**Optimization of the Synthetic Parameters of Lipid Polymer Hybrid Nanoparticles Dual Loaded with Darunavir and Ritonavir for the Treatment of HIV**

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**Abstract**

Human Immunodeficiency Virus (HIV) is a global health concern to which nanomedicine approaches provide opportunities to improve the bioavailability of existing drugs used to treat HIV. In this article, lipid polymer hybrid nanoparticles (LPHNs) were developed as a system to provide a combination drug delivery of two leading antiretroviral drugs; darunavir (DRV) and its pharmacokinetic enhancer ritonavir (RTV). The LPHNs were designed with a poly(D, L-lactide-*co*-glycolide) (PLGA) core, and soybean lecithin (SBL) and Brij 78 as the stabilizers. The LPHNs were prepared by modified nanoprecipitation and the effect of synthetic conditions on the particle properties was studied, which included the Z-average diameter and polydispersity index of LPHNs in water and phosphate buffered saline, and the morphology of the particles. This investigation aimed to prepare a formulation that could be stored in its dry and redispersible form, therefore avoiding the challenges associated with storage of dispersions. The optimum ratio of stabilizer to polymer core was established at 20 % w/w, and Brij 78 was found to be crucial in providing colloidal stability in physiological solutions; the minimum amount of Brij 78 required to provide stability in phosphate buffered saline was 70 % w/w of the total stabilizer mass. Viable formulations of LPHNs containing DRV and RTV in the clinically used 8:1 ratio were prepared containing 20 % w/w DRV with respect to the PLGA mass. The use of cryoprotectant, polyethylene glycol, combined with freeze-drying yielded LPHNs with a Z-average diameter of 150 nm when the particles were re-dispersed in water. The oral absorption behavior was assessed using an *in vitro* triple culture model. Whilst the use of cryoprotectant and freeze-drying led to no improvement of the transcellular permeability compared to the unformulated drugs. The non-freeze-dried samples with the highest soybean lecithin led to increased transcellular permeability, revealing the potential of LPHNs for enhancing HIV treatment.

# Introduction

In 2018, the World Health Organization (WHO) estimated that approximately 37.9 million people were infected with HIV worldwide and more than half of those infected did not receive antiretroviral therapy. If left untreated, HIV progresses to acquired immunodeficiency syndrome (AIDS) which causes severe damage to the immune system potentially resulting in death. (Mahajan et al., 2012; Panel on Antiretroviral Guidelines for Adults and Adolescents, 2012) Darunavir (DRV) is a highly effective first line antiretroviral agent that shows a high genetic barrier and has exceptional resistance profile against both wild type and mutant HIV strains. (Antoniou et al., 2017; Lascar and Benn, 2009; Seitz, 2016) DRV is a protease inhibitor that prevents the maturation of HIV virions, rendering them unable to infect new cells. (Ford, 2004) The main limitation of DRV is its low oral bioavailability, (Desai and Thakkar, 2017) which could be due to its low water solubility, high lipophilicity and it is also subjected to first pass metabolism. DRV is usually co-administered with ritonavir (RTV) as a pharmacokinetic enhancer, a protease inhibitor that inhibits the activity of both P-glycoprotein and Cytochrome P450 (CYP3A4), increasing the oral bioavailability of DRV from 37 % to 82 %. (Desai and Thakkar, 2016) DRV is usually co-administered with RTV at 600 mg with 100 mg RTV twice daily or 800 mg with 100 mg RTV once daily, which remains an extremely high dose. (Capetti et al., 2017; Lascar and Benn, 2009) DRV and RTV are used in combination with typically emtricitabine or prodrugs of tenofovir, however, these two drugs do not display the adsorption issues found for protease inhibitors such as DRV. Therefore, in order to improve the treatment of HIV it is important that approaches are identified that allow the enhanced delivery of DRV and RTV.

Nanomedicine offers the possibility to decrease the required dose of both drugs, enhance targeting to HIV reservoir sites and address pharmacokinetic limitations. Many examples of nanoformulations of HIV drugs have previously been found to offer enhanced drug delivery. (Desai and Thakkar, 2016; Giardiello et al., 2016; Gupta and Jain, 2010; McDonald et al., 2014a; Siccardi et al., 2013) However, amongst the many nanocarrier systems, lipid-polymer hybrid nanoparticles (LPHNs) was chosen as a new potential drug delivery system for the treatment of HIV in this study, to the best of our knowledge, there have not been any publications that involves using LPHNs as a nanocarrier for HIV drugs. LPHNs are core-shell nanoscale particles, composed of a core of a biodegradable non-toxic polymer, coated by a lipid monolayer and often a lipid-polyethylene glycol (PEG) shell. (Hadinoto et al., 2013; Mandal et al., 2013) These components provide different properties: the polymer core encapsulates the hydrophobic drug moieties, while the lipid coating which is usually composed of a layer of phospholipids, helps to stabilize the cores by electrostatic stabilization. (Li et al., 2017; Yu et al., 2017) Additionally, the lipid shell has been shown to enhance the entrapment efficiency of the drug (Zhang et al., 2008) and to increase oral bioavailability of hydrophobic drugs.(Chen et al., 2016; Jin et al., 2012; Li et al., 2015; Su et al., 2018) A lipid-PEG conjugate is often included in the LPHNs shell as this has been shown to improve the physical stability of the particles especially in electrolyte solutions present in the body. In experiments by Chan *et al*. to determine the role of the PEG shell in LPHNs stabilization, it was reported that large aggregates formed when LPHNs were dispersed in phosphate buffered saline (PBS) (a common model for biological ionic strength matrices) in the absence of the PEG shell despite the presence of high lipid coverage of the cores. While, LPHNs coated with lipid-PEG showed high stability in PBS, this was attributed to the steric stabilization provided by the PEG.(Chan et al., 2009) The lipid-PEG conjugates also offer LPHNs increased residence time in blood circulation due to the avoidance of nanoparticle removal by the reticuloendothelial system (RES), this is an important consideration in parenteral formulations. (Hadinoto et al., 2013; Zhang et al., 2008)

LPHNs combine many of the advantages of lipid-based nanomedicines such as enhanced cellular uptake and the inherent benefits of polymeric nanocarriers including: architectural integrity, high stability over prolonged times and a controllable drug release profile. (Dalmoro et al., 2018; Gao et al., 2013; Zhang and Zhang, 2010) The stabilizing physicochemical properties of LPHNs make them a promising drug delivery system to target HIV reservoir sites in the lymphatic system where, currently, the drug cannot be maintained at therapeutic concentrations. (Makwana et al., 2015) Lipid based formulations, including LPHNs, have the potential to be selectively taken up by the lymphatic route following oral administration. (Neves et al., 2014; Zhu et al., 2014) Their transport to intestinal lymphatics has been suggested to occur by various mechanisms including transcellular transport,(Mishra et al., 2014; Paliwal et al., 2009) or through the microfold cells (M cells) of Peyer patches. These are lymphoid follicles that are part of the gastrointestinal tract and particle uptake is believed to occur by endocytosis or transcytosis. (Prajapati et al., 2018) These same uptake processes can also occur in epithelial cells, which can also display a paracellular mechanism which occur between the tight junctions of the cells. (Ma et al., 2017; Paliwal et al., 2009) Much of the research on LPHNs focuses on parenteral dosing however, due to the chronic nature of HIV treatment oral dosing is currently the only viable route of frequent administration.

Brij 78 (C18, PEG20), a saturated polyethylene glycol octadecyl ether is a stabilizer that is widely used in lipid-based formulations. (Tagami et al., 2011a) Brij 78 has previously been used as surfactant in the synthesis of many formulations: liposomes, (S. Hsu et al., 2011; Tagami et al., 2011b, 2011a) micelles, (Ribeiro et al., 2012) solid lipid nanoparticles, (Zhang et al., 2007) active targeted nanoparticles, (Oyewumi and Mumper, 2003) lipid based nanoparticles (Dong et al., 2009) and nisomes, (Bayindir and Yuksel, 2010) and it has been used as a replacement of 1,2-distearoyl-sn-glycerol-3-phosphatidylethanolamine-N-[methoxy(polyethylene glycol)-2000] known as (DSPE-PEG2000) in the synthesis of liposomes. (S.-H. Hsu et al., 2011) The phospholipid soybean lecithin (SBL) is a compound that has been used as to form the lipid shell around the polymeric core, it is low cost, has good biocompatibility and is highly accepted in both food and pharmaceutical industry. (Su et al., 2018) Additionally, it can act as both an absorption enhancer which increase the bioavailability of poorly soluble drugs as it bio-mimics natural lecithin in the cell wall, (Chen et al., 2016; Jin et al., 2012; Li et al., 2015; Su et al., 2018) and non-irritant electrolytic stabilizer that prevent particles aggregation. (Zhang et al., 2009)

Drug delivery *via* oral administration can be modelled using an *in vitro* intestinal triple culture model containing Caco-2 cells, microfold cells (M cells) and HT-29-MTX mucus secreting goblet cells. Caco-2 cells are human intestinal adenocarcinoma cells (enterocytes) that offer morphological and physiological similarity to the human intestinal epithelium. (Schimpel et al., 2014) Transwells can be used to support Caco‑2 monolayers as the cells polarize, differentiate and form tight junctions. The polarized monolayer resembles the functional lining of the small intestine offering an *in vitro* model for absorption across the gut. The apical surface of the cells models the surface exposed inside the gut whilst the basolateral surface models the interface with the blood. The Caco-2 model provides exploratory data and is widely used to screen for absorption potential. (Elsby et al., 2008; Hatton et al., 2015; Savage et al., 2019) Despite these important features, one cell type cannot fully reflect the physiology of the intestine. An *in vitro* triple culture model comprising Caco-2 cells, HT-29-MTX mucus secreting goblet cells and Raji B lymphocytes can be used to stimulate the differentiation of Caco-2 cells into microfold cells (M cells) to study the absorption of nanocarriers. It has been suggested that goblet cells and M cells play a significant role in the uptake and permeation of nanoparticles in the intestine and inclusion of these cells provides a more representative *in vitro* model to assess and compare such materials. (Antunes et al., 2013; Beloqui et al., 2016; Tatham et al., 2015) Specifically, goblet cells continuously secrete mucus often limiting the ability of particles to permeate and gain access to the underlying epithelium. (Beloqui et al., 2014; Ferraretto et al., 2018) M cells located in the epithelium and overlaying the Peyer’s patches are responsible for the uptake of exogenous materials (*e.g.* bacteria) and delivering them to the lymphoid follicles.(des Rieux et al., 2007; Schimpel et al., 2014) Some studies suggest that nanoparticles enter the intestinal epithelium predominantly *via* the M cell route. (des Rieux et al., 2007; Rieux et al., 2005) Although the mechanisms that underpin particle permeation across the intestinal epithelium are not clearly understood, various processes have been described and these have been reviewed elsewhere. (Desai et al., 2012; Hunter et al., 2012; Tatham et al., 2015; Yameen et al., 2014)

A considerable challenge in the clinical use of nanoparticles for drug delivery is the ability to obtain a formulation that offers long-term storage stability. (Mukherjee et al., 2019a) This is particularly important given the supply chain challenges associated with administering therapies in low- and middle-income countries, where the capacity for temperature-controlled storage is limited. One possible solution is the ability to prepare dry formulations that can be formulated into capsules or redispersed upon administration for pediatric dosing.

In this work, the synthetic parameters of LPHNs prepared by nanoprecipitation loaded with both DRV and RTV designed for the treatment of HIV have been optimized. Several parameters that affect the formulation of LPHNs using the nanoprecipitation method were studied (Figure 1): mass percentage of the total stabilizer to the polymer core, the mass percentage of two different stabilizers (Brij 78 and SBL) and drug loading. It is shown that the LPHNs can be converted into dispersible formulations using freeze drying and that high drug loadings can be obtained. Finally, the composition of the surfactant mixture and the colloidal stability were investigated and the drug delivery behavior in an in vitro intestinal triple culture model for intestinal permeability was assessed.

**FIGURE 1**

# Materials and Methodology

## Materials

HPLC grade acetone (Fischer Scientific), ethanol (EtOH, Fisher Scientific), 90 % SBL (Alfa Aesar), DRV, RTV, PEG (Mn 2050 g mol-1), Brij 78, resomer® RG 503 H, PLGA, and PBS tablets, hydrochloric acid (HCl), sodium chloride (NaCl), dimethyl sulfoxide (DMSO), hydrogen peroxide, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), bovine serum albumin (BSA), Hank’s balanced salt solution (HBSS), Dulbecco’s Modified Eagle’s Medium (DMEM), RPMI and trypsin-EDTA were all purchased from Sigma-Aldrich. Amicon Ultra-0.5 Centrifugal Filter Unit (Merck). Caco-2 and HT-29-MTX cells were maintained in DMEM supplemented with 15 % fetal bovine serum (FBS) (Gibco, UK), 2 mM L-glutamine and 1 % non-essential amino acids (Sigma, UK). Raji B cells were maintained in RPMI supplemented with 10 % FBS, 2 mM L-glutamine and 1 % non-essential amino acids (Sigma, UK). [3H]-DRV was purchased from RC Tritec and [14C]-mannitol was purchased from American Radiolabeled Chemicals (US). Ultima Gold and ProSafe+ liquid scintillation fluid was purchased from Meridian biotechnologies (UK). Transwells with a 3 µM pore size were purchased from Corning (US). Caco-2, HT-29-MTX and Raji B cells were purchased from American Type Culture Collection (US). All reagents were used without further purification.

## Methodology

### Synthesis of LPHNs

LPHNs were prepared by a one-step optimized nanoprecipitation method (Figure 1), where the formation of the polymeric cores and the assembly of the lipid around them happens simultaneously. The procedure used in this study was based on the modified nanoprecipitation method reported by H. Fang *et al*. (Fang et al., 2010) and Pramual *et al.*(Pramual et al., 2017) Briefly, PLGA (2.5 mg) was dissolved in acetone (1.5 mL) to form an organic solvent polymeric solution. In the case of drug loaded LPHNs different amounts of DRV or DRV/RTV (8:1) were added to the organic solvent phase with varied drug/polymer mass percentage (5‑80 % w/w). For blank, unloaded LPHNs no drugs were added. The aqueous phase was prepared by dissolving different amounts of stabilizers, SBL and/or Brij 78, in 4 % v/v ethanolic aqueous solution (2.5 mL). In LPHNs where only one stabilizer was used, a total SBL or Brij 78 mass percentage of (3.75- 20 % w/w) with respect to the PLGA core was used. When mixtures of SBL and Brij 78 were used the total stabilizer/PLGA mass percentage was kept constant at 20 % w/w and the Brij 78/total stabilizer mass percentage was 10-90 % w/w. The aqueous phase was heated to 60 °C for 3 minutes to ensure that the SBL molecules do not self-assemble to form vesicular structure prior the addition of the PLGA solution, this temperature is above the gel-to-liquid transition temperature of SBL, which ensure the obtaining of a homogenously dispersed liquid crystalline phase, so the phospholipids molecules are not close enough to each other to self-assemble into vesicular structure.(Mukherjee et al., 2019b) The polymer solution was then injected dropwise (over approximately 1 minute) into the heated stirring lipid aqueous phase to form the LPHNs dispersion, which were then vortexed for 3 minutes and left to stir overnight, allowing for evaporation of acetone. The LPHNs were maintained as dispersions (4 mL) by addition of deionized water.

### Freeze drying

To obtain stable particles in solid form, the LPHNs dispersions were freeze dried using a VirTis Freeze Dryer. Samples were prepared for freeze drying by the addition of a cryoprotectant, 0.5 % w/v PEG aqueous solution. Diluted LPHNs dispersions were used to limit particles aggregation, and a volume ratio of LPHNs dispersion/deionized water/cryoprotectant solution (0.4:0.6:1) obtained. The LPHNs/cryoprotectant mixtures (2 mL) were placed in 14 mL vials and snap-frozen in liquid nitrogen, the solid mixture was then placed in the freeze-drier for 4 days resulting in a solid monolith in the form of white fluffy powder.

### Characterization of the size and morphology of LPHNs

The predominant analytical method used to determine the size of LPHNs was dynamic light scattering (DLS). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were also used for characterization of the morphology and size of selected samples. DLS measurements were performed using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) with a detector positioned at 173°. The concentration range of the LPHNs dispersions analyzed was (0.65- 1) mg/mL, when these samples were measured in PBS solution (pH 7.4) or simulated gastric fluid (SGF) (pH 1.2), the above-mentioned concentration range was halved, as 0.5 mL of the LPHNs dispersions was diluted with either 0.5 mL of PBS solution or SGF. The concentration of the re-dispersed freeze-dried formulations was 1 mg/mL. The measurements recorded at room temperature (25 °C). The measurements were carried out in triplicate to obtain the size average (Z-average) diameter and polydispersity index (PDI). PBS solution was prepared by dissolving 1 PBS tablet in 100 mL deionized water, while SGF was prepared by dissolving 2 g of NaCl and 7 mL concentrated HCl per 1000 mL deionized water.

Samples were prepared for SEM imaging by pipetting the LPHNs dispersions (0.75- 0.9 mg/mL) onto glass coverslips with 10 mm diameter which were attached to a carbon adhesive disc on top of an aluminum SEM specimen stub (12.5 mm diameter). The samples were left to dry overnight, this was followed by coating with gold (EMITECH K550X) with a deposition current of 25 mA for 100 seconds before imaging. The size and the morphology of LPHNs were then determined using a Hitachi S-4800 FE-SEM at 3 kV.  For TEM imaging, the sample was incubated on a 200-mesh copper formvar/carbon grid for 15 minutes. Excess sample was wicked off with filter paper before incubating the grid on 2% aqueous uranyl acetate (UA) for 1 minute. Excess UA was wicked off, grids left to dry for 10 minutes and then viewed at 120 kV in a Tecnai T12 bioTwin electron microscope with Gatan RIO16 camera.

### Measurement of entrapment efficiency and drug release using radiometric analysis

Radiolabeled DRV containing LPHNs (analogous to those described in section 2.2.1) were formulated with the addition of tritiated [3H]-DRV (specific activity 25 µCi/mg) to the organic solvent phase. Drug loading within LPHNs were determined *via* liquid scintillation counting (LSC) analysis (Packard Tricarb 3100TR liquid scintillation counter). To determine entrapment efficiency, (0.4 mL) of a dispersion of the LPHNs (0.9 mg/mL) was added to a centrifugal unit fitted with a 10,000 molecular weight cut-off (MWCO) filter and centrifuged (14,000 revolutions per minute (rpm), 60 minutes). Samples of the filtrate (3 x 100 µL) were taken and scintillation cocktail (10 mL) added. The ratio of drug which had crossed the filtration barrier determined.

Drug release behavior from the LPHNs was quantified by use of a dialysis method. LPHNs dispersions (1 mL, 0.9 mg/mL) were placed within a double-sided bio-dialyzer fitted with 3.5 kDa MWCO membranes. Dialyses were conducted within sink conditions (1:100) in deionized water at 37 °C, 300 rpm and DRV release was monitored at set time points of: 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 hours. Samples of the reservoirs (1 mL) were taken and scintillation cocktail (10 mL) added. At each time point the bio-dialyzer was removed from the reservoir and placed into fresh deionized water. Samples of water (1 mL) were taken to determine background of LSC, similarly to samples measuring drug release this was extrapolated to 100 mL.

### Transcellular permeability of Darunavir across a triple culture model

#### Cell culture and maintenance

Cells were incubated at 37 °C, 5 % CO2. Adherent cells were sub-cultured once ca. 85 % confluent and Raji B cells were sub-cultured every 3 days. Cell numbers and viability were assessed using a NucleoCounter NC-200 (Denmark).

The permeability of DRV and DRV loaded LPHNs was assessed across an *in vitro* intestinal triple culture model using a method adapted from Schimpel *et al.* (Schimpel et al., 2014) Briefly, transwells were seeded apically with Caco-2 and HT-29-MTX cells in a 7:3 ratio with 1.4 x 105 cells per well, respectively, and propagated over 21-days at 37 °C, 5 % CO2. Following 16-days of co-culture, 1.4 x 105 Raji B cells were added to the basolateral compartment of each well and the triple culture model propagated for a further 5-days at 37 °C, 5 % CO2. During the initial 16-days of propagation, the entire volume of media was aspirated from both apical and basolateral compartments and replaced with an equal volume of fresh pre-warmed media every other day. Following addition of the Raji B cells, 25 % of the media was aspirated and replaced from the basolateral compartment every other day whilst the entire volume was changed apically as previously described. Transepithelial electrical resistance (TEER) values of >800 Ω were observed for all cultured wells. After 21-days of culture, all the media was aspirated, wells washed twice with pre-warmed HBSS and replaced with either DMSO dissolved [3H]-DRV (<1 % total DMSO volume per well) or [3H]-DRV LPHN suspensions spiked into transport buffer to a final concentration of 10 µM DRV. Each suspension was added to either apical or basolateral compartments and transport buffer was added to the opposing chamber. DRV transport from apical-to-basolateral (A>B) and basolateral-to-apical (B>A) directions was assessed. The transwell plates were incubated at 37 °C, 5 % CO2 for the duration of the experiment and 0.1 mL was sampled hourly from the acceptor chamber over 4 hours and replaced with an equal volume of fresh pre-warmed transport buffer. Samples were placed into empty 5 mL scintillation vials before mixing with 4 mL of liquid scintillation fluid. Apparent permeability (Papp) and apparent oral absorption ((Papp (A>B) / Papp (B>A)) equations were used to calculate the rate of DRV permeation as previously described.(Savage et al., 2019) The integrity of the triple culture models were assessed following the 4 h incubation with each test condition. Transport buffer was aspirated, and the wells washed twice with pre-warmed HBSS. Subsequently, 0.1 mL of transport buffer containing [14C]-mannitol (50 µM, 2 µCi/mL) was added to the apical compartment of each well and 0.6 mL of transport buffer was added to the basolateral compartments. The transwells were incubated for 1 h at 37 °C, 5 % CO2 and 0.1 mL of the basolateral contents were sampled and placed into a 5 mL scintillation vial before mixing with 4 mL scintillation fluid for radiometric analysis as described above.

#### Statistical analysis

Statistical analysis was performed using GraphPad Prism v.8.2 (US). Data normality was assessed using a Shapiro-Wilk test and subsequently unpaired, two-tailed t-test were applied to the datasets. Differences were considered statistically significant at \*, P<0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; \*\*\*\*, P < 0.0001.

# Results and discussion

## Optimization of the synthesis of LPHNs

In order to optimize the formulation of LPHNs, blank (without drug) and DRV loaded LPHNs were prepared *via* nanoprecipitation. The aim of this optimization was to prepare nanoparticles that had the highest possible drug loading and possessed colloidal stability under physiological ionic strength. Three variables were tested: the effect of varying the mass percentage of the total stabilizer to the PLGA polymer core, the mass percentage of Brij 78 to total stabilizer and the drug loading. The first two variables were optimized using blank nanoparticles, then the drug loading was investigated once drug was incorporated. DLS was used to measure the Z-average diameter, the size distribution, and the polydispersity index of the particles in all formulations. The colloidal stability of the formulations was assessed *via* measurement of the diameter of the particles upon exposure to physiological ionic strength (in PBS) at pH 7.4. Poor colloidal stability would result in aggregation of the particles which would be observed as an increase in diameter and polydispersity.

## LPHNs formulations parameters

### The effect of varying the mass percentage of total stabilizer to the polymer core

The repulsive interactions between nanoparticles that are provided by the stabilizers (surfactants) used in the formulation of LPHNs are integral to their colloidal stability. The mass percentage of the stabilizer to the PLGA polymer core is a key variable as it must provide enough repulsion between particles to obtain colloidal stability. Initially, the effect of each stabilizer on the colloidal stability of the particles was studied. The two stabilizers SBL and Brij 78 were used as sole stabilizers to form blank nanoparticles at different stabilizer/PLGA mass percentage: (3.75- 20 % w/w), keeping the amount of PLGA constant. Control nanoparticles made of PLGA cores only with no stabilizer were synthesized, these particles had a Z-average of 66 nm and a PDI of 0.46, however they aggregated immediately upon dispersion in PBS.

#### Soybean lecithin as a stabilizer for LPHNs

When SBL was used on its own as a stabilizer (Table 1) for LPHNs, and the particles dispersed in water, mean diameters of 70-79 nm were observed. Smaller particles formed when higher amounts of SBL were used relative to the polymer core. If the particles were added to PBS at physiological ionic strength the particles aggregated at all SBL/PLGA mass percentages (0.375 – 20) % w/w. This was likely due to the electrostatic stabilization mechanism provided by SBL; electrostatic repulsion is greatly reduced in the presence of high ionic strength due to the electrostatic screening effect of the dissolved ions. The same behavior was reported for a different phospholipid by Chan *et al.* (Chan et al., 2009)their results showed that using phosphatidylcholine as a stabilizer for docetaxel LPHNs led to particle aggregation even at lipid/polymer mass percentage up to 20 % w/w. The zeta potential of the particles stabilized by SBL ranged from -54 to -67 mV when dispersed in water (Table 1), upon dispersion of the particles in a PBS, there was a decrease in the zeta potential value of the LPHNs (-29 to -35 mV) which affects the colloidal stability. It has been suggested by Hu *et al*. (Hu et al., 2013) that to obtain a full electrostatic stabilization a zeta potential more than ±30 mV and ideally more than ±60 mV is required. It was therefore concluded SBL as a lone stabilizer was not able to provide enough stabilization under physiological conditions.

**TABLE 1**

#### Brij 78 as a stabilizer for LPHNs

Brij 78 was used in a varying ratio to PLGA mass percentage, resultant LPHNs had a mean Z-average diameter of 62-85 nm with low PDIs (0.20-0.35). An increase in the Brij 78/PLGA mass percentage above 3.5 % resulted in a slight decrease in the Z-average of the particles (Figure 2). The particles had mean Z-average of 48-70 nm and PDI (0.23-0.25) when dispersed in PBS. Unlike SBL, the steric stabilization provided by Brij 78 allowed colloidal stability in PBS when > 7.5 % of the mass percentage of Brij 78/PLGA was used. Below this at a Brij 78/PLGA mass percentage of 3.75 %, aggregation of the particles occurred in PBS as the amount of surfactant was insufficient to give full steric coverage of the PLGA cores. Brij 78 surfactant/PLGA ratio of 20 % w/w was chosen for further studies, as it provided the particles with the smallest Z-average diameter following dispersion in PBS.

**FIGURE 2**

#### Using a combination of Brij 78 and soybean lecithin as stabilizers for LPHNs

In order to obtain LPHNs that offer the combination of enhanced pharmacokinetic behavior from a lipid shell with the colloidal stability under physiological ionic strength, the effect of varying the Brij 78 and SBL mass percentages were investigated. The mass percentage of the surfactant relative to the PLGA polymer core was kept fixed at 20 % w/w as this was the percentage that showed the lowest Z-average for LPHNs dispersions when dispersed in water and PBS for both SBL (Table 1) and Brij 78 (Figure 2).

LPHNs with different mass percentages of Brij 78 with respect to the total mass of surfactants (SBL and Brij 78) ranging from 10-90 % were investigated (Figure 3). The aim was to determine the optimum mass percentage that would allow LPHNs to maintain stability in PBS, with the highest amount of SBL that might enhance the biological behavior of the particles. Previously SBL has been shown to enhance the bioavailability of hydrophobic drugs such as curcumin in a mixed polymeric micellar system.(Chen et al., 2016) In water, all samples showed very similar mean diameters regardless of the composition, indicating that both stabilizers were equally effective surfactants. However, in PBS the effect of surfactant composition was much more pronounced, when the total mass of stabilizer contained less than 30 % Brij 78 then aggregation was observed. An increase in Brij 78/total stabilizer to greater than 50 % showed effective stability in PBS. The samples with Brij 78 content of either 70 or 90 % w/w were selected for further optimization of drug loading due to their small diameters. These results shows that the formulation requirements for LPHNs in terms of the ratio between Brij 78 and phospholipids mixture (7:3 and 9:1) compares closely to the work of Chan *et al*. who used a lipid to lipid-PEG conjugate at a 2.5:7.2 ratio.(Chan et al., 2009)

**FIGURE 3**

### Effect of Drug/PLGA mass percentage on particle properties and formation of dispersible formulation

As the role of each surfactant has been established in stabilizing blank LPHNs, drug loaded LPHNs were prepared to determine the highest possible drug loading. The Brij 78/total stabilizer percentage chosen for these studies were 70 and 90 % w/w, and a 100 % Brij 78 was used as a control. The drug mixture was composed of a mixture of DRV and RTV with a ratio 8:1 w/w as this is the clinically used ratio. Given the benefits that would be offered by a dispersible formulation, the effect of freeze drying was also investigated on the particle properties. Drug loaded LPHNs (DRV-RTV-LPHNs) with a range of different DRV/PLGA mass percentages were studied (5-80 % w/w). The DLS data shown in Table Supporting Information (SI) 1 shows that before freeze drying, the samples had similar Z-average diameters even with an increase of DRV/PLGA mass percentage from 5-40 % w/w at the three different Brij 78/total stabilizer mass percentage used (70, 90 and 100 % w/w). The Z-average diameter ranged from 45-109 nm and the PDI was 0.16-0.37 for LPHNs dispersed in DI water (Table SI1). Upon dispersion in PBS, the Z-average of LPHNs ranged from 50-98 nm and the PDI was 0.13-0.24 (Table SI2). Increasing DRV/PLGA mass percentage above 40 % w/w led to particle aggregation with poor quality DLS data. This showed that LPHNs with high drug loading of up to 20 % w/w could be formed with a range of stabilizer compositions.

The effect of freeze drying on the formulations was investigated using a cryoprotectant, 0.5 % w/v PEG, which is a significantly lower concentration of the cryoprotectant compared to that reported by Nidhi *et al.*(Nidhi et al., 2011) Lower concentration of excipients as cryoprotectants was targeted as it meant a relative higher concentration of drug loaded LPHNs can be achieved. After redispersion the effect of increasing the DRV/PLGA mass percentage on particles properties became apparent; increasing the DRV/PLGA mass percentage above 20 % w/w led to particle aggregation. For LPHNs with DRV/PLGA mass percentage between 5-20 % w/w, the Z-average for freeze-dried LPHNs dispersed in DI water ranged between 125-255 nm (Table SI1), while upon dispersion in PBS (Table SI2), the Z-average of freeze-dried LPHNs became larger ranging between 185-374 nm. After freeze drying all samples, independent of dispersion media, increased in diameter. Such increases in particle size have previously been shown to be due to the aggregation of particles in the freezing step. (Niu and Panyam, 2017) For drug loaded LPHNs (DRV-RTV-LPHNs), it was possible to incorporate relatively high amount of the drug into the polymeric cores (up to 20 % w/w of DRV/PLGA, the DRV/RTV ratio was kept at (8:1)) whilst maintaining a mean diameter below <330 nm. Varying the Brij 78/total stabilizer mass percentage between 70-100 % w/w did not show a significant difference on particle properties.

From the parameters tested (Table SI2), it was apparent that 20 % w/w DRV/PLGA mass percentage is the highest value that can be achieved among the studied mass percentages while maintaining good quality DLS data. The drug loading of these DRV-RTV-LPHNs is much higher than those reported in the literature. For example, Dalmoro *et al.*(Dalmoro et al., 2018) reported a drug loading of 10 % for indomethacin-loaded LPHNs synthesized using chitosan as a polymer. In another study*,* the drug loading of bupivacaine-LPHNs was found to be 8.6 % also prepared by a nanoprecipitation method. (Ma et al., 2017)

The optimum DRV-RTV-LPHNs formulas selected for further studies were referred to as LPHN70, LPHN90 and LPHN100, which refers to Brij 78/Total stabilizer % w/w of 70, 90 and 100, respectively. In all three formulations, the mass percentage of Total stabilizer/PLGA was 20 % w/w and DRV/PLGA was also 20 % w/w.

The stability of LPHN70, LPHN90 and LPHN100 in SGF was tested, DLS data in Table SI3 showed that all three formulations were stable in SGF as LPHNs dispersions (before freeze drying). The LPHNs had a mean Z-average diameter of 129- 138 nm and the PDI was (0.31-0.34). However, the freeze-dried formulations all aggregated once dispersed in SGF, the cause for this aggregation is unclear and will be investigated in our future work.

## Analysis of the morphology and size of the LPHNs by SEM and TEM

SEM and TEM were used to explore the structure and morphology of DRV-RTV-LPHNs with either SBL as the sole stabilizer or with a combination of surfactants Brij 78 70 % w/w and SBL 30 % w/w (Figure 4). SEM analysis provided images of spherical particles with smooth surfaces (Figure 4A and 4B). An average diameter of approximately 50 nm with narrow size distributions was determined, concordant with the DLS size distribution data shown in Figure 4E. TEM analysis was used to give an insight into the structure of the nanoparticles (Figure 4C and 4D), both samples had a dark ring surrounding the cores of the particles. These dark rings represent the lipid shell of the particles which were stained with uranyl acetate to increase the electron density in this peripheral region as was reported by Mandal *et al*.(Mandal et al., 2016) It is apparent that the use of mixture of Brij 78 and SBL result in a LPHN structure with a polymer core and a shell containing the lipid.

**FIGURE 4**

## Encapsulation efficiency and drug release

In order to assess the effect of surfactant composition on drug encapsulation and drug release behavior, three different LPHNs formulations were tested: LPHN70, LPHN90 and LPHN100. These formulations all contained DRV at 20 % w/w using both [3H]-DRV/DRV and unlabeled RTV in an 8:1 ratio into PLGA cores. Firstly, the encapsulation efficiency of the formulations was investigated using a spin filter method to separate the free drug from the encapsulated drug. The analysis of this showed that the DRV encapsulation efficiency of the three formulations was similar (62, 68.1 and 68.5 % w/w) for formulations LPHN70, LPHN90 and LPHN100 respectively, with a slight increase in encapsulation efficiency noticed with increasing Brij 78 content.

The effect of freeze drying and drug combination on the drug encapsulation efficiency of LPHN70 was also studied. The encapsulation efficiency of freeze-dried LPHN70 loaded with DRV/RTV (8:1) or DRV on its own was 90.8 and 95.7 % w/w, respectively. We can conclude that freeze drying increased the DRV encapsulation efficiency of LPHN70 from 62 to 90.8 %. This increase in the encapsulation efficiency may be caused by the cryogenic freezing of the samples resulting in the formation of solid drug nanoparticles from the non-encapsulated drug. Indeed, freezing and freeze drying in the presence of surfactants are key steps used in the process of some solid drug nanoparticles formulations. (Giardiello et al., 2016; McDonald et al., 2014b) These solid drug nanoparticles would have been unable to penetrate the dialysis membrane and would therefore be measured as encapsulated drug. The formation of such nanoparticles would be difficult to detect by DLS as this technique provides a limited ability to resolve separate nanoparticle populations unless the size differences are approximately 4 fold. (Filipe et al., 2010) Unfortunately, it was not possible to carry out useful SEM analysis on these samples after freeze drying in order to investigate the potential presence of solid drug nanoparticles, this was due to the film forming behavior of the PEG cryoprotectant which meant that it was not possible to resolve individual nanoparticles. It was also found that the incorporation of RTV led to an increase in the total drug content which caused a decrease in the encapsulation efficiency from 95.7 to 90.8 % w/w. Due to the role of RTV as a booster for DRV, the encapsulation efficiency of RTV in the formulations was not investigated in this study.

The drug release behavior from LPHNs was investigated using radiometric dialysis and the drug released quantified using radiolabeled [3H]-DRV Figure 5A. DRV is slightly soluble in water (0.15 mg/mL as listed on the FDA datasheet) and the concentration of DRV used in the release study was below this limit. The three formulations showed little burst release (measured at the first time point of 30 minutes) of less than 14 % which can be contributed to drug not encapsulated within the particles which may be adsorbed onto the surface of the particles. This value for burst release was lower than expected given that the encapsulation efficiency studies suggested that at least 30 % of the drug was not trapped within the particles. However, this discrepancy might be due to harsher separation conditions that occurred during the spin-filter separation. With regards to the drug release profile of the three formulations, they were relatively similar with over 75 % of the drug being released in the first 8 hours (Figure 5A). The sample containing the most SBL (30 %) and least Brij 78 (70 %) showed the fastest release. Increasing the Brij 78/total stabilizer mass from 70 to 100 % w/w led to slower release. This may be attributed to the PEG corona surrounding the PLGA cores derived from the Brij 78, as this corona layer becomes denser, it retards drug release from the cores, leading to slower release and higher encapsulation efficiency. Zhang and Chen (Zhang et al., 2014) showed that pegylated lipid nanoparticles displayed slower and sustained drug release compared to the non-pegylated ones. All three formulations showed low burst release and were selected for further biological studies to assess how the composition of the surfactant might influence the biological behavior. Interestingly, LPHNs post-freeze-drying (Figure 5B) showed that freeze-drying did not influence the release behavior of the particles.

**FIGURE 5**

## The effect of LPHNs surfactant composition on the absorption of DRV across a triple culture model

The three LPHNs formulations, namely LPHN70, LPHN90 and LPHN100 were investigated for their potential to deliver DRV in a model for the human intestinal epithelium. These formulations all contained DRV/PLGA at 20 % w/w using both [3H]-DRV/DRV and unlabeled RTV in an 8:1 ratio both before and after freeze drying. Radiolabeling was used to permit a quantitative assessment of DRV transcellular permeation and enabled a comparison to a conventional [3H]-DRV/DRV (<1 % DMSO) aqueous preparation. An *in vitro* triple culture model comprising; Caco-2 cells, HT-29-MTX mucus secreting goblet cells and Raji B lymphocytes which stimulate the differentiation of Caco-2 cells into microfold cells (M cells) were utilized to study the absorption of [3H]-DRV/DRV loaded LPHNs. This *in vitro* model has been shown to be a useful predictive model for intestinal permeability of small molecules (Lozoya-Agullo et al., 2017) while also providing a more physiologically accurate representation of the permeability of nanoparticles compared to Caco-2 monolayers alone. (Schimpel et al., 2014)

The permeation of DRV in both apical-to-basolateral and basolateral-to-apical directions was assessed. The results in Figure 6A show that LPHN70 displayed a significant increase in the Papp ratio of DRV equivalent to a 15 %, 84 % and 156 % increase following a 2, 3 and 4 h incubation respectively. Lower DRV apparent oral absorption at all time-points when formulated into LPHN90 (Figure 6B) and LPHN100 (Figure 6C) compared to the equivalent aqueous preparation was noted. The observed increase in *P*app ratio for LPHN70 appears to be facilitated by an increase in A>B permeation. Conversely, the reduced *P*app ratio for LPHN90 and LPHN100 appears to be primarily driven by an increased B>A permeation (efflux) Figure SI1). Previous studies have demonstrated enhancements in the oral bioavailability of poorly water-soluble drugs when co-formulated with lipids.(Lee M. Tatham, Alison C. Savage, Andrew Dwyer, Marco Siccardi, Trevor Scott, Manoli Vourvahis, 2018; McEvoy et al., 2017; Mohsin, 2012) It is possible that the greater mass percentage of SBL used in LPHN70 formulation permitted greater solubility enhancement and subsequent permeation of DRV in the triple culture model. However, the effect of reducing Brij in LPHN70 composition and increased *P*app ratio cannot be excluded and warrants further investigation. Due to the similar mean diameter (61-76 nm) and polydispersity index (0.13-0.21) for all three formulations, these particle properties can be excluded as the causation factor driving the differences in biological behavior. Integrity of the triple culture models were assessed using the low permeability marker [14C]-mannitol following incubation with each treatment. The integrity was assessed post-incubation to identify any potential cumulative damage over the 4 hours. The results in Figure 6D and Figure SI2B indicate mannitol *P*app values less than 0.953 x 10-6 cm s-1 and suggest that the triple culture model remains intact following each treatment. (Elsby et al., 2008) Therefore, the differences in the permeability of the different formulations was derived from the different surfactant compositions used in the formulations. The development of a LPHNs with 70 % Brij 78 and 30 % SBL as the surfactant provided excellent colloidal stability while also displaying enhanced apparent oral absorption in the triple culture model.

Subsequently, the biological behavior of freeze-dried LPHN70 was investigated using the triple culture model. The DRV *P*app ratio was markedly different compared to the non-freeze-dried preparation (Figure SI2) with increased *P*app ratio at 1 and 2 hours incubation but lower *P*app ratio at 3 and 4 hours incubation. No statistically significant differences in Papp between the unformulated DRV and LPHN70 formulated DRV were observed over the 4 hours which contrasts with the results observed for the non-freeze-dried preparation. It is difficult to be certain of the mechanism responsible for the marked difference in Papp due to freeze drying the formulation, however, the presence of the PEG cryoprotectant may have coated the LPHNs and inhibited their interaction with the cells. Additionally, the freeze drying processed resulted in an increased mean diameter (61 vs. 247 nm) and increased polydispersity (0.13 vs. 0.44) which may have influenced the interaction of the particles with the cells.

**FIGURE 6**

# Conclusion

This work shows that LPHNs can be prepared with high drug loadings of DRV/RTV (20 % w/w) without negatively impacting the particle properties. The particles encapsulate the clinically used antiretrovirals (DRV/RTV) and the surfactant composition was tuned to combine colloidal stability under physiological conditions with sufficient lipid (in the form of SBL) to offer enhanced permeability.

From the formulation ranges tested, our findings suggest that the optimum stabilizer to polymer core was 20 % w/w. Brij 78 was crucial in providing stability in biologically relevant media. We have shown that these LPHN formulations can be freeze-dried to obtain a solid, addressing many of the stability issues that are faced for the storage of liquid nanomedicine formulations. These solids can then be redispersed at the time of need, a considerable challenge in nanomedicine. However, for this formulation, the freeze-drying process reduced the permeation behavior in a triple culture model. The cause for this reduction in biological performance will need further research to understand the factors controlling this behavior. Our work shows that SBL plays an important role in the permeation of drug loaded particles across the intestinal epithelium. Formulations of DRV/RTV in LPHNs may offer improved drug delivery for the treatment of HIV.

# Acknowledgments

The authors would like to thank the Department of Chemistry at the University of Liverpool for supporting HE with an International Postgraduate Research Studentship. We very gratefully acknowledge financial support provide to HE through the Schlumberger Faculty for the Future grant program and funding from the EPSRC (Grant Number EP/S012265/1).

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**Highlights**

* Lipid polymer hybrid nanoparticles (LPHNs) were used as nanocarrier for oral delivery of HIV drugs: darunavir and ritonavir for the first time.
* Soybean lecithin was crucial for enhancement of the oral bioavailability of LPHNs and Brij 78 was a key component in maintaining particles stability at physiological pH.
* A high drug loading was achieved in the formulation (20 % w/w DRV/PLGA).
* Freeze drying using a comparatively low concentration of PEG as a cryoprotectant allowed dry, redispersible LPHNs formulations to be prepared.

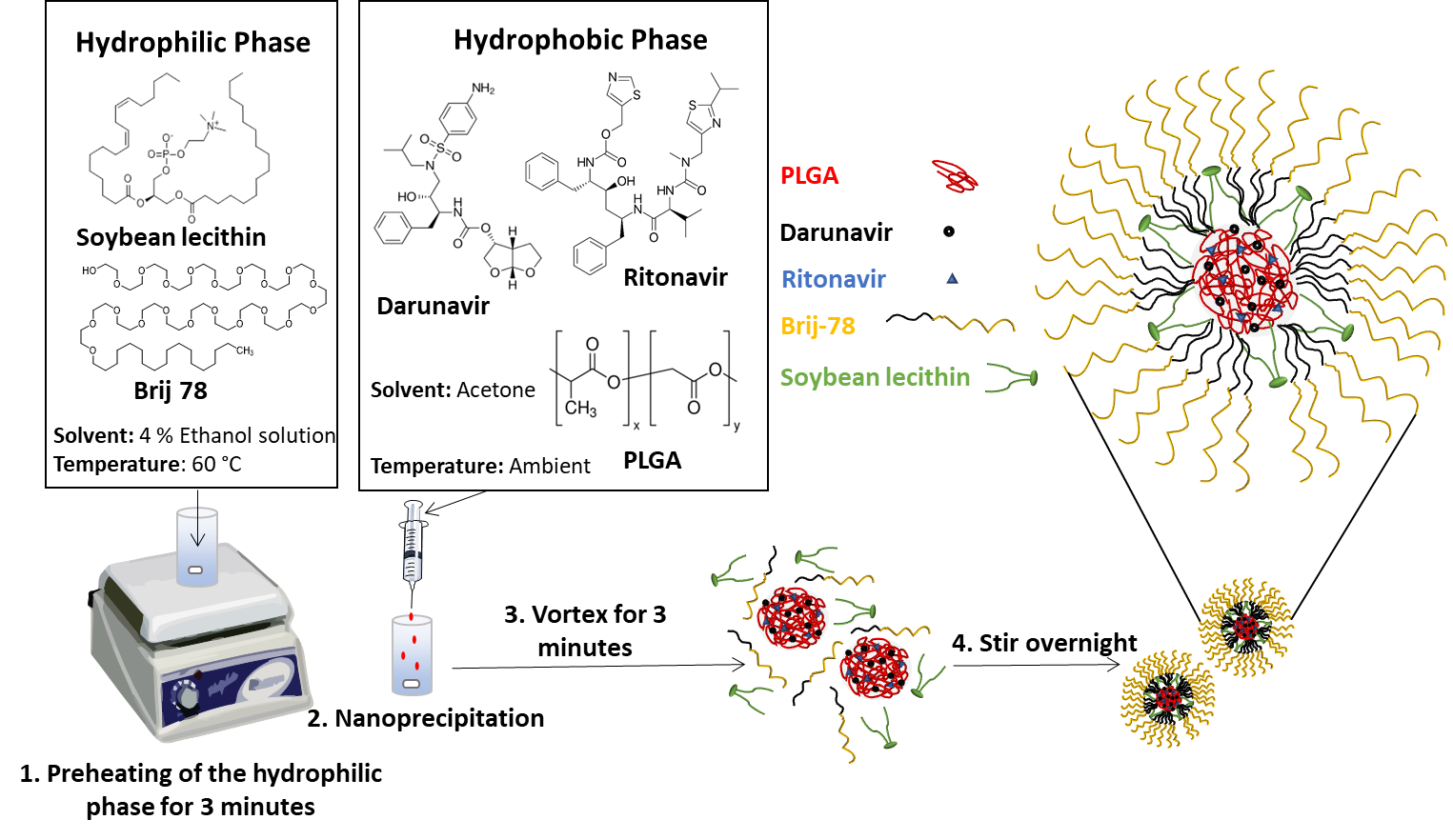
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Figure 1. Schematic representation of the synthetic steps of formulating drug loaded LPHNs by nanoprecipitation. DRV, RTV and PLGA were dissolved in acetone and added to an aqueous phase, under moderate magnetic stirring, containing SBL and Brij 78. Acetone evaporation at ambient temperature overnight allowed an aqueous suspension of LPHNs to be obtained. The LPHNs were composed of a core of PLGA containing the drugs and a shell of SBL and Brij 78.

Table 1. Summary of DLS data (Z-average, PDI and Zeta potential) of blank LPHNs dispersions synthesized with different mass percentages of SBL/PLGA dispersed in deionized water or PBS. The term “Aggregated” refers to particle aggregation that can be seen by naked eye that is accompanied by Z-average diameter above 1000 nm. Data are represented as mean ± SD (n =3), where SD is the standard deviation and n is the number of samples measured.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **SBL/PLGA % w/w** | **Deionized water** | | | **PBS** | |
| **Z-average diameter (nm)** | **PDI** | **Zeta potential (mv)** | **Z-average diameter (nm)** | **Zeta potential (mv)** |
| 3.75 | 79 ± 6 | 0.35 ± 0.09 | -54 ± 8 | Aggregated | -30 ± 6 |
| 7.5 | 79 ± 4 | 0.38 ± 0.05 | -60 ± 10 | Aggregated | -35 ± 8 |
| 15 | 71 ± 5 | 0.42 ± 0.07 | -67 ± 5 | Aggregated | -29 ± 3 |
| 20 | 70 ± 3 | 0.40 ± 0.03 | -59 ± 9 | Aggregated | -31 ± 9 |

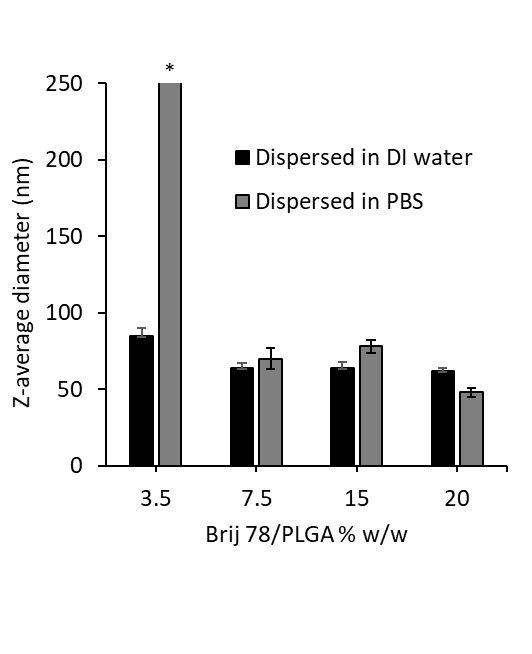


Figure 2. Comparison of Z-average of blank LPHNs dispersions synthesized using Brij 78 as a stabilizer at different Brij 78/PLGA % w/w. LPHNs were dispersed in two different media; DI water or PBS. (Asterisks (\*) indicates poor quality DLS data meaning that the measurements were unreliable likely due to particle aggregation). Data are represented as mean ± SD (n =3).

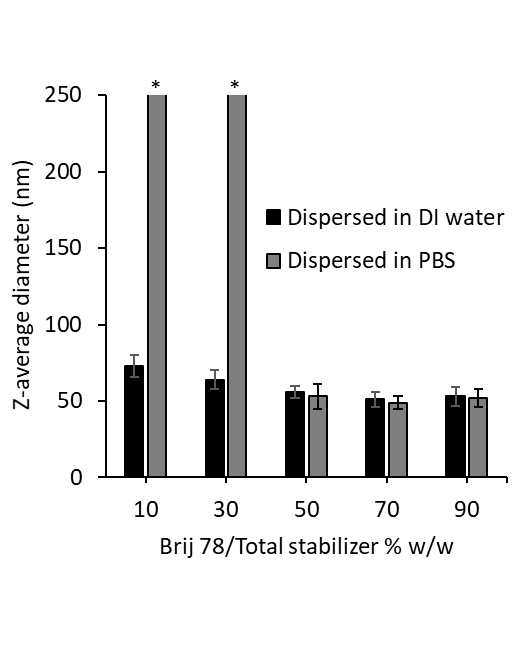


Figure 3. Comparison of Z-average of blank LPHNs synthesized using a mixture of Brij 78 and SBL with different Brij 78/Total stabilizer % w/w, (total stabilizer refers to the mass of both of (SBL and Brij 78). The measurements were carried out on the LPHNs dispersed in different media; DI water and PBS. (Asterisks (\*) indicates poor quality DLS data meaning that the measurements were unreliable likely due to particle aggregation). Data are represented as mean ± SD (n =3).



Figure 4. Particle characterization of DRV-RTV-LPHNs with 20 % w/w DRV/PLGA and 20 % w/w total stabilizer/PLGA have different stabilizers; (A) SEM image of LPHNs stabilized by SBL only. (B) SEM image for LPHNs with Brij 78/Total stabilizer 70 % w/w. (C) TEM image for LPHNs stabilized by SBL only. (D) TEM image for LPHNs with Brij/Total stabilizer 70% w/. (E) Size distribution graphs obtained by DLS for the same 2 formulations, the graph shows monomodal distribution of size with Z-average diameters below 100 nm.

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Figure 5. Drug release graphs of LPHNs containing DRV at 20 % w/w with respect to PLGA. (A) DRV-RTV-LPHNs (not freeze-dried) stabilized with varied Brij 78/total stabilizer mass percentage (70, 90 or 100 % w/w). (B) Freeze dried DRV- LPHNs and DRV-RTV-LPHNs stabilized with 70 % w/w Brij 78/total stabilizer. Error bars represent 3 times LSC background.

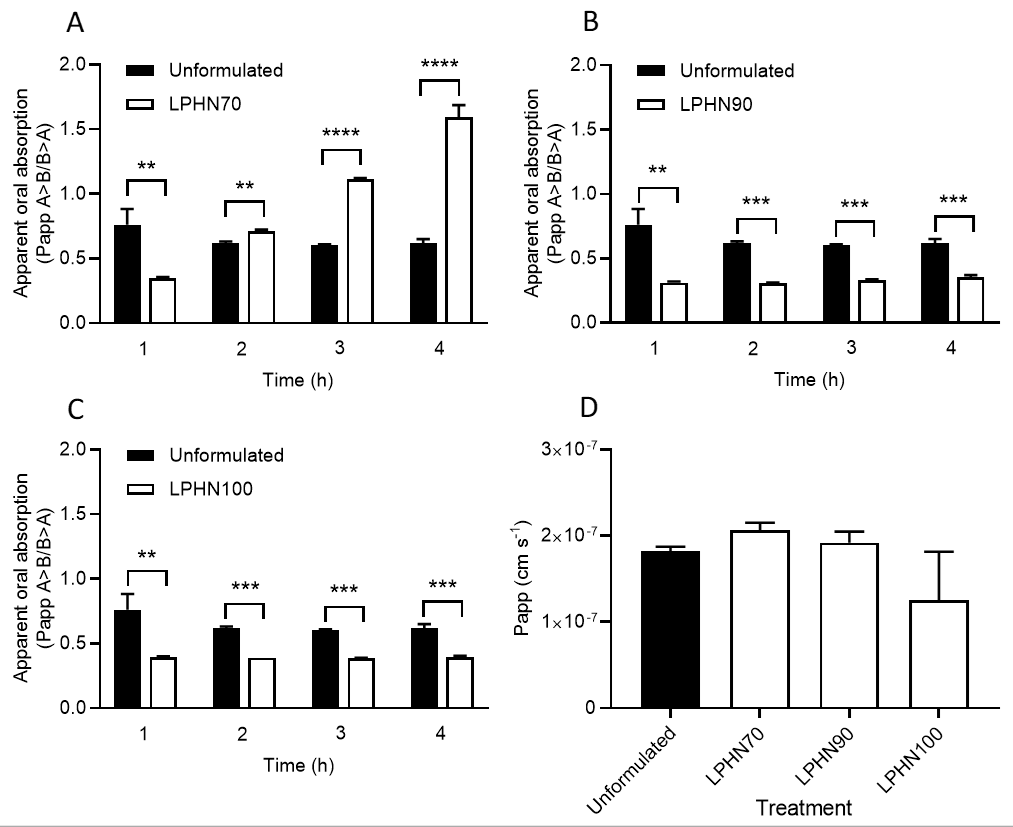


Figure 6. Biological assessment of the effect of surfactant formulation on permeability. (A-C) Apparent oral absorption (Papp ratio) of conventional [3H]-DRV/DRV (<1 % DMSO) and three [3H]-DRV-LPHN preparations across a triple culture permeability model over a 4 h incubation at 37 °C, 5 % CO2. (D) Mannitol apparent permeability (Papp cm s-1), post DRV treatment, following 1 h incubation at 37 °C, 5 % CO2. \*\*, P < 0.01; \*\*\*, P < 0.001; \*\*\*\*, P < 0.0001 (unpaired, two-tailed t-test) (n=4).