Development of a Hybrid Artificial Blood Vessel Using Polyurethane Modified with Extracellular Matrix

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By

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Abstract

The development of an artificial bypass graft for the treatment of peripheral vascular disease would overcome the limitations of using autologous veins. An ideal graft would be a tubular structure made from an elastomeric polymer. Layers of autologous cells would then be cultured on the inside of the tube in order to render the graft non-thrombogenic. The aim of this work was to modify the surface of a polyurethane by covalently attaching recombinant extracellular matrix proteins in order to gain a degree of control over the behaviour of smooth muscle cells.

A chemical modification protocol was used to attach the proteins to the polyurethane via a layer of dextran. The proteins used were an RGD-containing fragment of fibrillin-1 and full length tropoelastin. These are proteins found in the artery wall. Protein immobilisation was verified using Enzyme Linked Immunosorbent Assays. RGD-dependence of cell attachment was investigated by incubating the cells with a soluble RGD-containing peptide prior to seeding. The contribution of integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ to the attachment of the cells was determined using blocking antibodies. Cell proliferation and spreading were quantified. Gene expression and protein production were investigated using RT-PCR, immunostaining and Western blotting.

Attachment to the fibrillin-modified surface was found to be highly RGD-dependent and both integrins investigated were found to be involved. The integrin $\alpha_v\beta_3$ was also found to be involved in cell attachment to the tropoelastin surface. The level of spreading was higher on the fibrillin surface compared to the tropoelastin or the plain polyurethane. Immunostaining of integrins showed few classical focal adhesions and evidence of more fibrillar adhesions. Various extracellular matrix proteins were produced by the cells on both modified surfaces but only in low amounts.

The results of this work show that the modification protocol is an effective way to attach proteins to polymer surfaces whilst retaining their cell binding properties. The proteins investigated here initiated varying cellular responses.

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List of Abbreviations

ABTS - (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

APS - Ammoniumpersulphate

BSA – Bovine Serum Albumin

cDNA – Complementary DNA

DAPI – 4',6-diamidino-2-phenylindole

DCC - N,N'-dicyclohexylcarbodiimide

DMAc - Dimethylacetamide

DMEM – Dulbecco's Modified Eagles Medium

DNA – Deoxyribonucleic Acid

EBP – Elastin Binding Protein

EDC – 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

EDTA – Ethylenediaminetetraacetic acid

EGF – Epidermal Growth Factor

ELISA – Enzyme-Linked Immunosorbent Assay

FITC – Fluorescein isothiocyanate

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

HCASMC – Human Coronary Artery Smooth Muscle Cell

HRP – Horseraddish Peroxidase

IgG – Immunoglobulin G

IKVAV – Isoleucine-Lysine-Valine-Alanine-Valine

LDS – Lithium Dodecyl Sulphate

MAGP – Matrix Associated Glycoprotein

mRNA – Messenger RNA

MW – Molecular Weight

NHS – N-hydroxysuccinimide

NIH – National Institutes of Health

PAGE – Polyacrylamimide Gel Electrophoresis

PBS – Phosphate Buffered Saline

PCL – Poly(caprolactone)

PEG – Poly(ethylene glycol)

PEI - Polyethyleneimine

PF – Protein Fragment

PLLA – Poly(L-lactic acid)

PTFE – Poly(tetrafluoroethylene)

PU - Polyurethane

REDV – Arginine-Glutamic Acid-Aspartic Acid-Valine

RGD – Arginine-Glycine-Aspartic acid

RNA – Ribonucleic Acid

RT-PCR – Reverse Transcription-Polymerase Chain Reaction

SDS – Sodium Dodecyl Sulphate

SMC – Smooth Muscle Cell

SMGS – Smooth Muscle Cell Growth Supplement

TB – Transforming Growth Factor β-like

TBS-T – Tris-Buffered Saline with Tween-20

TEMED - Tetramethylethylenediamine

VGVAPG – Valine-Glycine-Valine-Alanine-Proline-Glycine

YIGSR – Tyrosine-Isoleucine-Glycine-Serine-Arginine

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Chapter 1 – Introduction

1.1 – Literature Review

1.1.1 - Peripheral Vascular Disease

Peripheral vascular disease is characterised by a build up of fatty deposits in the lining of the arteries of the limbs. As the plaques increase in size they disrupt the endothelial cell layer. This causes a phenotypic modulation of the underlying smooth muscle cells (SMCs) allowing them to proliferate and migrate into the intima. They also begin to secrete extracellular matrix proteins further decreasing the diameter of the blood vessel.[1-4]

The plaques increase in size over time reducing blood flow, and therefore oxygen delivery, to the limbs. At first this causes discolouration of the hand or foot (Figure 1). As the blood flow decreases further there will be intermittent claudication then pain at rest. In the absence of intervention eventually gangrene and tissue necrosis will develop resulting in the need for amputation.

Figure 1 – An ischaemic foot. Image provided by Dr Thien How, Clinical Engineering, University of Liverpool

Diabetes is a major risk factor for peripheral vascular disease. Reduced blood flow to the extremities along with diabetic nerve damage causes a high incidence of non-healing foot ulcers in people with the condition. These can also result in the need for amputation. One UK study showed that between 1999 and 2000 there were approximately 2.61 amputations per 1,000 people with diabetes in the local population.[5]

A number of diagnostic techniques are used to identify peripheral vascular disease, most commonly measurement of the ankle brachial pressure index followed by angiography (Figure 2).

Figure 2 – An angiogram showing occlusion of the left femoral artery (arrow). Image provided by Dr Thien How, Clinical Engineering, University of Liverpool

Treatment of the condition initially includes lifestyle changes i.e. cessation of smoking, lowering LDL cholesterol in the diet, lowering blood pressure and controlling diabetes. Cholesterol reducing drugs may be used. Angioplasty can be used to expand larger vessels; sometimes a stent is also deployed. Bypass grafting uses a graft that is sutured above and below the blockage to allow the blood flow to be resumed to the lower limb.

1.1.2 - Treatment by Bypass Grafting

1.1.2.1 – Autologous Graft

The most commonly used blood vessels used as autologous bypass grafts are the saphenous vein and the internal mammary artery. These have a high patency and are the preferred choice of graft at present. However, in some cases these vessels are not suitable or are unavailable. The vessel may be diseased or may not be the right size. It is also possible that the vessel has been taken for a previous graft. In addition, vein grafts have thin walls that may not be able to withstand the higher blood pressure found in arteries.[6]

1.1.2.2 – Xenogenic/Allogenic Graft

The availability of xenogenic grafts (from a different species) or allogenic grafts (from a donor of the same species) is not a problem. Umbilical veins that have been glutaraldehyde treated to reduce immunogenicity have been used as an alternative to autologous vessels.[7,8] The patency of these grafts is lower than for autologous grafts but is an adequate alternative in situations where life expectancy is low. After prolonged periods of implantation the graft is susceptible to aneurysm. Other problems include the incomplete removal of immunogenicity by the cross-linking and the presence of residual glutaraldehyde in the tissue.[6,9] Acellularised tissue has also been investigated. Methods employing a variety of detergents and enzymes are used

to remove the cellular component from an allograft or xenograft whilst leaving the extracellular matrix intact.[10] The thrombogenicity of these grafts can however be an issue.

1.1.2.3 – Synthetic Graft

Another option when an autologous graft is unavailable is a synthetic graft. These consist of a polymer tube, usually poly(ethylene terephthalate) (Dacron) or expanded poly(tetrafluoroethylene) (ePTFE).[11-16] However, these grafts have low patency for small diameter vessels such as those of the lower limbs. The surface of the polymers is thrombogenic and so blood clots are common. Also, they are rigid and so cause compliance mis-match at the anastomoses. This causes turbulent blood flow and this is thought to be a contributing factor to re-stenosis of the graft. The use of a more compliant polymer such as polyurethane could overcome this problem but these grafts are prone to aneurysm and have been shown to be even more thrombogenic than ePTFE grafts.[17] A number of methods have been investigated in order to reduce the thrombogenicity of synthetic grafts. One of these methods is to implant the graft into the peritoneal cavity of the patient in order to obtain a fibrous capsule that would reduce thrombus formation when the graft was used.[18,19] In a different approach heparin has been attached to the internal surface of a synthetic graft before implantation.[20-23] The occurrence of spontaneous endothelialisation of an implanted graft has also been investigated.[24] The endothelial layer would mimic the native intimal surface and therefore prevent thrombus formation. In animal studies complete

endothelialisation of the implanted grafts occurred. In contrast, in-growth into grafts implanted into human did not exceed 10mm even after extensive periods of time.[25]

1.1.2.4 - Cell-Seeded Graft

In the absence of spontaneous endothelialisation, seeding endothelial cells onto the internal surface of the graft prior to implantation has been investigated as a way to form an endothelial layer. A number of variations on this idea have been explored. Non-degradable polymers such as Dacron, ePTFE and polyurethane have been seeded with endothelial cells and their retention on the surface investigated under flow conditions either in vitro or in vivo.[15,16,26] Cell-seeding was shown to improve patency of the implanted grafts compared to non-seeded controls. Biodegradable polymers such as poly(lactic acid) have also been tried. Whilst cell adhesion to these surfaces did occur it would not be sufficient for a high level of retention in vivo. [27-29]

In order to achieve adequate cell adhesion to polymer surfaces so that the cells are retained under physiological conditions it is necessary to modify the surface in some way. One strategy is modification of the surface topography. Both nanometer and micrometer scale roughness have been shown to increase cell adhesion, proliferation and migration.[30-32] It is unlikely however that this modification alone would be adequate to retain the cells on implantation into the body.

Cell membranes consist of a phospholipid bilayer. This means that the cell surface is polar and therefore moderately hydrophilic. It follows that cells will bind more easily to hydrophilic surfaces. To this end research has been carried out to increase the wettability of polymer surfaces. This can be achieved by treating the surface to change its chemistry or by attaching hydrophilic molecules.[33-38] Again it is doubtful that the cell adhesion would be increased to the necessary level for in vivo conditions.

In 1984 the tri-peptide arginine-glycine-aspartic acid (RGD) was identified as the minimum cell adhesion motif recognised by integrins.[39] This sequence is found in fibronectin and a number of other proteins. Other short peptides have now also been identified as increasing cell attachment. These include REDV (fibronectin), YIGSR (laminin) and IKVAV (laminin). The covalent attachment of these peptides has since been shown by many groups to dramatically improve cell adhesion to surfaces (see section 1.1.3.2.2).

In their native environment cells are surrounded by extracellular matrix and basement membrane proteins. A great deal of research has concentrated on coating surfaces with these proteins in order to increase cell adhesion. Proteins such as fibronectin and laminin have been shown to successfully improve adhesion, proliferation and spreading on surfaces (see section 1.1.3.3.2). The adsorption of these proteins however is not adequate to promote the necessary strength of cell attachment as the protein has to withstand the contractile forces applied by the cells.[40] It has also been shown that the physical state of the protein i.e. adsorbed as opposed to immobilised, has an effect on the cytoskeletal organisation of the cells.[41]

1.1.3 – Polymer Surface Modification

1.1.3.1 – Surface Functionalisation

The majority of polymers that have been investigated as potential vascular graft materials have no functional groups available on their surface, PTFE for example. In order to attach cell adhesion molecules to these polymers it is necessary to first functionalise the surface. A commonly used method for introducing functional groups to surfaces is plasma treatment whereby a plasma is formed by applying a radiofrequency discharge to a gas filled chamber. The gas is ionised producing species with an energy high enough to break covalent bonds. If the polymer to be modified is placed in the plasma chamber its surface is bombarded by these species, breaking bonds to form functional groups.[33,42,43] By placing the polymer immediately into a protein solution the groups can react with carboxyl or amino functionalities directly therefore immobilising the protein on the surface.[44-48] Another method is to graft glutaraldehyde to the functionalities formed by the plasma treatment. This produces carboxyl groups on the surface that can then be used for binding cell adhesion molecules.[49] Alternatively acrylic or methacrylic acid can be grafted to the surface groups again producing carboxyl groups.[21,50-53]

Ozone can be used to oxidise polymer surfaces resulting in the formation of reactive peroxide groups. These groups can then react directly with a protein in solution or be used to graft acrylic acid to the surface in order to provide carboxyl moieties.[54,55]

In the case of some polymers it is possible to break bonds on the surface by hydrolysis. Treatment with concentrated sodium hydroxide has been used to form carboxyl groups on the surface of various polymers including poly(methyl methacrylate), poly(glycolic acid) and poly(ethyleneterephthalate).[56-62]

Aminolysis is another method that can be used to functionalise polyester based materials such as poly(lactide) or poly(caprolactone). An amine-containing compound is added to the polymer surface where it reacts at the ester bond to form an amide linkage. By using a diamine, 1,6-hexanediamine for example, as the reactant the surface becomes amine functionalised.[63-67]

Blending the polymer to be used with a functionalised polymer prior to scaffold formation can, in some instances, produce reactive groups on the surface but also maintain some of the physical properties of the polymer. In one study the carboxy terminal of poly(L-lactide) was functionalised with a diamine. This PLLA-NH_2 was then blended with non-modified PLLA before a porous scaffold was synthesised.[68] This method is only capable of providing a small number of reactive groups however.

In a similar manner, a functionalised polymer can be blended into the surface layer of another polymer. The surface is swelled using a solvent/non-solvent mixture allowing the functionalised polymer to diffuse into it. An excess of non-solvent then traps this polymer in the surface leaving active groups available. Polymers including poly(lysine) and poly(ethylene glycol) have been physically trapped in surface layers using this method.[69-72]

1.1.3.2 – Cell Adhesion Peptides

1.1.3.2.1 - Methods of Attachment

Carbodiimides are commonly used reagents that facilitate the reaction of an amine group with a carboxylic acid. They react with the acid group thus activating it and driving the reaction with the amine (Figure 3). The original carbodiimide used for these reactions was N,N'-dicyclohexylcarbodiimide (DCC). However, this is an allergen and the by-product of the reaction is insoluble. More commonly used now is the water soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC).

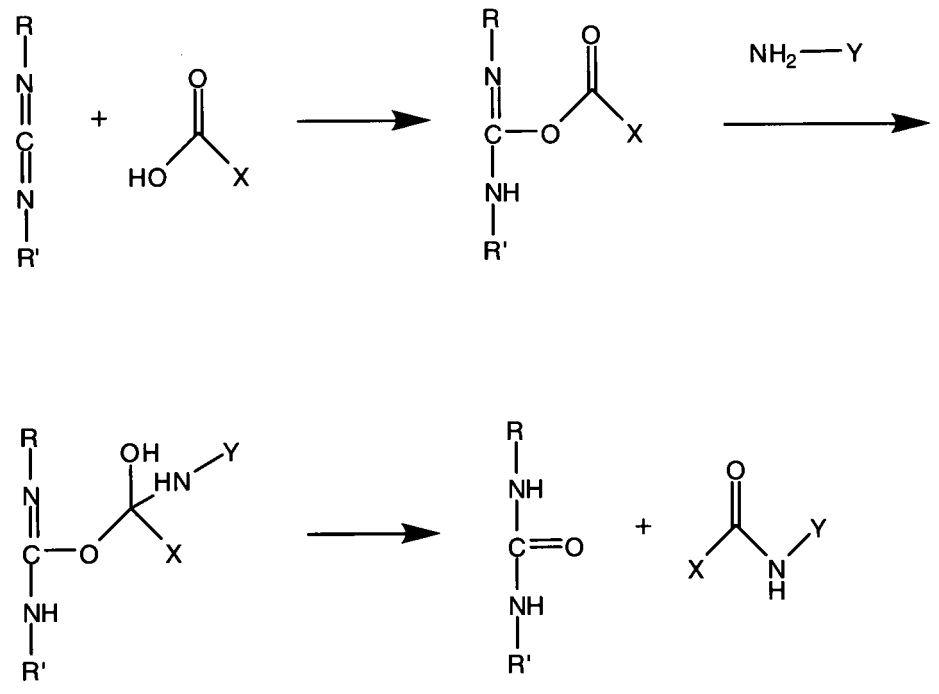

Figure 3 – Reaction scheme for the immobilisation of a peptide onto a carboxylated surface using a carbodiimide

Aminophase glass has been used to demonstrate the feasibility of using this reaction to couple peptides to a surface. For this, the EDC is used to activate the C-terminal of the peptide for reaction with the amine groups on the surface.[73-76] The carbodiimide can also be used to activate surface carboxyl groups for reaction with the N-terminal of the peptide. This method was used to attach adhesion peptides to oxidised poly(ethylene terephthalate),[77] carboxylated polyurethane[78,79] and poly(acrylic acid) grafted to polystyrene.[52]

N-hydroxy succinimide (NHS) is often used alongside carbodiimides to increase the product yield. When added to the reaction mixture, the NHS replaces the O-acylurea group formed by the reaction of the carbodiimide activated carboxyl with the amino group. This method has been used to attach cell adhesion peptides to silk films,[80] PEG-tethered poly(propylene fumarate),[81] poly(ethylene oxide),[82] poly(caprolactone)[63] and dextran-tethered polyurethane.[83]

Tresyl chloride (2,2,2-trifluoroethanesulphonyl chloride) is a coupling reagent that can be used to activate surface hydroxyl groups for reaction with the amino groups of peptides. This method has been used to immobilise various peptides to poly(tetrafluoroethylene-co-hexafluoropropylene)[84-86] and hydroxylated poly(ethyleneterephthalate) and poly(tetrafluoroethylene).[87]

In a different approach cell adhesion peptides can be derivatised with a phenyl azido moiety to enable photochemical immobilisation onto polymer surfaces. The derivatised peptide is adsorbed on to the surface before UV irradiation is used to

chemically bond it (Figure 4). This method has been used to graft RGD-containing peptides to polyurethane-PEG, poly(caprolactone)-PEG, poly(vinyl alcohol) and chitosan films.[30,88-92]

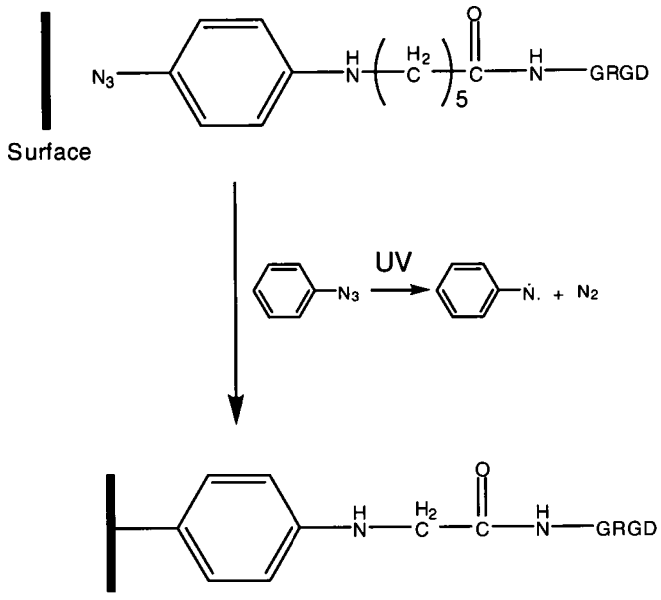

Figure 4 – Reaction scheme for the immobilisation of an azido-derivatised peptide onto a polymer surface

1.1.3.2.2 - Cellular Responses

The cellular responses to surfaces immobilised with cell adhesion peptides have been investigated extensively. The peptides have been shown to have an effect on cell adhesion,[49,52,63,74,78-80,83,87,90,91,93-100] spreading,[78,79,101-107] proliferation,[74,89-91,108-110] differentiation[49,68] and extracellular matrix production.[74,80,108] However, there are a

number of factors that affect cell behaviour on the surfaces. It has been shown that the amino acids that make up the peptide are very specific. For example, there is a significant difference in cell adhesion to RGD-modified surfaces and RGE-modified surfaces.[74,79,111,112] Replacing the aspartic acid residue with the structurally similar glutamic acid significantly reduced the adhesion and spreading of cells cultured on the modified surface. It was also observed that cells cultured on the RGE surfaces secreted considerably more extracellular matrix then those on the RGD surfaces.[73] In another study cell adhesion to the peptides YIGSE and YGGGR was found to be significantly lower than to the YIGSR motif found in laminin.[113]

By changing the sequence of the amino acids cell behaviour is altered extensively. RDG-modified surfaces showed reduced cell spreading and proliferation compared to RGD surfaces.[105]

The amino acid residues either side of the cell-adhesion motif also have an effect on cell behaviour. In fibronectin the amino acid directly after the RGD is a serine whereas in vitronectin it is a valine. A comparison of GRGDSY-modified surfaces with GRGDVY-modified surfaces showed greater cell attachment to the GRGDVY.[78,79] This was attributed to differing affinities of the peptides for the cell-surface receptors.

If the cell adhesion peptide is free in solution rather than immobilised on to a surface it will bind to the receptors of the cells and block them. This will prevent the cells from adhering to the surface. The level of inhibition of cell adhesion can therefore be

used as an indicator of the binding potential of the cells to the peptide. This principle has been used to investigate the effect of altering the conformation of one of the amino acids in the RGD motif. Substituting L-Arg for D-Arg made very little difference to the cell attachment. However, when the same substitution was carried out on the Asp residue, the peptide became inactive.[114] These results show that the conformation of the peptide alters its affinity for cell surface receptors.

The density of the cell adhesion peptides on a surface has also been shown to affect the behaviour of cells. The minimum amount of RGD needed on a surface to promote the formation of focal adhesions has been calculated.[99] Cells were fully spread on surfaces with a peptide density of 1fmol/cm^2 and at 10fmol/cm^2 they were found to have formed focal contacts and well organised stress fibres. These results show that only a very small amount of RGD peptide is needed to promote integrin-mediated cell adhesion. Another study used well-defined nano-patterned surfaces to show that cell adhesion to RGD was decreased when the peptide-covered nanodots were over 73nm apart from each other. Each nanodot was capable of binding one integrin and so the decrease in cell adhesion was attributed to the fact that the integrins were prevented from forming clusters.[115] Cell adhesion can be enhanced by increasing the concentration of peptide on a surface.[52,88,90,116] Peptide density also effects cell migration. The presence of low concentrations of peptide increases migration compared to non-adhesive control surfaces. However, at high peptide concentrations migration is suppressed.[74] For a cell to migrate it needs to form focal adhesions on the surface. The cell then uses these to pull itself in one direction. If the adhesions at the rear of the cell are too strong then they will not detach easily from the surface and so

the cell will not be able to move. It is for this reason that maximum cell movement occurs at intermediate peptide concentrations.[117]

The effect of clustering of the peptides on a surface has been investigated. It was found that clusters of RGD peptides enhanced the formation of stress fibres and increased the number of focal contacts compared to surfaces where the RGD was evenly spaced.[82] Effective focal adhesions require integrin clustering and so it is likely that this is facilitated by the peptides in this case. The results also showed an increase in cell migration caused by the clustering. This was likely due to the more effective focal adhesions and more organised stress fibres.

The RGD sequence in fibronectin has been shown to be at the tip of a loop formed by disulphide bonding. For this reason it was hypothesised that cyclising the peptides may make them more effective at increasing the adhesion of cells. In a number of cases it was found that the cyclic peptides were more efficient at increasing cell adhesion and spreading than their linear analogues.[94,101,118] This shows the importance of the conformation of the peptide in that it is more effective when presented to cells in a similar manner to when it's contained within the native protein.

Another method has been used to attempt to present the cell adhesion motif to the cells in the most natural conformation. By adding a tether or spacer molecule in between the peptide and the surface the peptide becomes less restricted in its movement. Its conformation is then determined solely by its structure with little effect from steric constraints due to the close proximity of the surface.[119] As

mentioned previously, clustering of the peptides enhances their effect on cell adhesion. A spacer group would allow the peptides, and therefore integrins, to cluster and so may be another reason for the increased adhesion.

It is clear from these results that the way that the adhesion peptide is presented to the cells greatly affects the cellular response. In summary, altering one of the amino acids in the sequence changes the conformation of the peptide and reduces cell attachment. Making the peptide cyclic rather than linear increases cell attachment, as does adding a tether or spacer group between the surface and the peptide. These alterations make the peptide available to cells in a more similar way to that in which they would be available in the native protein. It is therefore likely that better cell attachment would be achieved by using the native protein itself rather than a small fragment of it. For this reason the covalent attachment of proteins to a surface has been investigated extensively.

1.1.3.3 – Cell Adhesion Proteins

1.1.3.3.1 – Methods of Attachment

As well as being an effective method for attaching peptides to a surface, carbodiimides have also been used to immobilise proteins. The reagent can activate the C-terminal of the protein for reaction with an aminated surface or alternately activate a carboxylated surface for reaction with protein amino groups. Collagen,

gelatin, fibronectin, laminin, insulin and silk fibroin have all been attached to surfaces using carbodiimide chemistry.[50,51,56,57,65,120-125]

Glutaraldehyde is a dialdehyde that is often used as a cross-linker. It can be used to attach proteins to a surface by reacting one aldehyde group with an amino group on the surface and the other with an amino group in the protein. This method has been used to immobilise collagen, fibronectin and gelatin to surfaces.[64-67,126]

Another method used to immobilise proteins is to plasma treat the surface and then immediately immerse it in a solution of the protein. The functional groups created by the plasma react directly with amino or carboxyl groups on the protein thus anchoring it to the surface. Collagen and laminin have been successfully attached to polymer surfaces using this method.[44-46,125]

Collagen has also been attached to a surface by treating the polymer with ozone and grafting acrylic acid to it. The carboxylic acid groups then act as a crosslinker to immobilise the protein under acidic conditions.[55] Ozone treatment followed by direct immersion in a solution of collagen has also been used to attach the protein to a polymer surface.[54]

These modification methods have been used to successfully immobilise the mentioned proteins onto surfaces. These are the most commonly used proteins but there is no reason why the same methods couldn't be used to attach other proteins.

1.1.3.3.2 – Cellular Responses

The behaviour of cells cultured on protein-modified surfaces has been studied extensively. In order to get a more complete picture of the effects of different proteins, this section of the review will include the cellular response to adsorbed as well as covalently attached proteins.

Fibronectin is probably the most favoured protein used to control cell behaviour. It contains two well known cell binding motifs, RGD and REDV. These sequences are recognised by a number of cell surface integrins and so it is not surprising that significant increases in cell attachment to fibronectin-modified surfaces compared to controls have been seen in a number of studies.[1,57,126-129] Increases in cell proliferation have also been demonstrated in much of the same work. The modulation of SMCs from a contractile to a synthetic phenotype has been shown to be triggered by culturing cells on a fibronectin-modified surface.[2,129] Endothelial cells showed improved anti-thrombogenic and fibrinolytic properties when grown on fibronectin covalently attached to a polymer surface.[47] This demonstrates that the endothelial cell phenotype is maintained on this surface.

Collagen type I is the most abundant type of collagen in the body. It contains a number of sites recognised by integrins.[130] It is another protein that has been shown in many different studies to increase cell attachment to a surface. In each case the levels of cell adhesion were greater compared to unmodified control surfaces.[45,50,56,64,65,67,120,121,126-129] In addition, an increase in the rate of proliferation

has been identified on collagen-modified surfaces. However, there are also studies that show no change in cell growth.[56,126] These conflicting results could be due to the varying methods of attaching the protein to the surfaces. The ability of collagen to maintain the function of endothelial cells has also been demonstrated. Cells cultured on this protein secreted enhanced levels of von Willebrand Factor, a marker of endothelial cell phenotype.[64,65,67] In other work collagen was used to modify the surface of a porous polymer scaffold. Cells cultured on this infiltrated the pores and secreted large amounts of extracellular matrix. In contrast cells on the control scaffold stayed mainly on the surface.[45]

Collagen type II is the major form of collagen found in cartilage. For this reason its effect on chondrocytes is especially interesting. As with collagen I there are a number of cell binding sites within the protein. Crosslinked collagen II was found to increase cell proliferation when adsorbed on to a polymer surface compared to uncoated controls.[131] This effect was seen for up to 60 days. Attachment and spreading of chondrocytes has been shown to increase on surfaces coated with collagen II compared to a negative control.[132,133]

Collagen type IV is a major constituent of the basement membrane of cells. Both urothelial cells and fibroblasts have shown greater attachment to the protein compared to controls.[134,135] In addition to this, the effect of a collagen IV substrate on smooth muscle cells has been investigated. SMC phenotype is greatly affected by the surface on which the cells are cultured. The protein slowed down the modulation of the cells from a contractile to synthetic phenotype for approximately 4 days.[2] A

combination of collagen IV and laminin, the other main protein in the basement membrane, has also been shown to maintain the SMCs in their native contractile phenotype when cultured in vitro.[136]

Equally, SMCs cultured on laminin alone have also been shown to maintain a more contractile phenotype compared to uncoated surfaces and those coated with fibronectin or vitronectin.[1,2] However, one of these studies showed low initial attachment of the cells and this result is backed up by other work that shows low levels of attachment of urothelial cells.[134] In contrast fibroblasts have exhibited high levels of attachment to laminin compared to negative controls.[135] A number of cell adhesion peptide sequences have been identified in laminin. These include YIGSR and IKVAV. Integrins containing the β_1 sub-unit are major mediators of this cell adhesion.[137] Laminin has been shown to promote neurite outgrowth from cultured neurons.[118,138] It has also been shown to greatly increase hepatocyte, Schwann cell and olfactory ensheathing cell adhesion compared to un-coated controls.[139,140]

Elastin is a major constituent of the elastic fibres found in elastic tissue such as blood vessels and skin. It is formed by the cross-linking of its soluble precursor, tropoelastin. A number of cell binding sites on the protein have been proposed. Elastin-based surfaces have been shown to support the attachment of a number of different cell types.[141] In some studies there was evidence to show that this attachment was, in part, mediated by the elastin-binding protein on the cell surface.[142,143] The effect of elastin surfaces on SMC phenotype has been investigated in a number of studies. In each case the cells appeared to have a more contractile

phenotype, characterised by decreased proliferation and higher amounts of contractile filaments.[144,145] In one case endothelial cell proliferation was seen to increase whereas the rate of SMC proliferation decreased on surfaces with a fairly low elastin concentration.[146]

Fibrillin-1 is another protein of interest found in elastic fibres. This is a large glycoprotein that contains an RGD motif along with other cell binding areas. A number of cell types have been shown to attach to fibrillin-1 via the RGD motif. There is a great deal of evidence to suggest that this interaction is mediated, at least in part, by the $\alpha_v\beta_3$ integrin.[147,148] [149,150] Some studies have also demonstrated the involvement of the $\alpha_5\beta_1$ and, for epithelial cells, the $\alpha_v\beta_6$.[151-153] Cell adhesion and spreading were enhanced on the fibrillin compared to negative controls.

Fibulin-5, or DANCE, is a glycoprotein that is also a component of elastic fibres. It contains an RGD motif and cell attachment has been shown to be dependent on integrin binding to this sequence.[154,155] The involvement of the integrin $\alpha_5\beta_1$ and the subunit α_v in endothelial cell attachment has been demonstrated in one study.[152] In another, using smooth muscle cells, the contribution of the $\alpha_v\beta_3$ integrin was discounted. This study showed the major integrins involved in the attachment of SMCs to fibulin-5 were $\alpha_5\beta_1$ and $\alpha_4\beta_1$. The protein has was shown to support a degree of cell spreading whilst decreasing proliferation and migration compared to fibronectin.[156]

1.1.4 – Conclusions

Modifying polymer surfaces has been shown to be an effective way of increasing cell attachment. It has also been demonstrated that some proteins can control other aspects of cell behaviour such as proliferation, spreading, extracellular matrix production and cell phenotype. Surface modification with cell adhesion proteins is potentially an important tool for the development of a cell-seeded bypass graft.

1.2 – The Hybrid Artificial Blood Vessel

1.2.1 – Structure of the Native Artery

The outer layer of the artery is termed the tunica externa (Figure 5). It is made up of fibroblasts within a fibrous extracellular matrix consisting mainly of collagen. The function of this fibrous layer is to support and protect the vessel and to help anchor it to the surrounding tissue.

The tunica media consists of concentric layers of SMCs and elastic lamellae. The SMCs contract and relax in response to factors such as blood pressure and hormones. This allows control of the volume of blood flowing through the artery to be achieved by altering the lumen diameter. The elastic fibres are responsible for the elastic recoil property of arteries and they help prevent the over-extension of the vessel.

The innermost layer of the artery consists of an endothelial cell monolayer that is separated from the tunica media by a basement membrane. The endothelial cells secrete anti-thrombogenic molecules such as prostacyclin and nitric oxide. These prevent platelet adhesion to the luminal surface of the blood vessel and subsequent thrombogenesis.

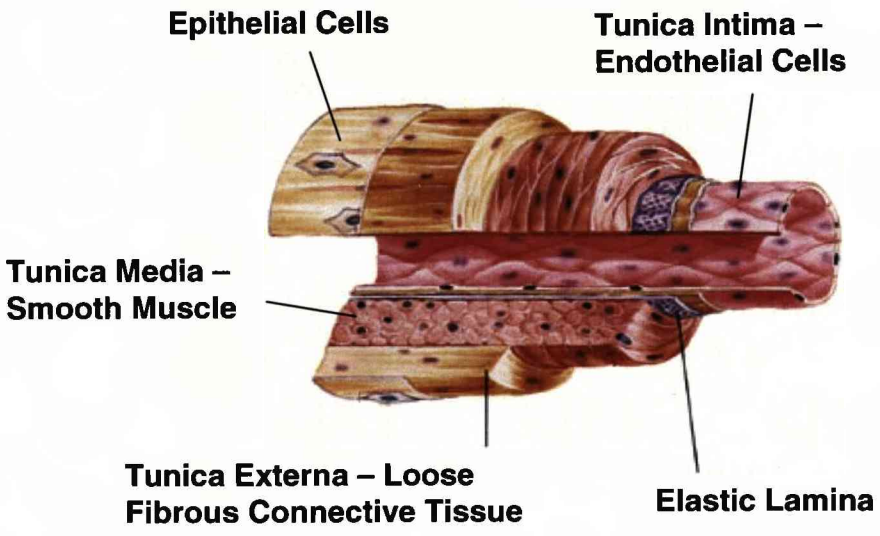

Figure 5 – The structure of an artery. Adapted from (S.I. Fox, Human Physiology, 4th Ed, Brown Publishers)

1.2.2 – Structure of the Hybrid Artificial Blood Vessel

The artificial blood vessel aspired to in this work is based on a porous, tubular scaffold (Figure 6). This is formed by the electrospinning of a non-degradable polyurethane elastomer. The polymer is biostable and would remain in the body permanently in order to remove the need for the newly grown tissue to withstand the large physiological pressures. The artery would therefore need less in vitro culture time making the process more cost effective.

Autologous vascular SMCs taken from a vein biopsy or derived from adult stem cells form a layer on the internal surface of the tubular scaffold. These are seeded on to the polymer surface in vitro and cultured in a bioreactor. The polymer is modified by covalently attaching recombinant proteins to the surface. These proteins serve to increase cell adhesion and gain a degree of control over the cells' behaviour including proliferation, protein production and phenotype. Endothelial cells are then cultured on top of the smooth muscle cells with the aim of forming a non-thrombogenic layer as the blood contacting surface. On the outside of the polymer tube is a layer of fibroblasts. The aim of these is to prevent aneurysm. This has previously been shown to be a problem with polyurethane grafts.[17]

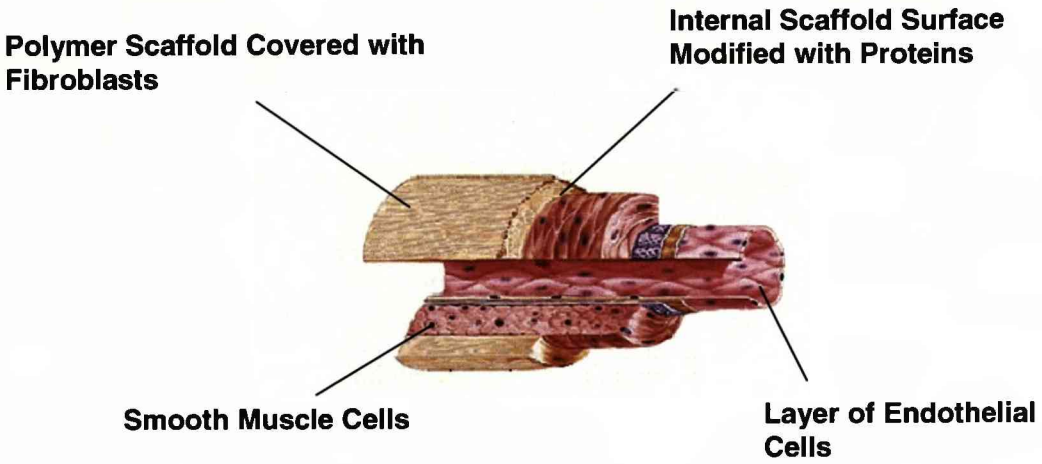

Figure 6 – Diagram of the proposed structure of the artificial artery. (Adapted from S.I. Fox, Human Physiology, 4th Ed, Brown Publishers)

This project will specifically focus on the interface between the polymer scaffold and the SMCs. Proteins will be covalently attached to the surface and the behaviour of the cells cultured on it investigated.

1.2.3 – Polyurethanes

Polyurethanes are a class of polymers that are formed from the reaction of a diisocyanate with a polyol (Figure 7). Different monomers can be used along with various chain extenders to give materials with a wide variety of chemical and physical properties.[157]

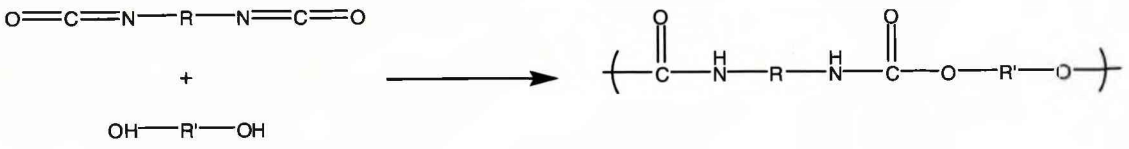

Figure 7 – General reaction to synthesise a polyurethane

The polyurethane structure consists of hard segments that are polar and immobile due to hydrogen bonding and soft segments consisting of non-polar, randomly coiled domains, often aliphatic polyethers or polyesters. The differences in the chemical properties of the segments cause them to phase separate. In response to an applied stress the coiled, soft segments partially unravel while the hard segments become aligned. Because of this just a small amount of stress can result in a large deformation of the polymer (Figure 8). Additional increases in stress cause further elongation of the polymer. When the stress is removed the polymer returns to its original state due to its elastic properties. The polymer has both viscous and elastic properties and so is termed viscoelastic.

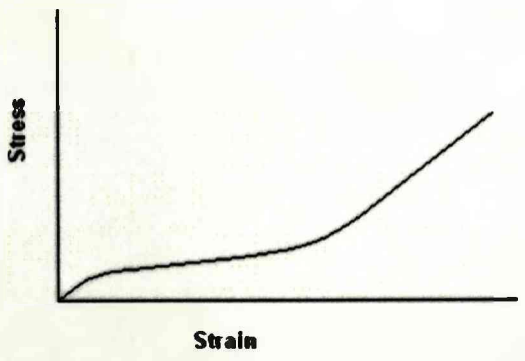

Figure 8 – Typical stress-strain behaviour of a polymeric elastomer

Compliance mis-match is thought to be a major factor in the failure of small diameter artificial bypass grafts. Dacron, for example, has almost no stretch in any direction in dramatic contrast to the native artery that it would be sutured to. This causes disruption of the pulsatile flow and increases the likelihood of neointimal hyperplasia. This is characterised by the migration and proliferation of smooth muscle cells from the tunica media and causes the diameter of the graft to decrease significantly. In order to reduce this problem it is necessary to use a polymer that has physical properties similar to those of the native artery i.e. viscoelasticity. For this reason a range of polyurethanes are being investigated as potential bypass graft materials.

In this work a polyether-urethane, b9 Z1A1 (Biomer Technologies Ltd), will be investigated. Polyether-urethanes are hydrolytically stable and so are good candidates for implantation into the body. This particular polyurethane has a high ultimate tensile strength and ultimate elongation. It therefore has the necessary strength and compliance for this application.

1.2.4 - Cell Adhesion Proteins

It is hypothesised that by modifying the polymer surface with proteins found in the artery wall, the cells cultured on it will be encouraged to secrete and organise extracellular matrix and produce an environment similar to that of a native artery. This environment may then control the phenotype of the SMCs preventing them from

over-proliferating and blocking the graft. It would also be beneficial for the produced extracellular matrix to support the formation of a confluent layer of endothelial cells.

The proteins investigated here are a fragment of fibrillin-1 and full length tropoelastin. These are major constituents of the elastic fibres found in the artery wall. Fibrillin-1 molecules assemble into microfibrils.[158-160] Bundles of these then form a template for tropoelastin deposition. Elastic fibre formation proceeds by the crosslinking of the tropoelastin to form insoluble elastin. Other molecules, including fibulin-5 and MAGP-1, associate with the microfibrils and the elastin facilitating the construction of the elastic fibres.[161-163]

1.2.4.1 - Fibrillin-1

Fibrillin-1 is a large glycoprotein (~350kDa) that consists of 47 epidermal growth factor-like domains, 43 of which are calcium-binding (cbEGF-like domains) and 7 8-cysteine transforming growth factor beta-like (TB) domains. Present in the 4th TB domain (exons 37-38) there's a cell binding domain, Arg-Gly-Asp (RGD). This has been found to be the minimum amino acid sequence necessary for integrin binding.[39] It is thought that over half of the known integrins recognise the RGD motif. A number of RGD-containing fragments of fibrillin-1 have been recombinantly expressed by researchers at the University of Manchester (Figure 9). These protein fragments have been adsorbed onto tissue culture plastic and cell attachment studies have been carried out. It was found that superior cell binding was achieved on fragments with domains present upstream of the RGD motif i.e. PF8 and PF14.[151] This suggests that there is a synergy binding site present in this area that is required

for efficient cell binding. Other work has shown that the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ are the major receptors involved in mediating cell attachment to these fibrillin-1 fragments.[148] In this work PF14 was chosen due to the presence of both the RGD sequence and the proposed upstream synergy site.

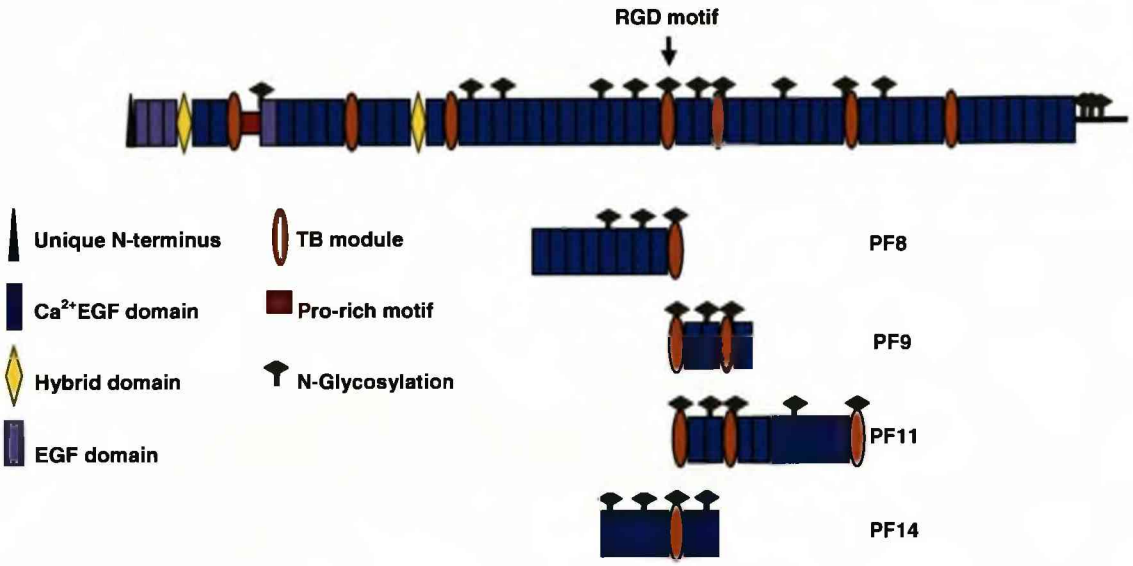

Figure 9 – Domain structure of recombinant fibrillin-1 fragments

1.2.4.2 - Tropoelastin

Tropoelastin is the soluble precursor to elastin. It is a 65-70 kDa protein that makes up approximately 90% of the elastic fibre.[164] It consists of alternating hydrophobic and lysine-rich cross-linking domains.[165] It is secreted by cells and deposited onto a lattice of fibrillin microfibrils. It is then cross-linked by enzymes of the lysyl oxidase family to form the insoluble elastin. As mentioned previously (section 1.3.3.2) a number of cell-binding sites on tropoelastin have been proposed.[166] One is said to be

near the C-terminus of tropoelastin with cell attachment mediated by the $\alpha_v\beta_3$ integrin.[167] In other work a binding site was identified, again at the C-terminus, but in this case cell attachment was not integrin mediated.[168] In a more central area of tropoelastin there is a binding site recognised by the cell surface elastin binding protein (EBP). The peptide VGVAPG is thought to be the sequence responsible.[169,170] Full length tropoelastin has been recombinantly expressed using E. coli.[171] This protein has been chosen due to its sizeable presence in the elastic fibres of the artery wall and its cell-binding properties.

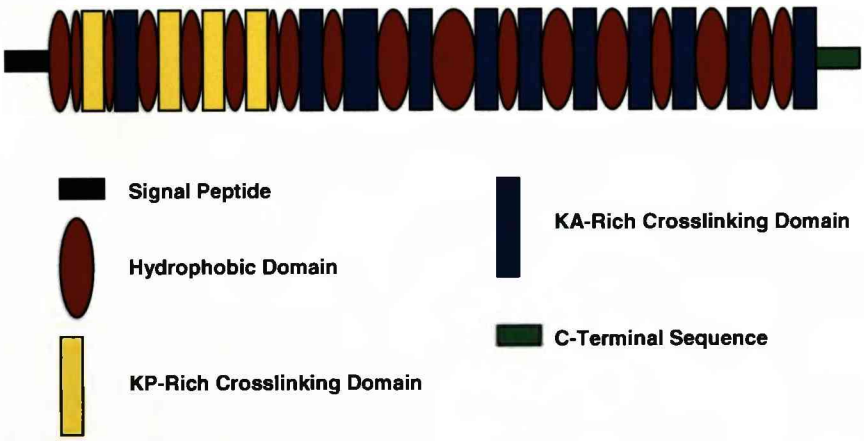

Figure 10 – Domain structure of tropoelastin

1.2.5 - Vascular Smooth Muscle Cells

Smooth muscle cells make up the medial layer of arteries. They form concentric layers alternating with elastic laminae. In vivo vascular SMCs have a contractile phenotype. They do not proliferate and produce very little extracellular matrix. Disruption of the SMC basement membrane triggers a phenotypic transformation. The cells acquire the ability to proliferate, migrate and synthesise extracellular matrix.[4,172]

This same transformation occurs during in vitro culture of SMCs.[173,174] In the first few days of culture the cells undergo a number of alterations. The number of myofilaments decreases and their ability to contract, either spontaneously or with chemical induction, disappears. There is an increase in ribosomes and rough endoplasmic reticulum indicating the cells have achieved the ability to synthesise extracellular matrix proteins. The cells change from bi-polar to a more fibroblast-like morphology.[1,175]

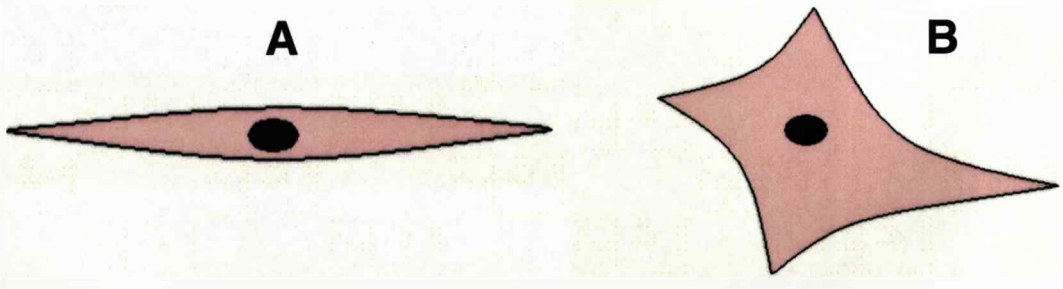

Figure 11 – Illustration of the morphology of the 2 different smooth muscle cell phenotypes; contractile (A) and synthetic (B)

The phenotype of SMCs in culture can be monitored by various methods. Observing the morphology of the cells is a basic but effective way of approximating their phenotypic state. Figure 11 shows the difference in appearance of the 2 distinct cell phenotypes. However, it is likely that a SMC in culture will be in an intermediate state and so this method is restricted. Looking at the rate of proliferation can also give a general idea of the level of transformation of the cells. Contractile SMCs do not proliferate whereas synthetic cells do. Again this method does not give a clear picture of the phenotypic state of the SMCs. To get a more accurate representation of the SMC transformation the observation of a number of protein markers is needed. This can either be achieved by semi-quantitative methods such as Western blotting or by imaging of the cells using immunostaining.

The level of smooth muscle α-actin decreases during the modulation of SMCs from a contractile to a synthetic phenotype.[176,177 178-180] In parallel to this the level of β-actin increases. [181] Smooth muscle myosin heavy chain and tropomyosin expression decrease.[177,179-181] The level of meta-vinculin increases during the transformation whereas the related protein vinculin decreases.[176,179-181] Desmin, calponin, SM22-α and the α_1 integrin have all been found to be at lower levels in synthetic SMCs.[176,177,179-183] Heavy caldesmon is decreased whereas light caldesmon increases.[176,177,179,180] Smoothelin is a protein only found in contractile SMCs and so is a good marker for this phenotype.[184-186]

1.2.6 – Specific Aims and Objectives

This project has four main aims.

1 – Can the fibrillin-1 fragment and the tropoelastin be covalently attached to the polyurethane via this surface modification protocol?

A protocol has previously been developed in order to covalently attach cell adhesion peptides such as GRGDSPK to a polyurethane surface.[187] This protocol has been adapted in order to attach proteins rather than peptides. In addition to this a different polyurethane is used. Each step of the modification was assessed and optimised using various chemical and biological techniques. The attachment of the proteins was verified using Enzyme-Linked Immunosorbent Assays.

2 – Is the conformation of the proteins adversely affected by the covalent attachment process?

The attachment of cells to these proteins adsorbed onto tissue culture plastic has been investigated. In the work presented here the attachment of cells to the covalently bound proteins was assessed. The results were compared in order to determine whether or not the modification protocol adversely affects the cell-binding properties of the proteins. If the conformation of the protein is altered a great deal by the attachment process then certain binding sites that are available on the adsorbed protein may not be available on the bound protein.

The RGD-dependence of cell attachment to fibrillin-1 was investigated as was the involvement of the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins. The involvement of the $\alpha_v\beta_3$ integrin in cell attachment to tropoelastin was also examined

3 – How is the behaviour of the cells affected by the protein-modified surfaces?

The behaviour of human coronary artery smooth muscle cells cultured on the modified surfaces was investigated qualitatively and quantitatively in detail. The effects of the two proteins were compared to each other and to plain, un-modified polyurethane.

Cell proliferation and spreading on the surfaces was investigated. RT-PCR was used to examine the expression of various genes by cells cultured on the surfaces including extracellular matrix proteins, basement membrane proteins and phenotypic markers. The synthesis of the same proteins was investigated using immunostaining and western blotting.

4 – What are the implications of this research for the development of an artificial bypass graft?

The results of the experiments were used to determine the value of this surface modification for use in developing a hybrid artificial artery.

Chapter 2 - Materials and Methods

2.1 – Materials

Human embryonal kidney 239-EBNA cells were transfected and grown at the University of Manchester. Nickel affinity chromatography columns were purchased from GE Healthcare, Buckinghamshire, UK. NuPAGE® lithium dodecyl sulphate buffer (4X), Sample Reducing Agent (10X), Novex 4-12% bis-tris gels and SimplyBlue™ SafeStain were purchased from Invitrogen, Paisley, UK. The Precision Plus Protein™ Standard was purchased from BIO-RAD, Hertfordshire, UK. The tropoelastin was a gift from Professor Anthony Weiss at the University of Sydney, Australia.

b9, Z1A1 polyurethane was obtained from Biomer Technologies Ltd, Runcorn, UK. Dextran (MW = 10,000) was purchased from PharmacosmosA/S, Denmark. Dimethylacetamide, polyethyleneimine (50%w/v in H_2O, MW = 750,000), sodium periodate, sodium cyanoborohydride coupling buffer, phosphate buffered saline tablets, fluorescamine, dextran-FITC (FITC:Glucose = 1:100), ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and bovine serum albumin were purchased from Sigma-Aldrich, Dorset, UK.

Cryopreserved human coronary artery smooth muscle cells were purchased from Cascade Biologics, Nottingham, UK, along with medium 231, smooth muscle growth supplement (SMGS) and trypsin/EDTA. Penicillin/streptomycin and GRGDSPK peptide were purchased from Sigma-Aldrich, Dorset, UK. Monoclonal antibody to fibrillin-1 was synthesised in the School of Biosciences at the University of Manchester, UK. Mouse anti-polyHistidine monoclonal antibody was purchased from R&D Systems, Abingdon, UK. Mouse anti-CD51 (272-17E6) and anti-CD61 (290.5C10) monoclonal antibodies were purchased from Calbiochem, Nottingham, UK. Mouse anti-CD49e (JBS5) monoclonal antibody was purchased from AbD Serotec, Oxford, UK. Mouse anti-CD29 (mAb13) monoclonal antibody was purchased from BD Biosciences, Oxford, UK. Mouse anti-CD51/61 (LM609) monoclonal antibody was purchased from Chemicon, Hampshire, UK. Rabbit anti-mouse IgG and goat anti-rabbit IgG polyclonal horseradish peroxidase conjugates were purchased from DAKO, Cambridgeshire, UK. Monoclonal antibodies to α-smooth muscle actin, elastin, calponin, laminin and collagen IV were purchased from Sigma-Aldrich, Dorset, UK. Monoclonal antibodies to caldesmon, fibulin-5 and smoothelin were purchased from AbCam, Cambridge, UK. Monoclonal anti-fibrillin-1 antibody was purchased from AbD Serotec, Oxford, UK. The Quant-iT™ PicoGreen® DNA quantification assay was purchased from Invitrogen, Paisley, UK. Primers for RT-PCR were purchased from Invitrogen, Paisley, UK. The DNase I Amplification Grade, Oligo(dT)20 Primer, dNTP Mix, RNaseOUT Recombinant Ribonuclease Inhibitor and SuperScript III Reverse Transcriptase PCR kits were purchased from Invitrogen, Paisley, UK. The iQ SYBR Green Supermix was purchased from BioRad, Hertfordshire, UK. The Western blot Mini Protean 3 system

was purchased from BioRad, Hertfordshire, UK. The Laemmli Sample Buffer was purchased from Sigma Aldrich, Dorset, UK. The MagicMark™ XP Western Standard and the SeeBlue® Plus2 Prestained Standard were purchased from Invitrogen, Paisley, UK.

The FLX800 and μQuant plate readers were purchased from BIO-TEK Instruments, VT, USA. Cell images were captured by a Zeiss Axioplan 2 microscope fitted with an axiocam digital camera. Confocal images were acquired using a Zeiss LSM500. The GeneGnome Western blot visualisation system was purchased from Syngene, Cambridge, UK.

2.2 - Recombinant Protein Synthesis and Purification

The fibrillin-1 fragment PF14 was produced using an episomal expression system and 293-EBNA cells as previously described.[148] The vector used was pCEP-pu/AC7 and this was modified with a polyHistidine$_6$ tag at the N-terminal to allow purification using nickel affinity chromatography. Cells were transfected at the University of Manchester, UK.

After transfection the 239-EBNA cells were cultured in DMEM-4 medium supplemented with 10% (v/v) foetal calf serum, 1% (w/v) penicillin/streptomycin, geneticin (300μg/ml) and puramycin (5μg/ml). When confluent, the medium was removed and the cells were washed with PBS. T6 medium was then added and the

cells were cultured for a further 3 days. After this time the medium was taken off and any dead cells were removed by centrifugation. Protease inhibitors were then added to the medium. The liquid was dialysed into 5litres of salt buffer (5.68g Na_2HPO_4, 5.52g NaH_2PO_4, 116.88g NaCl, 2.72g imidazole, 5l H_2O) for 3 days with a change of buffer every day. It was then pumped through a 1ml nickel column. The column was washed with 100ml of salt buffer before the protein was eluted as 1ml aliquots in elution buffer (1.42g Na_2HPO_4, 1.38g NaH_2PO_4, 29.22g NaCl, 34.04g imidazole, 1l H_2O).

Analysis of the protein fragment was carried out by analytical SDS polyacrylamide gel electrophoresis. The protein was analysed in both a reduced and non-reduced form. 10μl of 4X lithium dodecyl sulphate (LDS) buffer was added to 30μl of protein. 4μl of reducing agent was added to the samples to be reduced. All samples were placed in boiling water for 2 mins, mixed briefly and centrifuged for 2 mins at 8,000rpm. 40μl of each sample was run against a broad range molecular weight standard at 200V for 1 hr. The gels were then stained with SimplyBlue™ SafeStain and washed with distilled water before imaging.

The eluted aliquots that contained the protein as identified by SDS-PAGE were dialysed in PBS to remove the imidazole. The absorbance was read at 280nm and the corresponding protein concentration was calculated according to Beer's Law. ($A=\varepsilon cl$, taking ε as 1). The protein was then frozen at -80°C until use.

The tropoelastin was synthesised at the University of Sydney, Australia using a bacterial expression system.[188] The lyophilised protein was dissolved in phosphate buffered saline to the required concentration.

2.3 - Surface Modification Protocol

Polyurethane films were produced by spin-coating a 10% (w/v) solution of the b9 polymer in dimethylacetamide (DMAc) on to 13mm glass discs. The surface was then functionalised using a reversible swelling technique. A solution of 3.5% polyethyleneimine (w/v) in a 1:1 ratio of DMAc and water was prepared and added to the polymer-coated discs. The discs were left for 3 days at 40°C in the absence of light. After this time an excess of water (non-solvent) was added to trap the polyethyleneimine leaving amine groups present on the surface of the polyurethane. The discs were then washed thoroughly with distilled water and dried at 40°C to remove any residual solvent.

A solution of dextran (MW=10,000) in water was prepared (3g, 100ml H_2O). A cold solution of sodium periodate (3g, 100ml H_2O) was then added to this with vigorous mixing. The mixture was left stirring overnight at 4°C in the absence of light. A reductive amination reaction was used to attach the dextran to the amine groups. The partially oxidised dextran solution was added to the aminated polyurethane discs with an equal volume of sodium cyanoborohydride coupling buffer to quench the Schiff bases and stabilise the nitrogen-carbon bonds. The discs were then left overnight on a

mechanical shaker at room temperature. The dextran/$NaCNBH_3$ mixture was removed and the discs were washed thoroughly with distilled water (Figure 12).

A solution of $NaIO_4$ (0.6g, 100ml H_2O) was then added to the dextran-coated discs to form aldehyde groups on the dextran layer surface. The discs were immersed in the oxidising solution on a mechanical shaker overnight at room temperature before being washed thoroughly in distilled water.

The discs were then placed in the wells of a 24-well plate and sterilised by exposure to UV/O_3 for 20 seconds. The proteins were dissolved in sterile PBS to the required concentration. 500µl of this solution was added to each well along with an equal volume of $NaCNBH_3$. The plates were placed on a mechanical shaker overnight at room temperature. The solution was then removed from the wells and the discs were washed with sterile PBS (Figure 13).

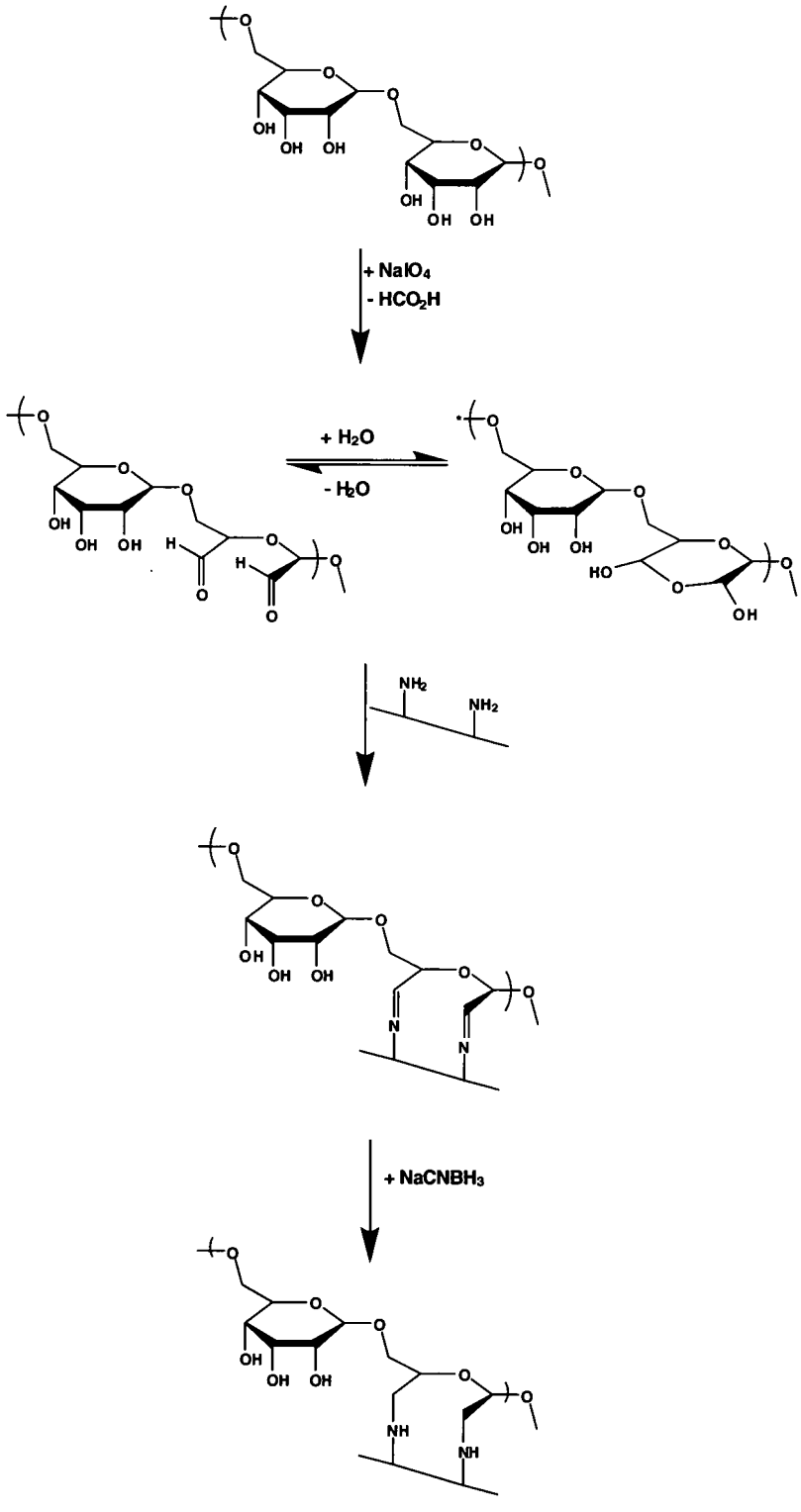

Figure 12 – Dextran attachment to the aminated polyurethane surface

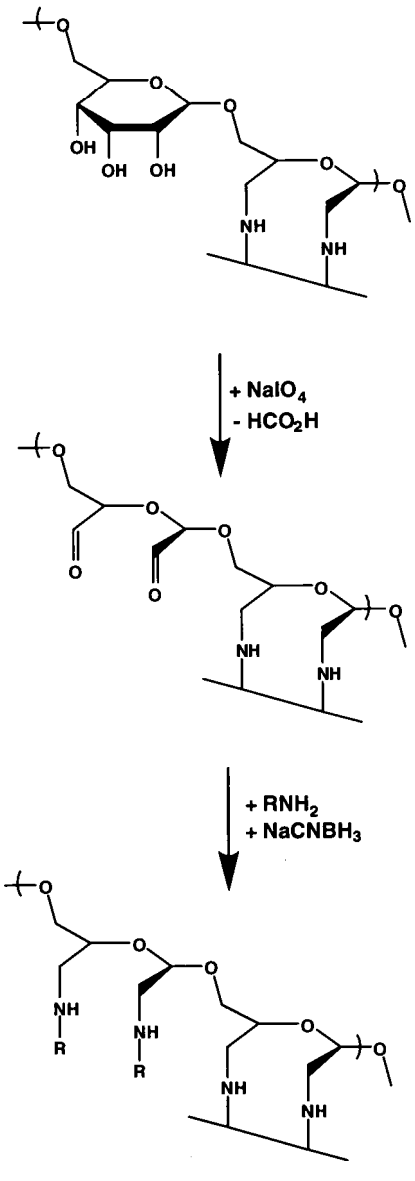

Figure 13 - Attachment of the protein to the dextran layer

2.4 – Cell Culture

Cryopreserved human coronary artery smooth muscle cells were cultured in medium 231 with the addition of a smooth muscle cell growth supplement (SMGS) (foetal bovine serum (4.9% (v/v) final concentration), human basic fibroblast growth factor (2 ng/ml), human epidermal growth factor (0.5 ng/ml), heparin (5 ng/ml), insulin (5 μg/ml), BSA (0.2 μg/ml)). 5ml of 50X penicillin-streptomycin (5,000 units/ml, 5mg/ml, respectively) was also added. Cells were passaged every 7 days using trypsin/EDTA (Cascade Biologics). They were grown in T75 tissue culture treated flasks seeded at a density of 5 x 10^3 cells/cm^2. Cells were used between passages 3 and 6.

2.5 - Surface Analysis Protocols

2.5.1 – Assessment of Amination

The presence of amine groups on the polyurethane surface was assessed using a solution of fluorescamine. This is a molecule that selectively reacts with primary amines to form a fluorescent conjugate (Figure 14).[189] Discs of aminated and plain polyurethane were added to a black 96-well plate (6 discs per surface). A solution of fluorescamine was prepared (4.5 x 10^{-3}M) and 100μl of the solution were added to each well. The fluorescence was measured at λ_{ex}=360nm, λ_{em}=460nm.

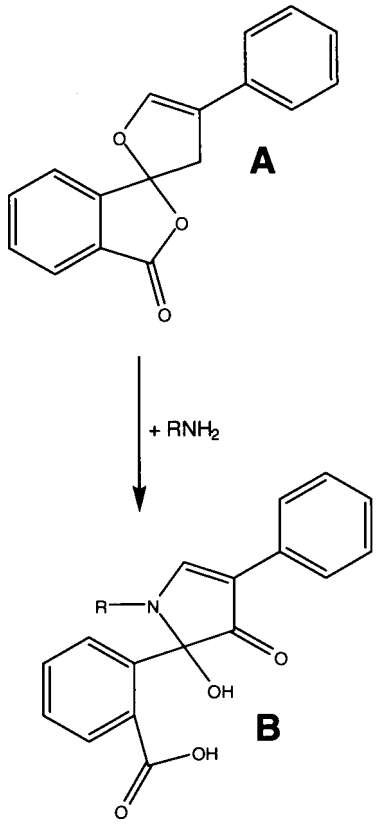

Figure 14 - Reaction of fluorescamine (A) with the amino terminal of a protein to form a fluorescent adduct (B)

2.5.2 – Assessment of Dextran Attachment

The efficacy of the covalent attachment of the dextran was investigated using a dextran-FITC conjugate. The dextran-FITC was dissolved in water (10mg, 2ml H_20). This solution was split into 2, an equal volume of sodium periodate was added to one sample to partially oxidise the dextran and an equal volume of water was added to the other to act as a control. The solutions were left to stir at 4°C in the absence of light for 1 hr. Discs of aminated polyurethane were placed in the wells of a black 24-well plate. The oxidised and non-oxidised dextran-FITC solutions were added to the discs

along with an equal volume of $NaCNBH_3$ (8 discs per solution). The discs were left on a mechanical shaker at room temperature for 3 hrs. After this time had elapsed the dextran solutions were removed and the discs were washed once with distilled water. The fluorescence of the discs was measured using a FLX800 fluorescence plate reader (λ_{ex}=485nm, λ_{em}=525nm). The discs were then washed repeatedly with the fluorescence being measured between each wash.

The optimal degree of dextran oxidation was determined as being the level at which cell attachment to the surface was at its lowest. 5ml aliquots of dextran were prepared, each containing 0.15g. An equal volume of different concentrations of $NaIO_4$ was added to each aliquot. The pH of each solution was measured after 30 mins, 1 hr and after being left to stir overnight. Each solution was then added to aminated polyurethane discs with an equal volume of $NaCNBH_3$ coupling buffer. The samples were then left overnight for the dextran to attach. The discs were washed and sterilised using UV/O_3 for 20 secs. Human coronary artery smooth muscle cells were seeded on to the surfaces at a density of 2.5 X 10^4 cells/well in serum-containing medium (see section 2.4 for details of the cell culture protocol). After 3 and 24 hrs the cells were fixed with formaldehyde/sucrose and stained with 0.4% (w/v) methylene blue for 10 mins. Representative images of the cells were taken at 10X magnification using an axiocam digital camera attached to an axioplan microscope.

2.5.3 – Enzyme-linked Immunosorbent Assays

Increasing concentrations of the protein were added to the dextran- coated discs in the final step of the modification (1, 2, 5 and 10μg/ml, 3 discs each). The first step of the

ELISAs was to block any non-specific antibody binding by adding 750µl of bovine serum albumin (5%w/v in PBS) to each well. The samples were left for 30mins at room temperature. After washing 3 times with PBS, 500µl of primary antibody at the required concentration was added to each well (fibrillin/elastin antibodies at 10µg/ml, RGD antibody at 5µg/ml). The samples were then left for 1hr at room temperature before washing 3 times with PBS. 500µl of 1:500 horseradish peroxidase-conjugated secondary antibody (polyclonal rabbit anti-mouse IgG) was added to each sample. The discs were then washed 3 times with PBS. A solution of ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was prepared as follows. 1ml of 40mM ABTS in water and 2µl of 30% hydrogen peroxide were added to 20ml of 0.1M sodium acetate/0.05M sodium dihydrogen phosphate (pH5). 500µl of the solution was then added to each sample and incubated at room temperature until a dark green colour appeared in the wells. The absorbance at 405nm was measured using a µQuant plate reader.

2.6 - DNA Quantification Assay

100µl of Triton-X100 (0.1% in carbonate buffer, pH10) was added to each well before freeze-thawing 3 times from -80°C to 37°C. 500µl of 200mM Tris-HCl, 20mM EDTA, pH 7.5 (TE) was added to each well followed by 500µl of the PicoGreen reagent diluted 200 fold in TE. 4 x 100µl aliquots were taken from each well and placed in a black 96-well plate. The fluorescence was read on a FLX800 plate reader

(λex = 480nm, λem = 520nm). A standard curve was generated by measuring the fluorescence generated by different dilutions of cells (Figure 15).

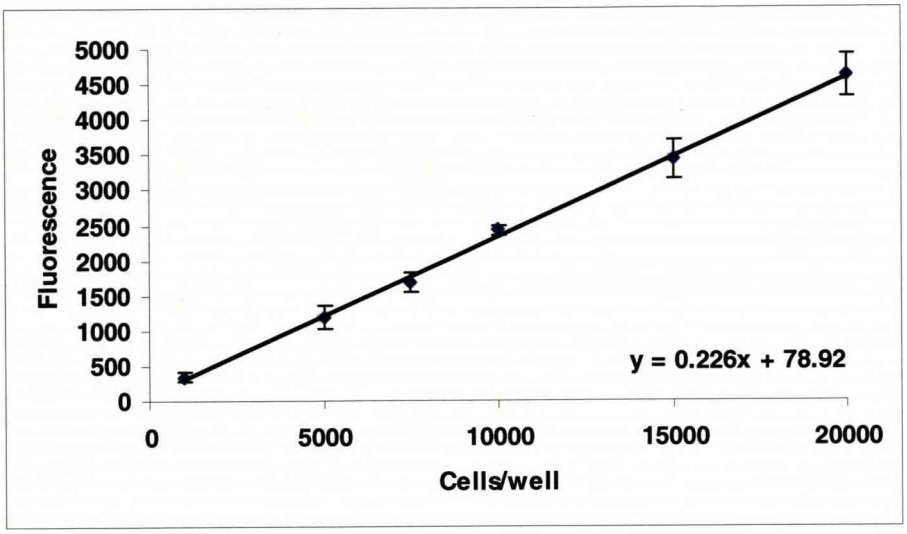

Figure 15 – Standard curve to relate measured fluorescence to cell number

2.7 – Integrin Blocking

2.7.1 – Soluble Peptides

The RGD-dependence of cell attachment to the fibrillin-modified surface was investigated by using a soluble RGD-containing peptide. HCASMCs were cultured as previously mentioned (section 2.4). Cells were trypsinised and re-suspended in serum-free medium. Viable cell number was determined using trypan blue staining to eliminate dead cells before counting using a haemocytometer. Cells were diluted to a concentration of 5 x 10^4 cells/ml with serum-free medium. 6ml of cell suspension

were placed into 4 vials. The peptide GRGDSPK was diluted with PBS and added to the vials to obtain concentrations of 0.5, 1, 5 and 10μg/ml. The cells were incubated with the peptide at 37°C for 30 minutes before seeding onto the fibrillin-modified surfaces at 1ml/well (6 discs per peptide concentration). Cells without peptide were seeded onto both glass and the modified surface as controls. The cells were left to attach for 1hr at 37°C. The number of cells attached to the surface after this time period was then determined as follows. The medium was removed and the cells were washed 3 times with PBS. The discs were then removed to fresh wells to eliminate any contribution from any cells attached to the surrounding tissue culture plastic. The DNA quantification assay, detailed in section 2.6, was used to calculate the amount of cells retained on each surface. The cell number was calculated using the equation generated from the graph in Figure 15. Attachment was presented as a percentage of the number of cells on the surface in the absence of any soluble peptide.

2.7.2 – Anti-integrin Antibodies

Antibodies were used to investigate integrin involvement in cell attachment to the modified surfaces. Antibodies to the α_v, β_3, α_5 and β_1 integrin sub-units and to the $\alpha_v\beta_3$ integrin were diluted with PBS. A cell suspension at a concentration of 5×10^4 cells/ml was prepared in serum-free medium and split into 6ml aliquots. Antibody was added to each aliquot to produce concentrations of 0.1, 0.5, 1 and 5μg/ml. The cells were incubated with the antibody for half an hour before they were seeded onto the modified surfaces (6 discs per antibody concentration). The fibrillin-modified surface was probed with the antibodies to the 4 integrin sub-units. For the tropoelastin just the antibody to the integrin $\alpha_v\beta_3$ was used due to the limited amount of

tropoelastin available to prepare the samples. Cells containing no antibody were seeded onto both glass and the modified surface as controls. Cells were left to attach for 1hr before the attachment was quantified using the PicoGreen reagent as before.

2.8 – Methylene Blue Staining

5 x 10^4 cells were seeded onto plain polyurethane and protein-modified surfaces in serum-free medium. After 3 hrs the medium in each well was exchanged for smooth muscle cell growth supplement -containing medium (SMGS). At 3 hrs, 1 day and 4 days the cells were fixed with formaldehyde/sucrose for 5 minutes at room temperature and stained with 0.4% methylene blue for 10 minutes before thorough washing with distilled water. Images of the cells on each surface were obtained using a Zeiss Axioplan 2 microscope fitted with an axiocam digital camera.

2.9 – Cell Spreading Assay

SMCs were cultured as in section 2.4. The cells were suspended in serum-free medium at a density of 5 x 10^4 cells/ml. They were then seeded on to plain PU and protein-modified PU surfaces at 1ml cell suspension per well. After incubation for 3 hrs the cells were washed with PBS, fixed with formaldehyde/sucrose for 5 mins and stained with 0.4% methylene for 10 mins. Images of the samples were taken at 20X magnification using a Zeiss Axioplan microscope fitted with an Axiocam digital

camera. 30 representative images were taken of the cells on each surface. Analysis of the images was performed with the aid of NIH image analysis software "Image J" as follows (Figure 16). The images were first converted to black and white (image B). Gaps in the cell images were present where the cells were at their most spread. To reduce the error due to this the gaps were first filled by the software (image C). They were then adjusted further by manually filling in gaps using the original image as a guide to ensure that any area covered by a cell was counted (image D). The number of cells in each image was then calculated by counting any objects containing 500 or more pixels. The total area covered by cells was also measured by the software. The area per cell was then calculated and an average taken for each surface.

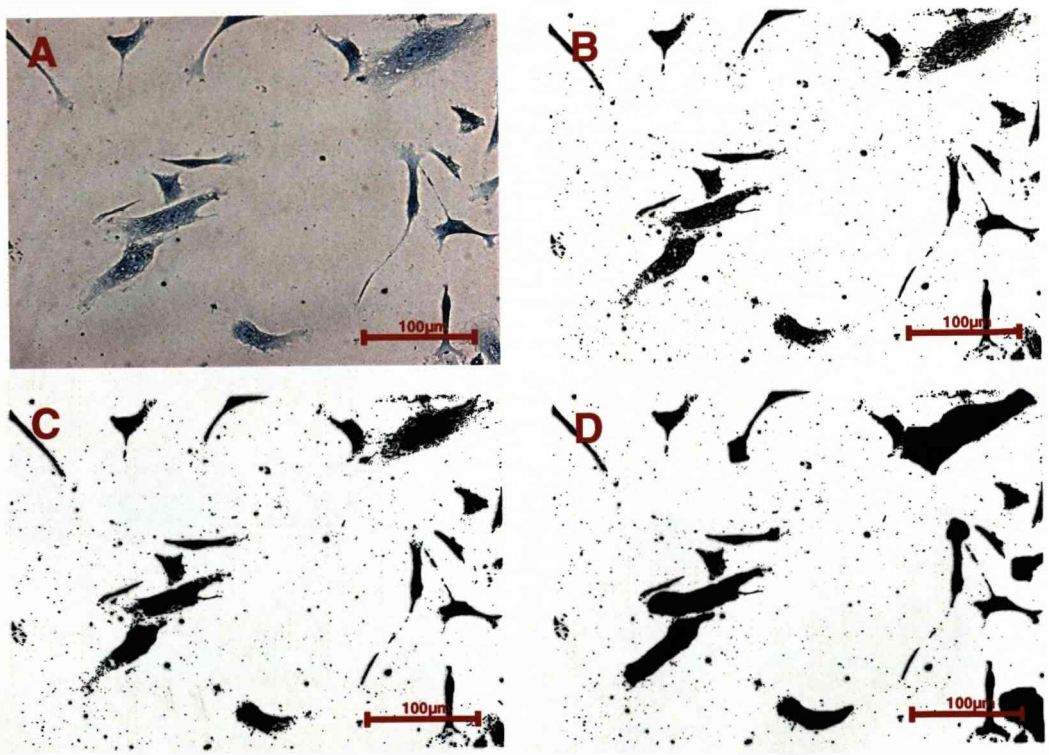

Figure 16 – Processing of images for analysis of cell spreading

2.10 – Cell Proliferation Assay

5 x 10^4 cells were seeded onto plain polyurethane and protein-modified surfaces in serum-free medium (8 discs per surface, per time point). After 3hrs the medium was exchanged for SMGS-containing medium. The Quant-iT™ PicoGreen® DNA quantification assay was used to determine the cell number after 3 hrs, 1, 2 and 4 days, (see section 2.6 for DNA quantification protocol).

2.11 – RT-PCR

Primers were designed for the extracellular matrix proteins fibronectin, fibrillin-1, elastin and laminin and the phenotypic markers smooth muscle α-actin and smoothelin using the Beacon Designer software (Table 1). The primers were optimised using cDNA dilutions and observing the melt curve to check for any primer dimers that would cause errors in the results.

Table 1 – Primer sequences and running temperatures

Primer		Sequence	Temperature
Fibronectin	Sense Anti-sense	5'-TCA TCC GTG GTT GTA TCA G-3' 3'-GTC TCA GTC TTG GTT CTC C-5'	54.2°C
Fibrillin-1	Sense Anti-Sense	5'-AGG CTG TGT AGA TGA GAA TGA ATG-3' 3'-GGC ACT CGT CCT GGT TGG-5'	56.5°C
Elastin	Sense Anti-sense	5'-GAG TTG GTG CTG GTG TTC-3' 3'-AGG TGC TGA GAG GAG GAG-5'	56.2°C
Laminin	Sense Anti-sense	5'-ACT ATT GCC TCA TAT TGT CCT CTG-3' 3'-CCA ACA CTG CTC ACT TCT TCC-5'	52.9°C
Smooth Muscle Alpha Actin	Sense Anti-sense	5'-TCC ACC TTC CAG CAG ATG-3' 3'-CCA CAG GAC ATT CAC AGT TG-5'	53.6°C
Smoothelin B	Sense Anti-sense	5'-GAG TCC ATG AAC GAT GTG-3' 3'-TCT CTT GAG CCA CTG TTG-5'	55.9°C
GAPDH	Sense Anti-sense	5'-GAA GGT GAA GGT CGG AGT-3' CAT GGG TGG AAT CAT ATT GGA A-5'	53.5°C

Cells were seeded on to glass, plain polyurethane, fibrillin and tropoelastin-modified surfaces at a density of 5 X 10^4 cells/well in 500µl of serum-free medium (3 discs per surface, per time point). After 3 hrs 500µl of SMGS-containing medium was added to each well. Cells were cultured for 3, 6 and 24 hrs. At each time point the medium was removed and the cells were washed with PBS. The samples were moved to fresh wells to eliminate DNA being harvested from the cells growing on the tissue culture plastic. 500µl of TRI-Reagent were added to each well. This was left at room temperature for 5 mins before being removed to DNase/RNase-free Eppendorfs.

100µl of chloroform was added to each sample before vortexing and then centrifuging at full speed for 5 mins. The clear upper liquid layer was removed to a fresh Eppendorf and 300µl of isopropanol was added. The samples were then centrifuged for 15 mins at full speed. After this time the supernatant was removed and 500µl of 100% ethanol was added. The samples were centrifuged at full speed for 5 mins, the ethanol was removed then 200µl of 70% ethanol was added. The samples were spun at full speed for 2 mins then the ethanol was removed and the RNA pellet was re-suspended in 10µl of nuclease-free water.

The RNA product was then DNase-treated. 1µl of 10X DNase I Reaction Buffer, 1µl DNase I, Amp grade and 2µl of nuclease-free water were added to up to 8µg of RNA. This mixture was then incubated at room temperature for 15 mins. The DNase I was then inactivated by the addition of 1µl of 25mM EDTA solution. This was then heated at 65°C for 10 mins.

cDNA was then synthesised from the RNA. 1µl of oligo(DT), 1µl of dNTP mix and 11µl of water were added to each RNA sample. The mixture was then heated at 65°C for 5 mins, cooled on ice for 1 min and briefly centrifuged. 4µl 5X First strand buffer, 1µl 0.1M DTT, 1µl RNaseOUT and 1µl superscript III RT were added to each sample before heating at 50°C for 45 mins then at 70°C for 15 mins. Each cDNA sample was diluted by 1:10 in water and then frozen.

The cDNA was then tested with each of the previously mentioned primers (Table 1). Each primer mix was first prepared by adding 10µl of sense and anti-sense to 80µl of nuclease-free water. For each well of the PCR plate to be used 7.5µl of SYBR Green, 4.5µl of water and1µl of primer mix were added to an ependorf. The plate was set up by adding 13µl of this mixture and 2µl of cDNA to each well. The samples were added in triplicate. The plate was then added to the PCR cycler and run at the required temperature sequence for 45 cycles.

ΔCt values were calculated by taking the average GAPDH Ct value of each sample from the average gene of interest Ct value e.g. $\Delta Ct = Ct_{laminin} - Ct_{GAPDH}$. An average ΔCt value was taken from the 3 repeats of each sample. The $2^{-\Delta Ct}$ value for each sample was taken from this and plotted on a graph.

2.12 – Immunostaining

2.5×10^4 cells per well were seeded on to plain and protein-modified PU surfaces in 500µl of serum-free medium. After 3 hrs 500µl of SMGS-containing medium was added to each well. The cells were cultured for 2 and 4 days. After each time point the cells were washed with PBS and fixed with formaldehyde/sucrose for 10 mins at 37°C. The cells were washed and then permeabilised by adding 100µl of 1% Triton-X100 in PBS and incubating for 5 mins at 4°C. The Triton solution was removed and the cells washed with PBS. The first primary antibody was diluted to the required concentration in 1% (w/v) BSA in PBS. Optimal antibody concentrations were determined by staining SMCs cultured on glass discs (Table 2). 200µl of the antibody was then added to each well. The samples were incubated at 37°C for 1 hr and then washed 3 times with PBS. The secondary antibody (AlexaFluor 488) was diluted to the required concentration with 1% BSA in PBS. 200µl of this was then added to each well and the samples were incubated at 37°C for 1 hr. The samples were mounted onto glass slides using a drop of vector shield mounting medium containing DAPI nuclear stain. The samples were then visualised using a confocal microscope.

Table 2 – Antibody Concentrations Used for Immunostaining

Antibody	Dilution
α5	1:100
β1	1:100
αvβ3	1:100
Vinculin	1:250
Laminin	1:100
Collagen IV	1:100
Fibulin-5	1:1000
Tropoelastin	1:100
Fibrillin-1	1:100
SMA	1:100
Calponin	1:100
Caldesmon	1:100
Smoothelin	1:100
AlexaFluor488	1:500

2.13 – Western Blot Analysis

SMCs were seeded on to modified and plain PU surfaces at a density of 5 X 10^4 cells/well in serum-free medium. After 3 hrs the medium was exchanged for growth supplement-containing medium. After a further 21 hrs, 500μl of SDS lysis buffer was added to the sample-containing wells. The cells were scraped from the surface and the solution removed to an Eppendorf. The samples were then heated at 100°C for 5 minutes.

A 10% polyacryamide gel was prepared. The running gel was made by mixing 4ml separating buffer, 5.3ml acrylamide, 6.7ml water, 75μl 10% ammonium persulphate (APS) and 15μl TEMED in a vial. The gel was then poured into a glass mould until it

was half full. The mould was then topped up with water. The gel was allowed to set at room temperature before the water was removed. The separating gel was then prepared and comprised of 1.5ml stacking buffer, 1ml acrylamide, 3.5ml water, 50µl 10% APS and 15µl TEMED. This was then added to the mould on top of the running gel. A comb was placed in the top of the gel and it was allowed to set at room temperature. The comb was removed and water was added to keep the wells intact.

Equal amounts of each sample and Laemmli buffer were added to Ependorfs and heated at 100°C for 5 mins. The wells of the gel were loaded with a SeeBlue® Plus2 pre-stained marker (10µl), MagicMark™ XP Western Standard (10µl) or sample (25µl). The gel was then secured in a tank filled with Tris-glycine buffer containing SDS and run at 80mA for 30 mins. The gel was then removed from the tank and placed in Tris-glycine buffer (without SDS).

The blot was then transferred on to a cellulose membrane. The membrane was soaked in methanol and then Tris-glycine buffer before being placed on top of the gel. These were then sandwiched between blotting paper and then sponge, both having been soaked in the Tris-glycine buffer. The gel sandwich was then secured in a tank containing Tris-glycine buffer and run at 300mA for 2hrs. After this time the membrane was placed into 3% (w/v) BSA in TBS-T buffer (10mM Tris buffer (pH7.4), 0.1M NaCl, 0.1% v/v Tween 20) for 1 hr at 4°C. The membranes were then probed by various antibodies.

The proteins examined in this way were the extracellular matrix proteins fibrillin-1, tropoelastin and fibulin-5; the basement membrane proteins laminin and collagen IV and the phenotypic markers smooth muscle α-actin, smoothelin, calponin and caldesmon. GAPDH was used as the control to normalise the protein concentrations calculated.

The primary antibody was diluted to 1:1000 in 3% (w/v) BSA in TBS-T. The membrane was placed into the solution and incubated at 37°C for 30 mins. The membrane was then washed 3 times with TBS-T for 5 mins. The horse radish peroxidase-conjugated secondary antibody was diluted to 1:2000 in 3% BSA and added to the membrane. This was then incubated at 37°C for 30 mins before being washed 3 times in the TBS-T buffer. The membrane was then placed in a solution of immobilon western chemiluminescent HRP substrate for 10 mins. The membrane was then placed on the tray of the GeneGnome chemiluminescent detection system. An image was collected for 5 mins. The contrast and brightness of the images were altered to obtain the clearest picture.

2.14 – Statistical Analysis

Statistical analysis was carried out using an unpaired t-test to compare two samples. A p value of ≤ 0.01 was considered to be significant and a p-value of ≤ 0.005 was considered to be highly significant.

Chapter 3 - Results

3.1 – Protein expression and purification

A fragment of fibrillin-1 (PF14) was recombinantly expressed and purified using nickel affinity chromatography. The molecular weight of the fragment was verified using SDS-PAGE. Figure 17 shows an image of the gels containing protein samples from the first 5 aliquots collected from the chromatography column. The figure shows both the reduced and non-reduced protein samples. It is clear that the highest concentration of protein was collected in aliquots 2-4. These aliquots were combined to give an overall protein concentration of 1.14mg/ml.

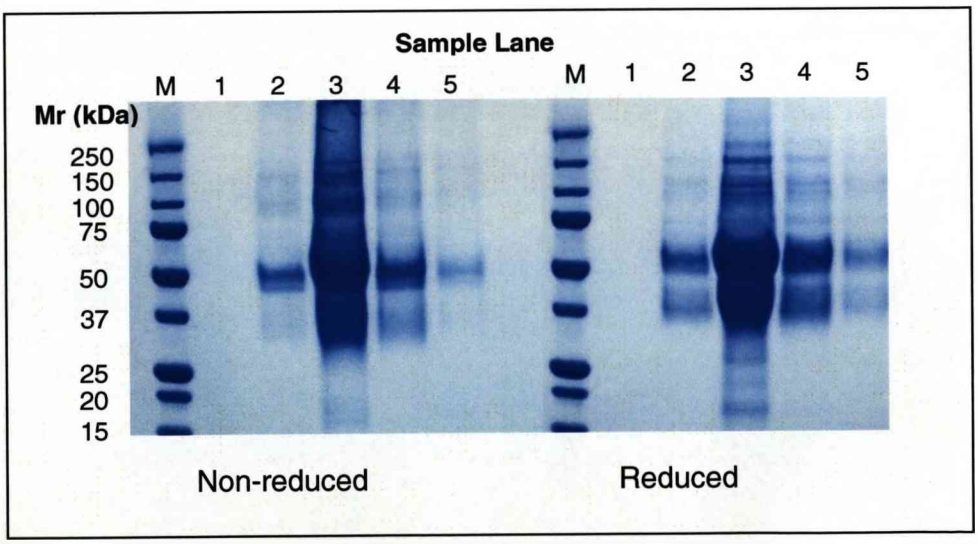

Figure 17 - SDS-PAGE gels of purified fibrillin-1 PF14 aliquots eluted from a nickel affinity chromatography column

3.2 – Surface analysis

3.2.1 – Assessment of amination

Primary amine groups were produced on the polyurethane surface by using a reversible swelling technique to incorporate polyethyleneimine into the surface layer. The level of amination was assessed by adding a solution of fluorescamine to plain and aminated polyurethane samples. Figure 18 shows the degree of fluorescence emitted from the plain and aminated surfaces on the addition of the fluorescamine. A degree of background fluorescence from the plain polymer is evident; however the difference between the plain and aminated surfaces is highly statistically significant ($p \leq 0.005$).

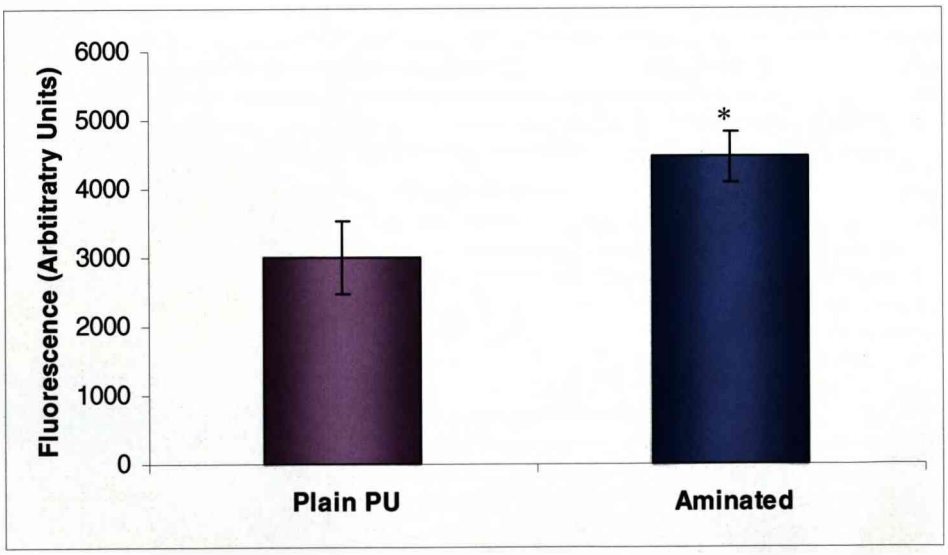

Figure 18 - Fluorescence emitted from discs of plain and aminated polyurethane on the addition of fluorescamine (*$p \leq 0.005$)

3.2.2 – Assessment of dextran attachment

A layer of dextran was attached to the aminated polyurethane by partially oxidising a solution of dextran and immobilising it via a reductive amination reaction. The presence of the dextran layer on the surface was verified using a dextran-FITC conjugate. Figure 19 shows the fluorescence emitted from the polymer discs modified with either oxidised or non-oxidised dextran-FITC. After the removal of the dextran solution the samples were washed a number of times and the fluorescence measured after each wash. For oxidised dextran the initial fluorescence recorded was extremely high. After approximately 4 washes the measurements even out at around 2,500 units. In contrast the fluorescence emitted from the discs modified with non-oxidised dextran drops significantly after just 2 washes and remains at a constant level of around 500 units.

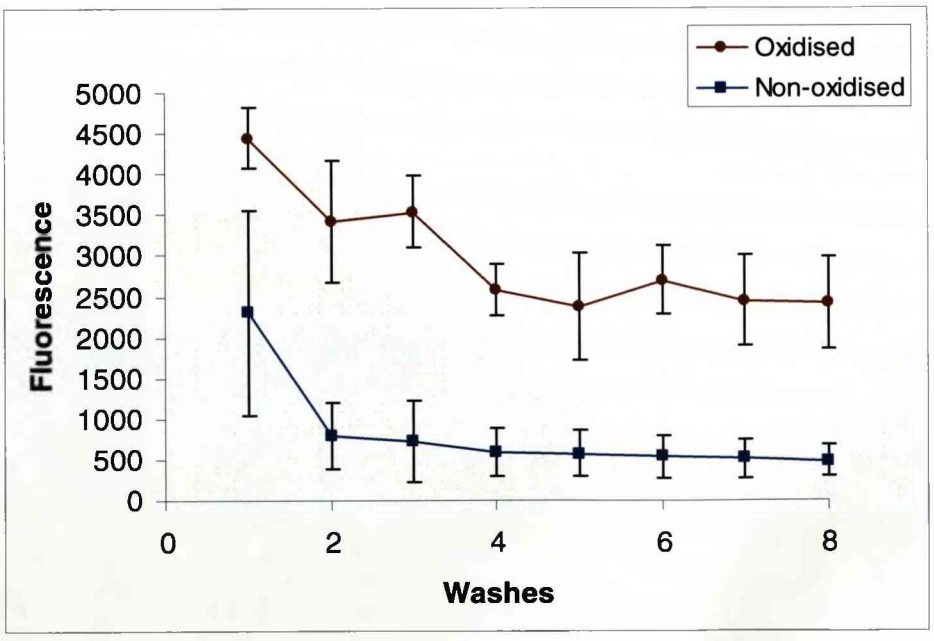

Figure 19 – Fluorescence levels due to the retention of oxidised and non-oxidised dextran-FITC by aminated polyurethane surfaces after washing

The level of oxidation of the dextran solutions was assessed by monitoring the change in pH caused by the formation of methanoic acid during the reaction. Figure 20 shows that the higher the concentration of $NaIO_4$ added, the lower the pH value was. This shows that there was more methanoic acid produced when the concentration of $NaIO_4$ was increased corresponding to a higher level of oxidation.

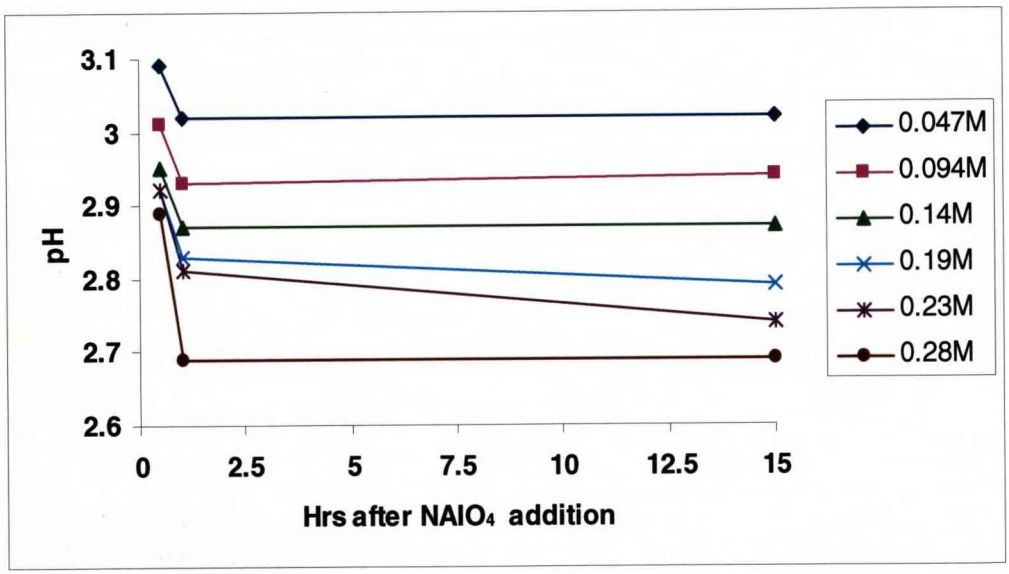

Figure 20 – Change in measured pH with time on the addition of varying concentrations of $NaIO_4$ to dextran solutions

Dextran solutions that had been oxidised to varying degrees were added to aminated polyurethane samples with an equal volume of $NaCNBH_3$ in order to attach the polysaccharide to the polymer surface. After washing, SMCs were cultured on the surfaces for 3 and 24hrs in serum-containing medium. Figure 21 shows light microscope images of methylene blue stained SMCs. After 3 hrs cell attachment was poor on all surfaces but was slightly better at the very low and very high $NaIO_4$

concentrations. At this time point cell attachment was least efficient when a 0.14M concentration of $NaIO_4$ was added. This concentration corresponds to equal masses of $NaIO_4$ and dextran in the reaction mixture. This trend is the same after 24 hrs. On the surfaces with the highest and lowest levels of oxidation, the cells have adhered and spread. At intermediate levels of oxidation the cells are mainly still rounded with very few having spread at all. Again cell adhesion was at its lowest level when the masses of dextran and $NaIO_4$ were equal.

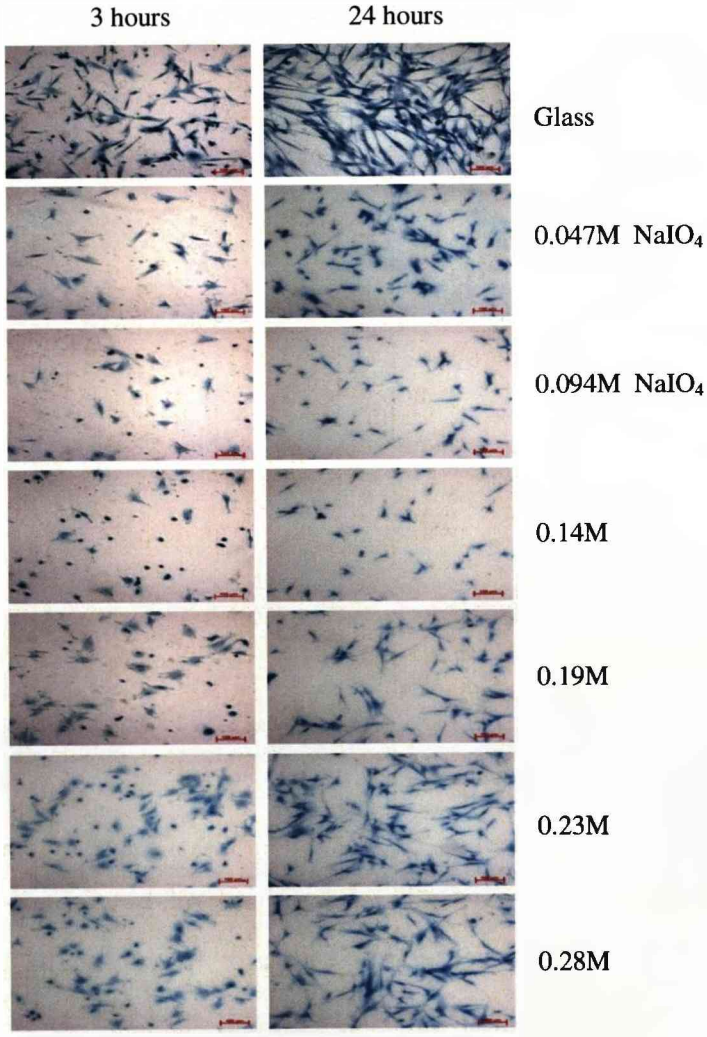

Figure 21 - SMC attachment to dextran surfaces produced by oxidising the dextran to different extents using varying concentrations of $NaIO_4$

3.2.3 - ELISAs

To assess the efficacy of the protein immobilisation protocol, an ELISA utilising an antibody to the section of fibrillin-1 that contains the RGD sequence was carried out. The concentration of protein added to the oxidised dextran surfaces was varied from 0 to 10 μg/ml. The results show that the attachment of protein increases as the concentration added increases (Figure 22). This trend levels off at the highest concentrations with the difference between 5 and 10μg/ml not being statistically significant ($p > 0.01$).

A second ELISA was carried out to determine whether or not the RGD sequence contained within the fibrillin fragment was available for integrin binding to the surface (Figure 23). The level of absorbance due to the amount of RGD increases as the concentration of added protein increases. Again, there is no significant difference in the amount of RGD present at the 5 and 10 μg/ml added protein concentrations ($p > 0.01$).

To assess the modification with tropoelastin, an ELISA was carried out using an antibody to elastin. As the concentration of added tropoelastin increased the amount of attached protein increased (Figure 24). The graph shows that the amount of attached protein does not increase further when the concentration of TE added is doubled from 5 to 10 μg/ml ($p > 0.01$).

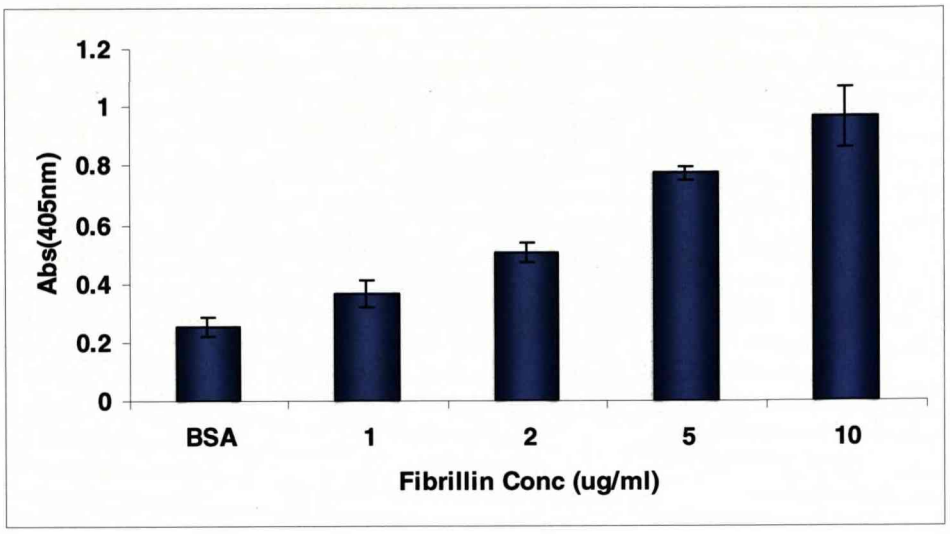

Figure 22 – An ELISA using an antibody to fibrillin-1 showing the change in amount of fibrillin present on the surface as the concentration of added fibrillin PF14 increases

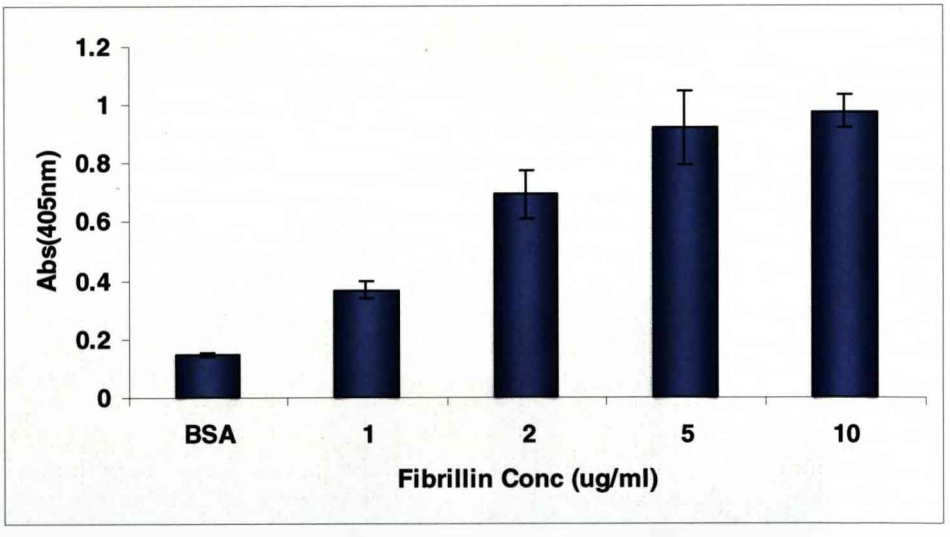

Figure 23 – An ELISA using an antibody to the RGD sequence showing the change in amount of RGD present on the surface as the concentration of added fibrillin PF14 increases

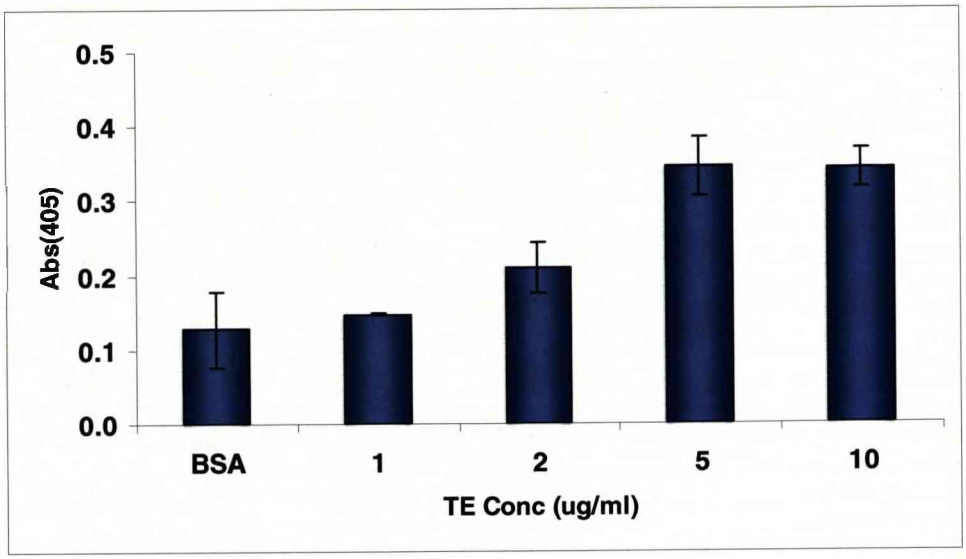

Figure 24 – An ELISA using an antibody to elastin showing the change in amount of elastin present on the surface as the concentration of added tropoelastin increases

3.3 – Integrin Blocking Studies

3.3.1 – Fibrillin-1

3.3.1.1 – Soluble Peptides

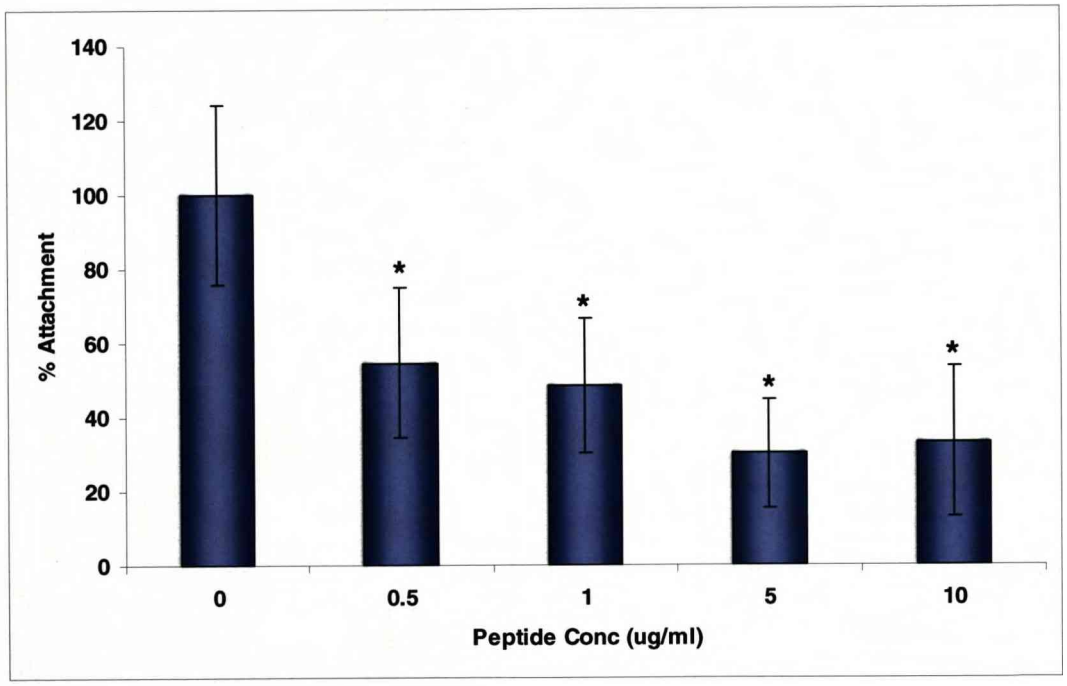

Figure 25 – Change in percentage cell attachment to fibrillin PF14-modified surface with an increasing concentration of RGD peptide added to the cell suspension prior to seeding (* indicates $p \leq 0.005$ compared to no antibody, n=2)

In order to determine whether cell attachment to the fibrillin-modified surface was dependent on the RGD sequence, soluble RGD peptide was added to the cell suspension prior to seeding. Figure 25 shows that the higher the concentration of soluble peptide in the culture medium the lower the degree of cell attachment to the surface. A 50% reduction in attachment compared to the control (no antibody) is

achieved with just 0.5μg/ml of soluble peptide with further decreases at higher concentrations.

3.3.1.2 – Anti-integrin Antibodies

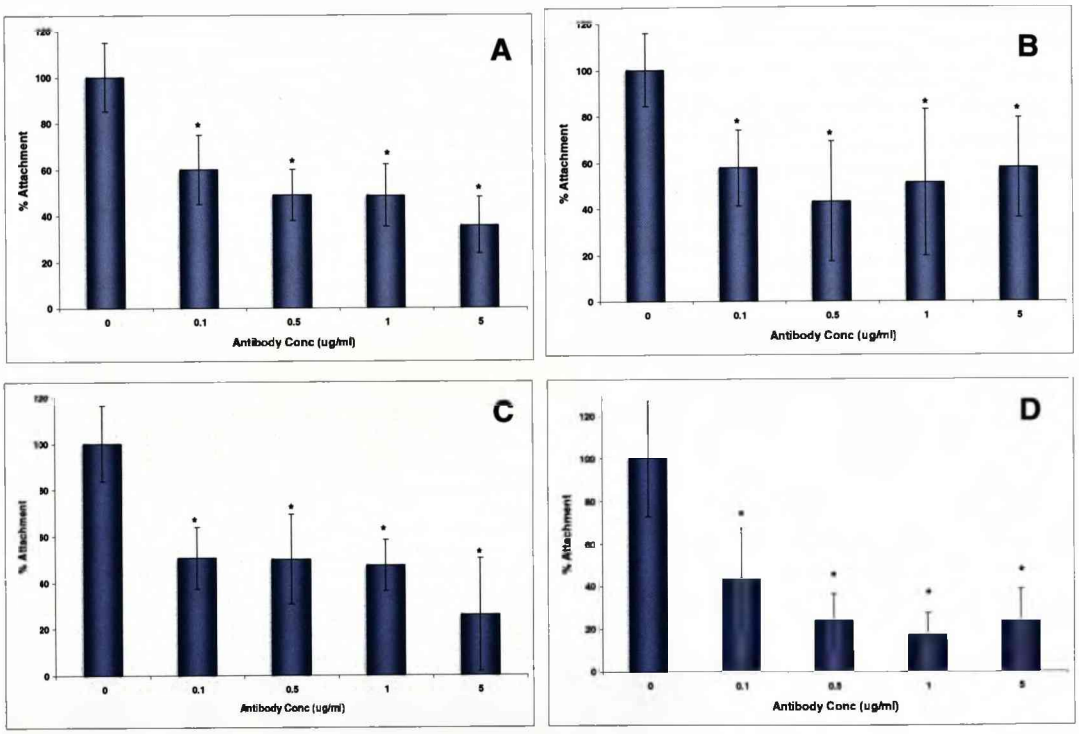

Figure 26 – Change in percentage attachment of cells to the fibrillin PF14 surface with increasing blocking-antibody concentration added to the cell suspension prior to seeding, anti-α_v (A), anti-β_3 (B), anti-α_5 (C) and anti-β_1 (D) (* indicates $p \leq 0.005$ compared to 0 antibody, n=2 except for β_3 where n=4)

The contribution of two different integrins to the attachment of cells to the fibrillin-modified surface was investigated by adding antibodies to the various integrin sub-units to the cell suspension prior to seeding. Figure 26 shows the effect of adding

antibodies to the α_v, β_3, α_5 and β_1 integrin sub-units on cell attachment. Each antibody reduced the cell attachment to approximately 50% of the control at a concentration of 0.5μg/ml. Further decreases in cell attachment were seen at higher concentrations with a reduction to around 20% for the anti-β_1 antibody.

3.3.2 – Tropoelastin

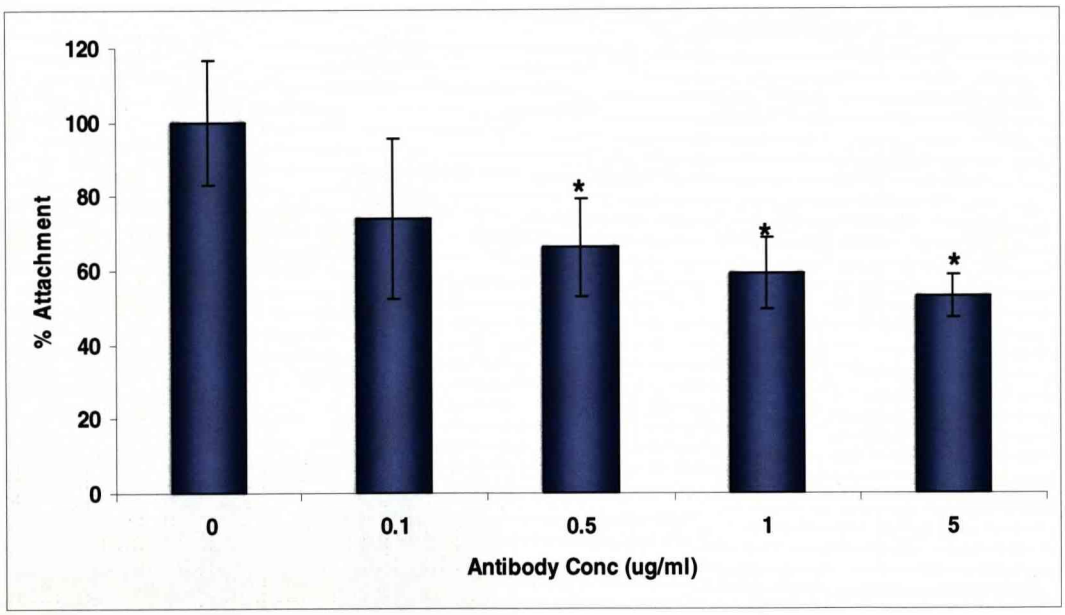

Figure 27 – Change in percentage cell attachment to tropoelastin-modified surfaces with an increasing concentration of anti-$\alpha_v\beta_3$ antibody added to the cell suspension prior to seeding (* indicates $p \leq 0.005$ compared to 0 antibody)

To determine the contribution of the $\alpha_v\beta_3$ integrin to cell attachment to the tropoelastin-modified surface an antibody to the integrin was added to the cell suspension prior to seeding. Figure 27 shows that cell attachment decreases on the addition of the anti-$\alpha_v\beta_3$ antibody to the culture medium. Compared to cell attachment

in the absence of antibody there are approximately 50% fewer cells attached to the modified surface in the presence of 5µg/ml of blocking antibody.

3.4 – Methylene Blue Staining

3.4.1 – Fibrillin-1

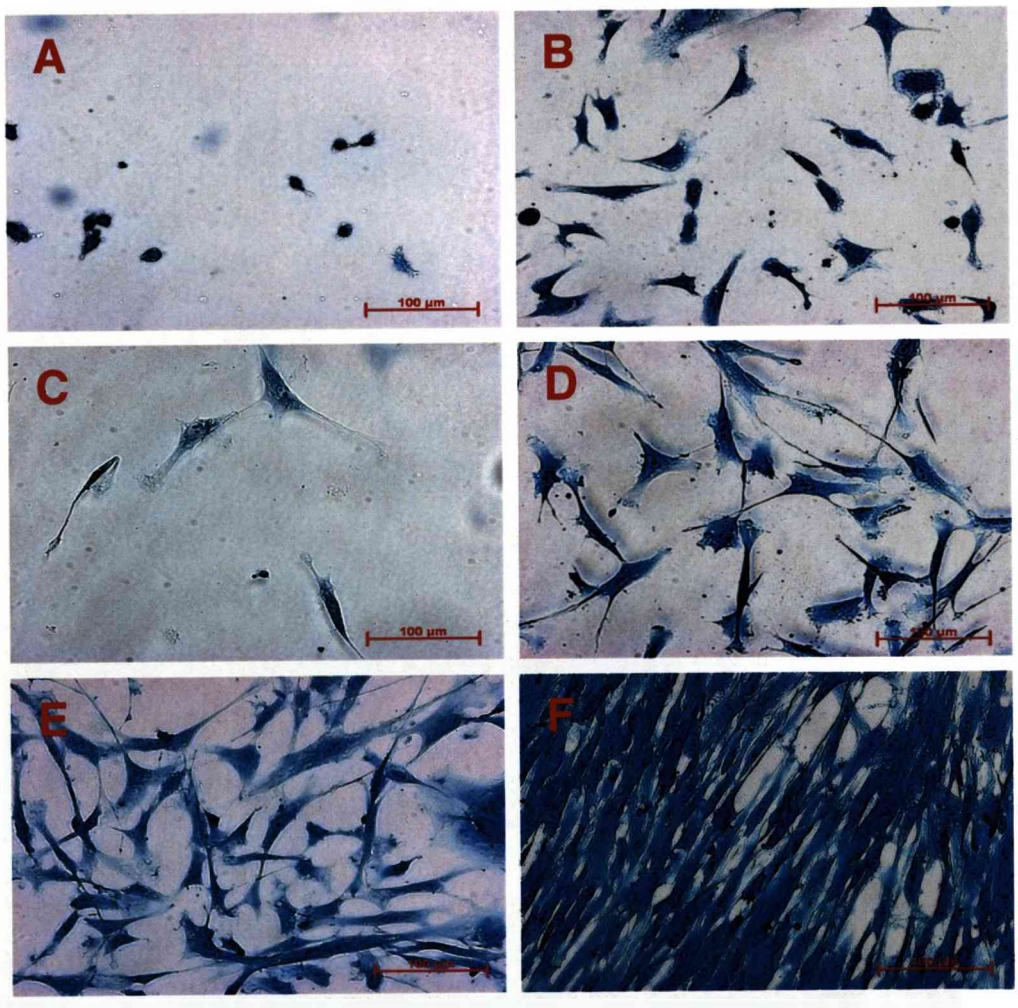

Figure 28 – Images of SMCs cultured for 3 hrs (A,B), 24 hrs (C,D) and 4 days (E,F) on plain polyurethane (A,C,E) and fibrillin PF14-modified polyurethane (B,D,F)

Images of cells cultured on plain and fibrillin-modified surfaces were obtained in order to study their morphology and growth over a 4 day time period. Figure 28 shows the difference in cell behaviour on the plain polyurethane and the fibrillin-1-modified surfaces. In the first 3hrs of culture the cells are in serum-free medium. The cells on the plain polyurethane surface are attached but remain rounded with very little spreading apparent. In contrast, the cells on the modified surface have attached and are well spread. After the first 3hrs the medium was exchanged for serum-containing medium. This enabled the cells on the plain surface to spread out more however not to the extent of those on the fibrillin-modified surface. These cells are extensively spread and an increase in cell number is evident. After 4 days significant cell proliferation has dramatically increased the number of cells on the modified surface to the extent that cell-cell contacts have caused the cells to align. It can be seen that there are a lot less cells on the plain surface.

3.4.2 - Tropoelastin

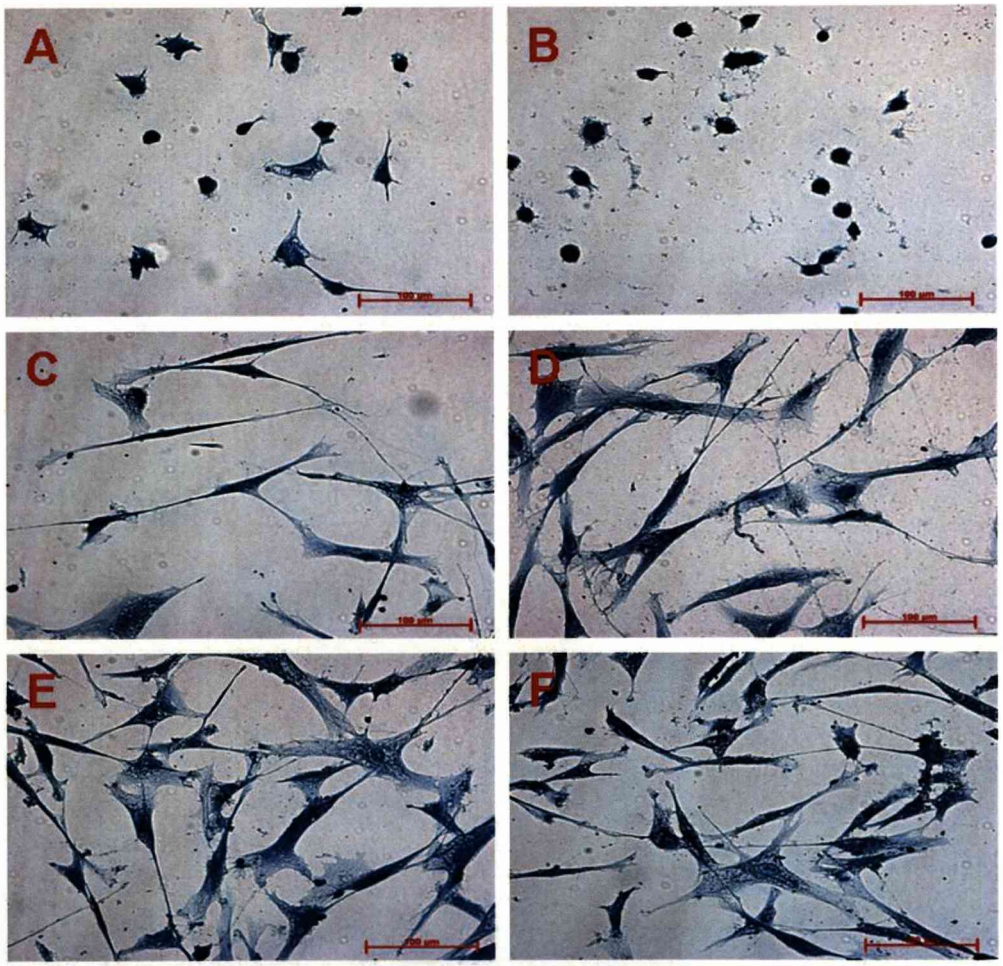

Figure 29 – Images of SMCs cultured for 3 hrs (A,B), 24 hrs (C,D) and 4 days (E,F) on plain polyurethane (A,C,E) and tropoelastin-modified polyurethane (B,D,F)

The images shown in Figure 29 are of SMCs cultured on plain and tropoelastin-modified polyurethane. As in Figure 28 the cells were cultured in serum-free medium for the first 3 hrs. After this time the cells can be seen to be weakly attached to both surfaces but neither has encouraged much adhesion or spreading. After 24 hrs the cells have spread considerably on both surfaces but little proliferation is evident.

After 4 days a low level of cell growth can be seen to have occurred. This level is similar on both the plain and modified surface.

3.5 – Cell Spreading

3.5.1 – Fibrillin-1

SMCs were cultured on plain and modified polyurethane. After 3 hrs the number of cells and the total area taken up by them were measured by taking images and analysing them using the Image J software. The average area per cell was calculated by dividing the total cell area by the number of cells. Figure 30 shows that cells cultured on the fibrillin-modified surface are considerably more spread than on the plain polyurethane surface ($p \leq 0.005$).

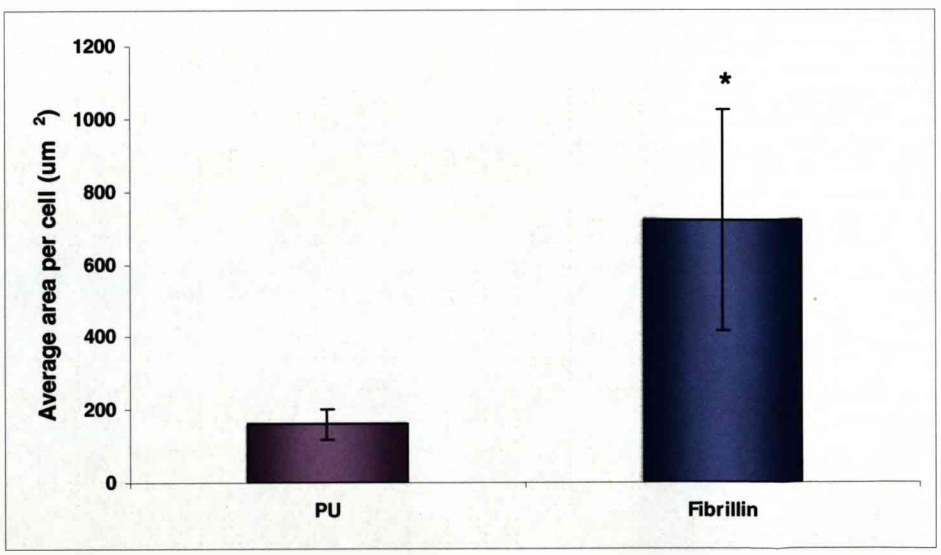

Figure 30 – Average area per cell of SMCs cultured on plain polyurethane and fibrillin-modified surfaces for 3 hours (* $p \leq 0.005$)

3.5.2 – Tropoelastin

Figure 31 shows the level of cell spreading for SMCs cultured on plain and tropoelastin-modified polyurethane. The graph shows that the cells cultured on the tropoelastin-modified surface spread out significantly less than those on the plain polyurethane ($p \leq 0.005$).

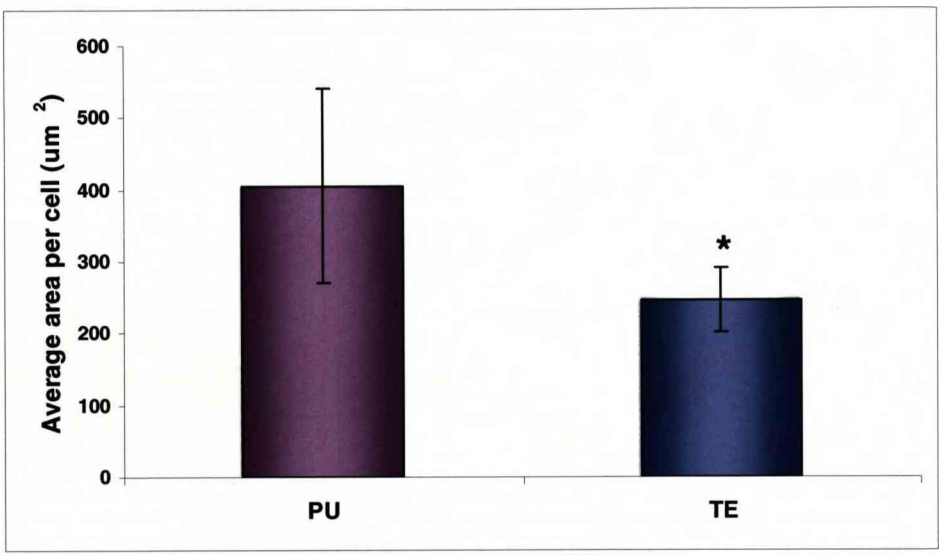

Figure 31 – Average area per cell of SMCs cultured on plain polyurethane and tropoelastin-modified surfaces for 3 hours (*$p \leq 0.005$)

3.6 – Cell Proliferation Assay

3.6.1 – Fibrillin-1

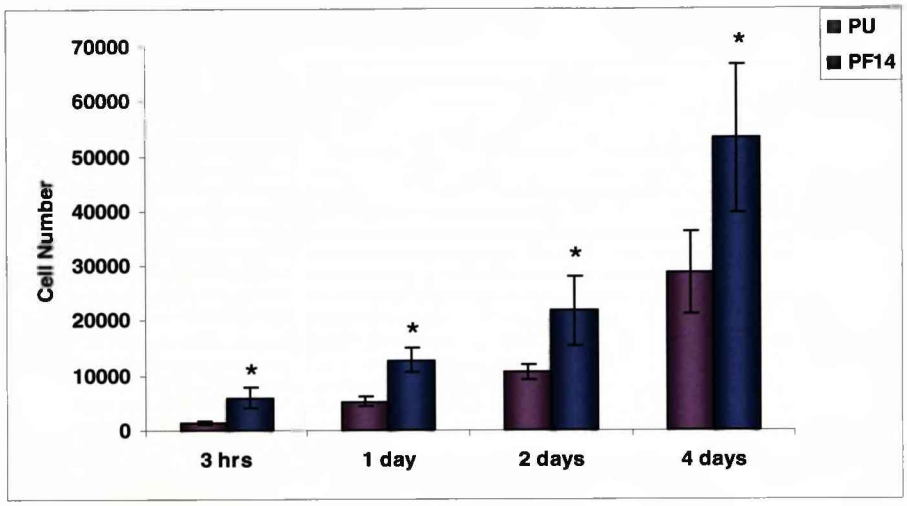

Figure 32 – Change in cell number on plain and fibrillin PF14-modified polyurethane over 4 days (*p ≤ 0.005 compared to plain polyurethane)

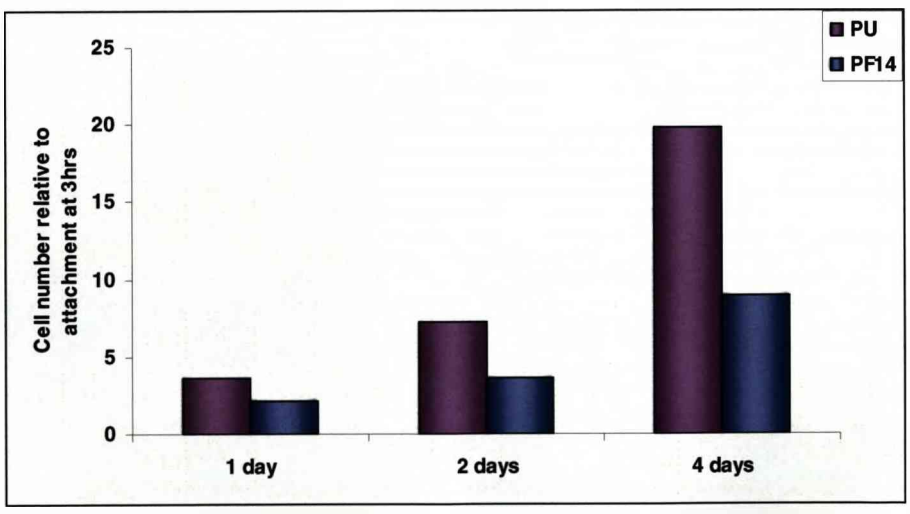

Figure 33 – Cell number calculated relative to the number of cells attached to each surface at the 3hr time point

The number of SMCs on plain and modified polyurethane was estimated by quantifying the DNA harvested from the cells at time points up to 4 days. A standard curve was then used to convert the data to cell number. Figure 32 shows the change in cell number on plain and fibrillin-modified polyurethane over the 4 day time period. The number of cells on both surfaces increased at a steady rate over the 4 days. There were statistically more cells on the fibrillin than on the plain surface at all time points. In order to show changes in the rate of cell proliferation, the cell number relative to the number of cells attached at 3hrs was calculated. Figure 33 shows that there were approximately 20 times more cells relative to initial attachment on the plain polyurethane at 4 days compared to 10 times more on the fibrillin-modified surface. The rate of proliferation was seen to be higher on the plain surface than the fibrillin surface at all time points.

3.6.2 – Tropoelastin

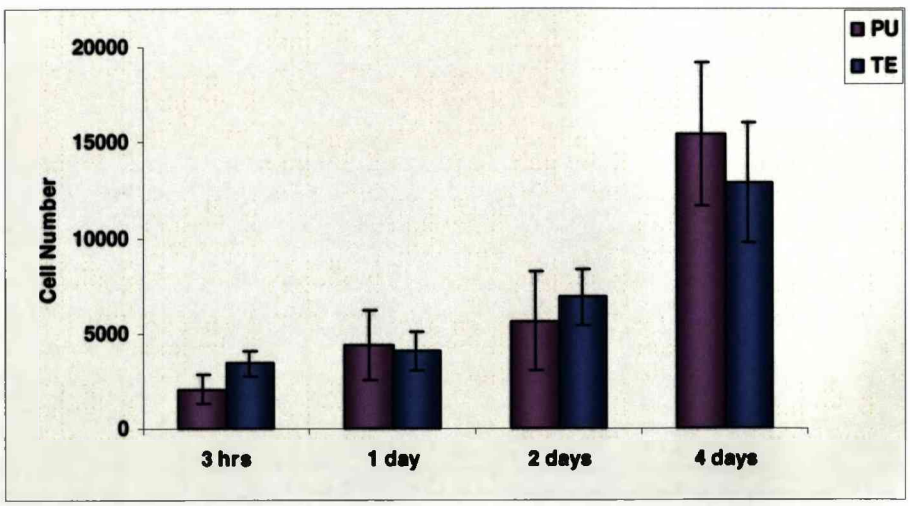

Figure 34 – Change in cell number on plain and tropoelastin-modified polyurethane over 4 days

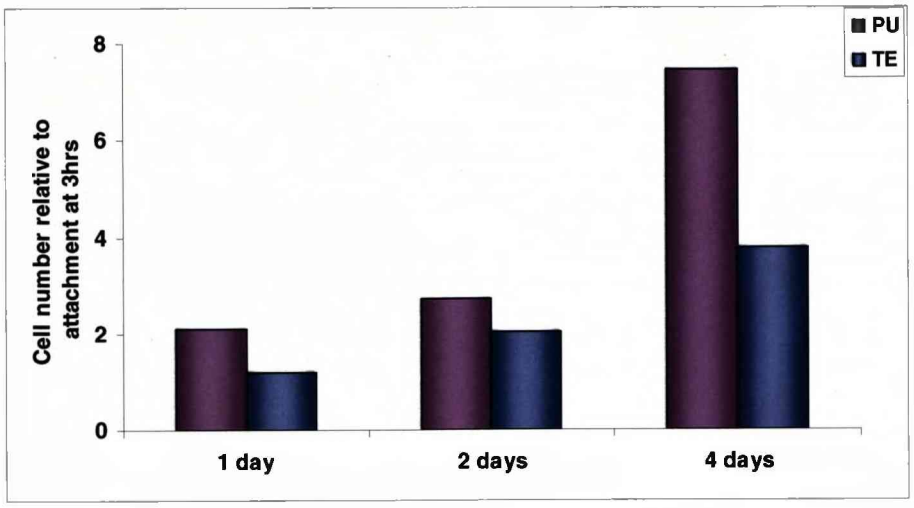

Figure 35 - Cell number calculated relative to the number of cells attached to each surface at the 3hr time point

As can be seen in Figure 34, cells proliferated on both the plain and tropoelastin-modified surfaces over the 4 day time period. The cell number was similar on both surfaces at all time points. At 4 days it appears that there are more cells on the plain surface than the tropoelastin. However, this difference is not statistically significant ($p > 0.01$). Figure 35 shows how the cell number increases relative to the number of cells attached at 3hrs. The graph shows that the cells are proliferating at a higher rate on the plain polyurethane surface.

3.7 – RT-PCR

Levels of gene expression of various proteins were investigated by reverse transcriptase RT-PCR. mRNA was harvested from cells cultured on glass, plain polyurethane and fibrillin and tropoelastin-modified surfaces at 3, 6 and 24 hrs. These levels were then normalised to the housekeeping gene GAPDH.

Laminin is a major constituent of the basement membrane. Its production was investigated in order to see if the cells were encouraged to produce this membrane by the modified surfaces. At the 3 hr time point there was no difference in the expression of laminin mRNA by the cells cultured on plain polyurethane compared to those on glass (Figure 36, Image A). The expression then increased at 6 hrs and 24 hrs. Cells cultured on the fibrillin-modified surface expressed slightly more laminin at all time points compared to glass (Figure 36, Image A). At 3 hrs the cells produced more laminin mRNA on the fibrillin than on the plain polyurethane (Figure 36, Image B). This level then decreased to below polyurethane levels at 6 hrs and then there was a further decrease at 24 hrs. The expression of laminin by cells on the tropoelastin surface was higher than on glass and plain polyurethane at 3 and 24 hrs but was lower at 6 hrs (Figure 36, Images A and B).

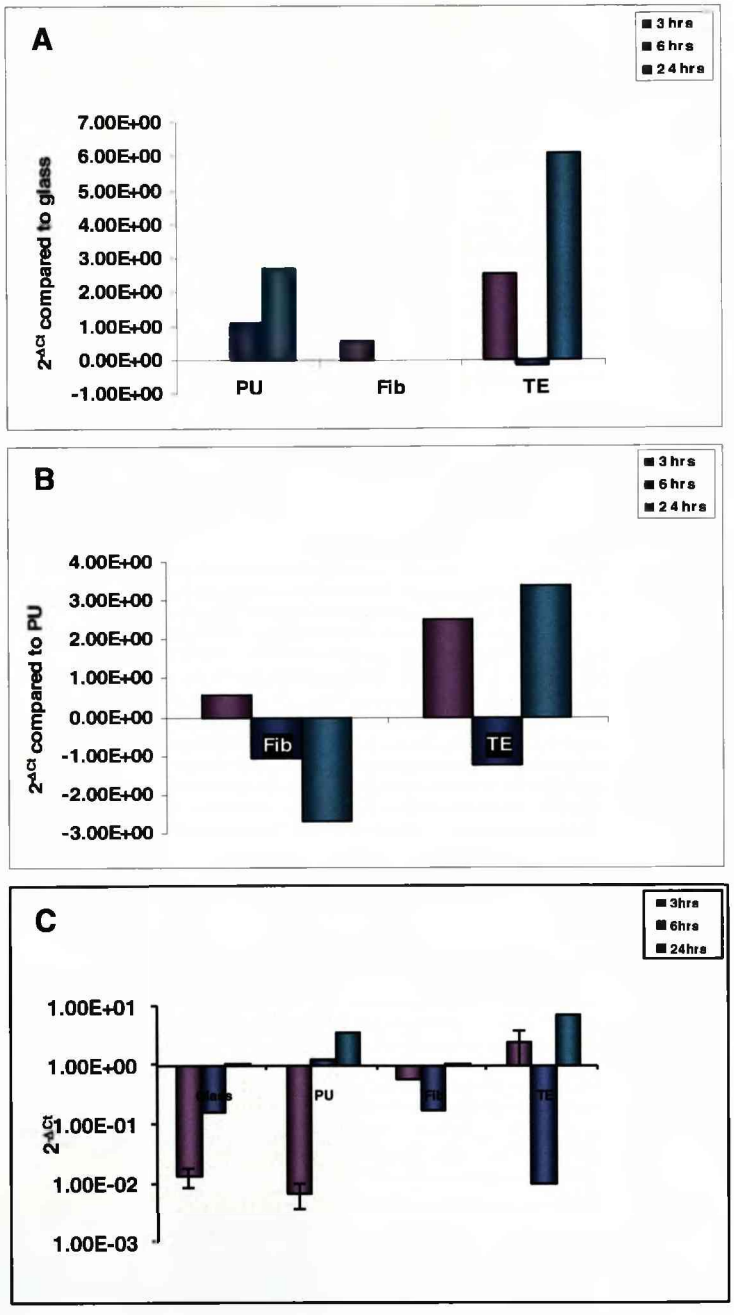

Figure 36 – Laminin mRNA levels compared to GAPDH expressed by cells cultured on the plain polyurethane, fibrillin and tropoelastin surfaces compared to glass (A), cells cultured on fibrillin and tropoelastin surfaces compared to plain polyurethane (B) and the raw mRNA levels from cells cultured on the glass, polyurethane, fibrillin and tropoelastin surfaces (C)

As it is a component of the elastic fibres in the artery wall, the expression of the gene for elastin was investigated. The production of elastin mRNA by the cells followed almost the same trend as for laminin. At 3 hrs there was no difference in elastin mRNA levels produced by cells on the glass or plain polyurethane surfaces (Figure 37, Images A and B). The expression then increased at 6 and then 24 hrs. mRNA levels were slightly higher on the fibrillin-modified surface than on glass at 3 hrs (Figure 37, Image A). They then decreased over time reaching a level lower than on glass by 24 hrs. Compared to cells cultured on the plain polyurethane cells on the fibrillin surface produced more elastin mRNA at 3 hrs with the level decreasing to a lower level at 6 hrs with a further decrease at 24 hrs (Figure 37, Image B). On the tropoelastin-modified surface expression of elastin mRNA was higher than on glass and plain polyurethane at 3 and 24 hrs but lower at 6 hrs (Figure 37, Images A and B).

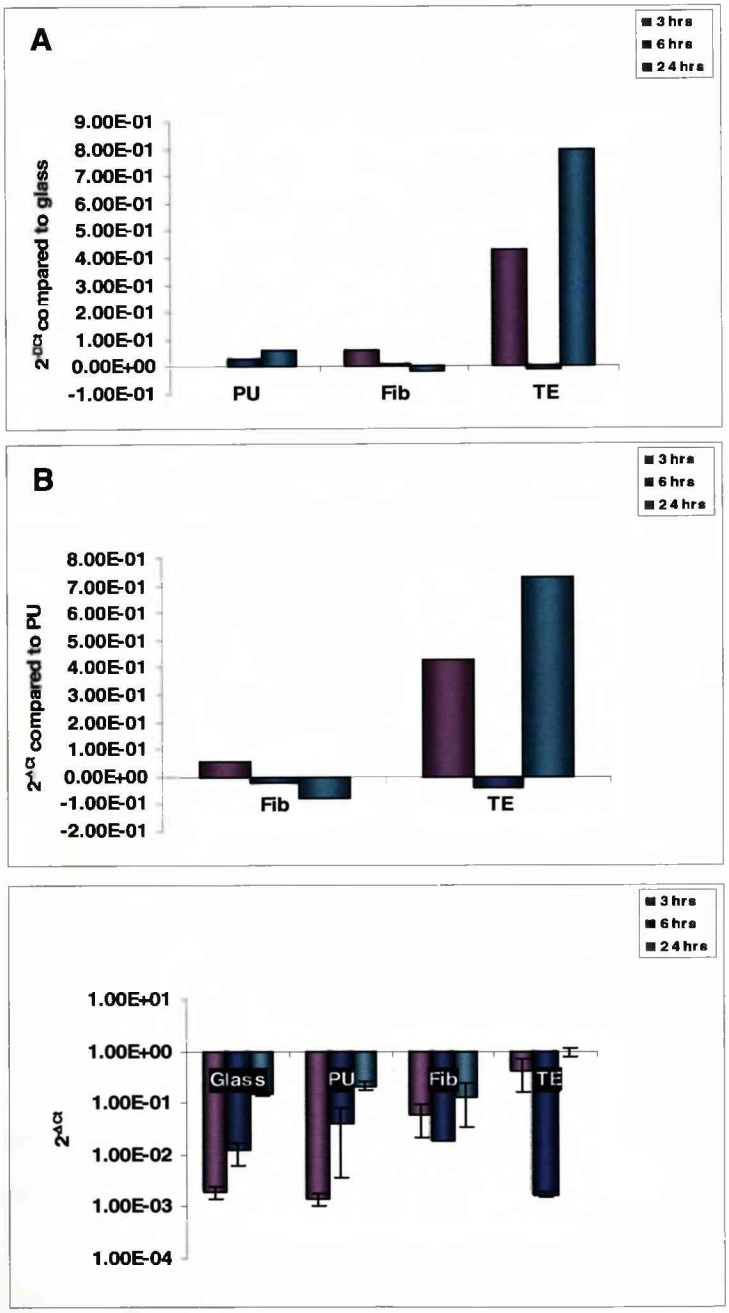

Figure 37 – Elastin mRNA levels compared to GAPDH expressed by cells cultured on the plain polyurethane, fibrillin and tropoelastin surfaces compared to glass (A), cells cultured on fibrillin and tropoelastin surfaces compared to plain polyurethane (B) and the raw mRNA levels from cells cultured on the glass, polyurethane, fibrillin and tropoelastin surfaces (C)

Fibrillin-1 is another important component of elastic fibres. At 3 and 6 hrs there was a lower expression of fibrillin-1 on the polyurethane compared to glass (Figure 38, Image A). The expression was then similar to glass at 24 hrs. On the fibrillin-modified surface the level of mRNA was again lower than on glass and also lower than on the plain polyurethane (Figure 38, Images A and B). At 6 hrs expression was lower than on glass but higher than on the polyurethane. By 24 hrs the levels of fibrillin mRNA on the glass, polyurethane and fibrillin surfaces are in distinguishable on the graphs. Compared to cells cultured on both glass and plain polyurethane the cells on the tropoelastin surface expressed less mRNA at 3 hrs but more at 6 hrs (Figure 38, Images A and B). By 24 hrs as with the fibrillin surface the expression was highly similar to the glass and polyurethane. The values of all these changes are extremely small with the scale only reaching from -2×10^{-6} to 2×10^{-6}.

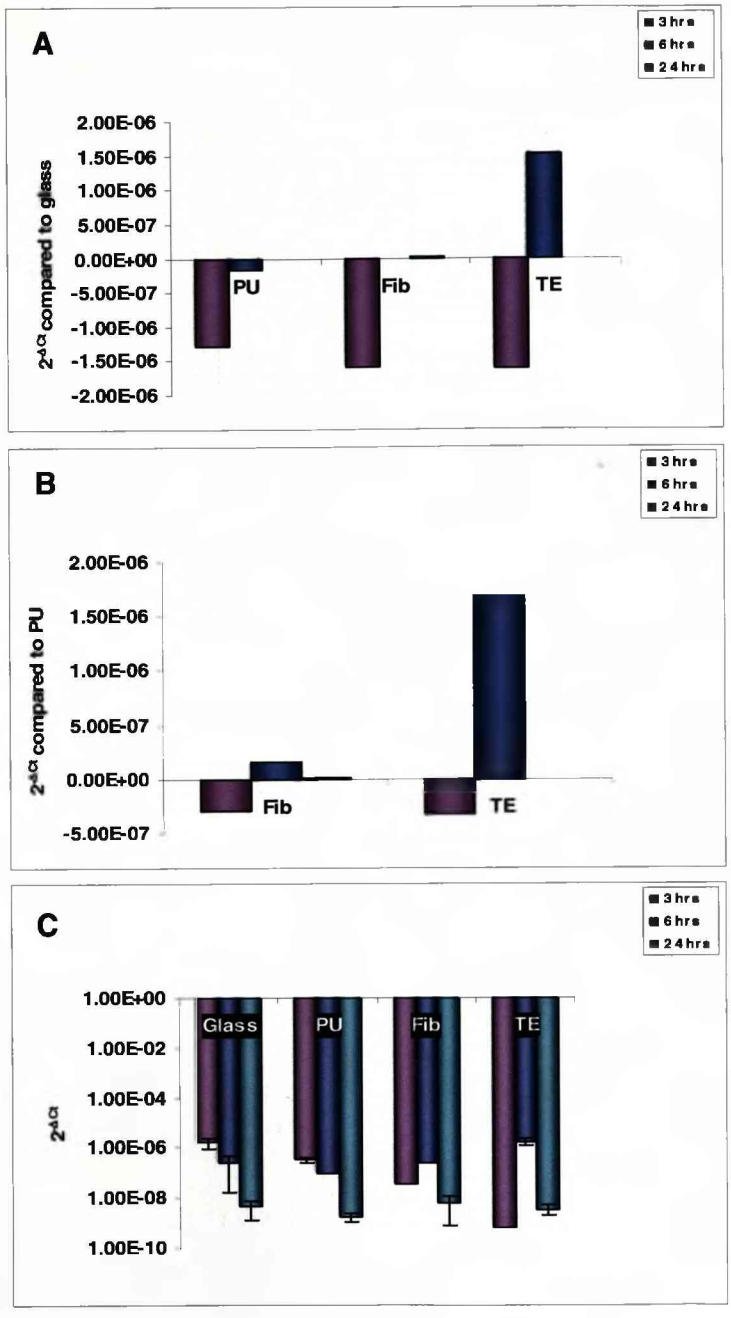

Figure 38 – Fibrillin-1 mRNA levels compared to GAPDH expressed by cells cultured on the plain polyurethane, fibrillin and tropoelastin surfaces compared to glass (A), cells cultured on fibrillin and tropoelastin surfaces compared to plain polyurethane (B) and the raw mRNA levels from cells cultured on the glass, polyurethane, fibrillin and tropoelastin surfaces (C)

The expression of the gene for fibronectin was investigated as a general indication of extracellular matrix synthesis. This is useful as measurement of protein synthesis is an important tool in determining the phenotype of SMCs. Cells on the plain polyurethane expressed higher levels of fibronectin mRNA at 3 and 24 hrs but lower levels at 6 hrs compared to glass (Figure 39, Image A). On the fibrillin-modified surface the cells produced less at all time points compared to glass. Compared to plain polyurethane the expression was slightly higher at 3 hrs but lower at 6 and 24 hrs (Figure 39, Image B). Cells on the tropoelastin-modified surface produced more fibronectin mRNA at 3 and 6 hrs compared to glass but less at 6 hrs (Figure 39, Image A). The level of expression was similar to that of the cells cultured on the polyurethane at 3 hrs it was then lower at 6 hrs and higher at 24 hrs (Figure 39, Image B).

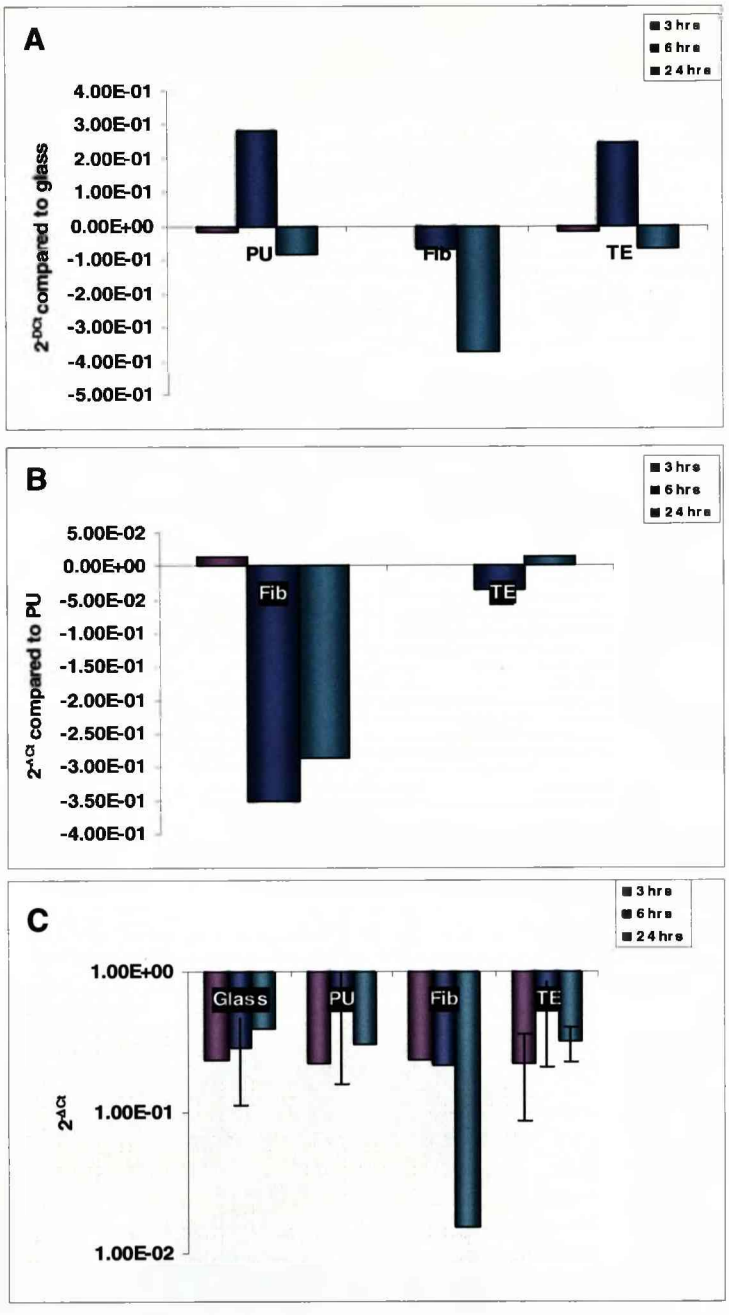

Figure 39 – Fibronectin mRNA levels compared to GAPDH expressed by cells cultured on the plain polyurethane, fibrillin and tropoelastin surfaces compared to glass (A), cells cultured on fibrillin and tropoelastin surfaces compared to plain polyurethane (B) and the raw mRNA levels from cells cultured on the glass, polyurethane, fibrillin and tropoelastin surfaces (C)

The expression of smooth muscle α-actin (SMA) mRNA was slightly higher on plain polyurethane than glass at 6 and 24 hrs but similar at 3 hrs (Figure 40, Image A). In contrast the expression was significantly higher at 3hrs on the fibrillin-modified surface. Compared to polyurethane the expression of SMA mRNA on the fibrillin surface was a lot higher at 3 hrs, slightly lower at 6 hrs and similar at 24 hrs (Figure 40, Image B). On the tropoelastin SMA gene expression is significantly higher than on glass and plain polyurethane at 3 hrs (Figure 40, Images A and B). These levels then decreased to nearly the same as those of glass at 6 and 24 hrs. Expression was lower than on polyurethane at 6 hrs but similar at 24 hrs. It should be taken into account that the scale of these variations is a great deal larger than the other graphs in this section.

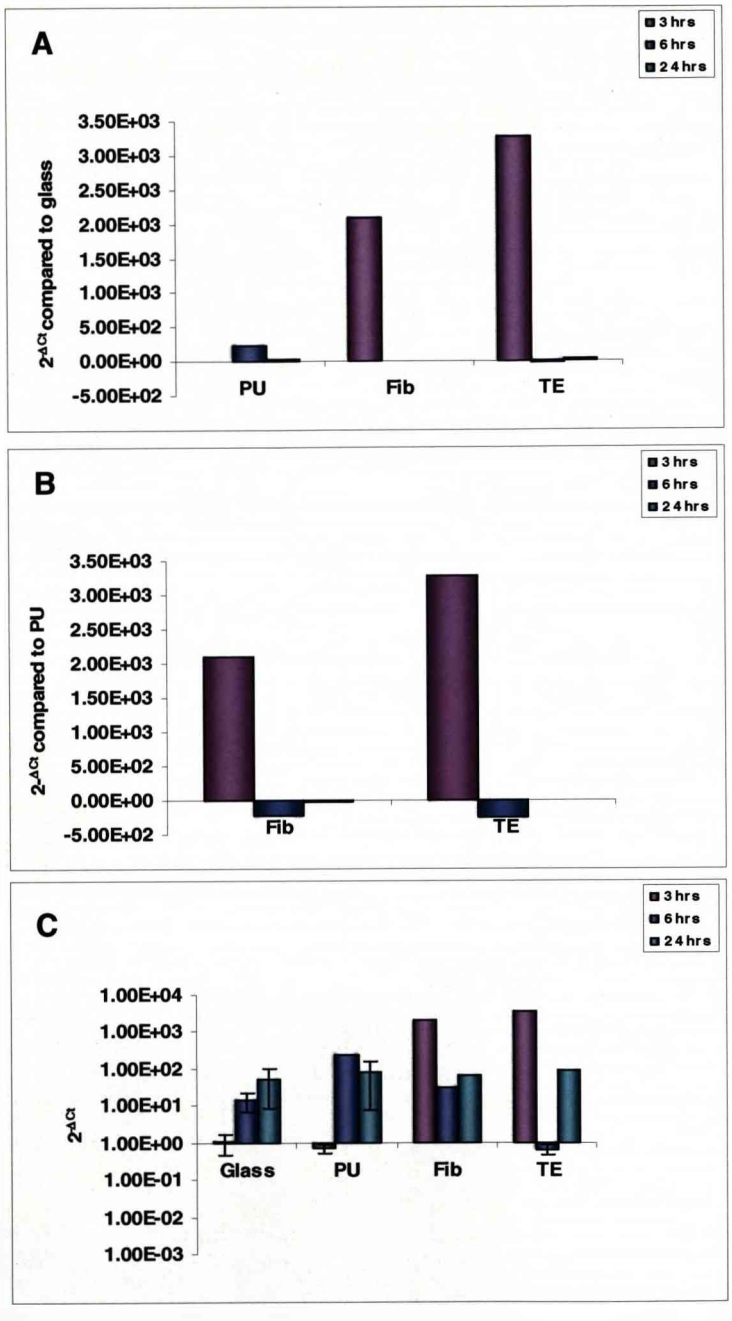

Figure 40 – Smooth muscle α-actin mRNA levels compared to GAPDH expressed by cells cultured on the plain polyurethane, fibrillin and tropoelastin surfaces compared to glass (A), cells cultured on fibrillin and tropoelastin surfaces compared to plain polyurethane (B) and the raw mRNA levels from cells cultured on the glass, polyurethane, fibrillin and tropoelastin surfaces (C)

On plain polyurethane smoothelin mRNA expression was similar to glass at 3 hrs and then increased up to a high level of expression at 24 hrs (Figure 41, Image A). On the fibrillin-modified surface smoothelin gene expression was higher than on glass and polyurethane at 3 and 6 hrs but then lower at 24 hrs (Figure 41, Images A and B). Cells cultured on the tropoelastin produced more smoothelin mRNA than those on glass and polyurethane at 3 hrs but less at 6 hrs (Figure 41, Images A and B). Expression on tropoelastin was higher compared to glass at 24 hrs but lower compared to polyurethane.

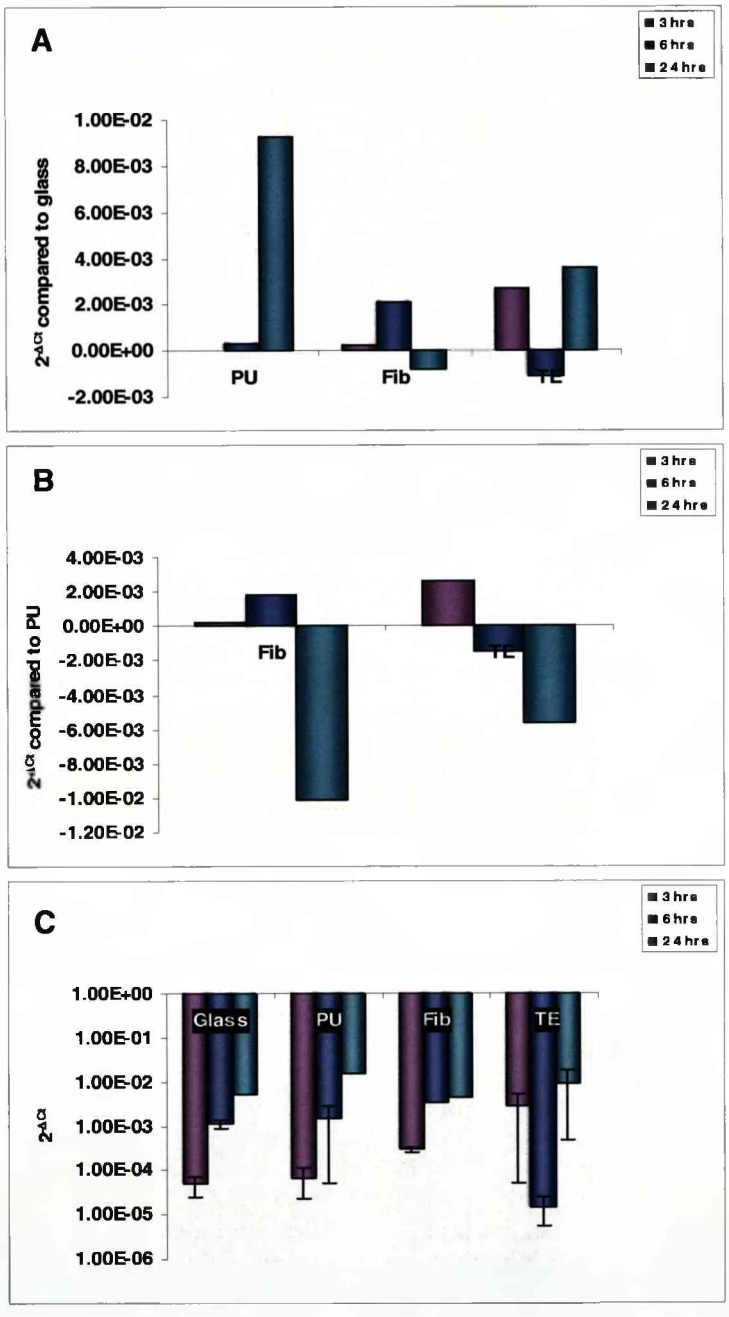

Figure 41 – Smoothelin mRNA levels compared to GAPDH expressed by cells cultured on the plain polyurethane, fibrillin and tropoelastin surfaces compared to glass (A), cells cultured on fibrillin and tropoelastin surfaces compared to plain polyurethane (B) and the raw mRNA levels from cells cultured on the glass, polyurethane, fibrillin and tropoelastin surfaces (C)

3.8 – Immunostaining

3.8.1 – Cell-matrix Adhesions

The amount and distribution of various integrins was investigated by staining for the α_5 and β_1 sub-units and the $\alpha_v\beta_3$ integrin. Figure 43 shows the presence of the α_5 sub-unit on all cells. It can be seen around the nucleus of the cells cultured on all of the surfaces. The cells grown on the modified surfaces have a more organised distribution of the sub-unit. It is highly concentrated at the periphery and is apparent in the many filopodia extending out from the cells. Similar results can be seen in Figure 44 where the green stain represents the β_1 integrin sub-unit. There is a high concentration of stain around the cell nuclei on all surfaces. On the glass, fibrillin and tropoelastin surfaces elongated structures can be seen to extend out from this area of high concentration. These are not as evident on the plain polyurethane surface.

Figure 45 shows positive staining for the integrin $\alpha_v\beta_3$. In contrast to the α_5 and β_1 sub-units this integrin is not seen at a high concentration around the nucleus. The cells cultured on the glass and plain polyurethane surfaces show a diffuse distribution of the integrin with brighter areas of staining at the edges of the cells. Cells on the modified surfaces exhibit strong staining along the cell periphery and extensive filaments are visible leading out from the cells. These filaments are particularly clear on the tropoelastin surface where they extend over a large area around the cell body.

Cells cultured on each surface were also stained for vinculin. This is a molecule found in focal contacts. If the staining was positive then short lines would be seen

aligned at the edges of the cells. Figure 46 shows a lack of any structures resembling these on the cells on all of the surfaces. Any staining is highly diffuse and the fluorescence could easily be due to non-specific binding of the primary antibody. Figure 42 shows a control image where the same staining protocol was used but the primary antibody was excluded. The image shows a low level of green fluorescence in the area of the cell cytoplasm. This represents non-specific binding of the AlexaFluor488 secondary antibody.

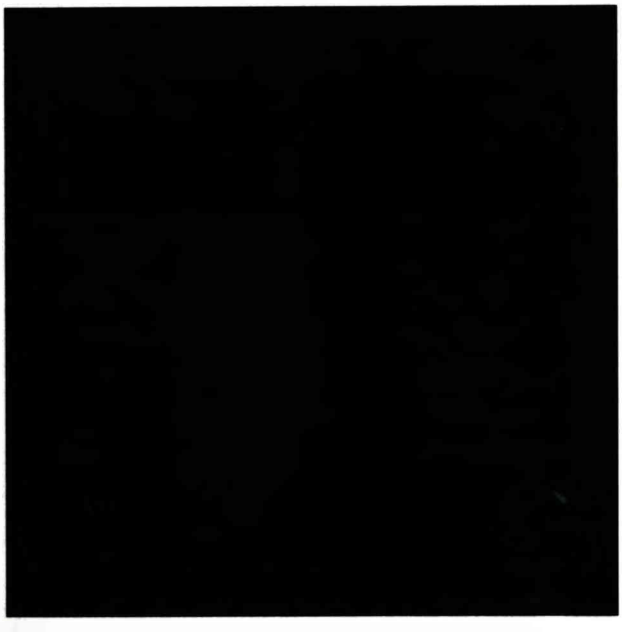

Figure 42 – SMCs stained using the protocol followed for all of the immunofluorescence tests but with the primary antibody excluded

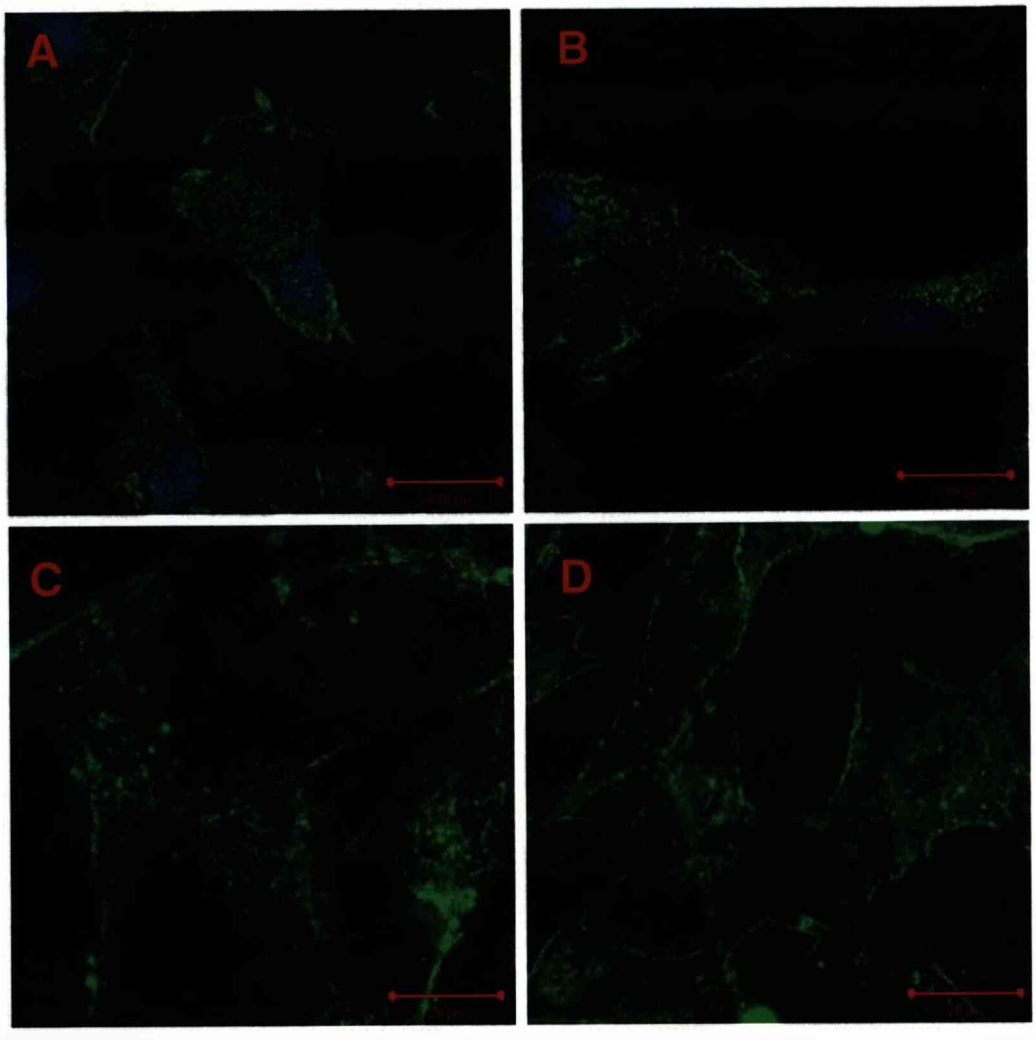

Figure 43 – SMCs stained for integrin α_5 after 2 days cultured on glass (A), PU (B), fibrillin PF14 (C) and tropoelastin (D) (Scale bars = 20μm)

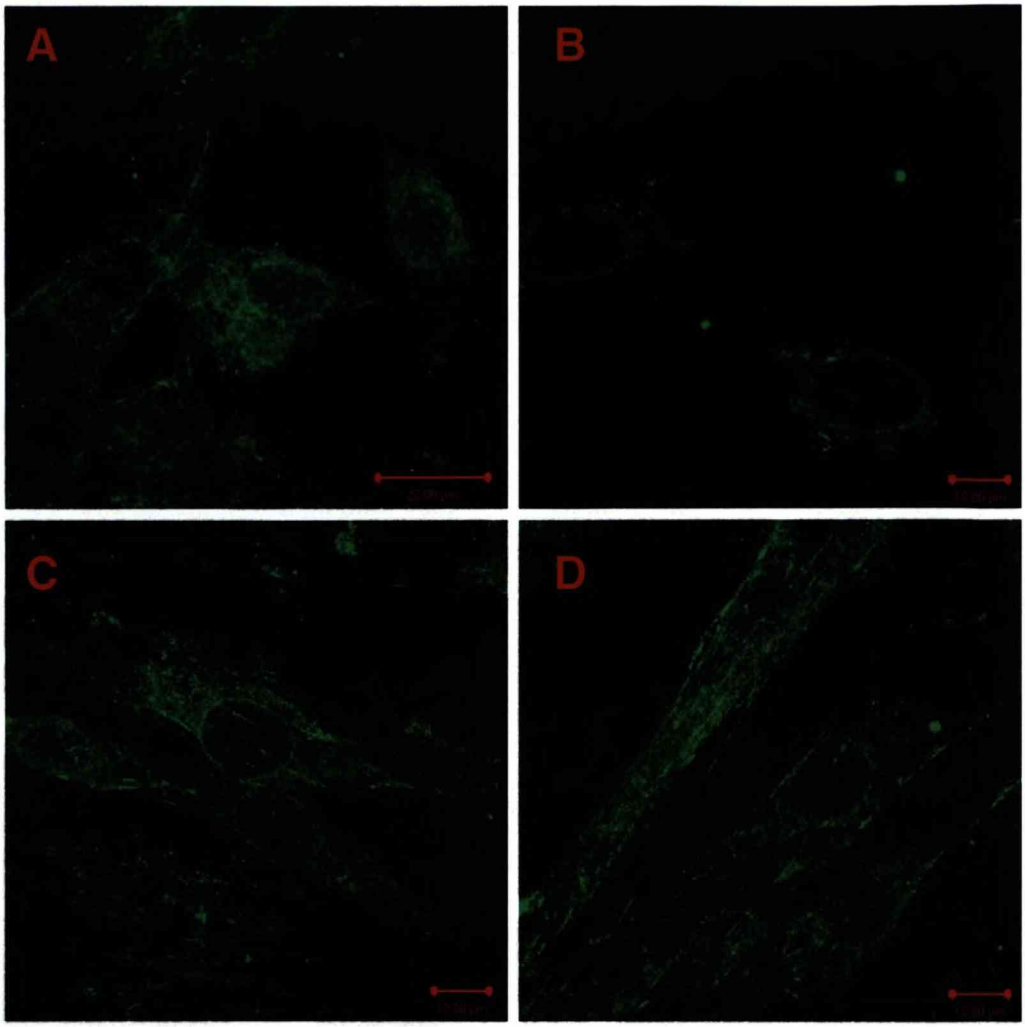

Figure 44 - SMCs stained for integrin β_1 after 2 days cultured on glass (A), PU (B), fibrillin PF14 (C) and tropoelastin (D) (Scale bars = 10μm apart from image A which is 20μm)

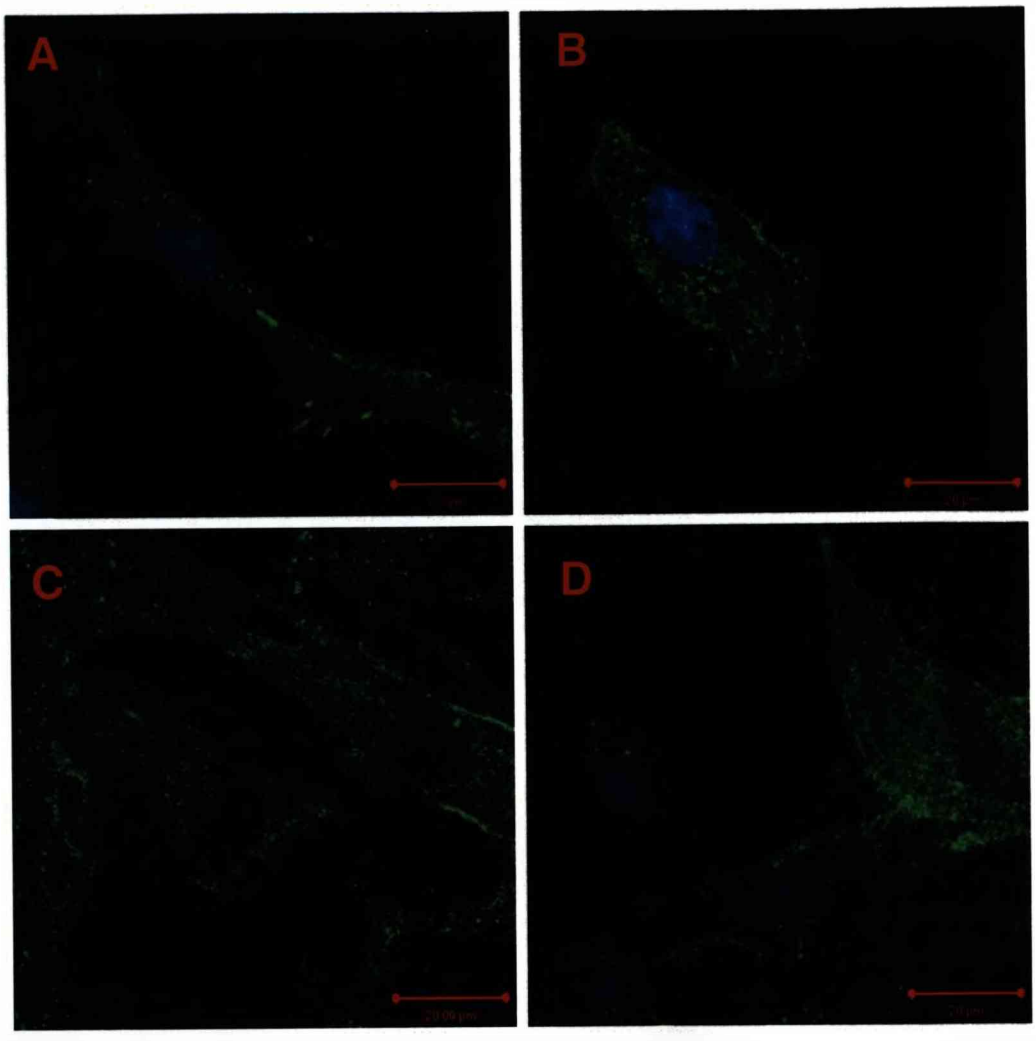

Figure 45 - SMCs stained for integrin $\alpha_v\beta_3$ after 2 days cultured on glass (A), PU (B), fibrillin PF14 (C) and tropoelastin (D) (Scale bars = 20μm)

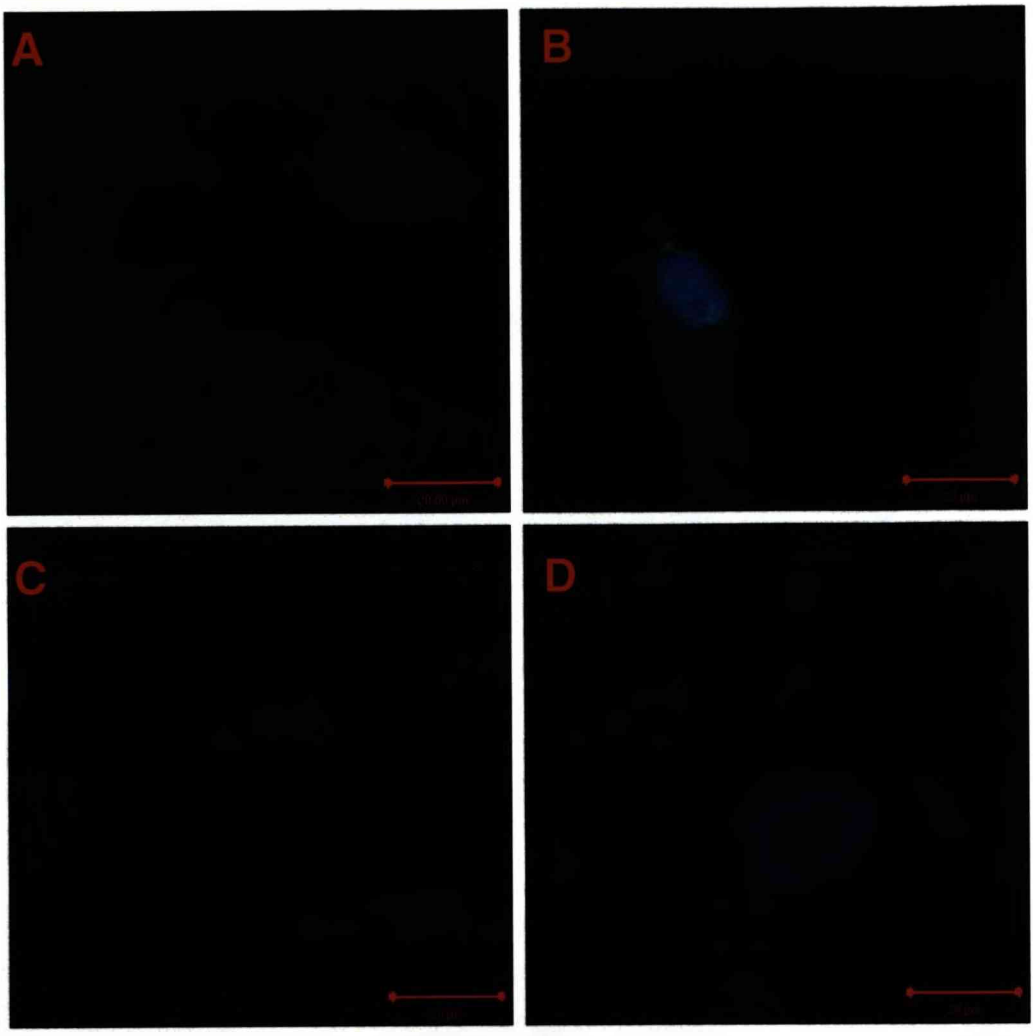

Figure 46 - SMCs stained for vinculin after 2 days cultured on glass (A), PU (B), fibrillin PF14 (C) and tropoelastin (D) (Scale bars = 20μm)

3.8.2 – Extracellular Matrix Proteins

The production of extracellular matrix proteins by cells cultured on the surfaces was investigated. The presence of laminin was found in the cells on all 4 surfaces. After 2 days bright green staining can be seen in the cytoplasm of each cell mostly around the nucleus (Figure 47). After 4 days the laminin is evident throughout the cells.

Figure 48 shows the presence of collagen IV in the cytoplasm of the cells cultured on all 4 surfaces. The amount of collagen is fairly low at both time points. It is evenly distributed throughout the cells with a speckled appearance.

The synthesis of fibulin-5 by the cells can be seen in Figure 49. After 2 days the protein can be seen mainly around the edge of the cells. After 4 days there appears to be a higher concentration in the cells cultured on the modified surfaces compared to the glass and plain polyurethane.

Figure 50 shows a high concentration of tropoelastin in the cells cultured on glass, plain polyurethane and fibrillin PF14. The staining is brightest around the nuclei but is present throughout the cells' cytoplasm.

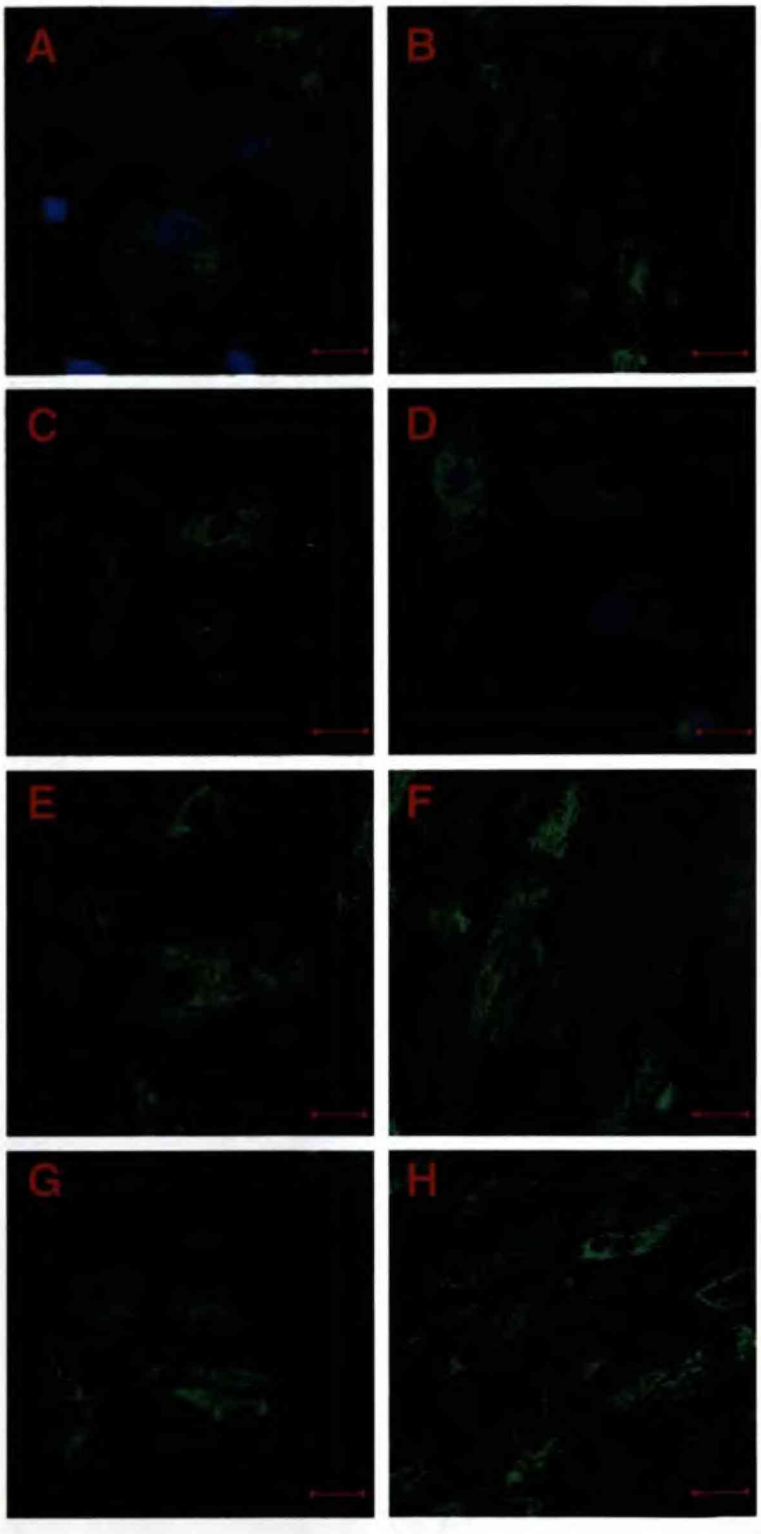

Figure 47 – SMCs stained for laminin after being cultured on glass (A,B), PU (C,D), fibrillin PF14 (E,F) and tropoelastin (G,H) for 2 days (A,C,E,G) and 4days (B,D,F,H) (Scale bars = 20μm)

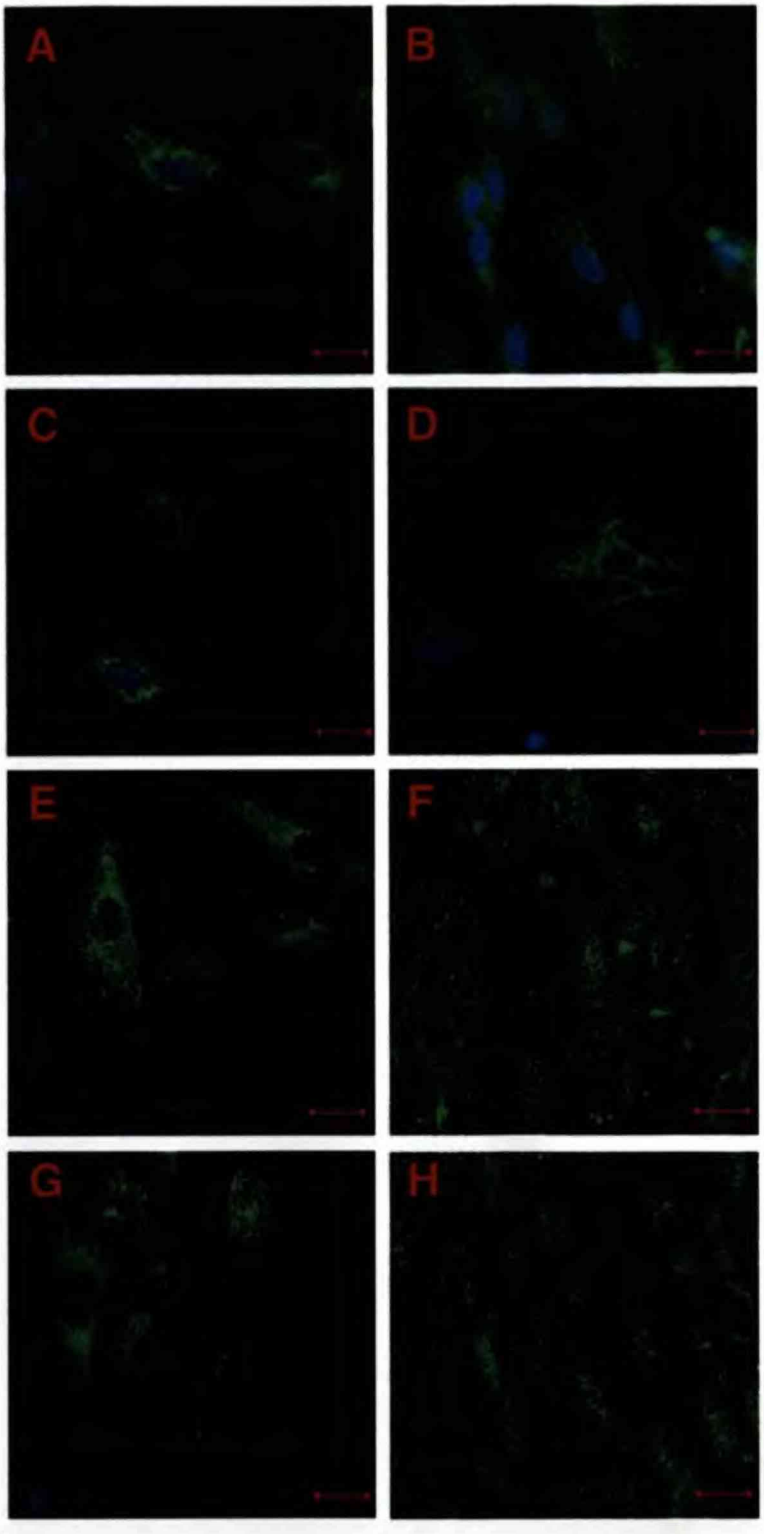

Figure 48 - SMCs stained for collagen IV after being cultured on glass (A,B), PU (C,D), fibrillin PF14 (E,F) and tropoelastin (G,H) for 2 days (A,C,E,G) and 4 days (B,D,F,H) (Scale bars = 20μm)

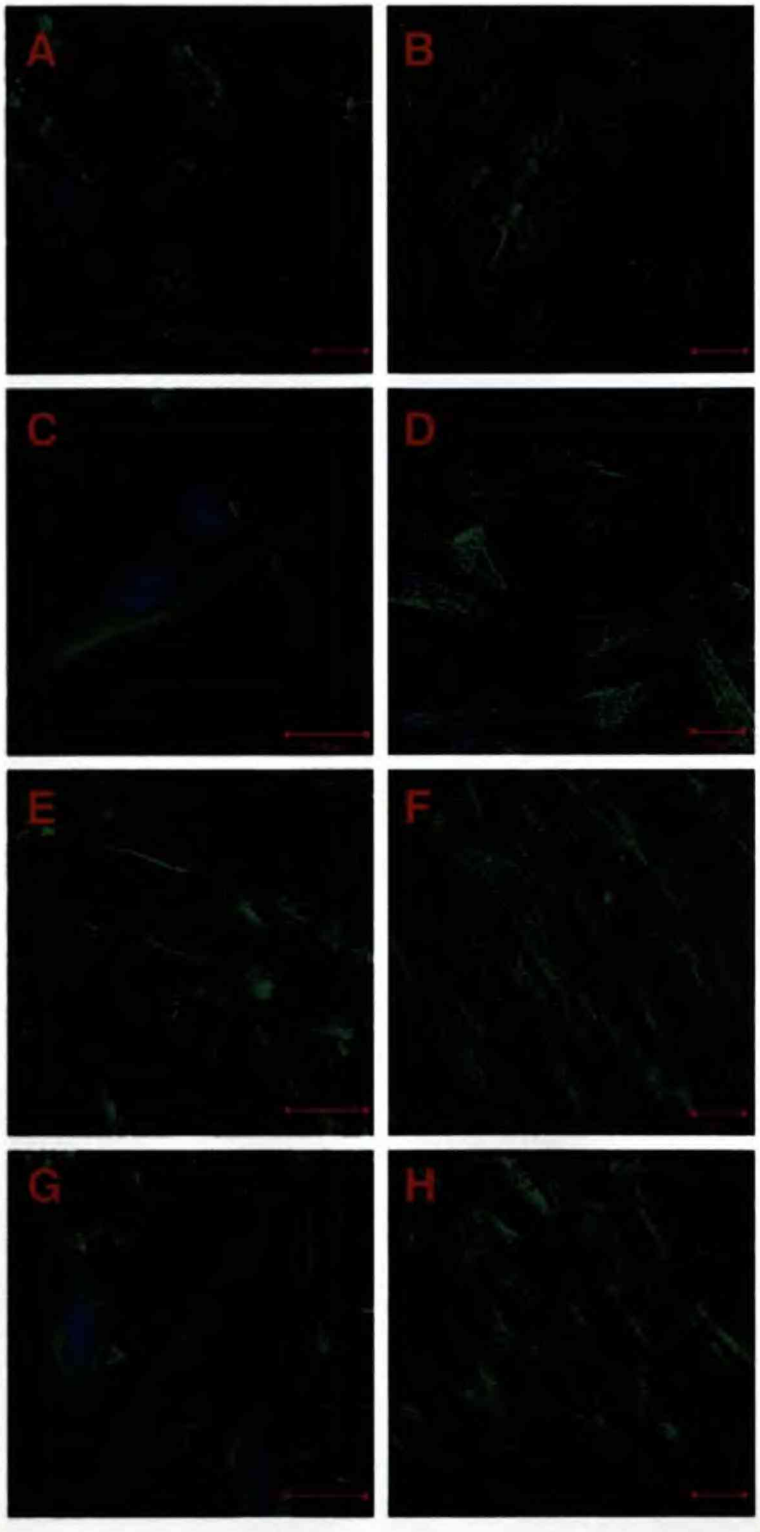

Figure 49 - SMCs stained for fibulin-5 after being cultured on glass (A,B), PU (C,D), fibrillin PF14 (E,F) and tropoelastin (G,H) for 2 days (A,C,E,G) and 4 days (B,D,F,H) (Scale bars = 20μm)

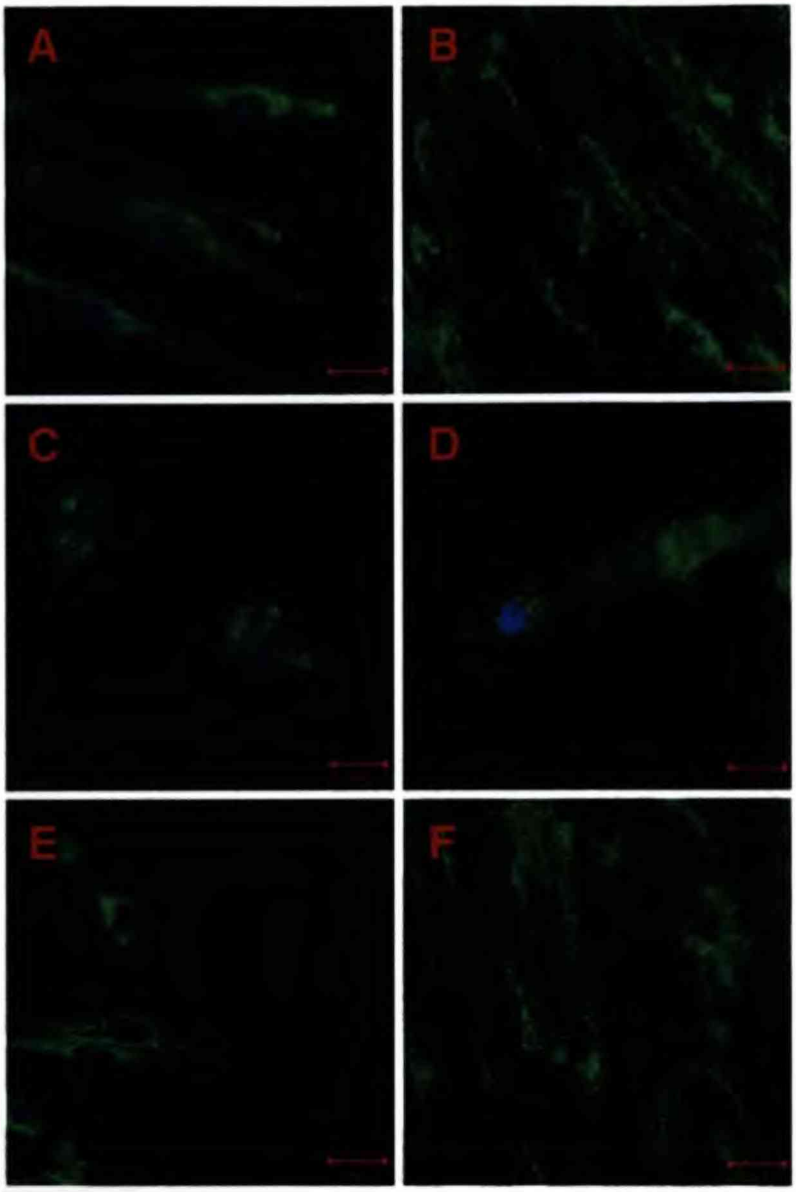

Figure 50 - SMCs stained for tropoelastin after being cultured on glass (A,B), PU (C,D) and fibrillin PF14 (E,F) for 2 days (A,C,E) and 4 days (B,D,F) (Scale bars = 20μm)

3.8.3 – Phenotypic Markers

Cells were stained for the SMC phenotype specific marker α-smooth muscle actin. Figure 51 shows the appearance of the cells after 4 days. Images A and B both show cells cultured on glass. Image A shows a cell with distinct actin fibres whereas image B shows both a cell with fibres and one without. The variation in these images is due to the high level of confluence of the cells after 4 days. The cell in image A and one of the cells in image B are growing on top of other cells whereas the cells in the other images are growing directly on the surfaces. The cells attached to the other surfaces show a diffuse pattern of smooth muscle actin and a clear lack of organised stress fibres.

Cells were also stained for smoothelin. This is a marker of the contractile phenotype of SMCs. Figure 52 shows that no cells stained positive for this protein on any surface.

Smooth muscle cells cultured on the surfaces were also stained for calponin and caldesmon. No cells stained positive for these proteins on any surface at either time point.

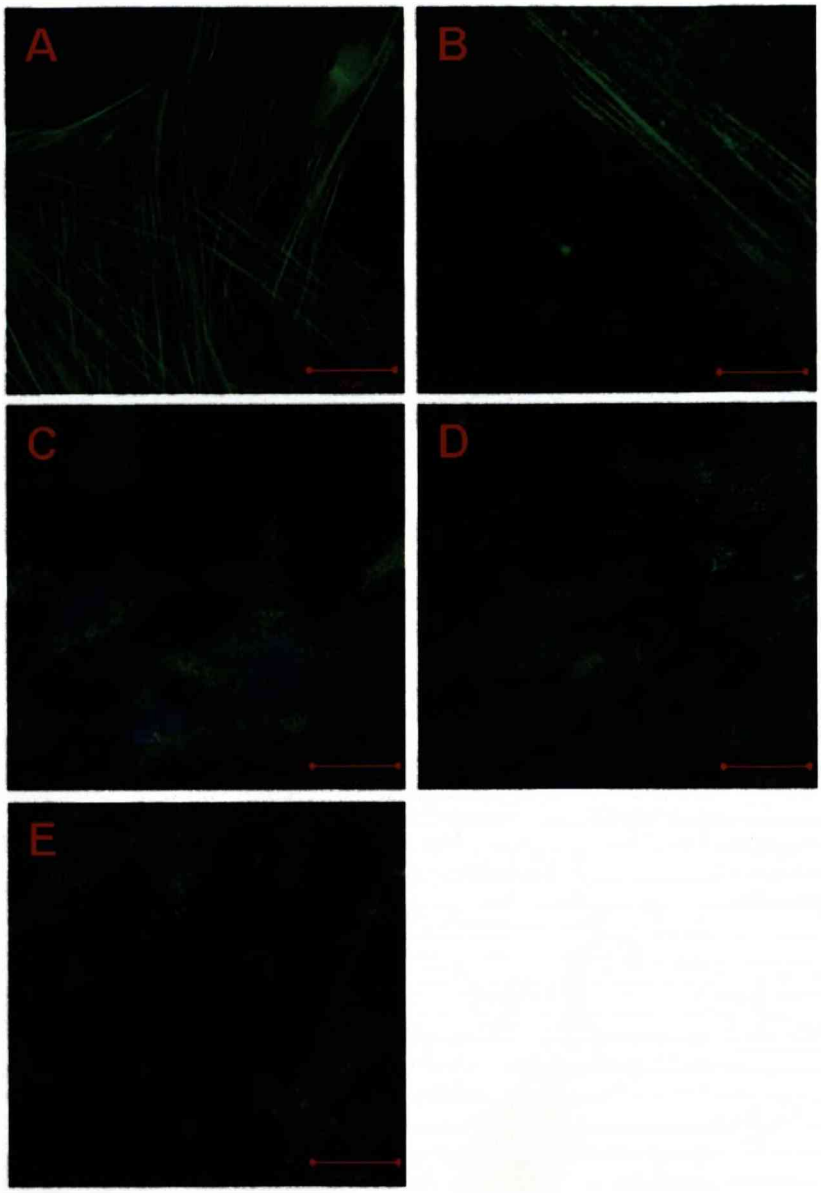

Figure 51 – SMCs stained for α-smooth muscle actin after being cultured for 4 days on glass (A,B), PU (C), fibrillin PF14 (D) and tropoelastin (E) (Scale bars = 20μm)

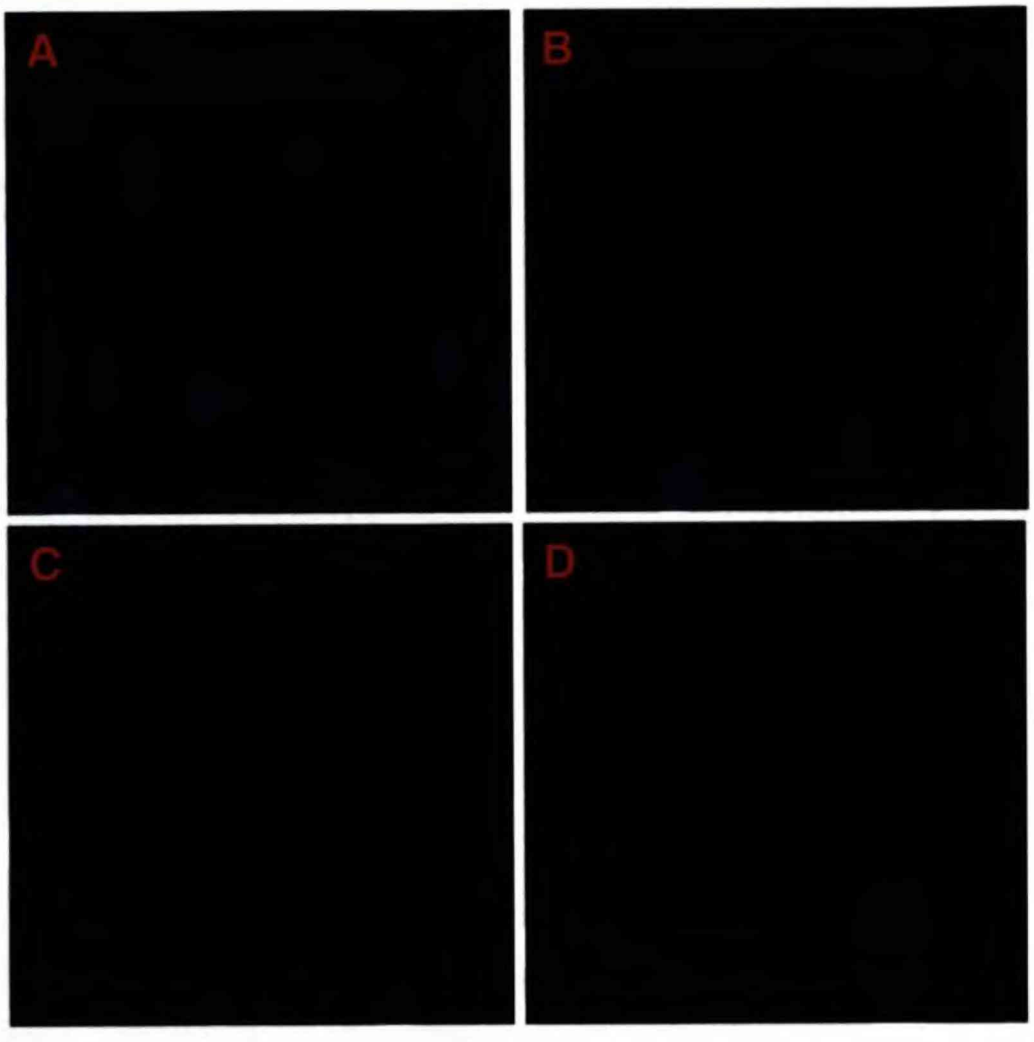

Figure 52 - SMCs stained for smoothelin after being cultured for 4 days on glass (A), PU (B), fibrillin PF14 (C) and tropoelastin (D) (Scale bars = 20μm)

3.9 – Western Blot Analysis

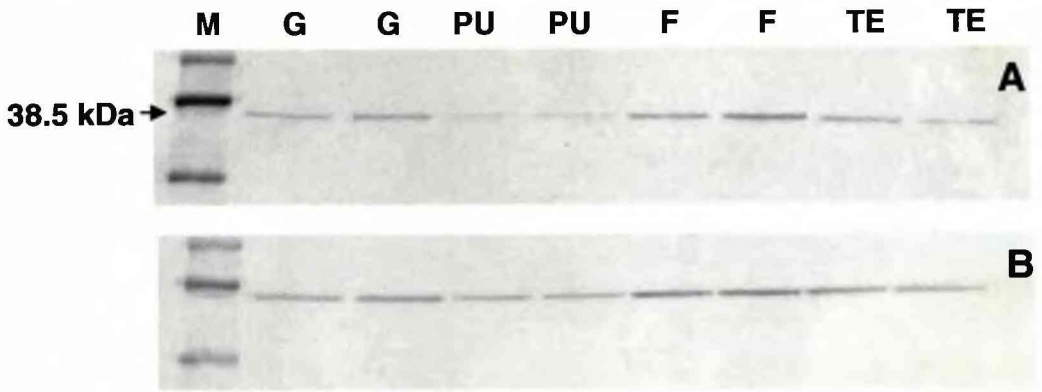

Figure 53 – Western blot gel images obtained from protein samples taken from SMCs cultured on glass (G), polyurethane (PU), fibrillin-modified polyurethane (F) and tropoelastin-modified polyurethane (TE) for 2 (A) and 4 (B) days. Samples were run against a molecular weight marker (M)

Levels of various proteins synthesised by cells cultured on glass, plain polyurethane and the modified surfaces were quantified using Western blotting. The gel images in Figure 53 show analysis of GAPDH production by cells cultured on glass (G), plain polyurethane (PU), fibrillin-1 PF14 (F) and tropoelastin (TE) after 2 and 4 days. Two repeats are shown for each surface. The production of laminin, collagen IV, fibrillin-1, tropoelastin, smooth muscle α-actin, smoothelin, calponin and caldesmon were also investigated, however there were no bands visible on the gels after processing.

Chapter 4 - Discussion

4.1 – Surface Modification

The initial functionalisation of the polyurethane was achieved by using a reversible swelling technique to incorporate polyethyleneimine (PEI) into the surface layer. This technique has been used to trap poly(ethylene oxide) and other water soluble polymers in the surface of scaffolds.[69-72,190] In previous work using a different polyurethane, the aminating solution consisted of 82% DMAc, 14.5% H_2O and 3.5% PEI.[187] In other unpublished work it was found that a similar degree of amination could be achieved on the b9 polyurethane used in this project by using a 1:1 ratio of DMAc to H_2O and 3.5% PEI. Using a lower amount of solvent is beneficial in that less of the polymer will be lost into solution. After the aminating treatment the presence of amine groups on the surface was verified using a solution of fluorescamine. This is a molecule that fluoresces on reaction with a primary amine. Figure 18 shows a higher level of fluorescence emitted from the aminated surface than the plain polyurethane surface ($p < 0.005$) indicating that there were a large number of primary amines present.

Dextran is a polysaccharide that has previously been shown to prevent protein adsorption and cell adhesion to surfaces to which it is attached.[48,191,192] The polysaccharide acts in a similar way to poly(ethylene glycol). It has highly hydrated,

randomly oriented chains, providing that it is pinned to the surface at a small number of points. Protein adsorption would compress the chains and restrict their motion therefore causing an unfavourable change in entropy.[193] Partially oxidised dextran was attached to the aminated polymer surface using a reductive amination reaction. The efficacy of this attachment was investigated using a dextran-FITC conjugate. This was partially oxidised and attached to the aminated surface in the same way as the un-conjugated dextran. To form a non-oxidised control, distilled water was added to the dextran in place of the $NaIO_4$ solution. Figure 19 confirms that only the oxidised dextran covalently attached to the aminated surface. The non-oxidised form was washed away as can be seen by the decrease in fluorescence with number of washes.

It is important that the properties of dextran that prevent protein adsorption and cell adhesion are not diminished by the immobilisation process. The polysaccharide needs to be oxidised enough to ensure adequate attachment. However, if too many reactive aldehyde groups are produced by the oxidation the dextran will be attached to the surface at so many points that the chains will be unable to hydrate and move sufficiently to prevent the adsorption of proteins. In order to determine the optimum level of oxidation, different concentrations of $NaIO_4$ were added to solutions of dextran and the pH of the solutions monitored over 24hrs. Figure 20 shows that the higher the concentration of $NaIO_4$ added to the dextran the lower the pH level reached. The drop in pH was due to the release of methanoic acid during the oxidation reaction. It therefore follows that the lower the pH the more aldehyde groups will be present on the dextran. Each of the solutions of oxidised dextran was

then added to aminated polyurethane surfaces with an equal volume of $NaCNBH_3$ and left overnight on a mechanical shaker. After washing, smooth muscle cells were cultured on each of the surfaces and images taken of them after 3 and 24 hrs. It is clear from Figure 21 that fewer cells appear to have adhered to the surface produced by the attachment of dextran oxidised by the 0.14M solution corresponding to an equal mass of $NaIO_4$. At low levels of oxidation it is likely that there weren't enough aldehyde groups formed to enable sufficient attachment of dextran to the surface. The polysaccharide would be removed during the washing step. This explains why cell attachment has not been discouraged. At the highest levels of oxidation there would be a large number of aldehydes and therefore the dextran would be attached to the surface at many points restricting its motion to the point where it is incapable of preventing cell attachment. It is also possible that at the highest $NaIO_4$ concentrations the majority of the monomeric units would undergo oxidation. This would completely disrupt the polysaccharide structure rendering it ineffective. Consequently an intermediate level of oxidation is necessary to ensure the effects of the dextran are optimised.

Further oxidation of the attached dextran layer with a dilute solution of $NaIO_4$ produced more aldehydes to which the proteins could be attached, again using a reductive amination reaction. The coupling occurs via amine groups present at the N-terminal of the protein or via lysine side chains. The ELISA that was carried out using the anti-fibrillin antibody confirmed the presence of the fibrillin fragment on the surface. Figure 22 shows that the higher the concentration of protein added to the oxidised dextran surface the higher the level of attachment was. This trend levels off

at higher concentrations of added protein. Doubling the concentration from 5μg/ml to 10μg/ml only gave a slight increase in protein attachment indicating that the surface was close to saturation and that were are no remaining aldehydes available for binding.

By using an anti-RGD antibody, the second ELISA showed that the RGD sequence was exposed on the protein surface and not concealed due to conformational restraints. This shows that the process of attaching the protein does not alter its conformation to the extent that the cell binding motif is unavailable for cell binding. The increase in the amount of RGD correlated with the increased concentration of protein added to the surface. Again this trend levelled off at the higher concentrations substantiating the idea that the surface was close to saturation at the 5μg/ml point (Figure 23).

ELISA assessment of the tropoelastin-modified surface using an anti-elastin antibody verified the presence of the protein. As in the case of the fibrillin fragment, an increase in the concentration of added protein resulted in an increase in the level of attachment. Figure 24 shows that an increase in tropoelastin concentration from 5μg/ml to 10μg/ml produced no increase in attachment. This suggests that the surface was saturated with protein when a concentration of 5μg/ml was used.

As the surfaces appeared to be saturated with protein when a concentration of 5μg/ml was used, this is the concentration that was used in all subsequent cell culture studies.

4.2 – Integrin Blocking Studies

Integrins are cell surface receptors that bind to extracellular matrix proteins (ECM).[194] They consist of two sub-units, an α chain and a β chain. In mammals 19 different α chains and 8 different β chains have been identified, these combine to make approximately 24 different integrins. They are transmembrane proteins that link the extracellular matrix to the cytoskeleton of the cell via various adapter proteins such as talin. Different integrins bind to different ECM ligands. The binding of an integrin to a ligand triggers intracellular signalling events that control functions such as cell migration, proliferation or differentiation.[195-200]

As mentioned in section 1.1.3.2.2, the amino acid sequence arginine-glycine-aspartic acid (RGD) has been shown to be a high affinity ligand for a number of cell-surface integrins. In this work an RGD-containing fragment of fibrillin-1 was covalently attached to a polyurethane surface. The mechanism of cell attachment to the modified surface was investigated. In order to determine the RGD-dependence of the attachment, the soluble peptide GRGDSPK was added to the cell suspension prior to seeding. This would bind to any integrins that recognise the RGD sequence therefore preventing them from binding to the protein on the surface. Figure 25 shows that cell attachment was diminished when the peptide was present in the cell suspension. This shows that cells bind to fibrillin-1 via the RGD motif. Increasing concentrations of added peptide caused further decreases in cell attachment by blocking increasing numbers of integrins. Percentage attachment compared to the control declined to around 20% at 10μg/ml indicating that cell attachment to the modified surface was

predominantly via the RGD motif contained within the protein. These results agree with previous work carried out on RGD-containing fibrillin-1 fragments adsorbed on to tissue culture plastic.[148,151]

These results also show that the RGD sequence was available at the protein surface and not concealed due to conformational restraints imposed by the surface modification procedure. By reacting the amino groups of the protein with the aldehyde groups on the surface it is possible that the intramolecular forces that control the protein conformation could be disrupted therefore altering the natural structure of the protein. As mentioned in the introduction, altering the conformation by just a tiny amount can alter the cell-binding properties of a protein. As similar results were obtained for the adsorbed and covalently attached protein it is clear that the modification protocol does not adversely affect the protein structure.

Various antibodies specific to certain integrins were utilised in order to determine which integrins were involved in SMC attachment to the fibrillin-modified surface (Figure 26). Each antibody was added to the cell suspension prior to seeding. The antibody would bind to the specific integrin preventing it from attaching to the fibrillin on the surface. Both the anti-α_v and the anti-β_3 antibodies caused a reduction in the number of cells attached to the modified surface after 1 hr compared to cells in the absence of any antibody. The anti-α_v antibody caused a reduction in cell attachment in a dose dependent manner with a decrease to approximately 35% at a 5μg/ml concentration of antibody. The anti-β_3 antibody caused a decrease to approximately 50% at all concentrations. This indicates that all of the β_3 integrin sub-

units may have been blocked at low antibody concentrations and so higher concentrations will have no additional effect. These results show that the $\alpha_v\beta_3$ integrin is involved in SMC attachment to the fibrillin-modified surface. Previous work has been carried out investigating cell attachment to fibrillin-1 PF14 adsorbed to tissue culture plastic[148,151] The results of that study agree with the results presented here and show that the $\alpha_v\beta_3$ integrin was involved in mediating cell attachment to the protein fragment.

The investigation into cell attachment to adsorbed fibrillin-1 PF14 also showed that the $\alpha_5\beta_1$ integrin was important. The α_5 sub-unit was blocked with an antibody prior to cell seeding. The results showed a higher decrease in cell attachment than when the $\alpha_v\beta_3$ integrin was blocked. To determine whether or not cell attachment to covalently bound PF14 was also mediated by the $\alpha_5\beta_1$ integrin, antibodies to the α_5 and β_1 sub-units were added to the cell suspension prior to seeding. It can be seen in Figure 26 that cell attachment was inhibited to a great extent by both of these antibodies with levels reaching approximately 20% of the control at 5μg/ml. It is clear that cell attachment to covalently attached PF14 was mediated by both the $\alpha_v\beta_3$ and the $\alpha_5\beta_1$ integrin but with the latter playing the most significant role. These results agree with the previous study on adsorbed PF14 and so it can be said that the covalent attachment procedure does not adversely alter the cell-binding properties of the protein.

It has previously been shown that for efficient cell attachment to both fibronectin and fibrillin-1 via the $\alpha_5\beta_1$ integrin a synergy site upstream of the RGD sequence is

necessary.[151,201-203] This site is thought to physically stabilise the binding between the integrin and the RGD motif. The PF14 fragment of fibrillin-1 has been identified as containing the synergy site. It is found within the 4 cbEGF-like domains upstream of the RGD motif. As it has been shown here in Figure 26 that the $\alpha_5\beta_1$ integrin is highly involved in the cell attachment to the PF14-modified surface it is clear that this synergy site is available on the surface in addition to the RGD sequence. This provides further evidence that the surface modification protocol does not change the protein conformation to any great extent.

Integrin-specific antibodies were used to investigate the mechanism of cell attachment to the tropoelastin-modified surface. In this instance an antibody to the $\alpha_v\beta_3$ integrin was used (LM609). The antibodies to the separate integrin sub-units were not used in this case due to the limited availability of the tropoelastin. Figure 27 shows a dose dependent decrease in cell attachment on the addition of the antibody to the cell suspension prior to seeding. Attachment was reduced by 50% at an antibody concentration of 5μg/ml. These results indicate that the $\alpha_v\beta_3$ integrin was involved in mediating cell attachment to the tropoelastin-modified surface. It should be noted that this particular experiment could only be carried out once, again due to the limited supply of tropoelastin. Previous work carried out on adsorbed tropoelastin has shown that there is an interaction between the $\alpha_v\beta_3$ integrin and an amino acid sequence located near to the C-terminus of tropoelastin.[167] The results presented here show that this interaction still occurs when the protein is covalently attached using this surface modification protocol.

The particular integrins that bind to proteins are of interest as they are responsible for triggering the intracellular signalling events that control the behaviour of the cell. As different integrins bind to different ligands, these signalling events will vary depending on the protein that the cell is in contact with. It is therefore possible that cell behaviour could be controlled by exposing the cells to a specific protein-covered surface. In this work the involvement of the $\alpha_v\beta_3$ integrin in cell attachment to both the fibrillin and tropoelastin surfaces has been shown. In addition to this, attachment to the fibrillin-modified surface was also seen to be mediated by the $\alpha_5\beta_1$ integrin. The subsequent behaviour of cells cultured on the modified surfaces will be discussed in the following sections however specific relationships between the different integrins and the intracellular signalling pathways have not been established during this project.

4.3 – Methylene Blue Staining

Smooth muscle cells were seeded on to plain and fibrillin PF14-modified polyurethane surfaces in serum-free medium. They were cultured for 3 hrs and then washed, fixed and stained with methylene blue. This is a stain that, at neutral pH, dyes the entire cell blue. It is evident from Figure 28 that the cells on the plain polyurethane surface did not adhere well. They attached only enough to prevent them being washed away in the staining procedure. There was no sign of the cells spreading out. This is due to a lack of protein for the cell surface integrins to bind to. The cells were unable to form focal adhesions and so remained rounded. In contrast

to this, cells on the fibrillin-modified surface attached and started to spread. The protein fragment on the surface allows for integrin binding and subsequent focal adhesion formation. This in turn enabled the cells to spread and adhere strongly to the surface.

Cells were also cultured on the surfaces for 24 hrs and 4 days. Again the cells were grown in serum-free medium for the first 3 hrs. However, after this time SMGS-containing medium was added to each well. Images C and D in Figure 28 show the cells' appearance after 24 hrs in culture. Cells on the plain polyurethane have now started to spread out. This was possible due to the adsorption of serum-proteins on to the surface from the growth supplement that was added at 3 hrs. The cells now have ligands for their integrins to bind to and so can form focal adhesions that enable the cells to spread out. Cells on the fibrillin-modified surface have spread out even more extensively. After 4 days a degree of proliferation has occurred on both surfaces. Cells on the plain polyurethane are well spread and the cell number is much higher than at 24 hrs. Cells on the fibrillin have proliferated to such an extent that cell-cell contact has caused them to align.

SMCs in vivo have a quiescent, contractile phenotype and a bipolar morphology. They do not proliferate or synthesise much extracellular matrix. In contrast, when the blood vessel is injured the disruption of the basement membrane causes a phenotypic transformation.[204] The cells become proliferative, start synthesising extracellular matrix and adopt a fibroblast-like morphology.[172-175,205] As mentioned in section 1.2.4, SMCs harvested from blood vessels and cultured in vitro also undergo this

phenotypic change, their morphology and proliferative capacity change rapidly in the unnatural environment.

In the images in Figure 28 the SMCs have a fibroblast-like morphology and are able to proliferate. It is clear that the cells cultured on both the plain and modified surfaces had a synthetic phenotype rather than a contractile one. The protein-modified surface was unable to maintain the cells in their in vivo phenotype even though fibrillin is a natural component of the artery wall. This is not unexpected as the fibrillin fragment contains cell-binding sequences similar to fibronectin and vitronectin. SMCs cultured on both of these proteins have been shown to rapidly adopt a synthetic phenotype.[3,129,206,207] It has also been shown that freshly isolated SMCs cultured on surface-immobilised GRGDSC peptide undergo a phenotypic transformation from contractile to synthetic.[208] It is therefore possible that binding of integrins to the RGD sequence triggers an intracellular signalling pathway that results in this modulation of SMC phenotype.

In the context of an artificial vascular graft, the proliferation of the SMCs would be necessary to form a confluent layer on to which endothelial cells could be seeded. However, this growth would need to be controlled to prevent vessel occlusion. The attachment of the fibrillin fragment alone would not be suitable as a surface modification. Other methods would need to be employed in order to gain the required level of control over the cells.

Separately, cells were seeded on to plain and tropoelastin-modified polyurethane. They were then stained with methylene blue at 3 hrs, 24 hrs and 4 days. As before, the cells were cultured in the absence of serum for the first 3 hrs. After this time cells on both surfaces had started to attach but very little spreading was evident (Figure 29). The appearance of the cells was similar on both the plain and modified surfaces. The lack of cell spreading on the polyurethane is likely due to the lack of protein ligands available for the cell-surface integrins to bind to. The presence of the tropoelastin on the modified surface would provide many potential ligands available to the cells. However, it is clear that these particular areas of the protein do not encourage cell adhesion to any great extent. After 24 hrs the cells on both surfaces were well spread. As before this is most likely due to the serum proteins adsorbed on to the surface from the SMGS-containing culture medium added after the first 3 hrs. It is also possible that cells on the tropoelastin needed more time to start spreading. The quality of the attachment at 3 hrs may not have been adequate for the cells to form strong focal adhesions. The extra time up to 24 hrs could have been necessary for these adhesions to form to allow the cells to adhere strongly and spread out. It is also possible that growth factors present in the SMGS-containing medium encouraged the cells to spread. To elucidate the reason for the poor spreading on the tropoelastin surface it would be necessary to culture the cells in the absence of serum for a slightly longer time period. Images E and F in Figure 29 show the cells after 4 days in culture. A small increase in cell number can be seen on both surfaces but it is clear that cell proliferation has not been encouraged by either surface.

The SMCs had a fibroblast-like morphology from 24 hrs onwards. Although their rate of proliferation was low it was clear that they had adopted a synthetic phenotype rather than a contractile one as on the fibrillin-modified surface. If the cell attachment that occurred was due to adsorbed serum proteins then it is likely that cell-surface integrins had bound to the RGD sequences present in the fibronectin and vitronectin. As discussed in section 4.3.1, binding to an RGD motif may trigger an intracellular signalling pathway that results in a synthetic phenotype being achieved.

The effects of the two proteins on the behaviour of the cells were very different. The fibrillin fragment allowed cells to attach and spread, even in the absence of serum. Conversely, cells on the tropoelastin didn't start to spread until after the 3 hr time point. Whether this was due to the addition of serum or to inadequate adhesion at this time is not clear. The difference in growth rate is unlikely to be due to the cells on the tropoelastin having a more contractile phenotype than those on the fibrillin as the morphology of the cells appears the same. It is more likely to be due to less efficient binding of the cells to the tropoelastin.

4.4 – Cell Spreading

When cells first attach to a surface they are rounded. The cells produce filopodia that then bind to the surface via integrins if suitable protein ligands are available. The bound integrins then cluster together followed by the formation of focal adhesions. These in turn cause organisation of the cytoskeleton that they are attached to and then

extension of the cell membrane. In order for cells to spread significantly efficient binding of the integrins to the extracellular matrix is necessary.

In this study the degree of cell spreading was estimated using image analysis. SMCs were cultured on the plain and modified surfaces for 3 hrs in serum-free medium. The cells were then fixed and stained with methylene blue. Images of the cells were taken using a digital camera attached to a light microscope. The number of cells in each field of view was counted and the total area taken up by cells was determined. The degree of cell spreading was calculated as the area divided by the cell number. This technique only gives an estimate of the average degree of spreading due to the sensitivity of the image analysis software. The areas at which the cells are most spread appear almost transparent and so are not recognised by the programme. To reduce the error caused by this, gaps within the cells were filled in, first by the software and then manually. This meant that a more complete cell image would be measured. A large number of images were analysed to reduce the error as much as possible and a number of sample repeats were used.

There was a highly significant difference in the level of spreading of the cells on the plain polyurethane compared to those on the fibrillin-modified surface (Figure 30). Cells seeded on to the modified surface had an average area per cell of around $720\mu m^2$ whereas cells on the plain surface only reached around $160\mu m^2$. The cells were unable to form adequate focal contacts on the plain polyurethane and so are unable to spread. It is clear that the cells on the fibrillin-modified surface had successfully formed strong focal contacts and these allowed the necessary

cytoskeletal organisation to take place for the cells to spread. The strength of these contacts is the result of strong binding of cell-surface integrins to various amino acid sequences found on the surface of the protein, including the RGD motif. Even though there are errors associated with this method of measuring the degree of spreading of the cells the results can be taken to be fairly accurate as they are backed up by the images in Figure 28. The images of the cells cultured on the surfaces for 3 hrs in serum-free medium show rounded cells on the plain polyurethane and well-spread cells on the fibrillin-modified surface.

Cells cultured on the tropoelastin surface did not spread well. They were even less spread out than on the plain polyurethane (Figure 31). It is clear that the tropoelastin on the surface did not encourage focal contact formation and so the cells were unable to organise their cytoskeleton in order to spread. The images in Figure 29 show the cells after 3 hrs in serum-free medium. These cells were rounded and not at all spread out, which agrees with the quantitative data. As mentioned in section 4.5.2 it is possible that cell attachment to the tropoelastin surface may be weak at the 3 hr time point and more time may be needed to form adequate focal adhesions.

The fibrillin and tropoelastin surfaces have very different effects on the behaviour of the smooth muscle cells. The fibrillin-modified surface caused the cells to spread out extensively in the 3 hr time period whereas on the tropoelastin-modified surface the cells remained rounded. These quantitative results are supported by the images in Figure 28 and Figure 29. The presence of cell binding sequences on the fibrillin surface enabled the SMCs to form strong focal contacts. Any cell-binding sequences

on the tropoelastin surface only formed weak interactions after 3 hrs and therefore the cells were unable to spread.

4.5 – Cell Proliferation

In order for SMCs to proliferate they need to be attached and spread out on the surface on which they are cultured. There are two main reasons for this. The first is the flattened shape of the cell. The more spread the cell is, the greater proportion of growth factor receptors are available on its surface. It has been shown that the height of a cell is inversely proportional to the amount of DNA synthesis that occurs in the cell cycle.[209] The second reason for the dependence of the cells on anchorage to a surface is the need for integrin engagement. The integrins bind to the ECM and become clustered. They then associate with a signal transduction complex that initiates actin filament formation and cytoskeletal organisation.[197] Integrin association with this signal transduction complex causes phosphorylation of various protein tyrosine kinases such as focal adhesion kinase (FAK). This activates a series of events that leads to DNA replication and cell division.[210,211] Without this integrin initiated signalling cascade cells are unable to undergo mitosis.

There is increasing evidence that integrins and growth factor receptors have a synergistic relationship. The binding of growth factors to their relevant receptors and the binding of integrins to the ECM are both required for the cell cycle to progress.[196,197,199,212] Various integrins have been shown to associate with growth

factor receptors and subsequently activate them. The $\alpha_v\beta_3$ integrin has been co-immunoprecipitated with the PDGF receptor and the β_1 integrin sub-unit has been co-immunoprecipitated with the EGF receptor.[200,213]

This shows that in order for cell proliferation to occur it is necessary for the cells to attach to the ECM via their integrins and subsequently spread out to maximise the number of growth factor receptors available for binding.

The proliferation of the SMCs cultured on plain and modified surfaces was assessed by estimating the cell number using DNA quantification. Cells were cultured in serum-free medium for the first 3 hrs. At this time point there were approximately 4 times more cells present on the fibrillin PF14 surface than on the plain polyurethane (Figure 32). This quantitative result agrees with the images taken of methylene blue stained cells after 3 hrs (Figure 28). The initial attachment of the cells was more efficient on the modified surface due to the presence of integrin-binding amino acid sequences present on the protein. The plain polyurethane had no protein due to the lack of serum in the culture medium and so cell attachment is very poor.

After the initial 3 hrs of culture the medium was replaced with SMGS-containing medium. The DNA was then quantified at the 1 day time point. The average number of cells had increased on both surfaces. As mentioned at the start of section 4, for cell growth to occur it is necessary for the cells to be attached to the surface via integrins and for the cell to be well spread. By again referring to the images in Figure 28 it can be seen that cells on both the plain and modified surfaces are well spread and so able

to proliferate. The spreading of cells on the plain polyurethane is possible due to adsorption of serum proteins from the culture medium. The cells can attach to these proteins via their integrins and therefore spread out and proliferate. There were more cells on the fibrillin surface than the plain surface as was seen at 3 hrs. However, the rate of proliferation of the cells on the plain surface was higher than on the modified surface (Figure 33). After 1 day there were only 2.5 times more cells on the fibrillin compared to 4 times more at 3 hrs. This could be due to the limited amount of growth factors and nutrients available in the medium. The greater number of cells on the fibrillin samples would use up the growth factors and nutrients a lot faster than the limited number of cells on the plain surface. These would have an excess and so be able to proliferate at a higher rate.

At the 2 day time point a higher rate of proliferation on the plain surface was again evident. This can be seen by comparing the cell number to the number of cells attached at the 3hr time point (Figure 33). By 4 days the cells were proliferating at a even higher rate on the plain polyurethane surface. The lower proliferation rate on the modified surface is again likely due to nutrient availability. As can be seen from the methylene blue images in Figure 28 the cells were almost confluent at 4 days on the fibrillin-modified surface and the quantitative data in Figure 32 shows that there were almost twice as many cells on this surface than on the plain polyurethane. This dramatic difference in cell number would result in a large difference in growth factor and nutrient availability. Another reason for the lower proliferation rate on the fibrillin is the change in cell shape caused by cell-cell contacts at this level of confluency. At this point the cells had become aligned and bi-polar. They were a lot

less well spread and so will have fewer growth factor receptors available for binding. This would reduce their rate of cell division.

It is clear that the fibrillin greatly enhanced the attachment and spreading of the cells in the absence of serum proteins. However, the cells were able to attach just as well to adsorbed serum when it is added. Two of the main serum proteins are fibronectin and vitronectin. Both of these contain RGD motifs as in the fibrillin fragment. These enable the cells to bind to the plain surface via their integrins and form strong focal adhesions. There doesn't appear to be any effect on the rate of cell proliferation by the fibrillin.

After 3 hrs in serum-free medium there were 1.7 times more cells attached to the tropoelastin surface than the plain polyurethane (Figure 34). The addition of SMGS-containing medium at this time did not encourage any cell proliferation on the tropoelastin surface but the number of cells on the plain surface doubled in the 24 hr time period. This put the cell number as almost identical after 1 day of culture. By referring to the images in Figure 29 it can be seen that the cells are still rounded after 3 hrs but have spread considerably by 24 hrs. This lack of spreading explains why the cells were unable to proliferate. They had not formed adequate focal adhesions and so could not initiate signal transduction in order to progress the cell cycle to the mitosis stage.

At 2 days there was a slight increase in cell number, this appeared greater on the tropoelastin but the difference between the surfaces was not statistically significant.

The rate of proliferation between 2 and 4 days was fairly high with the cell number increasing by 2.7 and 1.9 times in that period on the plain and tropoelastin-modified surfaces respectively. The rate of proliferation on the plain surface was much higher by the 4 day time point (Figure 35). The cells were well attached to the serum protein-coated plain polyurethane and so were able to proliferate extensively. Conversely, cells on the tropoelastin-modified surface were less able to grow, possibly due to less efficient adhesion.

These results show further evidence that the fibrillin and tropoelastin surfaces have very different effects on the behaviour of the SMCs. The fibrillin-modified surface caused the cells to proliferate extensively whereas the tropoelastin did not support any cell growth until SMGS-containing medium was added. These quantitative results are again supported by the images in Figure 28 and Figure 29. The presence of cell binding sequences on the fibrillin surface enabled the SMCs to form strong focal contacts and trigger signalling pathways that caused the cells to divide. Any cell-binding sequences on the tropoelastin surface only formed weak interactions and therefore the cells were unable to trigger the intracellular signalling pathways necessary for cell proliferation.

4.6 – RT-PCR

RNA was harvested from SMCs cultured on plain and modified polyurethane for 3 hrs, 6 hrs and 24 hrs. The mRNA was reverse transcribed to synthesise cDNA. This

was then analysed using PCR to determine whether or not the surfaces had an effect on the expression of various genes. Levels of expression were normalised to the house keeping gene for GAPDH.[214-216] The house keeping gene β-actin could not be used in this case as its levels change depending on the SMC phenotype.[181]

There are a number of limitations of using this technique to analyse cells that have been cultured on specific surfaces. It is impossible to know at what point after cell seeding any alteration in gene expression will occur at and how long it will last. The surface will alter gradually as the cells secrete their own matrix and so it is likely that gene expression will change at the same time. A large number of time points would have to be investigated to obtain more accurate results. This would push the number of samples to be processed to an impractical level. Due to the extensive processing required to synthesise the cDNA the number of sample repeats needs to be kept relatively low. This causes large standard deviation values. As can be seen in figures 36-41 (Images A) some of these values are higher than the mean and therefore cannot be plotted on the log scale. This illustrates the unreliability of the data and should be taken into account when inferring conclusions from the results. The short time periods investigated in this study were chosen as in the first few hours of culture any effect of the modified surfaces is more likely to be seen. At longer time points the cells will have started to produce their own matrix and so the effects of these proteins will also have an effect on the gene expression.

The expression of laminin mRNA by cells cultured on the plain polyurethane, fibrillin and tropoelastin-modified surfaces was compared to that of cells cultured on

glass (Figure 36, Image A). The cells on the plain polyurethane expressed a similar level to those on glass at 3 hrs but this increased at 6 and then 24 hrs. This may be due to an increase in protein adsorption from the culture medium allowing the cells to adhere to the surface more strongly. This in turn may induce the intracellular signalling pathways required for protein production. The serum proteins are likely to have adsorbed to the glass and polyurethane in slightly different conformations due to the different surface properties. It is therefore possible that the cells attached more strongly to the polyurethane compared to the glass. This may account for the difference in laminin gene expression. However, it can be seen in Figure 36, image C that the standard deviation of the data at 6 and 24 hrs is very high and this should be taken into account when drawing conclusions.

On the fibrillin-modified surface cells expressed slightly more laminin mRNA than those cultured on glass at all time points. Again, image C in Figure 36 shows a large standard deviation of the data at 24 hrs and at 3 and 6 hrs it was higher than the mean value and so could not be plotted on the graph. This conclusion is therefore unreliable. Laminin gene expression by cells cultured on the fibrillin surface was also compared to that by cells on the plain polyurethane (Figure 36, Image B). mRNA levels decreased on the fibrillin compared to the polyurethane over the 24 hrs. This is caused by a rise in expression on the polyurethane with little change on the fibrillin. However, due to the large standard deviation demonstrated in Figure 36, image C, this trend is unlikely to be accurate.

The expression of laminin mRNA by cells cultured on the tropoelastin-modified surface varied considerably with a higher level than on glass and plain polyurethane at 3 and 24 hrs but a lower level at 6 hrs (Figure 36, Images A and B). This may be due to the changing surface as the cells secrete their own matrix. It is also possible however that experimental error could be the cause and further repeats of the experiment would elucidate this. In this case repeats were not possible due to availability of samples.

The gene expression for elastin showed a similar trend to laminin (Figure 37). Cells on the plain polyurethane surface expressed gradually more mRNA over the 24 hrs. Again this may be due to increasing amounts of adsorbed protein and also variations in the conformation.

On the fibrillin-modified surface, elastin expression decreased over the 24 hr time period. By noting the scales of these graphs (Figure 37) it can be seen that the changes were extremely low and combining this information with the large standard deviation of the data in image C means that any difference to the glass or polyurethane surface is unlikely to be significant.

Elastin gene expression by cells on the tropoelastin-modified surface was higher than on glass and polyurethane at 3 and 24 hrs but lower at 6 hrs. These differences could be due to the constantly changing surface as the cells adhere and secrete proteins. The scales of the graphs demonstrate that these differences were fairly small and so the

observed changes may be due to experimental error caused by the large number of steps needed to prepare the cDNA and the subsequent processing for the PCR.

The levels of fibrillin-1 mRNA appear to be a lot lower on all of the surfaces compared to glass and polyurethane at 3 hrs (Figure 38, Images A and B). Again, the scale of the graphs shows that the differences are in fact extremely small. On plain polyurethane the expression at 6 hrs was similar to glass and by 24 hrs it was almost identical. This was the same for cells cultured on the fibrillin-modified surface. On the tropoelastin surface the cells expressed more fibrillin-1 mRNA than on glass at 6 hrs but this decreased to a level similar to it at 24 hrs. Expression was almost identical to glass and polyurethane on the modified surfaces at 24 hrs indicating that the type of surface had very little effect on fibrillin-1 gene expression.

As a general indicator of protein synthesis the levels of fibronectin mRNA were measured. SMCs with a more contractile phenotype produce very little extracellular matrix whereas synthetic SMCs produce a lot more. The level of fibronectin synthesis can therefore be a guide to the phenotype of the cells cultured on the different surfaces. Figure 39, image C shows the levels of fibronectin mRNA produced by the cells. The large standard deviation shows that this data is highly unreliable. In a number of cases it was so high that it could not be plotted on the graph. For this reason any conclusions drawn from the data would be meaningless. Further repeats of the experiment would need to be carried out to obtain more accurate data.

Smooth muscle α-actin (SMA) is the main type of actin found in SMCs. Its levels are decreased as the cell phenotype changes from a contractile to synthetic. Compared to cells on the glass surface the expression of SMA mRNA by cells on the plain polyurethane was similar at 3 hrs but slightly higher at 6 and 24 hrs (Figure 40, Image A). The variation at the 6 and 24 hr time points may be due to differences in serum protein conformation once adsorption on to the two surfaces had occurred. This may have caused a variation in the strength of cell attachment or in the cell adhesion molecules involved. Both of these differences have the potential to alter signalling pathways within the cells and therefore change gene expression.

Cells cultured on the fibrillin surface expressed considerably more SMA mRNA at 3 hrs than on glass or polyurethane (Figure 40, Images A and B). In theory this would correspond to the cells having a more contractile phenotype on the modified surface. However, this result is contradictory to those shown in figures 28, 30 and 32. These results strongly indicate that at 3 hrs on the fibrillin-modified surface the cells have a more synthetic phenotype rather than a contractile one. This in addition to the huge standard deviation of the data suggests that the cells are not in reality contractile and the measured gene expression is an inaccurate marker of the SMC phenotype in this instance. At 6 and 24 hrs the cells were seen to produce similar amounts of SMA mRNA to those cultured on glass (Figure 40, Image A).

At 3 hrs cells cultured on the tropoelastin-modified surface produced an extremely high level of SMA mRNA compared to those on the glass and polyurethane (Figure 40, Images A and B). Again, this is theoretically representative of a more contractile

phenotype. The images and data shown in figures 29, 31 and 33 indicate that at 3 hrs the cells had a fairly synthetic phenotype. Figure 40, image C shows high standard deviations in the PCR data and so the contrasting data from the other experiments is a more reliable indicator of the phenotype of SMCs cultured on the tropoelastin surface at 3 hrs. At 6 hrs the expression of the SMA gene decreases dramatically from that at 3 hrs. The level of mRNA is similar to that produced by cells cultured on glass and slightly less than that produced by cells on the plain polyurethane. This low level of gene expression correlates with the evidence of synthetic SMCs from other experiments.

Smoothelin is a specific marker of the contractile SMC phenotype. The expression of the gene by cells cultured on the plain polyurethane can be seen to increase over the 24 hrs (Figure 41, Image C). This suggests that the cells were becoming more contractile over this time period. Results from other experiments however contradict this conclusion (Figures 28-33). These experiments indicate a highly synthetic phenotype of SMCs cultured on the polyurethane. Figure 41, image A shows that levels of smoothelin mRNA production were also higher in cells on polyurethane compared to those on glass. Due to the small scale of the graphs these differences are very small and so unlikely to be significant.

Compared to glass and plain polyurethane cells cultured on the fibrillin-modified surface produced slightly more smoothelin mRNA at 3 and 6 hrs but less at 24 hrs. Again, the scale of the graphs show that these differences are small and so any conclusions drawn would be unreliable. The low level of smoothelin mRNA

production is expected as other experiments indicate that the SMCs have a synthetic phenotype when cultured on the fibrillin surface.

Levels of smoothelin gene expression by cells cultured on the tropoelastin-modified surface varied considerably over the 24 hr time period. Compared to cells on the plain polyurethane SMCs on the tropoelastin produced gradually less smoothelin mRNA. This is due to an increase in gene expression by cells on the polyurethane in conjunction with little change in expression by cells on the protein. High standard deviations of this data mean that accurate conclusions are not possible.

4.7 – Immunostaining

4.7.1 – Cell-matrix Adhesions

Three general categories of cell-matrix adhesions have been identified.[217] Classical focal adhesions are located at the cell periphery and are associated with actin stress fibres. These are rich in vinculin and paxillin and the integrin $\alpha_v\beta_3$. Fibrillar adhesions are elongated/beaded structures and are located more centrally in the cell. These contain high levels of tensin and the integrin $\alpha_5\beta_1$. The last category is mosaic adhesions, which contain a combination of the proteins. Previous research involving cell adhesion to fibronectin-modified surfaces has shown that the type of adhesion varies with the deformability of the protein.[41,218] Cells were seen to attach via classical focal contacts when the fibronectin was covalently immobilised whereas fibrillar adhesions were the method by which cells attached to adsorbed protein. The

immobilisation of the fibronectin causes a high level of tension along the cytoskeleton followed by the formation of large focal contacts. When the fibronectin is deformable there is less tension and this allows for translocation of the adhesions and the tension-independent recruitment of tensin and integrin $\alpha_5\beta_1$ resulting in the formation of fibrillar adhesions.

In section 3.3.1.2 it was shown that the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ were involved in SMC attachment to the fibrillin-modified surface. To investigate the mechanism of cell attachment further, cells were probed with antibodies to these integrins.

Image C in Figure 43 shows the result of staining cells that have been cultured on a fibrillin-modified surface for the integrin sub-unit α_5. There is definite positive staining indicating the presence of this sub-unit at the cell surface. More importantly it can be seen that the staining shows specific structures. The brightest areas are close to the nucleus of the cells. This might indicate the presence of fibrillar adhesions as these are known to contain a high concentration of the $\alpha_5\beta_1$ integrin. This theory is backed up by image C in Figure 44. This shows the cells cultured on the fibrillin surface but this time stained for the β_1 sub-unit. Again fluorescence is brightest near to the nucleus indicating the presence of fibrillar adhesions. Another point of interest is the presence of elongated structures in the nuclear regions of the cells stained for both the α_5 and β_1 sub-units. These are especially clear in for the β_1 sub-unit where the structures extend out from the nucleus towards the edge of the cells. The presence of organised structures rather than diffuse staining shows that the $\alpha_5\beta_1$ integrin is directly involved in the mechanism of cell adhesion to the fibrillin-modified surface

after 2 days. The presence of fibrillar adhesions could be further investigated by staining the cells for the protein tensin. This is found in fibrillar but not classical focal adhesions.

It has been said previously that fibrillar adhesions have been shown to form when cells are cultured on adsorbed fibronectin but not when it is covalently attached. It is a necessary requirement for fibrillar adhesions that the surface that the cells attach to is deformable. This is because fibrillar adhesion formation requires the translocation of integrins. Soon after cell seeding focal contacts form, these contain both the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins. After a length of time, if the protein surface is deformable, the $\alpha_5\beta_1$ integrin translocates towards the centre of the cell forming fibrillar adhesions.[219] Although these published results relate to fibronectin-modified surfaces it is likely that parallels can be drawn with the fibrillin-1 surfaces investigated here. With this in mind it would be expected that focal adhesions would be the main type of cell-matrix adhesion as the fibrillin is covalently attached rather than adsorbed. In fact it appears that fibrillar adhesions are a major source of attachment. This can be explained by the differences in the methods of covalently attaching the proteins. In this work the fibrillin is attached via a layer of dextran. From the results in section 3.2.2 it was deduced that the dextran was attached to the polymer surface at a small number of points. This allowed for random movement of the polysaccharide and was the reason for its ability to prevent protein adsorption. It is therefore possible that the covalent attachment method enables significant movement of the fibrillin and so fibrillar adhesions are able to form.

Cells were also stained for the integrin $\alpha_v\beta_3$ (Figure 45). Cells cultured on the fibrillin-modified surface showed diffuse staining and few definite structures apart from some bright areas along the periphery of the cells. It appears that the $\alpha_v\beta_3$ integrin is involved in SMC attachment to the modified surface but to less of an extent than the $\alpha_5\beta_1$ integrin. These results are backed up by the results of the integrin blocking studies shown in section 3.3.1.2. The graphs in Figure 26 show the involvement of both integrins but it is clear that blocking the $\alpha_5\beta_1$ integrin has a much greater effect than blocking the $\alpha_v\beta_3$.

Along with the $\alpha_v\beta_3$ integrin, the protein vinculin is highly concentrated in classical focal contacts. In order to elucidate whether or not this type of cell-matrix adhesion was present along with the fibrillar adhesions the cells were stained for vinculin (Figure 46). Cells on the fibrillin-modified surface showed no signs of any positive staining for vinculin. Positive staining would appear as short, bright lines at the edges of the cells. In this case the fluorescence was diffuse indicating that there were no vinculin-containing focal contacts present at this 2 day time point. These results point to fibrillar adhesions as the main way that these SMCs attach to the fibrillin-modified surface. It is possible that at shorter time points however that this might not be the case. If the cells were stained before translocation of the matrix adhesions had occurred then classical focal contacts may have been visible. Another reason for the lack of vinculin-containing adhesions could be that due to the synthetic phenotype of the cells the protein is down-regulated. As mentioned in section 1.2.4, during modulation of SMCs from a contractile to a synthetic phenotype, meta-vinculin levels increase whilst vinculin levels decrease.[181] Probing for meta-vinculin would elucidate

this. One published study has suggested that the majority of adhesion strength is due to integrin clustering with a smaller contribution from focal contact formation.[220] This would explain why the cells appear to be well attached and spread but there is no positive staining for the classical focal contact proteins.

In section 3.3.2 it was shown that the $\alpha_v\beta_3$ integrin was involved in mediating cell attachment to the tropoelastin-modified surface in agreement with previous work carried out on adsorbed protein. The involvement of the $\alpha_5\beta_1$ integrin was not investigated at this time due to the limited availability of the tropoelastin protein and the large number of samples necessary for the experiments. No previous research has been published that looks at whether or not this integrin binds to tropoelastin. As the immunostaining procedure requires relatively few samples, the tropoelastin surfaces were included in the experiments. More noteworthy conclusions about the method of cell attachment to the tropoelastin surface could be drawn if the integrin blocking studies involving the $\alpha_5\beta_1$ integrin were carried out.

Figure 43 shows positive staining for the α_5 sub-unit in and around the nucleus of the SMCs cultured on the tropoelastin-modified surface. There is also bright staining around the periphery of the cells and along the extensive filipodia that extend out from them. The presence of these structures and the lack of diffuse staining indicate that the α_5 sub-unit is involved in cell attachment to this surface. The β_1 sub-unit was also stained (Figure 44). The fluorescence is specific to elongated structures that are present in and extend out from the nucleus. These features are highly organised show that the β_1 sub-unit is also involved in the method of cell attachment. The bright

staining at the nucleus and the highly specific staining of the α_5 and β_1 sub-units suggest that fibrillar adhesions are a major type of cell-matrix adhesions on the tropoelastin surface. Further staining for tensin would elucidate this further.

The SMCs cultured on the tropoelastin-modified surface also stained positive for the $\alpha_v\beta_3$ integrin. Image D in Figure 45 shows lots of filipodia extending out from the cell body across the protein surface. These results show that the $\alpha_v\beta_3$ integrin is involved in mediating SMC attachment to the tropoelastin-modified surface and this is backed up by the integrin blocking study in section 3.3.2. The filipodia are visible when stained for both the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins. This indicates that they are co-localised and so points to mosaic adhesions as being important in the attachment of the cells to the surface.

As with the cells cultured on the fibrillin surface, there was no positive vinculin staining on the tropoelastin (Figure 46). This is further evidence that there is a lack of strong, classical focal adhesion formation.

Staining of cells cultured on glass and plain polyurethane also shows a high level of fluorescence indicating the presence of the $\alpha_5\beta_1$ integrin. Staining for the α_5 sub-unit is more diffuse than for the β_1. There are fewer definite structures. The images in Figure 43 and Figure 44 show that the staining is similar on the glass and fibrillin surfaces. Cells on the polyurethane have very little β_1 staining indicating that the attachment of the cells is less efficient. It is clear that the $\alpha_5\beta_1$ integrin is involved in the attachment of cells to both the glass and polyurethane surfaces or more correctly

to the serum proteins adsorbed onto these surfaces. This is expected as there is a lot of research that shows that cell attachment to fibronectin, one of the major serum proteins, is mediated partially by this integrin.

Cells cultured on the glass discs stained positive for the $\alpha_v\beta_3$ integrin. It can be seen in Figure 45, image A that there are features at the edge of the pictured cell that resemble classical focal contacts. However, it would be expected that these would also be visible when stained for vinculin. It is clear from Figure 46 that this is not the case. As with the cells cultured on the other surfaces the fluorescence is diffuse and no definite structures are visible.

Figure 45, image B shows a cell with a small number of short filipodia stained by the anti- $\alpha_v\beta_3$ integrin. This shows that this integrin does mediate cell attachment to the serum proteins adsorbed onto the polyurethane surface but that the attachment does not appear to be strong.

Neither the cells cultured on the glass nor the polyurethane surface showed any positive staining for vinculin. This could either be due to a lack of strong classical focal contacts or to low levels of the protein due to the synthetic phenotype of the cells.

4.7.2 – Extracellular Matrix Proteins

Laminin is one of the major proteins that make up the basement membrane of SMCs. The basement membrane is especially important for this cell type as it helps regulate the phenotype of the cell.[1,4,136,177,221] In vivo, disruptions of the membrane and subsequent exposure to other ECM proteins such as fibronectin are thought to trigger the transformation of SMCs from a contractile to a synthetic phenotype. The cells then proliferate and contribute to the narrowing of the blood vessel. SMCs were cultured on glass, plain polyurethane and the 2 modified surfaces. They were stained for laminin after 2 and 4 days in culture (Figure 47). There is positive staining on all of the surfaces at both time points. The laminin has a mottled appearance throughout the cell cytoplasm. At 2 days there are significant amounts of the protein in the cells cultured on the glass and modified. At 4 days there is no increase in the amount of laminin in the cells on these surfaces but it is clear that the cells have proliferated. This suggests that the rate of laminin production restricts the build up of the protein in the cytoplasm. This is backed up by observing the cells cultured on the plain polyurethane. There is more laminin evident in the cells at 4 days than 2 days. The lack of cell proliferation has enabled a build up of protein to occur. The PCR data presented in section 3.7 (Figure 38) shows an up-regulation of the laminin gene at the 24 hr time point. Although there are large errors associated with the PCR data, as mentioned previously, these 2 sets of results are consistent.

Collagen type IV is another major protein of the basement membrane. Positive staining for this protein was evident in the cells cultured on all of the surfaces at both 2 and 4 days (Figure 48). The collagen has a speckled appearance and is visible in

significant amounts throughout the cytoplasm of the cells cultured on the glass and modified surfaces. As with the laminin staining is brighter on the polyurethane after 4 days due to a lack of cell proliferation.

It is clear that the SMCs are actively synthesising these 2 basement membrane proteins when cultured on either of the protein-modified surfaces. The rate of synthesis of both laminin and collagen IV is a lot lower for the cells on the plain polyurethane. Both the fibrillin and the tropoelastin can be said to encourage the production of basement membrane proteins but to no greater extent than the glass. It is probably more accurate to say that the plain polyurethane discourages the protein synthesis. This is likely to be due to the poor cell attachment.

Immunostaining was also used to investigate synthesis of the proteins that make up the elastic fibres of the artery wall. Fibulin-5, tropoelastin and fibrillin-1 were all examined. Images of cells stained for fibulin-5 are shown in Figure 49. At the 2 day time point bright staining can be seen around the periphery of the cells cultured on glass and the two modified surfaces. Fluorescence coming from the cells on the plain polyurethane is diffuse and can be attributed to non-specific staining. After 4 days fibulin-5 staining can be seen in the cells on all of the surfaces including the plain polyurethane. The amount of fibulin-5 has increased most significantly in the cells cultured on the two modified surfaces especially the tropoelastin. On this surface there is stained protein throughout the cytoplasm with even brighter areas around the cell nuclei. This increase in brightness suggests that the rate of fibulin-5 synthesis

was high enough compared to the rate of proliferation to allow a build up of protein to occur.

The results of staining for tropoelastin production can be seen in Figure 50. It was not possible to investigate the effect of the tropoelastin-modified polyurethane as the antibody would bind to both the recombinant protein and any that was newly synthesised by the cells. There is positive staining of the cells cultured on all of the other surfaces at the 2 day time point. This is brightest around the cell nuclei. After 4 days the tropoelastin is evident throughout the cells. There is significantly more protein visible in the cytoplasm of the cells cultured on glass than on the fibrillin. The proliferation rate of the cells on these 2 surfaces appears to be the same and so it can be said that the glass surface has caused an increase in the production of tropoelastin. The proliferation rate of the SMCs on the plain polyurethane is a lot lower which accounts for the accumulation of protein in these cells.

Cells were also stained for fibrillin-1 however no images were taken. The staining was diffuse throughout the cells on all surfaces. It is unclear whether this was due to a lack of fibrillin-1 or to incorrect antibody concentrations as optimisation of the antibody was unsuccessful. This may have been due to the efficacy of the antibody or the absence of any protein on control samples. The RT-PCR data in Figure 36 shows an extreme down-regulation of the fibrillin-1 gene and so this would suggest that there is a negligible amount of this protein being produced.

At these time points it appears that the modified surfaces are not directing the SMCs to increase their production of these elastic fibre proteins compared to the glass control. The cells can be seen to be synthesising fibulin-5 and tropoelastin but no fibrillin-1 is evident. As fibrillin microfibrils form the template for tropoelastin deposition during elastic fibre formation it is highly unlikely that any fibres will be produced. Any of these proteins secreted by the SMCs will remain un-organised. It is possible that at longer time points the cells may start to produce fibrillin-1 and there may be a degree of fibre formation.

4.7.3 – Phenotypic Markers

Smooth muscle α-actin (SMA) is the major actin isoform of mature, fully differentiated SMCs.[172] It is an important constituent of the cell's contractile apparatus. The assembly of actin filaments is essential for cellular contraction. During foetal development the amount of α-actin increases, with a corresponding decrease in β-actin, and the SMCs acquire the ability to contract. In the event of modulation to the synthetic phenotype, either due to arterial injury in vivo or culture conditions in vitro, the amount of α-actin decreases.

Images A and B in Figure 51 show SMCs cultured on glass discs for 4 days. Well-organised actin filaments can be seen in the cells. At this 4 day time point the cells are almost confluent. The cells pictured in image A are in fact growing on top of other cells. There are 2 cells visible in image B. One is growing on the glass and the other is growing on top of other cells. There are no organised actin fibres visible in

the cells that are in direct contact with the glass only in those that are growing on top of other cells. The same phenomenon is true of the cells cultured on the plain and modified polyurethane surfaces (images C-E). No actin fibres are visible in any of the cells in direct contact with the surface, just a degree of diffuse staining. This could be due to a lack of sufficiently strong cell-matrix adhesions. As was discussed in section 4.9.1, there is no evidence of strong focal contact formation in the cells cultured on these surfaces. These are necessary for the assembly of actin stress fibres. As mentioned previously, the cells cultured on the modified surfaces have many of the characteristics of a synthetic phenotype rather than a contractile one. A low level of SMA is another of these characteristics and so it is not surprising that these cells are displaying a low level of positive staining for the protein. This lack of α-actin fibres is also seen when SMCs are cultured on a fibronectin substrate.[178]

Other phenotypic markers of SMCs include smoothelin, calponin and caldesmon. Smoothelin is associates with the actin cytoskeleton and is only present in contractile SMCs. It is therefore not surprising that the cells cultured on all of the surfaces stained negative for this protein (Figure 52). All of the results obtained during this work point to the cultured cells having a synthetic phenotype. Calponin and caldesmon are also associated with the contractile apparatus of SMCs. They are both up-regulated in cells of the contractile phenotype and only found in very low amounts in synthetic cells. As expected, the cells cultured on the plain and modified surfaces all stained negative for these proteins.

4.8 – Western Blot Analysis

Protein was harvested from cells cultured on glass, and plain and modified polyurethane for 2 and 4 days. Western blot analysis was then used to assess the amount of different proteins present in each sample. Images were obtained by chemiluminescent detection. They were then inverted and the contrast and brightness altered to obtain the clearest pictures. Figure 53 shows the images of the gels that were probed with antibodies for the GAPDH control and for smooth muscle α-actin. Bands are visible on all 4 surfaces at both time points on the GAPDH gels (images A and C). The size of these bands represents the amount of cells present on each sample. The amount of the other proteins is then normalised to this to eliminate variations in protein concentration due to different cell numbers. The size of the bands agree with both the qualitative results shown in Figure 28 and Figure 29 the quantitative results shown in Figure 32 and Figure 34. There are more cells on the glass and modified surfaces than on the plain polyurethane. There are also fewer cells on the tropoelastin than on the fibrillin surface. The bands can be seen to increase in size from 2 to 4 days as a result of the cell proliferation.

Images B and D in Figure 53 show gels stained with an antibody for smooth muscle α-actin. At 2 days there is nothing visible on the gel but after 4 days there are very slight bands in the fibrillin lanes. It is likely that the concentration of protein from most of the samples is too low to be detected. Bands can only be seen from the samples where the cell number is highest i.e. on the fibrillin-modified surface. Due to

the bands being so faint it was not possible to quantify the amount of protein using the Genenome software.

Gels were also probed with antibodies for laminin, collagen IV, fibrillin-1, tropoelastin, smoothelin, calponin and caldesmon. No bands were visible on any of these gels. From the results of the immunostaining experiments it might be expected that there would be some evidence of laminin, collagen IV and tropoelastin. It is likely that the level of these proteins was too low to be detected by this method. Larger samples would be needed to get a cell number high enough to get a protein concentration that could be detected.

4.9 - General Discussion and Further Work

The results presented in section 3.1 show that the surface modification previously used to attach peptides to the polyurethane Tecoflex can also be used, in an adapted form, to covalently attach proteins to the polyurethane b9 Z1A1.[187] In this work 2 different proteins were immobilised using this method. This indicates that other proteins could be successfully attached in this way. By using the dextran intermediate the cellular response to the modified surfaces could be attributed solely to the covalently immobilised protein.

The modification of the surface of flat films of polymer was investigated here. To use this method in the context of the hybrid artery the protocol would have to be

transferred to a porous scaffold. It has previously been shown that the aminating step of the modification does not disrupt the fibrous structure of an electrospun scaffold.[187] This is encouraging as it might be thought that the DMAc would dissolve the fibre surface to the extent that the porous structure would be lost. It should therefore be possible to use this protocol to immobilise proteins on an electrospun porous scaffold such as that necessary for the production of an artificial artery. This would need to be investigated by carrying out the modification and using ELISAs to determine its efficacy.

The attachment of SMCs to both proteins was found to be, at least in part, mediated by integrins. This was determined by integrin blocking studies and immunostaining. Blocking the $\alpha_v\beta_3$ integrin with antibodies resulted in reduced cell attachment to the fibrillin fragment. Fluorescent staining of the same integrin showed bright areas around the periphery of the cells indicating a degree of clustering. Together these results show that there is a contribution from the $\alpha_v\beta_3$ integrin in mediating cell attachment to the fibrillin-modified surface. Both the integrin blocking and immunostaining show that there is a more significant contribution from the $\alpha_5\beta_1$ integrin. Blocking antibodies caused a large decrease in cell attachment and fluorescent staining of both integrin sub-units shows lots of bright, organised structures indicating significant integrin clustering.

Cell attachment to the tropoelastin-modified surface was also seen to be mediated by the $\alpha_v\beta_3$ integrin. Antibody blocking caused a small but significant reduction in cell attachment. The immunostaining shows clear filipodia extending out from the body

of the cells, providing further evidence of the involvement of this integrin. No involvement of the $\alpha_5\beta_1$ integrin in attachment to tropoelastin has been reported in the literature. Due to the limited supply of the protein and the large number of samples needed for the integrin blocking experiments, this study could not be carried out in this project. However, as a much smaller number of samples are needed for immunostaining, this experiment was carried out. Staining of the α_5 sub-unit especially showed very bright structures around the periphery of the cells and also filipodia extending out from them. Staining for the β_1 sub-unit showed aligned, elongated structures within the cells. Together these images indicate that the $\alpha_5\beta_1$ integrin in addition to the $\alpha_v\beta_3$ is involved in cell attachment to tropoelastin. Carrying out the corresponding integrin blocking studies would elucidate this further.

In previous work, an area of tropoelastin containing the peptide sequence VGVAPG was shown to mediate cell attachment via a non-integrin cell-surface receptor termed the elastin binding protein (EBP). This is a 67kDa receptor that can be blocked in a similar manner to the integrins but by using galactose-based sugars rather than an antibody.[170,222-224] This method has been used to show the significant involvement of the EBP in mediating cell attachment to adsorbed tropoelastin. The presence of the VGVAPG sequence on the surface of the covalently immobilised tropoelastin could be determined by using soluble peptides or a sugar to block the EBP prior to seeding the cells onto the modified surface. If cell attachment was not reduced by the addition of the sugar then that would indicate that the modification protocol had altered the protein conformation and caused the VGVAPG cell-binding sequence to become concealed.

The integrin blocking and immunostaining studies have elucidated the involvement of certain integrins in the attachment of SMCs to the protein-modified surfaces. It is known that the binding of different integrins to ligands triggers different intracellular signalling pathways. This project did not investigate specific relationships between the integrins involved and the aspects of the cells' behaviour, although the previously reported link between RGD and the synthetic phenotype of SMCs was seen.[208] In order to do this the different integrins would be blocked as before prior to seeding of the cell on to the modified surfaces. Various aspects of the cell behaviour would be monitored and then compared to that of cells with all integrins available for binding. This would give a degree of understanding as to which integrins were associated with each particular cellular activity, proliferation for example.

The SMCs behaved differently on each of the surfaces. The fibrillin fragment dramatically increased cell adhesion compared to the plain polyurethane. Soon after seeding the cells attached and spread out, adopting a fibroblast-like morphology characteristic of SMCs in the synthetic phenotype. The cells then proliferated extensively on the surface, again indicating that they were more synthetic. Immunostaining showed that the cells were actively producing extracellular matrix proteins such as laminin and fibulin-5. As SMCs in the contractile phenotype synthesise very little matrix these results are further evidence that the cells are synthetic. This is backed up by the lack of proteins such as vinculin and calponin. These have been demonstrated to be down-regulated in synthetic SMCs.[180]

It is clear from the results that the fibrillin fragment encourages the SMCs to adopt the synthetic phenotype rather than the contractile state that they possess in vivo. The ability of the cells to proliferate is necessary for the production of the vascular graft. A confluent layer of SMCs on the internal surface of the polymer scaffold is required for the endothelial cells to grow on top of. A problem would occur however if the cells continued to proliferate after a confluent layer had formed. This may cause the graft to become blocked. Carrying out cell culture studies for longer time periods than were used in this work would give more information as to how the cells would behave once they were confluent. It is possible that the cell-cell contact would prevent over proliferation. It is also possible that if the cells continued to produce extracellular matrix then they may form an environment mimicking that of the native artery wall. This may cause the cells to transform into a more contractile phenotype and so cease proliferating. Again, studies using longer cell culture periods would elucidate this further. The formed extracellular matrix could be imaged using immunofluorescence to determine the extent of matrix re-modelling. It would be interesting to establish whether there was any elastic fibre formation.

If it was found that at longer time points the cells do not stop proliferating on their own then it may be possible to intervene and stop the growth by encouraging the cells to revert to a more contractile phenotype. Adding growth factors such as insulin-like growth factors I and II to culture medium is a possibility. This has been shown to maintain contractile cells in their phenotype.[177] Culturing SMCs on an elastomeric substrate allows for mechanical stretching of the cells. This has been shown to promote a more contractile phenotype.[215,225-230] These results are not surprising

considering the fact that in vivo the cells are constantly undergoing stretching due to the pulsatile nature of blood flow through the arteries. By culturing the artificial graft in a bioreactor and subjecting the cells to pulsatile stretch after a confluent SMC layer has been formed it may be possible to halt cell proliferation and transform the cells to a contractile phenotype. Research suggests that stretching also causes alignment of SMCs.[231-234] This would be an advantage for the artery as the cells would contract in the same direction enhancing the physical properties of the scaffold reducing the chance of compliance mis-match further. In addition to this if there was any elastic fibre formation then the mechanical stretching may also cause their alignment further strengthening the artery structure and improving its physical properties.

Cells cultured on the tropoelastin-modified surface had poor initial attachment and spreading. There was no improvement compared to the plain polyurethane. The addition of SMGS to the culture medium was necessary before the cells adhered properly. This may have been due to adsorption of serum proteins to the surface or a response to the growth factors now present in the medium. When the cells did adhere, they adopted a fibroblast-like morphology as on the fibrillin surface. They then proliferated at a similar rate to those on the plain polyurethane surface. Immunostaining showed that the cells on the tropoelastin were synthesising matrix proteins in similar amounts to the cells on the fibrillin fragment. The morphology of the SMCs, in addition to the fact that they were able to proliferate and produce extracellular matrix, indicates a synthetic morphology. The lack of cell growth in the first 3 hrs of culture is unlikely to be due to the quiescence found in contractile SMCs. It is more probable that this is caused by poor attachment to the tropoelastin.

The cells did not adopt the characteristic bipolar morphology of contractile cells at any point.

It was hypothesised that by using proteins native to the artery wall it may be possible to control the behaviour of SMCs cultured on the modified surfaces. The cells behaved differently on the 2 different protein-modified surfaces. For the purpose of the artificial vascular graft the fibrillin-1 fragment has potential as a surface-modifying agent as it was seen to promote proliferation of the SMCs. This is necessary for the formation of a confluent layer on which the endothelial cells would be seeded onto. Tropoelastin alone does not appear to be useful as a substrate as it neither encourages proliferation nor maintains the SMCs in a non-proliferative contractile phenotype.

It is clear that there is a need for carrying out cell culture studies for longer time periods in order to establish whether these proteins could be used for modifying the internal surface of an artificial vascular graft. It would also be necessary to culture the cell-seeded graft in a bioreactor with an applied pulsatile stretch in order to mimic the in vivo environment. Further experiments would include the culture of endothelial cells on top of the smooth muscle layer.

An implanted vascular graft has to withstand the mechanical stresses caused by the pulsatile blood flow immediately following the surgery. In this work a biostable polymer has been proposed as its strength and elasticity would prevent rupture of the vessel in the time period before significant tissue was established. This enables a

shorter in vitro culture time to be used and so renders the process more cost-effective. This is a vital issue in the development of regenerative medicine products. There are however certain problems that occur with long-term implantation of poly(ether urethanes). Whilst being hydrolytically stable they can be subject to oxidative degradation and stress cracking.[235]

The majority of tissue engineering strategies focus on using a biodegradable scaffold rather than a biostable one as proposed in this work. The scaffold gradually degrades in vivo and is replaced by new tissue. This has the advantage that the tissue can be re-modelled by the cells in response to a changing environment. Bone, for example, constantly re-models in vivo in response to changes in stress.

A more favourable situation would be a biodegradable polyurethane with the strength and elasticity of the b9 polymer used in this work. The material would be able to withstand the in vivo stresses in the period before the formation of adequate new tissue. It would then degrade slowly allowing the tissue to re-model.[236] The surface modification protocol assessed in this project should be able to be transferred to a different polyurethane with minimal adaptations.

Chapter 5 - Conclusions

The aim of this project was to answer 4 main questions.

1 – Can these proteins be covalently attached to the polyurethane via this surface modification protocol?

The results of the surface analysis experiments show that the surface modification protocol was successful in covalently attaching both the fibrillin-1 fragment and the tropoelastin to the polyurethane surface.

2 – Was the conformation of the proteins adversely affected by the covalent attachment process?

It was shown that cell attachment to the fibrillin fragment was RGD-dependent and that the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ were involved in mediating the cell attachment. These results indicate that both the RGD sequence and a downstream synergy site are available for cell binding. These results are similar to those obtained by studying the same protein fragment adsorbed onto tissue culture plastic and so it appears that the modification protocol does not adversely affect the protein conformation.

Cell attachment to the tropoelastin-modified surface was shown to be mediated in part by the αvβ3 integrin. This was in agreement with work carried out on adsorbed

protein. This indicates that the surface modification protocol does not adversely affect the tropoelastin conformation.

3 – How was the behaviour of the cells affected by the protein-modified surfaces?

The cells behaved differently on each of the surfaces. The fibrillin fragment increased cell attachment and spreading compared to the plain polyurethane surface. In contrast, the tropoelastin showed similar cell behaviour to the plain polyurethane surface. These surfaces demonstrated poor cell attachment, spreading and proliferation in the absence of serum-containing growth supplement. SMCs cultured on both protein-modified surfaces synthesised a number of extracellular matrix components.

All of the results point to the SMCs as having a highly synthetic phenotype as opposed to the quiescent, contractile phenotype maintained by cells in the artery wall. They proliferated, synthesised matrix and displayed no evidence of contractile phenotypic markers.

4 – What are the implications of this research for the development of an artificial bypass graft?

The fibrillin-1 fragment has potential for use in the formation of an artificial bypass graft. The covalent attachment of this protein encouraged adhesion and proliferation of the SMCs. This would be beneficial for covering the polymer surface

completely with cells. However, in order for this protein to be used, it would need to be determined whether the proliferation could be controlled once the cells had covered the polymer.

This work investigated two proteins found in blood vessel walls. However, the extracellular matrix is a highly complex 3D structure consisting of numerous proteins, proteoglycans and polysaccharides. The complexity of the matrix means that a truly biomimetic environment would be impossible to achieve using synthetic means. For this reason the modification of a surface with a protein is a good starting point for gaining some control over cell behaviour.

This adaptability of the modification protocol means that it is not restricted to the development of an artificial bypass graft. The majority of tissue engineering and regenerative medicine strategies involve the use of a scaffold to support the 3D culture of cells. By altering the polymer and/or proteins used, a wide variety of applications could be targeted.

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