymer was not possible, indicating very low
cytotoxicity towards Caco-2 cells over the concentration range investigated.

Additional cytotoxicity testing was carried out on the lead nanoprecipitated nanomaterial, AmG1^U-p(DEA50-co-EGDMA0.9) using Activated Tamm Horsfall Protein (ATHP-1) cells. ATHP-1 cells are monocyte-derived macrophage cells so it was important to ascertain the cytotoxicity of the materials with these cells, and ensure the NPs do not have any overt cytotoxicity to the host cell. The FA loaded sample showed increased cytotoxicity to ATHP-1 cells (35% (ATP) and 32% (MTT) cell viability) when compared with the aqueous sample of FA (76% (ATP) and 97% (MTT) cell viability).

Figure 6.10 Caco-2 cell MTT assay, 5 day incubation: A) Aqueous FA B) DMSO C) AmG2-p(DEA50-b-(BuMA65-co-EGDMA0.9)) D) AmG2-p(DEA50-co-EGDMA0.9) E) AmG1^U-p(DEA50-co-EGDMA0.9). Data shown as +/- standard deviation.
viability) during both assays over the 5 day incubation period (Figure 6.11 and 6.12). This may have been due to enhanced accumulation of FA in the ATHP-1 cells compared to the aqueous FA (cf. Caco-2 and macrophage cell accumulation studies), however, a cytotoxic effect or assay interference from the NPs cannot be ruled out.  

Figure 6.11 ATHP-1 cell ATP assay, 5 day incubation: A) Aqueous FA B) AmG1^U^-p(DEA50-co-EGDMA0.9). Data shown as +/- standard deviation.

Figure 6.12 ATHP-1 cell MTT assay, 5 day incubation: A) Aqueous FA B) AmG1^U^-p(DEA50-co-EGDMA0.9). Data shown as +/- standard deviation.

6.3.3 Caco-2 and ATHP-1 cell accumulation studies

AmG1^U^-p(DEA50-co-EGDMA0.9) containing encapsulated FA was utilised in experiments to measure the cellular accumulation ratio (CAR) in Caco-2 and ATHP-1 cells (Figure 6.13). It is important to note, that the CAR results may also include NPs associated with the cell membrane, without definite confirmation of cell entry. ATHP-1 can model accumulation of molecules in macrophages.
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Figure 6.13 Cellular accumulation ratio for aqueous FA and AmG1\textsuperscript{U}p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) containing encapsulated FA (9 wt%) in A) Caco-2 cells; and B) ATHP-1 cells. Data shown as +/- standard deviation; **, P < 0.01; and ***, P < 0.001 (ANOVA) (n=3).

Figure 6.13A shows that AmG1\textsuperscript{U}p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) provides an increased accumulation of FA in Caco-2 cells (>0.018) compared to the aqueous (>0.007). Figure 6.13B also shows AmG1\textsuperscript{U}p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) has an increased accumulation of FA in ATHP-1 cells (>0.024) compared to the aqueous (>0.005). The increased accumulation in Caco-2 cells suggests that the earlier observed permeation occurs via a transcellular, as opposed to paracellular, pathway. If this is the case, studies can be performed to understand whether it is an active or passive process through the cell.

6.3.4 Endocytosis inhibition

Polymeric complexes can enter mammalian cells through different endocytic pathways.\textsuperscript{40} For efficient optimisation of the nanocarrier it is important to profile its cellular uptake, because this largely determines its intracellular processing and subsequent permeation efficiency. Most of the current information on uptake of gene-delivery vehicles is based on data following the use of chemical inhibitors of endocytic pathways.\textsuperscript{41} Here, a detailed characterisation was performed using 5 commonly used endocytosis inhibitors: Chlorpromazine hydrochloride (Chl), Dansylcadaverine (Dan), Indomethacin (Ind), Genistein (Gen) and Dynasore hydrate (Dyn) on transcellular permeation and CAR in Caco-2 cells.

Initially, the % luminescence was measured with different levels of ATP depletion in Caco-2 cells, relative to an untreated control, using 2-Deoxyglucose and Rotenone. This was the control experiment to confirm that treating Caco-2 cells with 2-deoxyglucose
and rotenone depletes ATP, and was used to decide what concentrations to proceed with (Figure 6.14). In this instance, the measurement of ATP was performed using a luciferin-luciferase assay in a luminometer, which measures the production of ATP. Following this, cells were incubated with 2-Deoxyglucose/Rotenone for 20 min at 37°C, and encapsulated FA AmG1U-p(DEA50-co-EGDMA0.9) NPs were added to the well in order to gain an understanding of whether transport was potentially active (ATP-dependant) or passive (non ATP-dependant). Figure 6.15 shows the Caco-2 CAR % of AmG1U-p(DEA50-co-EGDMA0.9) encapsulated with FA (9 wt%), indicating a reduced, but not significant, CAR (48%) upon ATP depletion.

![Figure 6.14](image-url) % Luminescence relative to untreated control following treatment of varying concentrations of 2-deoxyglucose/Rotenone. Data shown as +/- standard deviation.
Figure 6.15 Caco-2 CAR % of AmG1\textsuperscript{U} -p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) containing encapsulated FA (9 wt%) with and without ATP depletion. Data shown as +/- standard deviation.

Following this, the effect on transcellular permeation (pApp) in the presence of each inhibitor was considered (Figure 6.16). Transport across the endocytosis pathway is an active process\textsuperscript{43} and when these processes were inhibited, there was no significant drop in pApp (P > 0.5) for AmG1\textsuperscript{U} -p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) containing encapsulated FA. This suggests that the movement is not dependant on ATP.

Figure 6.16 pApp of aqueous FA (blue) and AmG1\textsuperscript{U} -p(DEA\textsubscript{50}-co-EGDMA\textsubscript{0.9}) (9 wt% FA) (red) with various endocytosis inhibitors across the Caco-2 cell monolayer following a 4 hour incubation period. Data shown as +/- standard deviation.
Following this, the $^{14}$C-mannitol control experiment was repeated to investigate the integrity of the monolayer in the presence of the inhibitors (i.e. that the passage of FA is transcellular and not due to a compromised monolayer). Both experiments, aqueous FA (Figure 6.17A) and $\text{AmG}_{1}^{U-p(\text{DEA}_{50}^{\circ}-\text{co-EGDMA}_{0.9})}$ containing encapsulated FA (Figure 6.17B), suggest the monolayer remains intact throughout the incubation with aqueous FA and $\text{AmG}_{1}^{U-p(\text{DEA}_{50}^{\circ}-\text{co-EGDMA}_{0.9})}$ with the selected inhibitors.

**Figure 6.17** pApp of $^{14}$C mannitol following 1 hour incubation, after monolayer was exposed to aqueous FA (A) and $\text{AmG}_{1}^{U-p(\text{DEA}_{50}^{\circ}-\text{co-EGDMA}_{0.9})}$ containing encapsulated FA (9 wt%; B) with various endocytosis inhibitors (red-dotted line represents threshold value). Data shown as +/- standard deviation.
6.3.5 Encapsulation of an anti-retroviral therapy drug

\( \text{AmG}_1 \text{U-p(DEA}_{50}\text{-co-EGDMA}_{0.9}) \) was loaded with EFV using identical nanoprecipitation conditions for FA encapsulation. EFV is a non-nucleoside reverse transcriptase inhibitor which prevents the conversion of viral ribonucleic acid strands to deoxyribonucleic acid by the reverse transcriptase enzyme. EFV is considered a first-line global therapy for new cases of HIV infection and is well-tolerated by patients. Side-effects of EFV administration include neuropsychiatric disturbances (which may be persistent), low incidence of hepatotoxicity and metabolic alterations.\(^{44,45,46,47,48} \) EFV was successfully encapsulated within the \( \text{AmG}_1 \text{U-p(DEA}_{50}\text{-co-EGDMA}_{0.9}) \) hyp-polypendron NP dispersion at 9 wt%. This was confirmed by no observable macroscale precipitation of nanomaterial or drug, and a stable NP dispersion producing a monomodal size distribution (Figure 6.18).

![Figure 6.18](image)

**Table 6.3** DLS analysis of \( \text{AmG}_1 \text{U-p(DEA}_{50}\text{-co-EGDMA}_{0.9}) \) (blank) and containing encapsulated FA (9 wt%) or encapsulated EFV (9 wt%).

<table>
<thead>
<tr>
<th>pH=7.8</th>
<th>+ FA (9 wt%)</th>
<th>+ EFV (9 wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( D_z ) (nm)</td>
<td>PDI</td>
</tr>
<tr>
<td></td>
<td>134</td>
<td>0.148</td>
</tr>
</tbody>
</table>

The size of the encapsulated EFV NPs (\( D_z \) and \( D_n \)) was very similar to the encapsulated FA \( \text{AmG}_1 \text{U-p(DEA}_{50}\text{-co-EGDMA}_{0.9}) \) NPs, which were both noticeably smaller than the
un-encapsulated NPs (Table 6.3). This may suggest the dye or drug acts as a nucleation point and directs the aggregation process. Future studies for $\text{AmG}_1^\text{U}\text{-}p(\text{DEA}_{50}\text{-co-EGDMA}_{0.9})$ containing encapsulated EFV should include assessing pApp, cytotoxicity assessment, accumulation, and the efficacy of the nanocarrier against HIV, compared to aqueous EFV efficacy. Furthermore, in order to reduce the likelihood of HIV becoming resistant to a class of anti-retroviral drugs, healthcare providers recommend that people infected with HIV take a combination of anti-retroviral drugs in previously mentioned ART. Future studies may include the encapsulation of other drugs such as the protease inhibitors, Lopinavir and Ritonovir.\textsuperscript{49,50}

### 6.4 Conclusions

Overall, the research presented represents the study of previously synthesised materials within a pharmacological relevant manner. It is important to note, and issues have been addressed throughout, that there are limitations associated with these in vitro studies and how further investigations are essential to understand the behaviour of hyp-polydendron NPs. These results primarily suggest that the $\text{AmG}_1^\text{U}\text{-}p(\text{DEA}_{50}\text{-co-EGDMA}_{0.9})$ in particular, showed negligible cytotoxicity with a marked increase in transcellular pApp (A>B) without damage to the monolayer (membrane). The increased accumulation of encapsulated FA compared to the aqueous FA in Caco-2 and ATHP-1 cells could suggest uptake of the nano-carrier into primary lymphocytes and consequently, macrophages. This presents great advances for future developments that may allow oral dosing leading to circulating polymeric NPs. This may prove clinically desirable to many nonterminal or chronic diseases that utilise nanomedicines, but wish to avoid regular or repeated intravenous administration.

### 6.5 Experimental

**Transcellular permeation studies:** Cells were seeded at a density of $3.5 \times 10^4$ per well into 24 well Costar→HTS transwell plates, with 0.33 cm$^2$ surface area, and 3 μM pore size, and propagated to a monolayer over a 21 day period. Only wells with transepithelial electrical resistance (TEER) values $>800\,\Omega$ were used. 10 μM of FA hyp-polydendron or 10 μM aqueous FA (transport buffer spiked with DMSO dissolved FA, DMSO final volume <0.5% of total volume), was added to the apical or basolateral compartment of the wells to quantify transport in both A>B and B>A directions. Plates
were sampled following 4 hours incubation, A and B contents were stored at -30°C prior to analysis.

**Monolayer integrity studies:** To assess monolayer integrity following incubation, 100 μL of transport buffer containing 2 μL mL⁻¹ ¹⁴C mannitol was added to the apical compartment and incubated for 1 hour. 4 mL of scintillation fluid was added to 100 μL of the sampled contents and quantified on the scintillation counter.

**HPLC quantification of FA:** 150 μL of sample and prepared calibration for each hyp-polydendron material, were extracted using 9 volumes of acetone, sonicated for 6 min and centrifuged for 3 min prior to drying at 30°C on a vacuum centrifuge. Each sample was reconstituted using 150 μL of 25% DMSO. Samples were run on a Dionex HPLC using a Fortis C18 column (100 mm x 4.6 mm i.d., 3 μm). The mobile phase consisted of: (A) 95% H₂O; 5% ACN; 5 mM NH₄FA (B) 95% ACN; 5% H₂O; 5 mM NH₄FA. Elution peaks were monitored with a fluorescence detector at; 490 (ex), 530 (em) (Thermo Spectrasystem FL3000) and subsequently analysed using Chromeleon v.6.8. software.

**Caco-2 and macrophage cell accumulation studies:** Caco-2 cells were seeded into 6 well plates (Nunclon™) at a density of 4 x 10⁶ per well and incubated at 37°C 5% CO₂ for 24 hours. THP-1 cells were seeded at a density of 4 x 10⁶ cells per well in a 6 well plate and allowed to differentiate to ATHP-1 cells for 7 days in 10 nM PMA supplemented RPMI-1640 10% FBS prior to use. Following incubation, the media was aspirated and cells washed twice with HBSS (37°C) and subsequently replaced with pre-warmed (37°C) Transport Buffer containing either 10 μM (final concentration) aqueous FA or 10 μM (final concentration) hyp-polydendron formulated FA. Following 24 hours incubation at 37°C 5% CO₂, 150 μL of the extracellular media was sampled. The remaining media was aspirated and cells were washed twice with ice cold HBSS. The ice cold HBSS was aspirated and replaced with 500 μL of a 50% acetone 50% water solution and incubated for 24 hours at -20°C, 150 μL of the lysate was subsequently sampled. Finally, 9 volumes of acetone was added to each intracellular and extracellular sample to extract FA for quantification on the HPLC as previously described. Average cell volumes were previously determined using a Scepter 2.0 Automated Cell Counter (Millipore) and used to calculate Cellular Accumulation Ratios (CAR); (Intracellular concentration/Volume)/(Extracellular concentration/Volume).
Cytotoxicity assays: Caco-2 cells were seeded into 96 well plates (Nunclon™) at a density of 1 x 10⁴ per well and incubated at 37°C 5% CO₂ for 24 hours. THP-1 cells were seeded at a density of 1 x 10⁴ cells per well in 96 well plates and allowed to differentiate to ATHP-1 cells for 7 days in 10 nM PMA supplemented RPMI-1640 10% FBS at 37°C 5% CO₂ prior to use. Following incubation, the media was aspirated and replaced with media containing either varying concentrations (0.1 – 15 µM) of aqueous or hyp-polydendron formulated FA as determined by the molarity and mass of FA contained in each formulation. The treated cells were then incubated for either 24 or 120 hours at 37°C 5% CO₂. All cytotoxicity analyses were determined using both ATP and MTT cell viability assays.

ATP assays: ATP assays were conducted using CellTiter-Glo® Reagent (Promega) in accordance with manufacturer’s instructions. Briefly, the plates and contents were initially equilibrated to room temperature. Subsequently, all but 20 µL of media was removed from each well and 20 µL CellTiter-Glo® reagent was added to produce a 1:1 ratio. The contents were then mixed for 10 minutes on an orbital shaker to induce lysis and allow for stabilisation of luminescence signal prior to reading on a TECAN GENios plate reader. MTP assay: For MTT assays, 20 µL of a 5 mg mL⁻¹ MTT reagent was added to each well and incubated for 2 hours. Subsequently, 100 µL MTT lysis buffer (50% N-N-Dimethylformamide in water containing 20% SDS, 2.5% glacial acetic acid and 2.5% hydrochloric acid, pH 4.7) was added to each well and lysed overnight at 37°C 5% CO₂. Following incubation, the absorbance of each well was read using a TECAN GENios plate reader (560 nm).

ATP depletion of Caco-2 cells using varying concentrations of 2-Deoxyglucose and rotenone: Caco-2 cells were incubated with varying concentrations of 2-Deoxyglucose/rotenone (0/0, 1/50, 2/100 and 3/150 nM) as outlined for 20 min at 37°C 5% CO₂. Following incubation, ATP assays were carried out using CellTiter-GLO® kit as previously described to determine relative depletion of ATP compared to an untreated control.

Apparent permeability (Papp) of fluoresceinamine encapsulated in polymeric nanocarrier materials: Caco-2 cell monolayers were pre-incubated for 30 min in transport buffer containing either: 30 µM chlorpromazine (Chl); 200 µM dansylcadaverine (Dan); 10 µM genistein (Gen); 150 µM dynasore (Dyn); or 150 µM Indomethacin (Ind) prior to washing three times with pre-warmed (37°C) HBSS.
Following washing, the Caco-2 monolayers were co-incubated with the various endocytosis inhibitors as outlined, and either aqueous (DMSO spiked; <0.1% v/v) or nanocarrier formulated fluoresceinamine for 4 h at 37°C 5% CO₂.

**Statistical analysis:** Data was statistically analysed using Prism 5 (GraphPad) and StatsDirect statistical software (version 2.7.9).

Chapter 7

Synthesis and nanoprecipitation studies of biodegradable linear-dendritic ε-caprolactone polymer hybrids and hyp-polydendrons
CHAPTER 7

7.1 Introduction

7.1.1 Biodegradable nanoparticles

Biodegradable nanoparticles (NPs) are strongly desirable for drug delivery applications due to their enhanced biocompatibility and encapsulation efficiency,\(^1\) with reported controlled release and decreased toxicity concerns.\(^2\) The synthesis, encapsulation, release studies and overall enhanced therapeutic value of drug loaded biodegradable NPs, over non-biodegradable nanocarriers, have been discussed in several important review articles.\(^3,4\) Research is ongoing, with specific investigations targeting surface modification, targeted delivery and control of release profiles.\(^5\) Along with encouraging encapsulation and stabilisation in aqueous media, the covalent modification of NPs with cell-targeting motifs remains a key challenge for potential drug delivery vehicles.

NPs comprising \(p\)\((lactic-co-glycolic acid)\) \(p\)(LGA) are receiving increasing attention for drug delivery and tissue engineering applications. Opportunities exist for NP attachment to cells or organs in the body,\(^6\) for example, \emph{via} selectin-ligand chemistry.\(^7\) Functionalised surfaces also offer specific attachment to scaffolds to encourage control over cellular interactions, or allow delivery of encapsulated moieties to target sites. This functionality is particularly desirable as it may reduce adverse drug side effects and enhance antigen delivery when considering vaccination applications.

In many cases, problems have arisen during the coupling of ligands to polyester particles, due to the lack of functional chemical groups situated on the polymer backbone. This consequently hinders traditional conjugation methods and a collection of different methods have been developed to overcome this, including the synthesis of \(p\)(LGA) copolymers with either amine\(^8\) or acid\(^9\) chain-end functionality that can be further fabricated into particles. Alternatively, particles can be produced from polyester copolymers such as \(p\)(ethylene glycol) (PEG)\(-\)\(p\)(LGA), leading to the formation of biodegradable and biocompatible microspheres.\(^10\) Alternatively, plasma treatment of \(p\)(LGA) has proven to provide surface modification and introduce hydrophilic functional groups.\(^11,12\) Other strategies to achieve this include the inclusion of chemistries within the polymer backbone to render the materials more susceptible to modification. For example, alkyne groups have been introduced into aliphatic polyesters, \emph{via} co-polymerisation of \(\varepsilon\)-caprolactone (CL) with propargyl 3-methylpentenoate oxide, to allow copper catalysed azide “click” reactions.\(^13\)
The controlled release of drugs from biodegradable materials has been demonstrated via tablets,14,15,16 implants,17 patches18 and injectable microspheres.19 These offer drug release over varying periods extending from hours to years. Lupron Depot is a p(LGA) matrix-type mixture containing leuprolgin acetate,20,21 administered as injectable microspheres to treat endometriosis and palliative care for prostate cancer. The component polymer, polymer molecular mass, excipients or drug loading all have an impact on the stability of the drug, as well as the rate of release.

7.1.2 Degradable aliphatic polyesters

Polyesters find utility within the field of biomedical materials due to their intrinsic sensitivity to water, heat and enzymatic degradation. For p(LGA), not only is it biodegradable but hydrolysis in physiological media results in glycolic and lactic acids, which are non-toxic components eliminated as carbon dioxide and water via the Krebs cycle.22 Commercially, the most commonly and extensively used polyester NPs are made from p(lactic acid) (p(LA), p(glycolic acid) p(GA), p(CL)) or p(LGA) (Figure 7.1).

As mentioned above, degradable aliphatic polyesters provide the foundation for NP-based therapeutics, with each possessing different attractive properties. The slower degradation of p(LGA) in comparison to p(LA) makes it particularly useful in long-term implantable devices. The preparation of p(CL) NPs23 have also been reported, by methods such as nanoprecipitation, solvent displacement and solvent evaporation. Polyester NPs have been shown to evade the immune phagocytic and antigen-presenting systems due to their ability to mimic adhesive leukocyte behaviour.7,24 Administration, activity and therapeutic importance of drugs within each NP system display differences, hence the extensive research into each nano-system. For example, the chemotherapy drug Taxol may be loaded into p(LGA) with a 100% encapsulation efficiency25 in comparison to only 20% in p(CL).26 However, the stability and therapeutic activity
CHAPTER 7

associated with \( p(\text{CL}) \) outweighs that of \( p(\text{LGA}) \) nanomedicines.

### 7.1.3 Ring-opening Polymerisation

Ring-opening polymerisation (ROP) is a chain-growth polymerisation (Chapter 1, section 1.3), where the polymer chain end acts as the propagating centre for further cyclic monomers (opening the ring system) to add to the growing polymer chain. The ring-opening is driven by release of the bond-angle strain or steric repulsions between the ring atoms within the cyclic monomers.\(^{27}\)

The ROP of lactones is used worldwide to produce aliphatic polyesters for biomedical applications\(^{28}\) such as bioreorbable devices in surgery, and drug delivery vehicles in therapy. Few lactones spontaneously polymerise, simply on storage or through heating,\(^{29}\) and most require the presence of catalysts and initiators. Effective polymerisations have been performed using a range of organometallic catalysts (e.g. oxides, carboxylates, alkoxides), resulting in controlled ring-opening to synthesise a range of polyester structures;\(^{30,31}\) the polymerisation mechanism is dependent on the catalyst/initiator selected. A coordination-insertion mechanism has been proposed when using organometallic catalysts in combination with an alcoholic initiating species.

Tin (II) 2-octanoate, \( \text{Sn(Oct}_2\) ), is the most commonly reported catalyst for ROP. Despite its toxicity, it is effective, versatile, easy to handle and soluble in common organic solvents and lactone monomers. The full details of the polymerisation mechanism is rather complex and several specific mechanisms have been proposed,\(^{32,33,34,35}\) including the well reported coordination-insertion process (Scheme 7.1).\(^{36}\) Within this mechanism, the monomer coordinates to the Lewis acidic metal centre via the exocyclic oxygen, rendering the carbonyl group of the lactone susceptible to nucleophilic attack.\(^{37}\)

Metal-free polymerisation approaches are attractive when targeting polyesters for medicinal or microelectronic devices. These methods have been achieved via nucleophilic, basic or bifunctional activation\(^{38,39}\) in a variety of systems such as 4-aminopyridines,\(^{40}\) \( N \)-heterocyclic carbenes,\(^{41}\) thiourea/amine combinations,\(^{42,43}\) guanidines,\(^{44,45}\) and phosphazenes.\(^{36,47}\)
The application of a bifunctional thiourea-amine catalyst for the ROP of lactide was reported in 2005, with no observable transesterification side reactions following monomer consumption. The polymerisation proceeded by monomer activation via the hydrogen bond-donating thiourea and by the propagating alcohol initiator through the hydrogen bond-accepting tertiary amine (Scheme 7.2A). Investigations revealed that both the hydrogen bond donor and accepter were essential for the ROP to ensue, even upon separation of the hydrogen-bond donating/accepting activator groups into two independent molecules. Despite their benefits, the thiourea-amine catalyst systems are noticeably slow compared to reported “superbase” systems. These strongly basic amines, specifically the commercially available 1,8-diazabicyclo[5.4.0]undec-7-ene (Scheme 7.2B) have successfully polymerised lactide, without the requirement of a hydrogen-bond donor cocatalyst. The “superbase” system has also displayed high catalytic activity with >98% lactide monomer conversion (500 eq.) within 2 hours.

The increasing interest in acid-catalysed ROP of (di)lactones has led investigations of the influence of catalyst acidity on its activity. This growing curiosity stems from reports by Kricheldorf et al. demonstrating the feasibility of trifluoromethanesulfonic acid (HOTf)-catalyzed ROP of lactide. In the presence of a protic initiator, HOTf promotes and controls the ROP of lactide at room temperature. This combination also
proven efficient for the homopolymerisation of CL and for its co-polymerisation with lactide.\textsuperscript{53,54}

Comparatively, the ROP of (di)lactones promoted by Brönsted acids has been less well studied. Further studies include reports from Endo and co-workers\textsuperscript{55,56} and Jérôme and co-workers\textsuperscript{57} reporting the polymerisation of CL using hydrochloric acid (HCl·OEt\textsubscript{2}) as a catalyst in the presence of an alcohol initiator. These combinations provide a well-controlled polymerisation, but one that proceeds slowly despite the large amounts of acid used. Further to this, di- and tri-block copolymers of PEG and \(p\text{(CL)}\) have been successfully prepared \textit{via} a metal-free monomer-activated mechanism in the presence of (HCl·OEt\textsubscript{2}).\textsuperscript{58}

Studies have also investigated many variants of initiator for ROP, ranging from benzyl alcohols\textsuperscript{59} to aluminium alkoxides.\textsuperscript{60} The facile nature of ROP has also been extended to include microwave preparations of \(p\text{(CL)}\), without the need for a metal catalyst using benzoic acid as the initiator.\textsuperscript{61}

\textbf{7.1.4 Biodegradable linear-dendritic polymer hybrids}

Dendrimers have provided insights into the effect of branching on the physical and solution properties of macromolecules.\textsuperscript{62} Dendronised polymers and linear-dendritic polymer hybrids have been extensively investigated\textsuperscript{63} and shown to exhibit pharmacokinetic behaviour in bio-distribution studies that differs from non-dendritic linear materials of equivalent molecular weight.\textsuperscript{64} The synthesis of these materials is generally less complex than lengthy and expensive dendrimer preparation, with the potential utilisation of peripheral and pendant groups for drug attachment, targeting and enhanced solubilisation. Functionalisation of dendrons with drugs, fluorescent dyes, imaging moieties, targeting agents and stimuli-responsive components have all demonstrated a multi-valency benefit, including deoxyribonucleic acid (DNA) or protein binding and targeted drug delivery.\textsuperscript{65,66}

Fréchet and co-workers have reported combination of CL ROP with dendritic macromolecules containing primary alcohol functionality, derived from 3,5-bis(benzyloxy)-benzyl alcohol, serving as initiators to produce linear-dendritic \(p\text{(CL)}\) polymer hybrids (Figure 7.2i).\textsuperscript{67} These ideas have been progressed further, including reports of new synthetic routes to produce other dendritic initiators from 2,2-
bis(methylol)propionic acid (bis-MPA) based dendritic molecules. Malkoch and co-workers have reported libraries of linear-dendritic hybrids, produced by rapid and efficient orthogonal click chemistry, composed of $p$(CL) arms attached to bis-MPA-derived dendrimers with a linear component of PEG (Figure 7.2ii). Following successful micelle formation from these new amphiphilic dendritic structures, doxorubicin was encapsulated and the cellular toxicity was investigated on breast cancer cell lines and human macrophages.

Figure 7.2 Chemical examples of linear-dendritic biodegradable polymer hybrids that exist in the literature; i) Linear-dendritic CL polymer containing 3,5-bis(benzyloxy)-benzyl alcolate dendron chain end functionality (G4-CL); ii) Linear-dendritic PEG polymer comprised of bis-MPA dendritic functionality and $p$(CL) arms (PEG-G4-PCL).

Aside from the biodegradability and biocompatibility associated with these linear-dendritic polymer hybrids, they also offer distinctive hierarchical self-assembly behaviour, suggesting potential for stimuli-responsive nanomedicine benefits. Progress within this field includes linear-dendritic biodegradable block copolymer hybrids, DNA-/protein-dendritic bio-hybrids and the considered potential applications within bionanotechnology.

7.1.5 Research Aims

In previous work and chapters within this thesis, the successful preparation of linear-dendritic polymer hybrids and hyperbranched polydendrons ($hyp$-polydendrons) has been presented for methacrylic monomers polymerised via atom transfer radical
polymerisation (ATRP). High molecular weight materials with systematic architectural variation and comprising a variety of different monomer compositions have undergone nanoprecipitation to produce NPs whose behaviour in varying pH aqueous solutions have been studied. The same concepts have been applied to produce highly functional, linear-dendritic and branched polyesters within this chapter.

Dendron initiators were utilised in the ROP of CL to produce a series of linear-dendritic polymer hybrids (Figure 7.3i), with amine-functionality incorporated within the dendron initiators. The co-polymerisation of cyclic and bicyclic ester monomers was carried out to form high molecular weight branched polyester hyp-polydendrons (Figure 7.3ii), in order to investigate the effect of architecture, dendron generation and pH on behaviour of the polymeric NPs formed from these materials.

Figure 7.3 Diagrammatic representation of the polymerisation of CL using an amine-functionalised generation 2 dendron ROP initiator to produce a biodegradable linear-dendritic hybrid (i); and the co-polymerisation of CL and 4,4′-bioxepanyl-7,7′-dione to produce a biodegradable hyp-polydendron.

CL was chosen as the study monomer as its synthesis has been extensively studied with good levels of reported polymerisation control and high monomer conversions. \(P(\text{CL}_x)\) is a highly hydrophobic polymer, ideal for the encapsulation of poorly water soluble drugs and it naturally degrades via non-enzymatic hydrolytic ester cleavage.\(^{72}\) Sn(Oct\(_2\)) was utilised as the catalyst, as reaction conditions are well understood, it has been well documented in the successful production of \(p(\text{CL}_x)\)\(^{73}\) and, unlike organic catalysts (such as acid catalysts), it is unlikely to interact with the dendron initiators via the amine functionality.
Hyperbranched polymers of CL have typically been produced via AB$_2$ polycondensation reactions or a ring-opening multi-branching polymerisation. In the latter instance, the initiator is attached to the cyclic monomer ring.$^{74}$ The first instance of branched $p$(CL) was reported by Hedrick and co-workers using an AB$_2$ macromonomer derived from bis-MPA.$^{75}$ Hedrick and co-workers extended this synthesis approach with another branching strategy utilising benzyl ester protected bis-MPA, Sn(Oct)$_2$ and another AB$_2$ CL-based macromonomer.$^{76}$ The first preparation of CL branched polymers utilising ROP of CL was conducted by Thurecht and co-workers;$^{77}$ the solution co-polymerisation of CL was conducted with 4,4’-bioxepanyl-7,7’-dione (BOD), in the presence of a reversible addition-fragmentation transfer (RAFT) agent containing a hydroxyl group, leading to branched $p$(CL) with RAFT agents at the chain ends.

CL has also been polymerised in the presence of bifunctional degradable cyclic monomers such as BOD (Figure 7.4i) and 2,2-bis($\varepsilon$-CL-4-yl)propane$^{78}$ (Figure 7.4ii) in order to produce a range of selectively degradable cross-linked star polymers, which often possess improved solubility and a more globular structure with a higher density of functional groups, compared to linear CL polymers.$^{79}$

![Figure 7.4](image)

**Figure 7.4** Chemical structure of bifunctional degradable cyclic monomers: i) 4,4’-bioxepanyl-7,7’-dione and; ii) 2,2-bis($\varepsilon$-caprolactone-4-yl) propane.

In 2014, Irvine and co-workers reported the first investigations into the bulk ROP of CL and, in the presence of BOD, used an extension of the “Strathclyde Method” to control the growing polymer chains and produce high molecular weight, soluble CL polymers.$^{80}$ The concepts first discussed by Irvine and co-workers have inspired the work embodied within this research chapter, leading to polyester hyp-polydendrons.
7.2 Amine-functionalised dendron ROP initiator synthesis

The amine-functional hyp-polydendrons discussed in previous chapters, utilised new dendron ATRP initiators; the formation of amine-functional polyester hyp-polydendrons requires the modification of this approach to form new dendron-ROP initiators. The initiators were designed to contain the same numbers of tertiary amine functionality as the linear-dendritic hybrids and hyp-polydendrons previously produced via ATRP. The previously synthesised ATRP initiators were synthesised after the initial production of dendrons containing secondary alcohol focal point functionality, and subsequent esterification to produce the required bromo-initiators. The analogous initiators targeted for ROP comprise a primary alcohol focal group as the intended initiating centre. This was expected to allow efficient initiation and comparisons to be drawn against benzyl alcohol (BnOH), a well-known initiator of bulk CL ROP in the presence of Sn(Oct₂) catalyst.

As previously described, the synthesis of tertiary amine-functional dendritic ROP initiators (AmG₁'-OH and AmG₂'-OH) again utilised the exhaustive Michael addition of 2-(dimethylamino) ethyl acrylate (DMEA) to primary amine-containing molecules bearing primary hydroxyl functionality; the commercially available ethanolamine was used as the precursor for the synthesis of AmG₁'-OH, whilst 1-[N, N-bis(2-aminopropyl)-amino]-2-propan-1-ol (APAP') was used to synthesise AmG₂'-OH. The AmG₀'-OH was commercially available as 2-dimethylaminoethanol (Scheme 7.3).

![Scheme 7.3 Diagrammatic representation of amine-functionalised the dendron ROP initiators AmG₀'-OH (green), AmG₁'-OH (blue) and AmG₂'-OH (red).](image-url)
7.2.1 Synthesis of 1-[N, N-bis(2-aminopropyl)-amino]-propan-1-ol

The synthesis of 1-[N, N-bis(2-aminopropyl)-amino]-propan-1-ol (APAP’) was carried out in four synthetic steps. [tBOC_2-BAPA-G_1] was previously synthesised in Chapter 2, by reacting tertiary butanol (Scheme 7.4 1) with 1,1’-carbonyl diimidazole (CDI) (Scheme 7.4 2) producing the monosubstituted imidazole carboxylic ester (Scheme 7.3 3). This was reacted with bis(3-aminopropyl)amine (BAPA) to form [tBOC_2-BAPA-G_1] (Scheme 7.4 4), followed by N-alkylation to functionalise the focal point. [tBOC_2-BAPA-G_1] was refluxed with 2-bromo-ethanol (Scheme 7.4 5) in 1,4-dioxane, with potassium carbonate as the base, to yield the primary alcohol functional [tBOC_2-BAPA-OH’]. Analysis by \(^1\)H and \(^13\)C nuclear magnetic resonance spectroscopy (NMR) and electro-spray mass spectrometry (ES-MS) are shown in the Appendix, Figures A66-68.

![Scheme 7.4 Synthesis of 1-[N, N-bis(2-aminopropyl)-amino]-propan-1-ol.](image)

The protecting N-tertiary butoxycarbonyl (tBOC) groups were removed, as described in Chapter 2, to afford the tris-ammonium salt APAP’.3HCl. Finally, the salt was converted to the free amine AB_2 branching unit APAP’, using a strong base (4M sodium hydroxide) and extraction of the yellow product with chloroform (CHCl_3) led to the final
product. Characterisation of APAP’ was carried out by $^1$H and $^{13}$C NMR and ES-MS (Figure 7.5 and Appendix, Figures A69 and 70).

Figure 7.5 $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of APAP’.

The $^1$H NMR spectrum (Figure 7.5) confirms the removal of the 18 tBOC protons, previously seen at 1.41 ppm (Appendix, Figure A66). The $^{13}$C NMR shows 5 distinct peaks for the 5 different carbon environments (Appendix, Figure A69) and a molecular ion peak of [M+H]$^+$ = 176.2 Da was seen in the ES-MS (Appendix, Figure A70); calculated (C$_8$H$_{21}$N$_3$O) m/z = 175.1 Da.

7.2.2 Synthesis of tertiary amine functional dendron ROP initiators (AmG$_{1}'$-OH and AmG$_{2}'$-OH)

The synthesis strategy employed the simple exhaustive Michael addition of DMEA to the primary amine functional groups of either ethanolamine or APAP’ (Scheme 7.3i). Three tertiary amine functional ROP initiators were chosen for this study, ranging from the zeroth to the second generation (G$_0$-G$_2$), with the inclusion of 2-dimethylaminoethanol as the AmG$_{0}'$-OH initiator.

ES-MS and $^1$H NMR analysis confirmed the structures and purities of the products through monitoring the disappearance of the two singlet signals at ~6 ppm (Figures 7.6 and 7.7), corresponding to the protons on the double bond of the DMEA, confirming removal of unreacted acrylate. The $^1$H NMR of the AmG$_{1}'$-OH confirms an integration ratio of 12 protons (Figure 7.6a), for the terminal tertiary amine methyl groups to 2
protons corresponding to the methylene neighbouring the focal point hydroxyl group (Figure 7.6g). The $^{13}$C NMR shows a peak at 172.7 ppm corresponding to the carbonyls of the ester groups of the DMEA arms (Appendix, Figure A71). A molecular peak of \([\text{M+H}]^+ \text{ m/z = 348.2}\) and \([\text{M+Na}]^+ \text{ m/z = 370.2}\) was seen in the ES-MS (Appendix, Figure A72); calculated \((\text{C}_{16}\text{H}_{33}\text{N}_3\text{O}_5) \text{ m/z = 347.5 Da}\).

Figure 7.6 $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of AmG$_1$'-OH.

The $^1$H NMR spectrum for the AmG$_2$'-OH dendron initiator revealed a peak at 2.23 ppm, integrating to 24 protons and corresponding to the tertiary amine methyl group protons (Figure 7.7a). The signal corresponding to the methylene neighbouring the primary alcohol also integrated to 2 protons (Figure 7.7j) and the 8 methylene protons neighbouring the oxygen of the ester bonds in the DMEA residue are seen downfield at 4.11 ppm (Figure 7.7c). The carbonyl ester peaks are again seen at 172.6 ppm in the $^{13}$C NMR (Appendix, Figure A73) and an \([\text{M+H}]^+\) molecular ion peak at \text{m/z = 748.6 Da}\) was observed in the mass spectrometric analysis; calculated \((\text{C}_{36}\text{H}_{73}\text{O}_9\text{N}_7) \text{ m/z = 748.0 Da}\) (Appendix, Figure A74).
Figure 7.7 $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of AmG$_2$'-OH.

7.3 Ring-opening polymerisation of ε-caprolactone

$P$(CL) was first synthesised by van Natta et al. in 1934,$^8^4$ and received later recognition as a drug delivery vehicle in 1979 by Pitt et al.$^8^5,8^6$ in order to investigate the controlled release of narcotic antagonists, steroids and the delivery of ophthalmic drugs,$^8^7$ which are currently being developed as NPs.

The polymerisation of lactones has been reported under bulk, solution (e.g. tetrahydrofuran (THF), dioxane and toluene), emulsion$^8^8$ or dispersion$^8^9$ conditions. Polymerisation temperatures range from 100-150°C for bulk polymerisations and 0-25°C in solution polymerisations in order to minimize inter- and intramolecular transesterification reactions, but factors such as reaction time, the type and concentration of catalyst or initiator, and the nature of the lactone (or lactide)$^{9^0}$ also play important roles.
7.3.1 Linear polymerisation of ε-caprolactone using benzyl alcohol and amine-functionalised dendron ROP initiators

Linear polymers of CL were synthesised at 110°C in bulk, initiated by BnOH using the Sn(Oct₂) catalyst\(^{37}\) at the molar ratio [initiator]:[M]:[Sn(Oct₂)] = 1:30:1/350 (Scheme 7.5).

![Scheme 7.5 Diagrammatic representation of the linear polymerisation of ε-caprolactone (2) initiated by benzyl alcohol (1) to produce the linear polymer Bn-p(CL₃₀).](image)

After 24 hours at 110°C, the resulting Bn-p(CL₃₀) was dissolved in THF and purified by precipitation into hexane before analysis by triple detection gel permeation chromatography (GPC) and \(^1\text{H}\) NMR spectroscopy. The GPC refractive index (RI) chromatogram of the linear polymer showed a monomodal molecular weight distribution (Appendix, Figure A75); however, the analysis also revealed a relatively broad dispersity (\(\mathcal{D} = 1.71\)) (Table 7.1, entry 1). To attempt to optimise the reaction, the molar ratio of metal catalyst was altered from \([1/350]\) to \([1/200]\) relative to initiator; the variation of the Sn(Oct₂) molar ratio within aliphatic polyester polymerisations, has previously allowed control over the molecular weight and the molecular weight distribution.\(^{91}\)

Polymerisation at this higher ratio resulted in a broader molecular weight distribution, and produced a polymer with a number average molecular weight (\(M_n\)) significantly below the targeted values (Table 7.1, entry 2; Appendix, Figure A75). The breadth of the initial molecular weight distribution may be a result of transesterification, therefore the reaction time was shortened from 24 hours to 20 hours, at a metal catalyst molar ratio of \([1/350]\) relative to initiator (Table 7.1, entry 3). High monomer conversion was achieved, and a linear polymer with a narrower dispersity than the previous attempts was collected (\(\mathcal{D} = 1.50\); Figure 7.8). It is worth emphasising that reported CL polymers prepared in bulk are within a dispersity range \(\mathcal{D} = 1.4-1.7\) at high conversion.\(^{80}\)
### Table 7.1 GPC analysis of linear and linear-dendritic p(CL), branched CL-BOD copolymers and hyp-polydendrons synthesised in bulk at 110°C.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Polymer</th>
<th>[Sn(oct)]</th>
<th>Time (hrs)</th>
<th>Conversion (%)</th>
<th>$M_n$ Theory (g mol$^{-1}$)</th>
<th>$M_n$ (g mol$^{-1}$)</th>
<th>$M_w$ (g mol$^{-1}$)</th>
<th>$D$</th>
<th>$D_P$</th>
<th>$D_P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bn-p(CL$_{30}$)</td>
<td>1/350</td>
<td>24</td>
<td>99</td>
<td>3532</td>
<td>3060</td>
<td>5420</td>
<td>1.77</td>
<td>26</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Bn-p(CL$_{30}$)</td>
<td>1/200</td>
<td>24</td>
<td>99</td>
<td>3532</td>
<td>1780</td>
<td>4430</td>
<td>2.49</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Bn-p(CL$_{30}$)</td>
<td>1/350</td>
<td>20</td>
<td>98</td>
<td>3532</td>
<td>4050</td>
<td>6060</td>
<td>1.50</td>
<td>35</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>AmG$<em>0^\ast$-p(CL$</em>{30}$)</td>
<td>1/350</td>
<td>24</td>
<td>94</td>
<td>3513</td>
<td>2780</td>
<td>3950</td>
<td>1.42</td>
<td>24</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>AmG$<em>1^\ast$-p(CL$</em>{30}$)</td>
<td>1/350</td>
<td>48</td>
<td>95</td>
<td>3771</td>
<td>3090</td>
<td>6050</td>
<td>1.96</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>AmG$<em>2^\ast$-p(CL$</em>{30}$)</td>
<td>1/350</td>
<td>94</td>
<td>94</td>
<td>4172</td>
<td>5210</td>
<td>11460</td>
<td>2.20</td>
<td>39</td>
<td>36</td>
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<tr>
<td>7</td>
<td>AmG$<em>2^\ast$-p(CL$</em>{30}$)</td>
<td>1/150</td>
<td>66</td>
<td>80</td>
<td>3487</td>
<td>3910</td>
<td>9390</td>
<td>2.40</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>8</td>
<td>Bn-p(CL$<em>{30}$-co-BOD$</em>{0.6}$)</td>
<td>1/350</td>
<td>24</td>
<td>73</td>
<td>-</td>
<td>2850</td>
<td>4950</td>
<td>1.74</td>
<td>-</td>
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<tr>
<td>9</td>
<td>Bn-p(CL$<em>{30}$-co-BOD$</em>{1.0}$)</td>
<td>1/350</td>
<td>24</td>
<td>96</td>
<td>-</td>
<td>5600</td>
<td>108560</td>
<td>19.39</td>
<td>-</td>
<td>14</td>
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<tr>
<td>10</td>
<td>AmG$<em>0^\ast$-p(CL$</em>{30}$-co-BOD$_{0.6}$)</td>
<td>1/350</td>
<td>120</td>
<td>55</td>
<td>-</td>
<td>4400</td>
<td>8650</td>
<td>1.97</td>
<td>-</td>
<td>25</td>
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<td>11</td>
<td>AmG$<em>0^\ast$-p(CL$</em>{30}$-co-BOD$_{0.8}$)</td>
<td>1/350</td>
<td>192</td>
<td>58</td>
<td>-</td>
<td>7100</td>
<td>20400</td>
<td>2.87</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>12</td>
<td>AmG$<em>0^\ast$-p(CL$</em>{30}$-co-BOD$_{1.0}$)</td>
<td>1/350</td>
<td>120</td>
<td>98</td>
<td>-</td>
<td>4540</td>
<td>26500</td>
<td>5.83</td>
<td>-</td>
<td>33</td>
</tr>
<tr>
<td>13</td>
<td>AmG$<em>1^\ast$-p(CL$</em>{30}$-co-BOD$_{0.6}$)</td>
<td>1/350</td>
<td>120</td>
<td>68</td>
<td>-</td>
<td>5730</td>
<td>24600</td>
<td>4.29</td>
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<td>22</td>
</tr>
<tr>
<td>14</td>
<td>AmG$<em>1^\ast$-p(CL$</em>{30}$-co-BOD$_{0.8}$)</td>
<td>1/350</td>
<td>144</td>
<td>83</td>
<td>-</td>
<td>3250</td>
<td>92600</td>
<td>28.50</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>15</td>
<td>AmG$<em>2^\ast$-p(CL$</em>{30}$-co-BOD$_{0.8}$)</td>
<td>1/150</td>
<td>72</td>
<td>75</td>
<td>-</td>
<td>6030</td>
<td>40730</td>
<td>6.75</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>16</td>
<td>AmG$<em>2^\ast$-p(CL$</em>{30}$-co-BOD$_{1.0}$)</td>
<td>1/150</td>
<td>89</td>
<td>81</td>
<td>-</td>
<td>13450</td>
<td>31350</td>
<td>2.33</td>
<td>-</td>
<td>59</td>
</tr>
</tbody>
</table>

*a* Triple detection GPC (THF + TEA 2% v/v); *b* $M_n$ Theory = (($M_n$ CL)*$DP_n$ targeted) + $M_w$ chain end; *c* Determined by GPC; *d* Determined by NMR
Figure 7.8 GPC RI chromatogram overlays of Bn\textsubscript{-}\textit{p}(CL\textsubscript{30}) (yellow), AmG\textsubscript{0}'\textsubscript{-}\textit{p}(CL\textsubscript{30}) (green), AmG\textsubscript{1}'\textsubscript{-}\textit{p}(CL\textsubscript{30}) (blue) and AmG\textsubscript{2}'\textsubscript{-}\textit{p}(CL\textsubscript{30}) (red).

The reaction proceeded to high monomer conversion (98\%) as confirmed by \textsuperscript{1}H NMR analysis of the crude polymerisation media. \textsuperscript{1}H NMR analysis of the purified sample also allowed assessment of the number average degree of polymerisation (DP\textsubscript{n}), by comparison of the aromatic signal attributing to the benzyl chain end group (Figure 7.9a), with clear resonances assigned to \textit{p}(CL\textsubscript{x}) proton peaks residing in the polymer backbone.

Figure 7.9 \textsuperscript{1}H NMR spectrum (CDCl\textsubscript{3}, 400 MHz) of Bn\textsubscript{-}\textit{p}(CL\textsubscript{30}).
\textbf{AmG}_0'^{-}\text{OH}, \textbf{AmG}_1'^{-}\text{OH} \text{ and } \textbf{AmG}_2'^{-}\text{OH} \text{ dendron ROP initiators were also used to polymerise CL under identical conditions (Figure 7.10)} ([\text{initiator}]:[\text{M}]:[\text{Sn(Oct)}_2] = 1:30:1/350, in bulk at 110^\circ \text{C}), \text{ with the resulting linear-dendritic polymers purified and analysed by GPC (Table 7.1, entries 4, 5 and 6).}

\textbf{Figure 7.10} Diagrammatic representation of the linear polymerisation of CL initiated by the \textbf{AmG}_0'^{-}\text{G}_2'^{-} \text{ dendron ROP initiators to produce the linear-dendritic } p(\text{CL}) \text{ polymer hybrids, } \textbf{AmG}_0'^{-}\text{p}(\text{CL}_{30}), \textbf{AmG}_1'^{-}\text{p}(\text{CL}_{30}) \text{ and } \textbf{AmG}_2'^{-}\text{p}(\text{CL}_{30}).

The linear-dendritic } p(\text{CL}) \text{ hybrids reached high monomer conversion (>94 %) in noticeably longer reaction times (Table 7.1) which increased considerably with increasing dendron initiator generation. The } \textbf{AmG}_0'^{-}\text{p}(\text{CL}_{30}) \text{ and } \textbf{AmG}_1'^{-}\text{p}(\text{CL}_{30}) \text{ possessed an } \text{M}_n \text{ close to the targeted theoretical values, with the } \textbf{AmG}_0'^{-}\text{p}(\text{CL}_{30}) \text{ showing a narrower molecular weight distribution than any } \textbf{Bn}-p(\text{CL}_{30}) \text{ polymers previously produced (Figure 7.8). In comparison, the } \textbf{AmG}_1'^{-}\text{p}(\text{CL}_{30}) \text{ displayed a broad molecular weight distribution (} \text{Đ} = 1.96) \text{ suggesting a lack of control and the recovered } \textbf{AmG}_2'^{-}\text{p}(\text{CL}_{30}) \text{ equally exhibited a broad distribution of molecular weights and high } \text{M}_n \text{ values. In an attempt to establish a higher level of control, the } \textbf{AmG}_2'^{-}\text{OH} \text{ ROP was carried out using an increased amount of Sn(Oct)}_2\text{, namely [initiator]:[M]:[Sn(Oct)}_2\text{] =}}
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1:30:1/150, in bulk at 110°C (Table 7.1, entry 7). An $M_n$ closer to the theoretical value was obtained (3910 g mol$^{-1}$), with a broader dispersity, $D = 2.40$ (Figure 7.8). The polymerisation only reached 80% monomer conversion within 66 hours. These initial studies for the production of linear-dendritic CL polymer hybrids suggest that improvements could be made to optimise each particular polymerisation.

The AmG$_1$'-p(CL$_{30}$) $^1$H NMR spectrum (Figure 7.11B) shows a peak at ~2.27 ppm integrating to 72 (Figure 7.11 a,b,e,f,h), corresponding to the polymer protons neighbouring the carbonyl group and all protons neighbouring N atoms in the dendron chain end (22 protons). Subtraction of the dendron protons reveals a DP$_n$=26 monomer units, similar to the DP$_n$=24 monomer units predicted by GPC. The $^1$H NMR spectrum of AmG$_2$'-p(CL$_{30}$) (Figure 7.11C) allows integration of the protons residing in the dendron chain end. The protons neighbouring the oxygen atom within the DMEA ester arms integrates to 8 protons (Figure 7.11c) and the peak at ~2.23 ppm predicts a DP$_n$=36 monomer units, following subtraction of the 24 protons from the surface methyl groups.

Previously reported kinetic studies for BnOH-initiated $p$(CL) synthesis, targeting chain lengths of DP$_n$ = 50 monomer units at 150°C in solvent free conditions, have confirmed controlled ROP of CL; similar kinetic studies were performed for the bulk linear CL polymerisations (target DP$_n$ = 30 monomer units, 110°C) initiated by BnOH and the three dendron ROP initiators. All variants of initiator indicated first order kinetics for the linear polymerisation and linear evolution of $M_n$ with conversion (Figure 7.12), at least within the early stages of detailed examination. Accurate dn/dc values of the final polymers were used to determine the earlier $M_n$ values (Appendix, Table A3), which may lead to inaccuracy in reported $M_n$ values. The Bn-p(CL$_{30}$) and AmG$_0$'-p(CL$_{30}$) reached high monomer conversion within similar reaction times of 22 and 24 hours respectively (Figure 7.12A and C). The AmG$_1$'-p(CL$_{30}$) and AmG$_2$'-p(CL$_{30}$) polymerisations took longer to reach high monomer conversion; 48 and 94 hours for the AmG$_1$'- (Figure 7.12E) and AmG$_2$'- (Figure 7.12G). In all cases, dispersities remained low until reaching high monomer conversion.
Figure 7.11 $^1$H NMR spectra (CDCl$_3$, 400 MHz) of AmG$_9'$.p(CL$_{30}$) (A), AmG$_1'$.p(CL$_{30}$) (B) and AmG$_2'$.p(CL$_{30}$) (C).
Figure 7.12 Kinetic plots for linear DP30 polymers. A) and B) Bn-p(CL30), C) and D) AmG0'-p(CL30), E) and F) AmG1'-p(CL30), G) and H) AmG2'-p(CL30). A, C, E and G) Conversion (blue squares), \( \ln([M]/[M]) \) (red circles); B, D, F and H) \( M_n \) (red circles) and \( \bar{D} \) (blue lines).
7.3.2 Co-polymerisation of CL and 4,4’-bioxepanyl-7,7’-dione using benzyl and AmG_{0-2} functional dendron ROP initiators

7.3.2.1 Synthesis of BOD

BOD has been synthesised by several research groups, with reports including its use for the cross-linking of lactide and CL polymerisations in order to generate selectively degradable star polymers.\textsuperscript{92} Further investigations have used the bicyclic ester in the production of selectively degradable core-crosslinked shell micelles.\textsuperscript{78} BOD is synthesised via the Baeyer-Villiger rearrangement (Scheme 7.6) of bis(4-cyclohexanone) (BCH), carried out according to the method of Kirschke.\textsuperscript{93} The BCH (Scheme 7.7 3) is treated with a peroxy-acid, produced from urea/hydrogen peroxide (Scheme 7.7 2) and formic acid (Scheme 7.7 1), which inserts an oxygen next to the carbonyl group, to produce an ester.

Scheme 7.6 Mechanism of Baeyer-Villiger reaction between bicyclohexanone and peroxy-acid formed from urea hydrogen peroxide and formic acid.

Scheme 7.7 Synthesis of BOD via the initial reaction of formic acid (1) with urea hydrogen peroxide (2) to produce a peroxy-acid for the subsequent reaction with bis(4-cyclohexanone) (3).

BOD was characterised using ES-MS (Appendix, Figure A76) and \textsuperscript{1}H (Figure 7.13) and \textsuperscript{13}C NMR (Appendix, Figure A77) spectroscopy. The protons neighbouring the oxygen atom of the ester group integrate to 4 protons between 4.08-4.44 ppm, and those neighbouring the carbonyl group are showed between 2.51-2.82 ppm. The \textsuperscript{13}C NMR
spectrum shows a peak at 181.3 ppm, accounting for the lactone carbonyl group amongst the other carbon environments. The ES-MS shows a molecular ion peak \([\text{M+NH}_4]^+ = 244.2\ \text{Da}\) (calculated \([\text{M+H}]^+ (\text{C}_{12}\text{H}_{18}\text{O}_4)\ m/z = 226.3\)).

![1H NMR spectrum (CDCl₃, 400 MHz) of BOD.](image)

**Figure 7.13**

### 7.3.2.2 Co-polymerisation of \(\varepsilon\)-caprolactone and \(4,4'\)-bioxepanyl-7,7’-dione

In order to produce highly branched CL polymers in bulk, the optimal BOD concentration has been reported to be 0.6 equivalents of brancher per initiating species.⁸⁰ Therefore, initially, the co-polymerisation of CL and BOD was carried out at 110°C, using \(\text{BnOH}\) as the initiator and \(\text{Sn(Oct}_2)\) catalyst in the molar ratio \([\text{initiator}]:[\text{BOD}]:[\text{M}]:[\text{Sn(Oct}_2)] = 1:0.6:30:1/350\) (Scheme 7.8).

![Diagrammatic representation of the co-polymerisation of CL (2) and BOD (3) initiated by BnOH (1) to produce the branched copolymer Bn-\(p\)(CL₃₀-co-BOD₈₁).](image)

**Scheme 7.8**

Upon analysis by triple detection GPC, the co-polymerisation appeared to have failed to produce a branched copolymer as the determined \(M_n\) and average molecular weight (\(M_w\)) values were not indicative of branching between chains; 2850 g mol\(^{-1}\) and 4950 g mol\(^{-1}\) respectively (Table 7.1, entry 8). The BOD to initiator ratio was, therefore,
increased to 1.0 eq. (Table 7.1, entry 9) and a highly branched \( p(\text{CL}_{30}-\text{co-BO}_{D1.0}) \) sample was generated with \( M_w = 108560 \text{ g mol}^{-1} \); a very broad molecular weight distribution was also seen (Figure 7.14) and a final high monomer conversion (97%) was achieved. Branched \( p(\text{CL}) \) has been reported in the literature (\( \text{Bn-}p(\text{CL}_{30}-\text{co-BO}_{D0.6}) \)) possessing \( M_w \) values > 90,000 g mol\(^{-1}\) when prepared in bulk.\(^{80}\) The \(^1\)H NMR spectrum of the purified, branched copolymer is displayed in Figure 7.15, with identical peaks present compared to those observed in the \( \text{Bn-}p(\text{CL}_{30}) \) spectrum (Figure 7.9).

![Figure 7.14 GPC RI chromatogram overlays of \( \text{Bn-}p(\text{CL}_{30}-\text{co-BO}_{D0.6}) \) (yellow-dotted) and \( \text{Bn-}p(\text{CL}_{30}-\text{co-BO}_{D1.0}) \) (yellow).](image)

**Figure 7.14** GPC RI chromatogram overlays of \( \text{Bn-}p(\text{CL}_{30}-\text{co-BO}_{D0.6}) \) (yellow-dotted) and \( \text{Bn-}p(\text{CL}_{30}-\text{co-BO}_{D1.0}) \) (yellow).

![Figure 7.15 \(^1\)H NMR (CDCl\(_3\), 400 MHz) spectrum of \( \text{Bn-}p(\text{CL}_{30}-\text{co-BO}_{D1.0}) \) copolymer.](image)

**Figure 7.15** \(^1\)H NMR (CDCl\(_3\), 400 MHz) spectrum of \( \text{Bn-}p(\text{CL}_{30}-\text{co-BO}_{D1.0}) \) copolymer.
Following the successful production of highly branched $\text{Bn-p}(\text{CL}_{30}\text{-co-BOD}_{1.0})$, the dendron ROP initiators were utilised in the co-polymerisation of CL and BOD (Figure 7.16).

![Figure 7.16 Diagrammatic representation of the co-polymerisation of CL and BOD using AmG$_0$-G$_2'$ dendron ROP initiators.](image)

The AmG$_0'$-OH and AmG$_1'$-OH initiators were used to co-polymerise CL and BOD at 0.6 and 0.8 molar eq. reactive to initiator, at 110°C in bulk, using identical conditions to those utilised in the linear polymerisations. The AmG$_0'$-OH failed to produce highly branched hyp-polydendrons using either ratio of BOD:initiator (Table 7.1, entries 10 and 11), containing only on weight average $>2$ and $5$ linear-dendritic chains joined together respectively. A highly branched hyp-polydendron, AmG$_1'$-p(CL$_{30}\text{-co-BOD}_{0.8}$) (Table 7.1, entry 14 and Figure 7.17) was collected.

The BOD content was increased further to 1.0 molar equivalent, producing AmG$_0'$-p(CL$_{30}\text{-co-BOD}_{1.0}$) with an $M_w = 26500$ g mol$^{-1}$ (Table 7.1, entry 12 and Figure 7.17), but still low compared to the $M_w$ values achieved when using BnOH and AmG$_1'$-OH initiators. Following failure to produce highly branched hyp-polydendrons initiated by AmG$_0'$-OH and AmG$_1'$-OH using 0.6 molar equivalents of BOD per initiator, the
**AmG₂⁻¹-OH** was used to initiate the co-polymerisation of CL and BOD at 0.8 and 1.0 molar equivalents (Table 7.1, entries 15 and 16), with the former producing a hyp-polydendron with a $M_w = 40730$ g mol$^{-1}$ (Figure 7.17).

![Figure 7.17 GPC RI chromatogram overlays of AmG₀⁻¹-p(CL₃₀-co-BOD₁₀) (green), AmG₁⁻¹-p(CL₃₀-co-BOD₀₈) (blue) and AmG₂⁻¹-p(CL₃₀-co-BOD₀₈) (red).](image)

The dendron chemistry clearly plays a role in the successful production of highly branched CL hyp-polydendrons. Monomer conversions were not as high as seen previously for ATRP hyp-polydendron synthesis (Chapter 3), which may be a limitation of bulk ROP polymerisation. The AmG₀⁻¹-p(CL₃₀-co-BOD₁₀) achieved 98% monomer conversion, compared to the AmG₁⁻¹-p(CL₃₀-co-BOD₀₈) reaching 83% and the AmG₂⁻¹-p(CL₃₀-co-BOD₀₈) reaching only 81% before termination of the reactions; if the evolution of high molecular weight structures follows the trends seen in ATRP branched vinyl polymerisations, very high conversions ($> 90\%$) would be required to generate highly branched structures and these were not achieved for first and second generation polymerisations. Despite several hyp-polydendrons not reaching high monomer conversion, all excess monomer was successfully removed, confirmed by $^1$H NMR analysis of the final polymer following purification (Figure 7.18).
Figure 7.18 $^1$H NMR (CDCl$_3$, 400 MHz) spectra of AmG$_8$$^\ast$$^{-}$$^p$(CL$_{30}$-co-BOD$_{1.0}$) (A), AmG$_1$$^\ast$$^{-}$$^p$(CL$_{30}$-co-BOD$_{0.8}$) (B) and AmG$_2$$^\ast$$^{-}$$^p$(CL$_{30}$-co-BOD$_{0.8}$) (C) hyp-polydendrons.
The branched copolymer and hyp-polydendrons taken forward for nanoprecipitation studies contained on weight average > 18 (Bn-p(CL\textsubscript{30-co}-BOD\textsubscript{1.0})), > 7 (AmG\textsubscript{0.9}-p(CL\textsubscript{30-co}-BOD\textsubscript{1.0})), > 15 (AmG\textsubscript{1.5}-p(CL\textsubscript{30-co}-BOD\textsubscript{0.8})) and > 4 AmG\textsubscript{2.9}-p(CL\textsubscript{30-co}-BOD\textsubscript{0.8}) primary polymer chains; with 7, 30 and 16 tertiary amine surface groups for the hyp-polydendrons respectively.

7.4 Nanoprecipitation of linear polymers, linear-dendritic hybrids, branched copolymers and hyp-polydendrons

Stabilisation of NPs in aqueous media has been successfully achieved by two main strategies: (i) surface coating with hydrophilic surfactants\textsuperscript{94} and (ii) development of biodegradable copolymers with hydrophilic segments, for example, PEG.\textsuperscript{95} These provide increased stability within the body, longer circulation times and a means to control the release rate of encapsulated or conjugated drugs.\textsuperscript{4}

The preparation of \(p(\text{CL})\) NPs has been successfully achieved by a variety of nanoprecipitation, solvent displacement and solvent evaporation techniques.\textsuperscript{3} The encapsulation of the antihypertensive agent Isradipine within \(p(\text{CLx})\) NPs has been reported via nanoprecipitation with Pluronic F68 as a stabiliser within the aqueous antisolvent. NPs with diameter size values in the range 110-208 nm were generated,\textsuperscript{94} noticeably larger than NPs prepared with \(p(\text{LAx})\) or \(p(\text{LGAx})\) under similar conditions.\textsuperscript{3} Results from these reports suggested potential for drug delivery via oral administration.

Amphiphilic triblock copolymers, \(p(\text{CLx-b-PEGy-b-CLz})\), have been reported in the literature to produce stable micelles where both PEG chains are anchored within the main bulk of the nano-object.\textsuperscript{95} Alteration of the block polymer lengths resulted in a change in micelle size, drug loading capacity, critical micelle concentrations and drug release behaviour. Increased \(p(\text{CL})\) block lengths resulted in larger micelles and decreased drug release rates. Malkoch and co-workers have successfully produced stable micelles of \(~100\) nm from linear-dendritic polymer hybrids comprised of \(p(\text{CL})\) arms and a linear PEG component. Overall, investigations into the self-assembly of linear-dendritic biodegradable polymer hybrids to produce stable NPs are few, and the formation of stable nanoprecipitates from branched CL polymers are not widely reported.
Chapter 2 described the production of stable NPs in aqueous media from amine-functionalised hyp-polydendrons comprising 2-hydroxypropyl methacrylate (HPMA) and ethylene glycol dimethacrylate (EGDMA) primary chains; the linear-dendritic HPMA hybrids only succeeded to produce nanoprecipitates in aqueous HCl due to charge-stabilisation from protonation of the dendron groups. Nanoprecipitation studies of linear-dendritic polymers containing 2-(diethylamino) ethyl methacrylate (DEA) revealed the requirement of additional charge to produce monomodal size distributions, with the branched \( p(\text{DEA}-\text{co}-\text{EGDMA}) \) equivalent NPs exhibiting narrow polydispersities from high levels of stabilisation in both neutral and acidic water, confirmed by the additional stability observed in physiologically-relevant media (Chapter 6).

The selected linear, \( \text{Bn}-p(\text{CL}_{30}) \), \( \text{AmG}_0'-p(\text{CL}_{30}) \), \( \text{AmG}_1'-p(\text{CL}_{30}) \) and \( \text{AmG}_2'-p(\text{CL}_{30}) \) and branched equivalent materials, \( \text{Bn}-p(\text{CL}_{30}-\text{co}-\text{BOD}_{1.0}) \), \( \text{AmG}_0'-p(\text{CL}_{30}-\text{co}-\text{BOD}_{1.0}) \), \( \text{AmG}_1'-p(\text{CL}_{30}-\text{co}-\text{BOD}_{0.8}) \) and \( \text{AmG}_2'-p(\text{CL}_{30}-\text{co}-\text{BOD}_{0.8}) \) were dissolved in THF at a concentration of 5 mg mL\(^{-1}\). All nanoprecipitations were performed by adding the polymer solution (2 mL) to aqueous media (10 mL) to obtain a NP dispersion at a concentration of 1 mg mL\(^{-1}\) following evaporation of THF overnight. Analysis of the stable NP dispersions was conducted by dynamic light scattering (DLS) to assess NP hydrodynamic diameters \( (D_z) \), number average diameters \( (D_n) \) and polydispersities (PDI).

All linear and linear-dendritic CL-containing polymers failed to produce stable NPs and underwent observable macroscale precipitation at pH = 7.8 (Table 7.2). Chapters 2 and 4 reported the production of stable NPs from HPMA and tertiary butyl methacrylate (tBuMA) linear-dendritic hybrids bearing dendritic surface functionality in acidic media, resulting from protonation of the tertiary amines providing charge stabilisation. In an attempt to produce stable NPs from linear-dendritic CL-containing polymers, the polymer solutions were nanoprecipitated into aqueous HCl (pH = 4); successful NP dispersions were formed for all linear-dendritic polymer hybrids with only the \( \text{Bn}-p(\text{CL}_{30}) \) precipitating out of solution (Table 7.2). The linear-dendritic \( p(\text{CL}_{30}) \) NPs displayed increasing size values, with narrower size distributions as the size of the dendron chain end decreases (Figure 7.19). The \( \text{AmG}_2'-p(\text{CL}_{30}) \) additionally, failed to produce a monomodal size distribution.
Table 7.2 DLS analysis of nanoprecipitated linear and linear-dendritic CL polymer hybrids and CL-BOD branched copolymers and hyp-polydendrons.

<table>
<thead>
<tr>
<th>Branched Polymer</th>
<th>Linear Polymer</th>
<th>pH=7.8&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pH=4.0&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( D_z ) (nm)</td>
<td>PDI</td>
</tr>
<tr>
<td>-</td>
<td>AmG&lt;sub&gt;0&lt;/sub&gt;-</td>
<td>581</td>
<td>0.280</td>
</tr>
<tr>
<td></td>
<td>( p(\text{HPMA}_{50}) )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>AmG&lt;sub&gt;1&lt;/sub&gt;-</td>
<td>246</td>
<td>0.193</td>
</tr>
<tr>
<td></td>
<td>( p(\text{HPMA}_{50}) )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>AmG&lt;sub&gt;2&lt;/sub&gt;-</td>
<td>491</td>
<td>0.165</td>
</tr>
<tr>
<td></td>
<td>( p(\text{HPMA}_{50}) )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Bn-( p(\text{CL}_{30}) )</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>AmG&lt;sub&gt;0&lt;/sub&gt;-( p(\text{CL}_{30}) )</td>
<td>123</td>
<td>0.140</td>
</tr>
<tr>
<td>-</td>
<td>AmG&lt;sub&gt;1&lt;/sub&gt;-( p(\text{CL}_{30}) )</td>
<td>130</td>
<td>0.259</td>
</tr>
<tr>
<td>-</td>
<td>AmG&lt;sub&gt;2&lt;/sub&gt;-( p(\text{CL}_{30}) )</td>
<td>36</td>
<td>0.260</td>
</tr>
<tr>
<td>-</td>
<td>( \text{Bn-} p(\text{DEA}_{50}) )</td>
<td>65</td>
<td>0.381</td>
</tr>
<tr>
<td>-</td>
<td>( \text{Bn-} p(\text{CL}<em>{30}-c o- \text{BOD}</em>{1.0}) )</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>AmG&lt;sub&gt;0&lt;/sub&gt;-( p(\text{CL}<em>{30}-c o- \text{BOD}</em>{1.0}) )</td>
<td>145</td>
<td>0.197</td>
</tr>
<tr>
<td>-</td>
<td>AmG&lt;sub&gt;1&lt;/sub&gt;-( p(\text{CL}<em>{30}-c o- \text{BOD}</em>{1.0}) )</td>
<td>151</td>
<td>0.081</td>
</tr>
<tr>
<td>-</td>
<td>AmG&lt;sub&gt;2&lt;/sub&gt;-( p(\text{CL}<em>{30}-c o- \text{BOD}</em>{1.0}) )</td>
<td>133</td>
<td>0.186</td>
</tr>
<tr>
<td>-</td>
<td>Bn-( p(\text{CL}_{30}) )</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>AmG&lt;sub&gt;0&lt;/sub&gt;-( p(\text{CL}_{30}) )</td>
<td>147</td>
<td>0.125</td>
</tr>
<tr>
<td>-</td>
<td>AmG&lt;sub&gt;1&lt;/sub&gt;-( p(\text{CL}_{30}) )</td>
<td>169</td>
<td>0.149</td>
</tr>
<tr>
<td>-</td>
<td>AmG&lt;sub&gt;2&lt;/sub&gt;-( p(\text{CL}_{30}) )</td>
<td>217</td>
<td>0.173</td>
</tr>
<tr>
<td>-</td>
<td>( \text{Bn-} p(\text{CL}<em>{30}-c o- \text{BOD}</em>{1.0}) )</td>
<td>182</td>
<td>0.118</td>
</tr>
</tbody>
</table>

<sup>a</sup> Initial pH of water. <sup>1</sup> Italics indicate a bimodal size distribution measured by DLS.

<sup>2</sup> All co-nanoprecipitations were carried out at 90:10 wt% branched copolymer:linear-dendritic hybrid.
CHAPTER 7

The observed zeta-potential ($\zeta$) values were all highly positive (Table 7.2), with a decreasing trend witnessed as the size of the dendron chain end increased. This suggests that the AmG0'-p(CL30), that displayed the highest positive surface charge (+84 mV), contains the most protonated tertiary amine groups on the surface of the nanoprecipitates.

The linear-dendritic HPMA polymers (Chapter 2), produced NPs with large $D_z$ size values and an observable decreasing PDI values as the dendron size increased. The linear-dendritic CL hybrids formed NPs with considerably smaller sizes, with increasing PDI values as the dendron size increases. The surface charge decreases significantly as the dendron size increases, with the AmG2'-p(CL30) producing the smallest NPs with the smallest $\zeta$. This is also observed in the linear-dendritic HPMA series, where the smallest nanoprecipitates (AmG1'-p(HPMA50), $D_z$=246 nm) contained the smallest $\zeta$ value (+40 mV) and the largest nanoprecipitates (AmG0'-p(HPMA50), $D_z$=581 nm) had the most positive surface potential (+52 mV).

![Figure 7.19 DLS size distribution analysis of AmG0'-p(CL30) (green), AmG1'-p(CL30) (blue) and AmG2'-p(CL30) (red) prepared in acidic water at a concentration of 1 mg mL$^{-1}$.](image)

The branched copolymer Bn-p(CL$_{30}$-co-BOD$_{1.0}$) and hyp-polydendrons AmG0'-p(CL$_{30}$-co-BOD$_{1.0}$), AmG1'-p(CL$_{30}$-co-BOD$_{0.8}$) and AmG2'-p(CL$_{30}$-co-BOD$_{0.8}$) subsequently underwent nanoprecipitation into deionised water. Only the AmG1'-p(CL$_{30}$-co-BOD$_{0.8}$) produced stable nanoprecipitates in neutral water ($D_z$=151 nm), displaying a narrow and monomodal size distribution (PDI = 0.081 and Figure 7.20). The branched copolymers and other hyp-polydendrons failed to form stable nanoprecipitates with observable polymer precipitation. Although it is unclear why this difference arises, there is a significant difference of molecular weight of the AmG1'-p(CL$_{30}$-co-BOD$_{0.8}$) ($M_w$ = 92600 g mol$^{-1}$) compared to the AmG0'-p(CL$_{30}$-co-BOD$_{1.0}$) ($M_w$ = 26500 g mol$^{-1}$) and
AmG\textsubscript{2}\textsuperscript{+}*-p(CL\textsubscript{30}-co-BOD\textsubscript{0.8}) \textsubscript{(M\textsubscript{w} = 41730 g mol\textsuperscript{-1})}. Unpublished results from the Rannard group have indicated the critical nature of a small fraction of very highly branched polymer and this may be further supported by this anomalous result.

To the best of our knowledge, this is the first example of stabilised \(p(\text{CL})\) NPs in aqueous solution, without the need for additional stabilisers.

![Figure 7.20 DLS size distribution of AmG\textsubscript{1}\textsuperscript{+}*-p(CL\textsubscript{30}-co-BOD\textsubscript{0.8}) NP dispersion prepared in neutral water at a concentration of 1 mg mL\textsuperscript{-1}.](image)

Following nanoprecipitation into aqueous HCl, stable NP dispersions formed for all three hyp-polydendrons. As the size of the dendron surface group increases, a decrease in \(D_{z}\), with concomitant increase in polydispersity and decrease in \(\zeta\) values was observed; the AmG\textsubscript{0}\textsuperscript{+}*-p(CL\textsubscript{30}-co-BOD\textsubscript{1.0}) NPs were the largest with the largest positive surface charge; and the AmG\textsubscript{2}\textsuperscript{+}*-p(CL\textsubscript{30}-co-BOD\textsubscript{0.8}) produced the smallest nanoprecipitates, the broadest size distribution, with the lowest surface charge (Table 7.2 and Figure 7.21).
Figure 7.21 DLS size distribution analysis of CL-BOD hyp-polydendron NPs produced in acidic water at a concentration of 1 mg mL⁻¹: AmG₀⁺⁺'-p(CL₃₀-co-BOD₁.₀) (green), AmG₁⁺⁺'-p(CL₃₀-co-BOD₁.₈) (blue) and AmG₂⁺⁺'-p(CL₃₀-co-BOD₁.₈) (red).

Compared to the linear-dendritic equivalents, nanoprecipitates with very similar sizes formed from the hyp-polydendrons (123 and 145 nm (AmG₀⁺⁺') and 130 and 133 nm (AmG₁⁺⁺')). The AmG₂⁺⁺'-containing materials produced NPs with similar sizes and surface charge, due to the low molecular weight of the hyp-polydendron. Similar trends are noticed, with the AmG₀⁺⁺' particles containing the highest surface charge, with the AmG₂⁺⁺' particles displaying the highest polydispersity values.

A correlation between the sizes of the hyp-polydendron nanoprecipitates in aqueous HCl and the surface charge was observed, a behaviour that was also noticeable within the NPs generated by the series of HPMA-EGDMA hyp-polydendrons. The AmG₀⁺⁺'-p(CL₃₀-co-BOD₁.₀) produced NPs with a $D_z=145$ nm and a $\zeta=+53$ mV, compared to the AmG₂⁺⁺'-p(CL₃₀-co-BOD₁.₈) nanoprecipitates exhibiting a $D_z=48$ nm and a $\zeta=+22$ mV (and the AmG₁⁺⁺'-p(CL₃₀-co-BOD₁.₈) residing in between with a $D_z=133$ nm and a $\zeta=+48$ mV). Equally, the AmG₁⁺⁺'-p(HPMA₅₀-co-EGDMA₉₉.₉) exhibited the smallest $D_z (=102$ nm) and smallest $\zeta (=+45$ mV); and the AmG₂⁺⁺'-p(HPMA₅₀-co-EGDMA₉₉.₉) exhibited the largest $D_z (=196$ nm) and largest $\zeta (=+61$ mV) in aqueous HCl.

### 7.5 Co-nanoprecipitation studies of linear and linear-dendritic polymers with branched, hydrophobic CL copolymers

Previously, co-nanoprecipitated linear-dendritic HPMA-based hybrids with branched $p$(HPMA-co-EGDMA) copolymers have yielded stabilised NP dispersions (Chapter 5) in neutral water (pH=7.8). The co-nanoprecipitation of the linear-dendritic tBuMA polymer AmG₂⁺⁺'-p(tBuMA₅₀) with EBiB⁻⁻-p(tBuMA₅₀-co-EGDMA₀.₉₅) failed to produce stable NPs,
however, combined to form stable nanoprecipitates in aqueous HCl with the aid of protonation of the dendron chain ends (Chapter 5). The combination of EBiB-p(HPMA<sub>50</sub>-co-EGDMA<sub>0.95</sub>) with AmG<sub>2</sub>-p(HPMA<sub>50</sub>) produced particles that varied considerably from the nanoprecipitation of AmG<sub>2</sub>-p(HPMA<sub>50</sub>-co-EGDMA<sub>0.9</sub>) alone, with a larger \(D_z\) (=210 nm, compared to 149 nm), a reduced size distribution (PDI=0.072, compared to 0.099) and identical \(\zeta\) values (=21 mV, compared to -22 mV). A similar co-nanoprecipitation study was conducted using the linear-dendritic \(p\)(CL) hybrids and the branched benzyl alcohol initiated copolymer (Figure 7.22).

![Figure 7.22 Diagrammatic representation of the co-nanoprecipitation of linear-dendritic CL polymer hybrids (AmG<sub>2</sub>-p(CL<sub>30</sub>) (i)) with Bn-p(CL<sub>30</sub>-co-BOD<sub>1.0</sub>) (ii).](image)

### 7.5.1 Co-nanoprecipitation studies of amine-functionalised linear-dendritic CL hybrids with Bn-p(CL<sub>30</sub>-co-BOD<sub>1.0</sub>)

The selected linear and linear-dendritic \(p\)(CL) polymers were co-nanoprecipitated with the branched copolymer Bn-p(CL<sub>30</sub>-co-BOD<sub>1.0</sub>) into neutral (pH = 7.8) and acidic (pH = 4) water, in a 10:90 wt% linear polymer:branched copolymer ratio. Stable NPs formed for all linear-dendritic hybrid combinations in acidic water (Table 7.2), with the Bn-p(CL<sub>30</sub>)/Bn-p(CL<sub>30</sub>-co-BOD<sub>1.0</sub>) combination resulting in macroscale precipitation.

The AmG<sub>0</sub>-p(CL<sub>30</sub>), AmG<sub>1</sub>-p(CL<sub>30</sub>) and AmG<sub>2</sub>-p(CL<sub>30</sub>) co-nanoprecipitations with Bn-p(CL<sub>30</sub>-co-BOD<sub>1.0</sub>) all produced monomodal size distributions (Figure 7.23). An increased particle size was observed as the size of the dendron increased, the opposite behaviour that was seen for the nanoprecipitation of the hyp-polydendrons (a decreasing
particle size as the dendron size increases). This additionally correlates to an increasing ζ as the dendron size increases, again the opposite behaviour observed when the hyp-polydendrons were nanoprecipitated individually. This suggests that the co-nanoprecipitation method leads to a lessened amount of dendrons situated in the main bulk of the nanoprecipitate during the growth stages, resulting in an increased level of dendron on the nanoprecipitate surface, explaining the increased zeta potential values.

Figure 7.23 DLS size distribution analysis of co-nanoprecipitated NPs of Bn-p(CL$_{30}$-co-BOD$_{1.0}$) with AmG$_{2'}$-p(CL$_{30}$) (green), AmG$_{1'}$-p(CL$_{30}$) (blue) and AmG$_{2'}$-p(CL$_{30}$) (red).

7.5.2 Co-nanoprecipitation and pH studies of AmG$_{2'}$-p(DEA$_{50}$) and Bn-p(CL$_{30}$-co-BOD$_{1.0}$)

Following the successful production of co-nanoprecipitated NPs from linear-dendritic CL hybrids and Bn-p(CL$_{30}$-co-BOD$_{1.0}$), the co-nanoprecipitation of Bn-p(CL$_{30}$-co-BOD$_{1.0}$) with the pH-responsive linear-dendritic DEA hybrid, AmG$_{2'}$-p(DEA$_{50}$), synthesised in Chapter 3, (Figure 7.24) was investigated.
**Figure 7.24** Diagrammatic representation of the co-nanoprecipitation of Bn-\(p(\text{CL}_{30}-\text{co}-\text{BOD}_{1.0})\) (ii) with the pH-responsive, linear-dendritic AmG\(_2\)-\(p(\text{DEA}_{50})\) polymer hybrid (i).

AmG\(_2\)-\(p(\text{DEA}_{50})\) has previously been co-nanoprecipitated with EBiB-\(p(\text{HPMA}_{50}-\text{co}-\text{EGDMA}_{0.95})\) to produce NPs with a \(D_z=144\) nm, PDI=0.102 and \(\zeta=+52\) mV (Chapter 5). Upon the addition of acid, the EBiB-\(p(\text{HPMA}_{50}-\text{co}-\text{EGDMA}_{0.95})\) precipitated out of solution as large polymeric aggregates following solubilisation of the AmG\(_2\)-\(p(\text{DEA}_{50})\). Additionally, AmG\(_2\)-\(p(\text{DEA}_{50})\) was co-nanoprecipitated with EBiB-\(p(\text{tBuMA}_{50}-\text{co}-\text{EGDMA}_{0.95})\) forming stable nanoprecipitates (\(D_z=234\) nm, PDI=0.051 and \(\zeta=+40\) mV) that remained stable for a short period upon acid addition, requiring further time to solubilise the AmG\(_2\) dendron chain end, presumably residing in the bulk of the nanoprecipitate. The co-nanoprecipitation of EBiB-\(p(\text{tBuMA}_{50}-\text{co}-\text{EGDMA}_{0.95})\) with AmG\(_2\)-\(p(\text{DEA}_{50})\) was the only example throughout this work, of a stabilised branched \(\text{tBuMA}\)-derived polymer NPs in neutral water.

Co-nanoprecipitation of AmG\(_2\)-\(p(\text{DEA}_{50})\) and Bn-\(p(\text{CL}_{30}-\text{co}-\text{BOD}_{1.0})\) was carried out into acidic and neutral water (Table 7.2) at a linear-dendritic polymer:branched copolymer ratio of 10:90 wt%. A stable NP dispersion formed (Table 7.2) in neutral water, producing a monomodal size distribution (Figure 7.25), with a positive \(\zeta\) suggesting \(p(\text{DEA})\) chains residing towards or on the nanoprecipitate surface. A stable NP dispersion failed to form in acidic water presumably due to solubilisation of AmG\(_2\)-\(p(\text{DEA}_{50})\) in pH 4. Although Chapter 4 suggested the insolubility of AmG\(_2\)-\(p(\text{DEA}_{50})\),
confirmed by the formation of nanoprecipitates at a concentration of 1 mg mL\(^{-1}\), with slight protonation confirmed by the positive \(\zeta\) values, solubility might be possible at a concentration of 0.1 mg mL\(^{-1}\).

![Figure 7.25](image)

**Figure 7.25** DLS size distribution analysis of co-nanoprecipitated AmG\(_2\)-p(DEA\(_{50}\)) with Bn-p(CL\(_{30}\)-co-BOD\(_{1.0}\)) prepared in neutral water at a concentration of 1 mg mL\(^{-1}\).

The stabilised NPs formed from Bn-p(CL\(_{30}\)-co-BOD\(_{1.0}\)) and AmG\(_2\)-p(DEA\(_{50}\)) underwent the addition of acid to establish any pH-responsive behaviour. Upon the addition of acid, the NPs failed to solubilise and remained as intact nanoprecipitates, confirmed by DLS analysis \((D_z=208\text{ nm}, \text{PDI}=0.111; \text{Figure 7.26})\). The NPs were re-analysed after 7 days following an excess of acid addition, and continued to retain integrity \((D_z=197\text{ nm}, \text{PDI}=0.128)\). This suggests dendron chain ends may be trapped within the main bulk of the nanoprecipitates, and the close packed nature of the co-nanoprecipitated NPs prevents the penetration of acid or diffusion of the trapped chains.

![Figure 7.26](image)

**Figure 7.26** DLS size distribution analysis of co-nanoprecipitated AmG\(_2\)-p(DEA\(_{50}\)) with Bn-p(CL\(_{30}\)-co-BOD\(_{1.0}\)) (green) following acid addition (red) after 7 days (red-dotted).
7.6 Degradation studies of \( p \)(caprolactone)

As previously mentioned, the hydrolysis of \( p \)(CL\(_x\)) in physiological media results non-enzymatic cleavage of the ester bonds,\(^{96,97}\) resulting in non-toxic components that are eliminated from the body as carbon dioxide and water via the Krebs cycle. A preliminary study was devised to study the hydrolysis of CL in phosphate buffer solution (PBS). \textbf{Bn-} \( p \)(CL\(_{30}\)) (1.00 g) was left in PBS for 8 weeks. Samples were taken after 4, 6 and 8 weeks and analysed by GPC to observe the break-down of the linear polymer. Figure 7.27 shows the overlay of the initial polymer (blue), overlain with the samples after 4 (red), 6 (green) and 8 (orange) weeks. An increasing amount of smaller material is witnessed at \( \sim 22 \) mL suggesting an increasing amount of degradation over the time period. The samples continue to contain \textbf{Bn-} \( p \)(CL\(_{30}\)), confirmed by the exact overlay of the untreated material eluting between 17.5-20.5 mL.

![Figure 7.27 GPC RI chromatograms of Bn-\( p \)(CL\(_{30}\)) (blue) after 4 (red), 6 (green) and 8 (orange) weeks in PBS.](image)

7.7 Conclusions

Following the synthesis of a new AB\(_3\) branching molecule, and new amine-functionalised ROP dendron initiators, CL was successful polymerised to produce linear-dendritic polymer hybrids with varying dendron generation chain ends. \textbf{AmG}_{1'}- and \textbf{AmG}_{2'}-initiated polymerisations displayed broad molecular weight distributions, and further work needs to be done to continue optimising the ROP conditions. The effect of catalyst amount and reaction time demonstrated an effect on the level of control, and variables such as temperature and solution ROP are yet to be resolved. The
incorporation of BOD to produce novel biodegradable hyp-polydendrons was achieved for all dendron initiators, reaching $M_w > 92,000$ g mol$^{-1}$. Again, detailed understanding into the branched co-polymerisation conditions are lacking, and optimisation improvements may be necessary to reach higher monomer conversions, and consequently, higher molecular weights.

The formation of linear-dendritic CL NPs was achieved via nanoprecipitation into acidic water. Equally, the AmG$_0^\prime$-p(CL$_{30}$-co-BOD$_1$) and AmG$_2^\prime$-p(CL$_{30}$-co-BOD$_{0.8}$) required additional charge to produce stable nanoprecipitates; although stable AmG$_1^\prime$-p(CL$_{30}$-co-BOD$_{0.8}$) NPs formed in neutral water. These introductory results are encouraging, particularly due the Bn-p(CL$_{30}$) and Bn-p(CL$_{30}$-co-BOD$_{1.0}$) undergoing observable macroscale precipitation upon introduction into any aqueous media. The hyp-polydendron structure, clearly offers benefits in terms of NP production, without the need for stabilisers. A preliminary degradation study suggests degradation in physiologically-relevant media.

7.8 Experimental

[BOC$_2$-BAPA-G$_1$] – CDI (19.55 g, 0.121 mol, 2 eq.) was added to an oven-dried 500mL 2-neck round-bottomed flask (RBF) fitted with a reflux condenser, magnetic stirrer and a dry N$_2$ inlet. 350 mL of anhydrous toluene was added and the flask purged with N$_2$ for 10 minutes. The solution was stirred at 60°C and tertiary butanol (17.83 g, 23 mL, 0.241 mol, 4 eq.) added via a warm syringe. The mixture was left stirring at 60°C for 6 hours under a positive flow of nitrogen. Bis(3-aminopropyl)amine (7.88 g, 8.4 mL, 0.060 mol, 1 eq.) was added dropwise, and the reaction was left stirring for a further 18 hours at 60°C under a positive flow of nitrogen. Following this, the solution was allowed to cool to room temperature, and the pale yellow solution was filtered to remove any solid imidazole, and concentrated in vacuo. The resulting viscous oil was dissolved in dichloromethane (200 mL) washed with distilled water (3 x 200 mL) and once with brine (150 mL). The organic layer was dried with anhydrous Na$_2$SO$_4$, filtered, and concentrated in vacuo. Yield: 16.63 g, white solid, (84%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 1.43 (s, 18H), 1.63 (m, 4H), 2.64 (t, 4H), 3.20 (t, 4H), 5.19 (s, br, -NH). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 28.5, 29.9, 39.0, 47.7, 79.2, 156.2. Calcd: [M+H]$^+$ (C$_{16}$H$_{33}$N$_3$O$_4$) m/z = 332.3. Found: ESI-MS: [M+H]$^+$ m/z = 332.3. Anal. Calcd for C$_{16}$H$_{33}$N$_3$O$_4$: C, 58.00; H, 10.00; N, 12.69. Found: C, 57.78; H, 9.92; N, 12.82.
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\[\text{[BOC}_2\text{-APAP-OH}'] - \text{[BOC}_2\text{-BAPA-G1}'] \] (15.38 g, 0.046 mol, 1 eq.), bromoethanol (5.81 g, 3.3 mL, 0.046 mol, 1 eq.), sodium iodide (150 mg), potassium carbonate (19.27 g, 0.139 mol, 3 eq.) and 1,4-dioxane (150 mL) was added to a 500 mL 2-necked RBF fitted with a reflux condenser and magnetic stirrer. The reaction was refluxed overnight. After this time, water (150 mL) was added to the reaction mixture and the product was extracted with ethyl acetate (2 x 225 mL). The combined extracts were washed with water (1 x 150 mL), dried over sodium sulfate and concentrated \textit{in vacuo}. The crude product purified by liquid chromatography (silica gel, eluting with EtOAc:MeOH, 80:20). Yield: 7.55 g, pale yellow oil at ambient temperature, solidifying to an off white solid upon cooling (43 %). \( \text{H NMR (400 MHz, CDCl}_3\):}\( \delta = 1.41 \text{ (s, 18H), 1.62 (m, 4H), 2.47 (t, 4H), 2.54 (t, 2H), 2.85 (s, br, OH), 3.16 (m, 4H), 3.57 (t, 2H), 5.09 (s, br, NH).} \) \( \text{13C NMR (100 MHz, CDCl}_3\):}\( \delta = 27.2, 28.4, 38.9, 51.7, 56.0, 58.9, 79.1, 156.2. \) Calcd: [M+H] + (C18H37N3O5) m/z = 376.5. Found: ESI-MS: [M+H] + m/z = 376.3. Anal. Calcd for C18H37N3O5: C, 57.52; H, 9.85; N, 11.18. Found: C, 56.97; H, 9.81; N, 11.02.

\[\text{1-[N,N-bis (2-aminopropyl)-amino]-2-propan-1-ol (APAP')}\] – To a 500 mL RBF, \[\text{[BOC}_2\text{-APAP-OH}'] \] (7.49 g, 0.02 mol, 1 eq.) was dissolved in ethyl acetate (80 mL), and had concentrated HCl (12.14 g, 10.3 mL, 36% active) added very slowly. CO\textsubscript{2} began to rapidly evolve. The reaction vessel was left open to the atmosphere, heated to 50°C and stirred for 24 hours. After removal of ethyl acetate \textit{in vacuo}, the crude oil was dissolved very slowly in 4M NaOH (80 mL), and reduced by approximately half its volume on the rotary evaporator (60°C). A yellow oily substance formed on the surface of the NaOH solution. The mixture was extracted with CHCl\textsubscript{3} (2 x 80 mL), the organic layers combined, dried with anhydrous Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated \textit{in vacuo}. Yield: 2.98 g, pale yellow oil (85%). \( \text{H NMR (400 MHz, CDCl}_3\):}\( \delta = 1.54 \text{ (m, 4H), 2.48 (m, 6H), 2.70 (t, 4H), 3.53 (t, 2H).} \) \( \text{13C NMR (100 MHz, CDCl}_3\):}\( \delta = 30.58, 40.37, 52.01, 56.02, 59.77. \) Calcd: [M+H] + (C\textsubscript{18}H\textsubscript{37}N\textsubscript{3}O\textsubscript{3}) m/z = 175.5. Found: CI-MS: [M+H] + m/z = 176.2. Anal. Calcd for C\textsubscript{18}H\textsubscript{37}N\textsubscript{3}O\textsubscript{3}: C, 54.86; H, 12.00; N, 24.00%. Found: C, 53.47; H, 12.06; N, 23.67%.

\text{AmG}_1'-\text{OH} – 2-(Dimethylamino)ethyl acrylate (DMEA) (6.0 g, 42 mmol, 6 eq.) was added to a 50 mL round 2 necked RBF containing propan-2-ol (IPA) (12 mL). The flask was deoxygenated under a positive N\textsubscript{2} purge for 10 minutes. Ethanolamine (0.4266 g, 7.0 mmol, 1 eq.) dissolved in IPA (12 mL) was added drop wise while the solution was stirring in an ice bath under a positive flow of N\textsubscript{2}. The final mixture was stirred for a further 10 minutes at 0°C before being allowed to warm to room temperature and left...
stirring for 48 hr. The solvent was removed and the product left to dry in vacuo overnight. Yield: 2.33 g, yellow oil (96%). $^1$H NMR (400 MHz, CDCl$_3$) δ 2.27 (s, 12H), 2.44-2.61 (m, 10H), 2.81 (t, 4H), 3.57 (t, 2H), 4.18 (t, 4H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 32.6, 45.6, 49.4, 56.2, 57.8, 59.5, 62.0, 172.7. Calcd [M+H]$^+$ (C$_{16}$H$_{33}$N$_3$O$_5$) m/z = 347.5. Found: ESI-MS: [M+H]$^+$ m/z = 348.2, [M+Na]$^+$ m/z = 370.2. Anal. Calcd for C$_{16}$H$_{33}$N$_3$O$_5$: C, 55.26; H, 9.50; N, 12.09%. Found C, 57.09; H, 9.47; N, 11.02%.

AmG$_2$'-OH – DMEA (6.0 g, 0.042 mol, 6 eq.) was added to a 50 mL round 2 necked RBF containing IPA (12 mL). The flask was deoxygenated under a positive N$_2$ purge for 10 minutes. APAP’ (1.2222 g, 0.007 mmol, 1 eq.) dissolved in IPA (12 mL) was added drop wise while the solution was stirring in an ice bath under a positive flow of N$_2$. The final mixture was stirred for a further 10 minutes at 0°C, allowed to warm to room temperature and left stirring for 48 hours. The solvent was removed and the product left to dry in vacuo overnight. Yield: 4.84 g, yellow oil, (93%). $^1$H NMR (400 MHz, CDCl$_3$) δ 1.52 (m, 4H), 2.23 (s, 24H), 2.41 (m, 16H), 2.51 (t, 10H), 2.72 (t, 8H), 3.50 (t, 2H), 4.11 (t, 8H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 24.62, 32.24, 45.68, 48.94, 51.53, 51.89, 55.76, 57.82, 59.48, 62.15, 172.6. Calcd [M+H]$^+$ (C$_{36}$H$_{73}$O$_9$N$_7$) m/z = 748.01. Found: ESI-MS: [M+H]$^+$ m/z = 748.6. Anal. Calcd for C$_{36}$H$_{73}$O$_9$N$_7$: C, 57.75; H, 9.76; N, 13.10%. Found: C, 57.50; H, 9.76; N, 13.01%.

[4,4'-bioxepanyl-7,7'-dione (BOD)] – Urea hydrogen peroxide (10 g) was added to a 250 mL RBF containing formic acid (81.97 g, 100 mL). The solution was stirred for 2 hr at room temperature. The flask was then immersed in an ice bath and BCH (5 g) was slowly added to the solution. The reaction mixture was stirred for 4 hr. Water (100 mL) was then added to the mixture and the product was extracted with chloroform (3 x 100 mL). The organic fractions were collected and washed with saturated aqueous sodium bicarbonate solution then dried over Na$_2$SO$_4$. After removing the solvent, a white powder was isolated and dried under vacuum overnight. Yield: 3.26 g, white solid, (56%). $^1$H NMR (400 MHz, CDCl$_3$) δ 1.50 (m, 2H), 1.66 (m, 4H), 1.87 (m, 4H), 2.51-2.82 (d of t, 4H), 4.08-4.44 (d of t, 4H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 29.6, 35.9, 37.1, 49.7, 72.4, 181.3. Calcd [M+H]$^+$ (C$_{12}$H$_{18}$O$_4$) m/z = 226.3. Found: ESI-MS: [M+H]$^+$ m/z = 228.2. Anal. Calcd for C$_{12}$H$_{18}$O$_4$: C, 63.64; H, 7.96%. Found: C, 61.67; H, 7.72%.

[General procedure for Bn-, AmG$_0$'-, AmG$_1$'- and AmG$_2$'-p(CL$_x$)]; In a typical experiment, Sn(Oct)$_2$ (0.0025 g, 0.0062 mmol, 1/350 eq.) was added using a dry syringe to a RBF equipped with a magnetic stirrer bar flushed with dry nitrogen. Following this,
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CL (7.39 g, 6.86 mL, 0.0648 mol, 30 eq.) was added using a dry syringe. The reaction mixture was degassed for a further 15 minutes and then immersed in a silicon oil bath at 110°C. AmG$_2$'-OH (1.62 g, 0.0022 mol, 1 eq.) was added via a dry syringe and the polymerisation left for 66 hr. The polymerisation was stopped by removing the reaction mixture from the heat and immersing it in an ice bath. The crude product was dissolved in 50 mL of THF and precipitated from 600 mL of hexane. The precipitated polymer was dried under vacuum for 24 hr.

[General procedure for Bn- AmG$_0$'-, AmG$_1$'- and AmG$_2$'-p(CL$_{30}$-co-BOD$_x$)]; In a typical experiment, Sn(Oct)$_2$ (0.0018 g, 0.005 mmol, 1/350 eq.) and BOD (0.2849 g, 0.0013 mol, 0.8 eq.) were added to a RBF equipped with a magnetic stirrer bar flushed with dry nitrogen. Following this, CL (5.39 g, 5 mL, 0.047 mol, 30 eq.) was added using a dry syringe. The reaction mixture was degassed for a further 15 minutes and then immersed in a silicon oil bath at 110°C. AmG$_1$'-OH (0.5469 g, 0.0016 mol, 1 eq.) was added via a dry syringe and the polymerisation left for 144 hr. The polymerisation was stopped by removing the reaction mixture from the heat and immersing it in an ice bath. The crude product was dissolved in 50 mL of THF and precipitated from 600 mL of hexane. The precipitated polymer was dried under vacuum for 24 hr.

[General procedure for aqueous nanoprecipitation of p(CL$_{30}$) and p(CL$_{30}$-co-BOD$_x$)]; The materials were dissolved in THF at a concentration of 5 mg mL$^{-1}$. 2 mL of this solution was then subjected to a rapid solvent switch through drop wise addition into 10 mL of water, to give a final polymer concentration of 1 mg mL$^{-1}$ in water after THF removal by evaporation overnight.

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8.1 Conclusions

8.1.1 Hyp-polydendron synthesis

The synthesis of amine-functionalised dendron atom transfer radical polymerisation (ATRP) initiators was achieved, with consequent controlled formation of linear-dendritic polymer hybrids and hyp-polydendrons. The dendron-functional nanoprecipitates derived from 2-hydroxypropyl methacrylate $p$(HPMA$_{50}$) and the co-polymerisation of HPMA and ethylene glycol dimethacrylate $p$(HPMA$_{50}$-co-EGDMA$_x$) displayed behaviour that appeared to benefit from both the presence and generation of the dendron end groups and the branching of the methacrylate polymer chains. The linear-dendritic hybrids and hyp-polydendrons exhibited varying behaviour when nanoprecipitated into aqueous media, presumably from a different arrangement of dendrons and vinyl polymer segments within the nanoprecipitates. This appears to be directed predominantly by the branched polymer architecture and the chemical dissimilarity of the charged chain-ends and the uncharged and collapsed polymer chains. These differences also appeared to modify the ability of the nanoparticle (NP) surfaces to protonate and deprotonate.

Synthetic exploration of this new polymeric material class was carried out, in order to demonstrate the chemical and architectural versatility available. The hyp-polydendron structure has been altered by variation of multiple components, including the monomer chemistry, hyp-polydendron primary chain architecture, surface functionality, the use of multiple initiators and the incorporation of a new pH-sensitive brancher. This ultimately led to a variety of new polymer architectures including linear-dendritic polymer hybrids and hyp-polydendrons derived from tertiary butyl methacrylate ($t$BuMA) or pH-responsive 2-(diethylamino) ethyl methacrylate (DEA); statistically copolymers of DEA and either HPMA or $t$BuMA; diblock copolymers of DEA and $t$BuMA with varying overall primary polymer chain lengths; multiple dendron/non-dendron initiators incorporated into the same hyp-polydendron; variation within the dendron surface and linker chemistry; and the incorporation of a pH-responsive brancher. Synthetically, all polymerisations and co-polymerisations that were monitored proceeded via first order kinetics. The production of linear-dendritic materials resulted in mono-modal molecular weight distributions, and a much broader disperse polymer distribution was observed when a divinyl monomer was incorporated and a branched polymer was targeted.
Finally, new amine-functionalised ring-opening polymerisation (ROP) dendron initiators were synthesised, in order to produce new caprolactone-based (CL) linear-dendritic polymer hybrids and hyp-polydendrons with varying generation dendron chain ends. These studies were conducted as a proof-of-concept to extend the hyp-polydendron concept to polyesters and produce biodegradable linear-dendritic polymers and hyp-polydendrons. A more detailed study and optimisation of polymerisation conditions will be required to enhance polymerisation control and produce materials with higher molecular weights. Nevertheless, an AmG1*-initiated polyester hyp-polydendron exhibiting an $M_w > 92,000$ g mol$^{-1}$ was recovered. The formation of linear-dendritic CL NPs was achieved via nanoprecipitation into acidic water and stable AmG1*-p(CL$_{30}$-co-BOD$_{0.8}$) NPs were formed in neutral water following analysis by DLS and scanning electron microscopy. These introductory results are encouraging, particularly due to the Bn-p(CL$_{30}$) and Bn-p(CL$_{30}$-co-BOD$_{1.0}$) undergoing observable macroscale precipitation upon introduction into any aqueous media. The hyp-polydendron structure, clearly offers benefits in terms of NP production, without the need for stabilisers. Additionally, a preliminary degradation study was carried out, suggesting degradation in physiologically-relevant media.

8.1.2 Nanoparticle formation and behaviour

Following the synthetic exploration into the chemical and architectural variables within hyp-polydendron structures, the ability of these materials to form stable nanoprecipitates in aqueous media was investigated. Generally, the hyp-polydendrons produced stable nanoprecipitates with narrow size distributions, compared to the linear-dendritic hybrids that typically resulted in broad and bimodal size distributions. The proposed explanation for this behaviour includes the rapid formation of large nuclei originating from the high molecular weight fraction of the hyp-polydendron collapsing to form larger particles that contain a significant number of conjoined chains, as opposed to the linear materials slowly producing nuclei from much smaller structures. The inability of the statistical and block hyp-polydendrons derived from DEA and tBuMA to form stable NPs in neutral water was overcome via manipulation of the primary polymer chain length, and the change of precipitation media to acidic water. The inclusion of DEA within the hyp-polydendron allowed for the stable production of nanoprecipitates via protonation of the tertiary amines residing in the pendant polymer groups. The pH-responsive effects of the DEA-containing nanoprecipitates upon the addition of acid was studied, indicating the existence of soluble, hydrated polymers at low pH; confirmed by observable clear
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solutions, and noticeable decreases in particle sizes and derived count rates (DCR) measured by dynamic light scattering (DLS).

Acid hydrolysis of the brancher within 1,4-butanediol di(methacryloxyloxy)-ethyl ether (BDME)-containing hyp-polydendron NP dispersions, derived from DEA and statistical copolymers of DEA and HPMA, resulted in complete degradation of the NPs and component hyp-polydendrons to linear-dendritic polymer chains as confirmed by gel permeation chromatography (GPC) analysis displaying mono-modal molecular weight distributions. The AmG2-p(DEA17-b-(tBuMA33-co-BDME2.0)) failed to solubilise upon the addition of acid, suggesting a NP packing that prevents acid entry into the core of the particle. Successful encapsulation of a model dye guest molecule was achieved within various pH-responsive hyp-polydendron NPs, and release studies revealed a more rapid release rate based on modifications within the hyp-polydendron i.e. upon incorporation of the acid-cleavable brancher.

Following the stable NP formation from nanoprecipitation of hyp-polydendrons, the co-nanoprecipitation concept was applied to produce stabilised NPs from linear-dendritic polymer hybrids and branched copolymers. Overall, the co-nanoprecipitated particles prepared with linear-dendritic polymers produced nanoprecipitates that were noticeably different compared to the nanoprecipitates prepared from similarly composed hyp-polydendrons. The facile construction of NPs via co-nanoprecipitation produces a hydrophobic domain, with the opportunity to introduce specific characteristics and easily tailored surface functionality, to provide enhanced properties and stability.

8.1.3 Pharmacological assessment

A selection of the nanoprecipitated and co-nanoprecipitated NP dispersions were progressed to pharmacological testing. A range of pharmacological evaluations were conducted including; passage of NPs across a model gut epithelium, predicting the behaviour after oral administration, studying cellular uptake and measurement of cytotoxicity. These were carried out in order to evaluate the potential for drug delivery benefits. The pharmacological experiments were carried out by Dr. Lee Tatham and Prof. Andrew Owen in the Department of Molecular and Clinical Pharmacology at the University of Liverpool.

The pharmacology results suggested negligible cytotoxicity within the cell lines tested.
with an increase in transcellular permeability, without damage to the model gut epithelium monolayer, for NPs comprised of $\text{AmG}_{1}\text{U}^{\text{p}}(\text{DEA}_{50}\text{co}-\text{EGDMA}_{0.9})$ containing the model drug molecule, fluoresceinamine (FA). The increased accumulation of encapsulated FA compared to the aqueous FA in Caco-2 and ATHP-1 cells could suggest uptake of the nano-carrier into primary lymphocytes and, consequently, macrophages. The NPs comprising $\text{AmG}_{1}\text{U}^{\text{p}}(\text{DEA}_{50}\text{co}-\text{EGDMA}_{0.9})$, which exhibited the most encouraging behaviour as orally administered NPs, were also used to encapsulate the anti-retroviral drug, Efavirenz (EFV) (cf. Section 8.2.1), demonstrating the potential for therapeutic drug loading.

Overall, a variety of linear-dendritic polymer hybrids and hyp-polydendrons have been produced, demonstrating the versatility available within the modification of chemical and architectural components of this new macromolecular architecture. The ability to produce stable nanoprecipitates has been demonstrated to depend on a variety of these different chemical and architectural properties, allowing modification of these structural properties to encourage stable NP formation. The co-nanoprecipitation method has also been successfully applied to produce stable NPs from linear-dendritic hybrids and branched copolymers. The facile construction of NPs via co-nanoprecipitation provides the opportunity to introduce specific characteristics and easily tailored surface functionality. The encouraging pharmacology data presents opportunities for future development that may allow oral dosing leading to circulating polymeric NPs. This may prove clinically desirable to many non-terminal or chronic diseases that utilise nanomedicines, but wish to avoid regular or repeated intravenous administration.

8.2 Future Work

The hyp-polydendron structure and the co-nanoprecipitation concept has revealed some of the impact that manipulation of the chemical and structural components has on the ability to form NPs, and their subsequent behaviour. Future studies may include the incorporation of other methacrylate or styrenic monomers using ATRP, variation of surface functionality for cell targeting, multiple dendron surface functionality within single structures or subsequent dendron functionalisation following hyp-polydendron synthesis.

Pharmacological testing described within the thesis was carried out on a small selection of hyp-polydendron NPs containing encapsulated FA. In vitro studies for other
nanoprecipitated particles would be useful to build a database of activity; particularly NPs comprised of BDME-containing hyp-polydendrons and co-nanoprecipitated particles. In particular, the comparison of hyp-polydendron NPs containing block copolymers of DEA and tBuMA (and EGDMA) that showed stability in neutral water (Chapter 4) and the co-nanoprecipitates of EBiB-\(p\)(tBuMA\(_{50}\)-co-EGDMA\(_{0.95}\)):\(\text{AmG}_2-p\)(DEA\(_{50}\)) that have showed stability in buffered media (Table 8.1) would be ideal to study.

**Table 8.1** DLS analysis of co-nanoprecipitated 10:90 wt% \(\text{AmG}_2-p\)(DEA\(_{50}\)):\(\text{EBiB}-p\)(tBuMA\(_{50}\)-co-EGDMA\(_{0.95}\)) 10:90 wt% before and after the addition of transport buffer solution.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>pH=7.8(^a)</th>
<th>+TBS (aq.)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{AmG}<em>2-p)(DEA(</em>{50})):(\text{EBiB}-p)(tBuMA(<em>{50})-co-EGDMA(</em>{0.95}))</td>
<td>194 0.109 155</td>
<td>343 0.216 253</td>
</tr>
</tbody>
</table>

\(^a\)Initial pH, \(^b\)TBS (aq.) (1 mL) added to 10 mL (1 mg mL\(^{-1}\)) NP dispersion

In addition, the EBiB-\(p\)(DEA\(_{50}\)-co-EGDMA\(_{0.95}\)):\(\text{AmG}_2-p\)(DEA\(_{50}\)) 90:10 wt% co-nanoprecipitates would provide insight into the direct comparison of *in vitro* studies of covalently-bound DEA\(_{50}\)-co-EGDMA\(_{0.9}\) hyp-polydendrons that have already exhibited encouraging behaviour as a potential drug delivery vehicle. Future studies for the AmG\(_1\)U-\(p\)(DEA\(_{50}\)-co-EGDMA\(_{0.9}\)) containing encapsulated EFV should include assessing apparent permeability, cytotoxicity, accumulation, and nanocarrier efficacy against human immunodeficiency virus (HIV), compared to EFV in an aqueous vehicle. Furthermore, as anti-retroviral drugs are only taken as combinations, future studies would require the encapsulation of other multiple drug cocktails.\(^{1,2}\)

### 8.2.1 pH studies on linear-dendritic polymer hybrids and hyp-polydendrons containing DEA, tBuMA and EGDMA

The pH-responsive behaviour of statistical and block copolymer linear-dendritic polymer hybrids comprising DEA and tBuMA and the corresponding hyp-polydendrons (Chapter 3) that produced stable nanoprecipitates in acidic water, has been investigated. The NP behaviour, size and surface charge for these materials in acidic water has already been discussed in Chapter 4.
The stable NPs containing statistical HPMA and DEA containing hyp-polydendrons were observed to undergo full solubilisation upon the addition of significant amounts of acid, therefore the response of similar materials containing tBuMA and DEA was studied. In this case, the NPs prepared in aqueous HCl were subjected to a further (rapid) addition of HCl. Due to the neutralisation during initial NP formation in acid, the final pH of the original aqueous dispersions was measured to vary between pH = 5.9-7.4. Addition of 1M HCl to dispersions generated from the linear-dendritic statistical copolymer hybrids led to a rapid switch of pH and a noticeable decrease in the number size distribution ($D_n$) values and measured DCR (Table 8.2), consistent with dissolution in all cases.

Table 8.2 DLS analysis of acid addition on nanoprecipitated particles prepared from statistical and block DEA-tBuMA linear-dendritic polymer hybrids and hyp-polydendrons

<table>
<thead>
<tr>
<th>pH of water</th>
<th>pH = 4.0$^a$ (Final pH = 5.9-7.4)</th>
<th>+ 1M HCl (Final pH = 2.6-3.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer</td>
<td>$D_c$ (nm)</td>
<td>PDI</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{33}$-co-tBuMA$_{17}$)</td>
<td>44</td>
<td>0.167</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{25}$-co-tBuMA$_{25}$)</td>
<td>37</td>
<td>0.181</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{17}$-co-tBuMA$_{33}$)</td>
<td>65</td>
<td>0.172</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{33}$-co-tBuMA$<em>{17}$-co-EGDMA$</em>{0.9}$)</td>
<td>51</td>
<td>0.222</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{25}$-co-tBuMA$<em>{25}$-co-EGDMA$</em>{0.9}$)</td>
<td>134</td>
<td>0.284</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{17}$-co-tBuMA$<em>{33}$-co-EGDMA$</em>{0.9}$)</td>
<td>129</td>
<td>0.222</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{33}$-b-tBuMA$_{17}$)</td>
<td>38</td>
<td>0.192</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{25}$-b-tBuMA$_{25}$)</td>
<td>40</td>
<td>0.136</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{17}$-b-tBuMA$_{33}$)</td>
<td>40</td>
<td>0.112</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{33}$-b-(tBuMA$<em>{17}$-co-EGDMA$</em>{0.9}$))</td>
<td>23</td>
<td>0.322</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{25}$-b-(tBuMA$<em>{25}$-co-EGDMA$</em>{0.9}$))</td>
<td>84</td>
<td>0.197</td>
</tr>
<tr>
<td>AmG$<em>2$p(DEA$</em>{17}$-b-(tBuMA$<em>{33}$-co-EGDMA$</em>{0.9}$))</td>
<td>28</td>
<td>0.366</td>
</tr>
</tbody>
</table>

$^a$ Initial pH
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Figure 8.1 DLS size by intensity analysis of A) AmG$_2$-$p$(DEA$_{33}$-co-tBuMA$_{17}$) and B) AmG$_2$-$p$(DEA$_{17}$-co-tBuMA$_{33}$-co-EGDMA$_{0.9}$) before (green) and after (red-dotted) acid addition; C) AmG$_2$-$p$(DEA$_{25}$-co-tBuMA$_{25}$) and D) AmG$_2$-$p$(DEA$_{25}$-co-tBuMA$_{25}$-co-EGDMA$_{0.9}$) before (orange) and after (red-dotted) acid addition; E) AmG$_2$-$p$(DEA$_{33}$-co-tBuMA$_{17}$) and F) AmG$_2$-$p$(DEA$_{33}$-co-tBuMA$_{17}$-co-EGDMA$_{0.9}$) before (red) and after (red-dotted) acid addition.

All linear-dendritic polymer hybrids exhibited $D_n$ values representative of fully solvated, individual linear polymer chains at this low pH. The hyp-polydendrons did not fully solubilise, particularly the AmG$_2$-$p$(DEA$_{17}$-co-tBuMA$_{33}$-co-EGDMA$_{0.9}$), where the $D_z$
and \( D_n \) values remained relatively high, although the PDI and the \( D_z \) did increase for two of the materials with higher DEA content. This is relatively surprising as all linear-dendritic hybrids dissolved under these conditions. \( \text{AmG}_2\text{-p}(\text{DEA}_{17}\text{-co}-\text{tBuMA}_{33}\text{-co-EGDMA}_{0.9}) \) contained the greatest hydrophobic monomer content, which may prevent complete solubilisation at low pH. Alternatively, this could imply a \( \text{hyp}-\text{polydendron} \) arrangement during nanoprecipitation and aggregation, with a compact assembly of branched material that prevents acid access to the pH-responsive primary chains within the main bulk of the nanoprecipitate (Figure 8.1).

The linear-dendritic block copolymer hybrid and block copolymer \( \text{hyp}-\text{polydendron} \) nanoprecipitates prepared in aqueous HCl, where studied in similar experiments to those used to evaluate the statistical copolymer architectures; namely rapid addition of acid to the aqueous NP dispersions initially produced in acid environments (Table 8.2). After the pH change, the NP dispersions prepared from the \( \text{AmG}_2\text{-p}(\text{DEA}_{33}\text{-b}-\text{tBuMA}_{17}) \) and \( \text{AmG}_2\text{-p}(\text{DEA}_{25}\text{-b}-\text{tBuMA}_{25}) \) linear-dendritic block copolymer hybrids ceased to exist as stabilised nanoprecipitates, becoming solvated chains at low pH as confirmed by the dramatic decrease in DCR and reduction in \( D_n \) values (5 and 4 nm respectively) (Table 8.2); the \( \text{AmG}_2\text{-p}(\text{DEA}_{33}\text{-b}-\text{tBuMA}_{17}) \) and \( \text{AmG}_2\text{-p}(\text{DEA}_{25}\text{-b}-\text{tBuMA}_{25}) \) linear-dendritic polymer hybrids contain the highest DEA content. The other linear-dendritic polymer, \( \text{AmG}_2\text{-p}(\text{DEA}_{17}\text{-b}-\text{tBuMA}_{33}) \), was stable even at such a low pH. A limited swelling was seen, accounting for the small increase in \( D_z \) and \( D_n \) values, and corresponding increase in DCR, presumably due to additional polymer/dendron protonation occurring at the NP surface (Figure 8.2).

The \( \text{AmG}_2\text{-p(DEA}_{33}\text{-b-}(\text{tBuMA}_{17}\text{-co-EGDMA}_{0.9})) \) and \( \text{AmG}_2\text{-p(DEA}_{17}\text{-b-}(\text{tBuMA}_{33}\text{-co-EGDMA}_{0.9})) \) \( \text{hyp}-\text{polydendrons} \) initially nanoprecipitated into aqueous media at pH = 4, generated bimodal NP distributions measured by DLS. Following acid addition, these distributions coalesced into single NP populations (Figure 8.2B and F) and no obvious dissolution was seen.
As mentioned above, the hyp-polydendron nanoprecipitates failed to fully solubilise under the very low pH conditions. The $\text{AmG}_2\text{p}(\text{DEA}_{33}-b-(\text{BuMA}_{17}-co-\text{EGDMA}_{0.9}))$ increased in $D_n$ and DCR values, potentially as the increased levels of protonation led to solvated polymer chains extending from the NP surface. The $\text{AmG}_2\text{p}(\text{DEA}_{25}-b-(\text{BuMA}_{25}-co-\text{EGDMA}_{0.9}))$ DCR values decreased significantly, resembling a value
more similar to the other block hyp-polydendron nanoprecipitates. The AmG2\textsubscript{2}-p(DEA\textsubscript{17-}b-\(t\text{BuMA}_{33}\text{-co-EGDMA}_{0.9}\)) also increased in \(D_n\) and DCR values, suggesting a lack of solubilisation, potentially requiring further aggregation in order to maintain a stabilised NP dispersion. These observations suggest collapsed and closely packed polymeric nanoprecipitates that prevent acid penetration into the main bulk of the NP. This behaviour was also observed for the AmG2\textsubscript{2}-p(DEA\textsubscript{17-}co-\(t\text{BuMA}_{33}\text{-co-EGDMA}_{0.9}\)) nanoprecipitates, where the high content of hydrophobic \(t\text{BuMA}\) led to a lack of solubilisation.

Further investigations could include increasing the primary polymer chain length across the range of these materials, in attempts to produce stable NPs in neutral water. This has initially been demonstrated for the AmG2\textsubscript{2}-p(DEA\textsubscript{50-}co-\((t\text{BuMA}_{65}-b\text{-EGDMA}_{0.9})\)), which not only formed stable NPs, but retained stability in transport buffering vehicle media (Chapter 6).

8.2.2 Preliminary co-nanoprecipitation studies of linear-dendritic CL polymer hybrids of varying DP\(_n\) with Bn-\(p(CL_{30}\text{-co-BOD}_{1.0})\)

The co-nanoprecipitation method has produced a variety of stabilised NPs from a range of differently-composed linear and branched polymers at 10:90 wt\%. All the linear-dendritic polymers \(p(\text{HPMA}_{50}), p(\text{DEA}_{50})\) and \(p(t\text{BuMA}_{50})\) and branched copolymers, \(p(\text{HPMA}_{50}\text{-co-EGDMA}_{0.95}), p(\text{DEA}_{50}\text{-co-EGDMA}_{0.95})\) and \(p(t\text{BuMA}_{50}\text{-co-EGDMA}_{0.95})\) prepared by ATRP contained targeted number average degrees of polymerisation (DP\(_n\)) = 50 monomer units. The co-nanoprecipitation process could be extended to include linear-dendritic hybrids of varying DP\(_n\). This was preliminarily studied using linear-dendritic CL polymer hybrids of DP\(_n\) = 20, 30 and 50 monomer units: AmG0'-\(p(CL_{20}), AmG0'-p(CL_{30}), AmG0'-p(CL_{50}), AmG1'-p(CL_{20}), AmG1'-p(CL_{30}), AmG1'-p(CL_{50}), AmG2'-p(CL_{20}), AmG2'-p(CL_{30})\) and AmG2'-\(p(CL_{50})\). Co-nanoprecipitation was conducted with Bn-\(p(CL_{30}\text{-co-BOD}_{1.0})\) previously synthesised in Chapter 7.

All materials were dissolved in tetrahydrofuran (THF) at a concentration of 5 mg mL\(^{-1}\). The linear-dendritic polymer solution (0.2 mL, 1 mg polymer) was combined with the Bn-\(p(CL_{30}\text{-co-BOD}_{1.0})\) polymer solution (1.8 mL, 9 mg polymer) prior to co-nanoprecipitation into acidic (pH=4) and neutral (pH=7.8) water (10 mL) to produce a final NP dispersion with a concentration of 1 mg mL\(^{-1}\) following THF evaporation.
overnight and subsequent analysis by DLS.

Figure 8.3 Diagrammatic representation of AmG0'-p(CL20), AmG0'-p(CL30), AmG0'-p(CL50), AmG1'-p(CL20), AmG1'-p(CL30), AmG1'-p(CL50), AmG2'-p(CL20), AmG2'-p(CL30) and AmG2'-p(CL50).

The AmG0’-initiated linear-dendritic polymers produced stable NPs, exhibiting a decreasing $D_z$ value as the polymer chain length increases. The AmG0'-p(CL20) produced NPs with the broadest size distribution (Table 8.3), suggesting a reduced level of controlled NP aggregation with the polymer containing the lowest DPn. This could be due to the longer polymer chains aggregating less to achieve stabilisation. The AmG1’-initiated polymers all produced NPs of similar sizes, with the DP50 linear-dendritic hybrid displaying an increased level of controlled assembly suggested by the narrowest PDI (0.148). Across the co-nanoprecipitation of the DP20 series of linear-dendritic CL NPs, a decreasing NP diameter with narrower size distributions are witnessed as the size of the dendron chain end decreases (Figure 8.4). Contrastingly, the co-nanoprecipitated particles prepared using the DP50 linear-dendritic hybrids (AmG0'-p(CL50) and AmG1'-p(CL50)) showed increasing sizes with narrowing polydispersities as the dendron size increases; the AmG2'-p(CL50) failed to produce stabilised nanoprecipitates.
Table 8.3 DLS analysis of nanoprecipitated linear and linear-dendritic CL polymer hybrids and co-nanoprecipitated linear-dendritic CL polymer hybrids with *Bn*-p(CL<sub>30</sub>-co-BOD<sub>1.0</sub>) 10:90 wt% linear-dendritic hybrid:branched copolymer.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>pH=7.8&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pH=4.0&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D&lt;sub&gt;z&lt;/sub&gt; (nm)</td>
<td>PDI</td>
<td>ζ (mV)</td>
<td>D&lt;sub&gt;z&lt;/sub&gt; (nm)</td>
<td>PDI</td>
</tr>
<tr>
<td><strong>Bn</strong>-p(CL&lt;sub&gt;30&lt;/sub&gt;)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;0&lt;/sub&gt;*-p(CL&lt;sub&gt;20&lt;/sub&gt;)</td>
<td>133</td>
<td>0.307</td>
<td>+48</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;0&lt;/sub&gt;*-p(CL&lt;sub&gt;30&lt;/sub&gt;)</td>
<td>123</td>
<td>0.140</td>
<td>+84</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;0&lt;/sub&gt;*-p(CL&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>97</td>
<td>0.184</td>
<td>+51</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;1&lt;/sub&gt;*-p(CL&lt;sub&gt;20&lt;/sub&gt;)</td>
<td>121</td>
<td>0.241</td>
<td>+68</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;1&lt;/sub&gt;*-p(CL&lt;sub&gt;30&lt;/sub&gt;)</td>
<td>130</td>
<td>0.259</td>
<td>+66</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;1&lt;/sub&gt;*-p(CL&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>122</td>
<td>0.148</td>
<td>+62</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;2&lt;/sub&gt;*-p(CL&lt;sub&gt;20&lt;/sub&gt;)</td>
<td>37</td>
<td>0.301</td>
<td>+27</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;2&lt;/sub&gt;*-p(CL&lt;sub&gt;30&lt;/sub&gt;)</td>
<td>36</td>
<td>0.260</td>
<td>+20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;2&lt;/sub&gt;*-p(CL&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;0&lt;/sub&gt;*-p(CL&lt;sub&gt;20&lt;/sub&gt;)</td>
<td>181</td>
<td>0.155</td>
<td>+81</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;0&lt;/sub&gt;*-p(CL&lt;sub&gt;30&lt;/sub&gt;)</td>
<td>160</td>
<td>0.126</td>
<td>+42</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;0&lt;/sub&gt;*-p(CL&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>141</td>
<td>0.138</td>
<td>+35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;1&lt;/sub&gt;*-p(CL&lt;sub&gt;20&lt;/sub&gt;)</td>
<td>177</td>
<td>0.167</td>
<td>+69</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;1&lt;/sub&gt;*-p(CL&lt;sub&gt;30&lt;/sub&gt;)</td>
<td>169</td>
<td>0.149</td>
<td>+51</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;1&lt;/sub&gt;*-p(CL&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>137</td>
<td>0.127</td>
<td>+40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;2&lt;/sub&gt;*-p(CL&lt;sub&gt;20&lt;/sub&gt;)</td>
<td>235</td>
<td>0.212</td>
<td>+82</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;2&lt;/sub&gt;*-p(CL&lt;sub&gt;30&lt;/sub&gt;)</td>
<td>217</td>
<td>0.173</td>
<td>+71</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AmG&lt;sub&gt;2&lt;/sub&gt;*-p(CL&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>192</td>
<td>0.148</td>
<td>+79</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Initial pH of water
Figure 8.4 DLS size distribution analysis of co-nanoprecipitated \( \text{Bn-} \beta \text{-}(\text{CL}_{30}\text{-co-BOD}_{10}) \) with A) \( \text{AmG}_{0}{'}\beta \text{-}(\text{CL}_{20}) \) (yellow), \( \text{AmG}_{0}{'}\beta \text{-}(\text{CL}_{50}) \) (brown); B) \( \text{AmG}_{1}{'}\beta \text{-}(\text{CL}_{20}) \) (yellow), \( \text{AmG}_{1}{'}\beta \text{-}(\text{CL}_{50}) \) (brown); and C) \( \text{AmG}_{2}{'}\beta \text{-}(\text{CL}_{20}) \) (yellow), \( \text{AmG}_{2}{'}\beta \text{-}(\text{CL}_{50}) \) (brown) prepared in acidic water at a concentration of 1 mg mL\(^{-1}\) at a ratio of 10:90 wt\% linear-dendritic hybrid:branched copolymer.

Further studies involving CL NPs could include the co-nanoprecipitation of linear-dendritic polymers of varying DP\(_{n}\) with branched copolymers of varying DP\(_{n}\); and the stability of these biodegradable NPs in a physiologically-relevant media. The degradation of these materials could be investigated further to include the degradation of co-nanoprecipitated particles compared to the degradation rates when the polymers are covalently-bound. Furthermore, investigations of the clearance and interaction within the body of the hydrolysed products for these materials, and for the pH-responsive materials could be studied.

8.2.3 Synthesis and applications of CL copolymers

The composition and architecture of polyesters has been explored in various literature reports to include a range of statistical and block co-polyesters in the hopes of improved
mechanical properties, degradability and hydrophilicity. Copolymers of CL include the production of macroinitiators via ROP, followed by polymerisation techniques such as reversible-addition fragmentation chain transfer and ATRP. The latter has been successfully reported with the synthesis of A-B-A triblock copolymers with pH-responsive di(methylamino)ethyl methacrylate A blocks polymerised from a bifunctional p(CL) macroinitiator. A range of selectively degradable core cross-linked star polymers has been reported via a two-step process, where the linear polymers are able to further polymerise due to their active terminal functionality. These are capable of initiating polymerisation of monofunctional and bifunctional monomers in such a way that the active linear polymers are coupled together, forming a star-shaped polymer held together by the branched polymer core. In this particular instance, the core cross-linked polymers were designed so that either the arm or the core was degradable, allowing for polymers with a selective target degradation profile, either by initial linear ROP and subsequent ATRP or initial linear ATRP and subsequent ROP.

These strategies could be utilised to produce linear-dendritic CL polymer arms, following by conversion of the hydroxyl chain end of the polyester into an ATRP initiator by a facile esterification reaction. This macroinitiator could then be used to initiate DEA or DEA and EGDMA to produce block hyp-polydendrons composed of biodegradable and pH-responsive polymers (Figure 8.5).

![Diagrammatic representation of the proposed ROP of CL using a dendron initiator, followed by the ATRP of DEA/DEA and EGDMA using the CL macroinitiator.](image)

**Figure 8.5** Diagrammatic representation of the proposed ROP of CL using a dendron initiator, followed by the ATRP of DEA/DEA and EGDMA using the CL macroinitiator.

### 8.2.4 Preliminary studies of thermo-responsive hyp-polydendrons

The range of methacrylic and styrenic monomers on offer for utilisation in ATRP is
vast, and, as already demonstrated, the hyp-polydendron structure has included HPMA, DEA and tBuMA within the polymer core. The polymerisation of oligo ethylene glycol methyl ether methacrylate (OEG) provides opportunity for the production of a thermo-responsive hyp-polydendron. Thermo-responsive polymers are considered as one of the most widely utilised stimuli-responsive polymers, as they are easy to apply both in vitro and in vivo.6,7,8,9,10,11 The EBiB and AmG1U-Br ATRP initiators were used to co-polymerise OEG and EGDMA (Figure 8.6) at 20°C in isopropanol using CuCl/bpy as the catalyst system in the molar ratio [Initiator]:[M]:[CuCl]:[bpy]=1:50:1:2. Both polymers were purified by passing through a basic alumina column to remove the catalytic system, followed by precipitation from THF into cold hexane before analysis by GPC (Table 8.4).

Figure 8.6 Diagrammatic representation of the co-polymerisation of OEG and EGDMA initiated by EBiB and AmG1U dendron ATRP initiators.
A high molecular weight branched copolymer, \( \text{EBiB-}\text{p}(\text{OEG}_{50}\text{-co-EGDMA}_{0.95}) \) and hyp-polydendron \( \text{AmG}_{1}\text{U-}\text{p}(\text{OEG}_{50}\text{-co-EGDMA}_{0.9}) \) were successfully collected containing broad size distributions typical of branched polymers, with \( M_w \) values of 170,800 and 208,650 g mol\(^{-1}\) respectively.

The \( \text{EBiB-}\text{p}(\text{OEG}_{50}\text{-co-EGDMA}_{0.95}) \) and \( \text{AmG}_{1}\text{U-}\text{p}(\text{OEG}_{50}\text{-co-EGDMA}_{0.9}) \) were dissolved in THF at 5 mg mL\(^{-1}\) and nanoprecipitated into neutral water (pH=7.8) to produce an end concentration of 1 mg mL\(^{-1}\) after evaporation of THF overnight. Both branched polymers existed as solubilised objects in aqueous media, confirmed by the very low \( D_n \) values and low derived count rates (Table 8.5). Upon heating to 70°C, large stabilised nanoprecipitate aggregations formed with narrow size distributions (Table 8.4 and Figure 8.7).

### Table 8.4 GPC analysis of \( p(\text{OEG}_{50}\text{-co-EGDMA}_{x}) \) branched copolymer and hyp-polydendron.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>( M_n ) (g mol(^{-1}))</th>
<th>( M_w ) (g mol(^{-1}))</th>
<th>( D )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{EBiB-}\text{p}(\text{OEG}<em>{50}\text{-co-EGDMA}</em>{0.95}) )</td>
<td>33600</td>
<td>170800</td>
<td>5.09</td>
</tr>
<tr>
<td>( \text{AmG}<em>{1}\text{U-}\text{p}(\text{OEG}</em>{50}\text{-co-EGDMA}_{0.9}) )</td>
<td>82940</td>
<td>208650</td>
<td>2.52</td>
</tr>
</tbody>
</table>

\( ^a \) Triple detection using THF/TEA (2% v/v) eluent

### Table 8.5 DLS analysis of \( p(\text{OEG}_{50}\text{-co-EGDMA}_{x}) \) branched copolymer and hyp-polydendron in different temperature conditions in neutral water (pH=7.8).

<table>
<thead>
<tr>
<th>Temperature</th>
<th>( D_z ) (nm)</th>
<th>PDI</th>
<th>( D_z ) (nm)</th>
<th>DCR (kcps)</th>
<th>( D_z ) (nm)</th>
<th>PDI</th>
<th>( D_z ) (nm)</th>
<th>DCR (kcps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>19</td>
<td>0.368</td>
<td>6</td>
<td>794</td>
<td>625</td>
<td>0.113</td>
<td>564</td>
<td>634790</td>
</tr>
<tr>
<td>70°C</td>
<td>50</td>
<td>0.512</td>
<td>9</td>
<td>2150</td>
<td>222</td>
<td>0.119</td>
<td>193</td>
<td>451460</td>
</tr>
</tbody>
</table>

\( \text{EBiB-}\text{p}(\text{OEG}_{50}\text{-co-EGDMA}_{0.95}) \) | 33600 | 170800 | 5.09 |
| \( \text{AmG}_{1}\text{U-}\text{p}(\text{OEG}_{50}\text{-co-EGDMA}_{0.9}) \) | 82940 | 208650 | 2.52 |

\( ^a \) Triple detection using THF/TEA (2% v/v) eluent
The volume phase transition occurs because of competing hydrogen-bonding properties depending on whether the intra- and intermolecular hydrogen bonding within the polymer is more favourable than the water-polymer interaction. The solubility in water arises when the interactions between the water and polymer are preferred. Once again, a difference in NP size is witnessed between the branched copolymer and hyp-polydendron, establishing different NP behaviour as a consequence of the surface functionality; the $\text{AmG}_1^\text{U}$-$p(\text{OEG}_{50}-\text{co}-\text{EGDMA}_{0.9})$ produced smaller NPs, suggesting less aggregation is required to achieve stabilisation.

Further work could include the co-polymerisation of DEA and OEG to produce a series of dual-responsive hyp-polydendrons (Figure 8.8), for NP behaviour in different pH and temperatures. Equally, the co-nanoprecipitation of $\text{AmG}_2^\text{U}$-$p(\text{DEA}_{50}):\text{EBiB}$-$p(\text{OEG}_{50}-\text{co}-\text{EGDMA}_{0.95})$ could be conducted.

![Diagram](image-url)  
**Figure 8.8** Proposed diagrammatic representation of dual-responsive hyp-polydendrons containing varying amounts of OEG and DEA.

---

**Figure 8.7** DLS size by intensity analysis of $p(\text{OEG}_{50}-\text{co}-\text{EGDMA}_{0.9})$. A) $\text{EBiB}$-$p(\text{OEG}_{50}-\text{co}-\text{EGDMA}_{0.95})$ at 25°C (blue) and 70°C (red); B) $\text{AmG}_1^\text{U}$-$p(\text{OEG}_{50}-\text{co}-\text{EGDMA}_{0.9})$ at 25°C (blue) and 70°C (red).
8.3 Experimental

Typical synthesis of branched copolymer and hyperbranched polydendron utilising the co-polymerisation of OEG and EGDMA, (EBiB- and AmG\textsuperscript{1U}-p(OEG\textsubscript{50-co-}
EGDMA\textsubscript{0.9})) – In a typical synthesis, targeting a DP\textsubscript{n} = 50 monomer units (p(OEG\textsubscript{50-co-}
EGDMA\textsubscript{0.9})), bpy (83.3 mg, 0.5333 mmol, 2 eq.), OEG (4 g, 13.3 mmol, 50 eq.), EGDMA (47.6 mg, 0.2401 mmol, 0.9 eq.) and IPA/H\textsubscript{2}O (33.3% v/v based on OEG) were placed into a 25 mL round-bottomed flask. The solution was stirred and deoxygenated using a N\textsubscript{2} purge for 15 minutes. Cu(I)Cl (26.4 mg, 0.2667 mmol, 1 eq.) was added to the flask and left to purge for a further 5 minutes. AmG\textsuperscript{1U}-Br (0.1590 g, 0.2667 mmol, 1 eq.) was added to the flask under a positive flow of N\textsubscript{2} and the solution was left to polymerise at 20°C. Reactions were terminated when >99% conversion was reached, as judged by \textsuperscript{1}H nuclear magnetic resonance spectroscopy, by exposure to oxygen and addition of THF. The catalyst residues were removed by passing the mixture over a basic alumina column. THF was removed under vacuum to concentrate the sample before precipitation into cold hexane.

Appendix
Chapter 2 – Synthesis and nanoprecipitation studies of 2-hydroxypropyl methacrylate linear-dendritic polymer hybrids and hyperbranched polydendrons

Figure A1 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of [tBOC$_2$-BAPA-G$_1$]

Figure A2 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of [tBOC$_2$-BAPA-G$_1$]
Figure A3 ES-MS of [tBOC₂-BAPA-G₁]

Figure A4 ¹H NMR (CDCl₃, 400 MHz) spectrum of [tBOC₂-BAPA-OH]
Figure A5 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of [tBOC$_2$-BAPA-OH]

Figure A6 ES-MS of [tBOC$_2$-BAPA-OH]
Figure A7 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of APAP

Figure A8 ES-MS of APAP
Figure A9 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of AmG$_1$-OH

Figure A10 ES-MS of AmG$_1$-OH
Figure A11 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of AmG$_2$-OH

Figure A12 ES-MS of AmG$_2$-OH
APPENDIX

**Figure A13** $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of AmG$_0$-Br

**Figure A14** ES-MS of AmG$_0$-Br
Figure A15 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of AmG$_1$-Br

Figure A16 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of AmG$_2$-Br
Figure A17 ES-MS of AmG₁-Br

Figure A18 ES-MS of AmG₂-Br
APPENDIX

Figure A19 $^1$H NMR (MeOD, 400 MHz) spectrum of EBiB-$p$(HPMA$_{50}$).

Figure A20 $^1$H NMR (MeOD, 400 MHz) spectrum of AmG$_{0}$-$p$(HPMA$_{50}$).
Figure A21 $^1$H NMR (DMSO, 400 MHz) spectrum of AmG$\text{I}_{1-p}$(HPMA$_{50}$).

Figure A22 $^1$H NMR (MeOD, 400 MHz) spectrum of EBiB$\text{p}(HPMA_{80-co-EGDMA}_{0.95})$. 

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Figure A23 $^1$H NMR (MeOD, 400 MHz) spectrum of AmG$_{0-1}$p(HPMA$_{50}$-co-EGDMA$_{0.9}$).

Figure A24 $^1$H NMR (MeOD, 400 MHz) spectrum of AmG$_{1-7}$p(HPMA$_{50}$-co-EGDMA$_{0.9}$).
Chapter 3 – Linear polymer, linear-dendritic polymer hybrid, branched copolymer and hyp-polydendron synthesis
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Figure A27 $^1$H NMR (MeOD, 400 MHz) spectrum of AmG$_9$-$p$(DEA$_{50}$).

Figure A28 $^1$H NMR (MeOD, 400 MHz) spectrum of AmG$_1$-$p$(DEA$_{50}$).
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Table A1 Average dn/dc values of $p$(DEA$_{50}$) linear/linear-dendritic polymers and $p$(DEA$_{50}$-co-EGDMA$_x$) copolymer/hyp-polydendrons

<table>
<thead>
<tr>
<th>Target polymer</th>
<th>dn/dc$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBiB-$p$(DEA$_{50}$)</td>
<td>0.0705</td>
</tr>
<tr>
<td>AmG$<em>0$-$p$(DEA$</em>{50}$)</td>
<td>0.0812</td>
</tr>
<tr>
<td>AmG$<em>1$-$p$(DEA$</em>{50}$)</td>
<td>0.0710</td>
</tr>
<tr>
<td>AmG$<em>2$-$p$(DEA$</em>{50}$)</td>
<td>0.0713</td>
</tr>
<tr>
<td>AmG$<em>1$U-$p$(DEA$</em>{50}$)</td>
<td>0.0897</td>
</tr>
</tbody>
</table>

$^a$ THF eluent containing 2% TEA (v/v)
Figure A29 Kinetic plots for branched DP₅₀ polymers. A) and B) EBiB-p(DEA₅₀-co-EGDMA₀.₉₅), C) and D) AmG₁-p(DEA₅₀-co-EGDMA₀.₉), E) and F) AmG₂-p(DEA₅₀-co-EGDMA₀.₉). A, C and E) Conversion (blue squares), ln([M]₀/[M]) (red circles); B, D and F) $M_n$ (red circles) and $M_w$ (black diamonds).
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Figure A30 Kinetic plots for linear EBiB-\textit{p}(DEA_{50}). A) Conversion (blue squares), ln([M]/0/[M]) (red circles); B) Mn (red circles) and D (blue lines).

Figure A31 $^1$H NMR (MeOD, 400 MHz) spectrum of EBiB-\textit{p}(DEA_{50-co-EGDMA_{0.95}}).
Figure A32 $^1$H NMR (MeOD, 400 MHz) spectrum of $\text{AmG}_{\theta-p}(\text{DEA}_{50-c-o-\text{EGDMA}_{0.9}})$.

Figure A33 $^1$H NMR (MeOD, 400 MHz) spectrum of $\text{AmG}_{1-p}(\text{DEA}_{50-c-o-\text{EGDMA}_{0.9}})$. 
Figure A34 $^{1}$H NMR (MeOD, 400 MHz) spectrum of EBiB-$p$(tBuMA$_{50}$).

Figure A35 $^{1}$H NMR (MeOD, 400 MHz) spectrum of EBiB-$p$(tBuMA$_{50}$-co-EGDMA$_{0.95}$).
Figure A36 Kinetic plots for linear EBiB-p(tBuMA50) and branched EBiB-p(tBuMA50-co-EGDMA0.95). A and C) Conversion (blue squares), ln([M]/[I]) (red circles); B and D) Mₙ (red circles) and Ð (blue lines), M_w (black diamonds).

Table A2 Average dn/dc values of EBiB and amine-functionalised tBuMA linear polymer, lineardendritic polymer hybrid, branched co-polymer and hyp-polydendron.

<table>
<thead>
<tr>
<th>Target polymer</th>
<th>dn/dc^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBiB-p(tBuMA₅₀)</td>
<td>0.0589</td>
</tr>
<tr>
<td>AmG₂-p(tBuMA₅₀)</td>
<td>0.0708</td>
</tr>
<tr>
<td>EBiB-p(tBuMA₅₀-co-EGDMA₀.₉₅)</td>
<td>0.0541</td>
</tr>
<tr>
<td>AmG₂-p(tBuMA₅₀-co-EGDMA₀.₉)</td>
<td>0.0568</td>
</tr>
</tbody>
</table>

^a THF eluent containing 2% TEA (v/v)
Figure A37 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_{27}$p(DEA$_{25}$-co-HPMA$_{25}$).

Figure A38 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_{27}$p(DEA$_{17}$-co-HPMA$_{33}$).
Figure A39 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG2-p(DEA$_{33}$-co-HPMA$_{17}$-co-EGDMA$_{0.9}$).

Figure A40 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG2-p(DEA$_{17}$-co-HPMA$_{33}$-co-EGDMA$_{0.9}$).
Figure A41 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$p(DEA$_{33}$-co-tBuMA$_{17}$).

Figure A42 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$p(DEA$_{25}$-co-tBuMA$_{25}$).
Figure A43 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$-$p$(DEA$_{25}$-co-tBuMA$_{25}$-co-EGDMA$_{0.9}$).

Figure A44 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$-$p$(DEA$_{17}$-co-tBuMA$_{33}$-co-EGDMA$_{0.9}$).
Figure A45 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$-p(DEA$_{33}$-b-tBuMA$_{17}$).

Figure A46 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$-p(DEA$_{17}$-b-tBuMA$_{33}$).
Figure A47 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$-$p$(DEA$_{33}$-$b$-(tBuMA$_{17}$-$co$-EGDMA$_{0.9}$)).

Figure A48 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$-$p$(DEA$_{17}$-$b$-(tBuMA$_{33}$-$co$-EGDMA$_{0.9}$)).
Figure A49 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$-p(DEA$_{a5}$-b-(tBuMA$_{a5}$-co-EGDMA$_{0.9}$)).

Figure A50 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of AmG$_1$-OH
Figure A51 ES-MS of AmG$_1$$^u$-OH

Figure A52 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of AmG$_1$$^u$-Br
Figure A53 ES-MS of AmG\textsubscript{1}•-Br

Figure A54 \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 100 MHz) spectrum of BnG\textsubscript{2}-OH
Figure A55 ES-MS of BnG2-OH

Figure A56 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of BnG$_2$-Br
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Figure A57 ES-MS of BnG$_2$-Br

Figure A58 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_1^{14}$-p(DEA$_{50}$).
Figure A59 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_1^U$-[p(DEA$_{80}$-co-EGDMA$_{10}$)].

Figure A60 $^1$H NMR (MeOD, 400 MHz) spectrum of EBiB$_{a,s}$-(AmG$_2$b$_{1}$p(BuMA$_{80}$-co-EGDMA$_{10}$)).
Figure A61 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of BDME.

Figure A62 ES-MS of BDME
Figure A63 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$-p(DEA$_{33}$-co-HPMA$_{17}$-co- BDME$_{2.0}$).

Figure A64 $^1$H NMR spectrum (MeOD, 400 MHz) of AmG$_2$-p(DEA$_{17}$-b-(tBuMA$_{33}$-co- BDME$_{2.0}$)).
Chapter 4 – Nanoprecipitation and pH studies of linear polymers, linear-dendritic hybrids, branched copolymers, terpolymers and hyp-polydendrons

Figure A65 Calibration curve for FA in acidic water.
Chapter 7 – Synthesis and nanoprecipitation studies of biodegradable linear-dendritic ε-caprolactone polymer hybrids and hyp-polydendrons

Figure A66 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of [BOC$_2$-BAPA-OH$'$]
Figure A67 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of [BOC$_2$-BAPA-OH$'$]

Figure A68 ES-MS of [BOC$_2$-BAPA-OH$'$]
Figure A69 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of APAP'.

Figure A70 ES-MS of APAP'.
Figure A71 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of AmG$_1$'-OH.

Figure A72 ES-MS of AmG$_1$'-OH
Figure A73  $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of AmG$_2'$-OH

Figure A74 ES-MS of AmG$_2'$-OH
Figure A75 GPC RI chromatogram overlays of Bn-p(Cl₃₀) polymerised in 24 hours (black) and using [1/200] molar ratio of Sn(Oct₂) (brown).

<table>
<thead>
<tr>
<th>Target polymer</th>
<th>dn/dc⁰</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bn-p(Cl₃₀)</td>
<td>0.0825</td>
</tr>
<tr>
<td>AmG₀⁺-p(Cl₃₀)</td>
<td>0.0776</td>
</tr>
<tr>
<td>AmG₁⁺-p(Cl₃₀)</td>
<td>0.0798</td>
</tr>
<tr>
<td>AmG₂⁺-p(Cl₃₀)</td>
<td>0.0711</td>
</tr>
</tbody>
</table>

⁰ THF eluent containing 2% TEA (v/v)

Figure A76 ES-MS of BOD
Figure A77 $^{13}$C NMR (CDCl$_3$, 400 MHz) spectrum of BOD