

Developing artificial cells for delivering drug molecules to micro-engineered heart tissue

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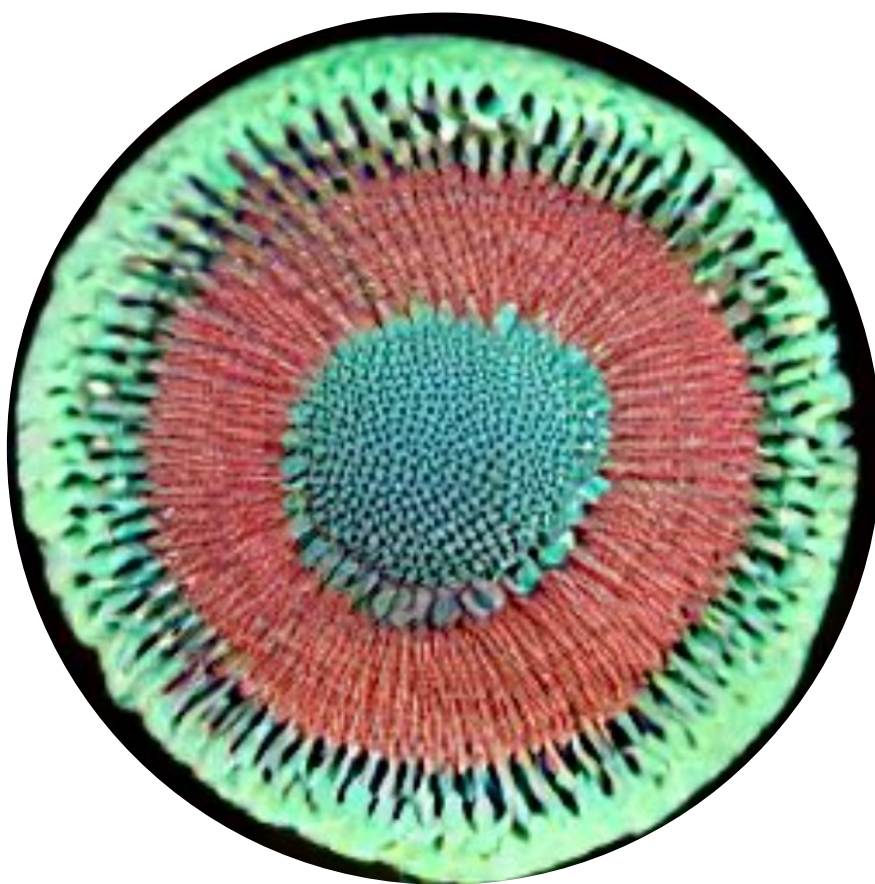


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In memory of Björn Sydney Gugu (1995–2017)

Declaration of originality

I declare that all work within this thesis has been undertaken by myself, unless otherwise stated, and work undertaken by others is acknowledged within main body text, figures, and references.

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Abstract

Batch-based liposome production methods are widely applied within academia. Although batch-based methods offer poor mechanical control, limited size accessibility, and low batch size, therefore, their usage in the study of drug delivery liposomes can present challenges. Additionally, ever increasing specificity of drug delivery liposomes means more representative models of the human micro-environment are required to facilitate efficacy testing. This is especially pertinent in the field of cardiology, where current drug success rates are low. Herein, this work attempts to further understand these two challenges and first present a means for higher throughput, continuous liposome production that has scope for size exploration. Through the application of liposomes produced by this method, the efficacy of drug delivery in both conventional cardiac tissue culture and bioprinted cardiac tissue models can be studied.

Initially addressed are the limitations of batch-based liposome synthesis by employing the previously reported method of microfluidic hydrodynamic flow focusing, a flow-based production method with well-defined mechanical formation. This is extended to another reported method of vertical flow focusing to achieve greater throughput, through the application of a larger channel aspect ratio. Increased throughput ensures the application to larger cell-based validation studies, without sacrificing sample quality. The vertical flow focusing device used in this work employed a differing approach of polymethylmethacrylate multilayer assembly method. This fabrication was designed to be achieved in non-specialist institutions, thus presenting a fully accessible method. Additionally, the multilayer assembly method is amenable to incorporating further functionality. This device was shown to produce liposomes in a therapeutically relevant size window (< 200 nm diameter). In further exploration of the device both the impact of liposome formulation and residual ethanol were explored. Lipid formulation trends shown within the same device instruct how to achieve desired liposome size and distribution, empowering size control within the device application. The exploration into ethanol using molecular rotors is the first attempt at evaluating the impact of residual solvent on the lipid bilayer, this suggested the use of a high flow rate ratio (> 25) for producing minimally perturbed lipid bilayers.

Next, the development of tissue-on-chip models was explored, ranging from polydimethylsiloxane base devices to a bioprinted tissue model. The design and application of a bioprinted tissue model showed potential scalability and ease of analysis. Bioprinted models also enable the study of extracellular matrix, this is especially important for liposomal drug delivery systems as it presents another recognised barrier to liposomal delivery that is currently largely unaddressed. A study of liposomal drug delivery through model extracellular matrixes could further the development of liposome formulations for penetrating specific tissue compositions.

Finally, the performance of targeting (I-1 peptide) and non-targeting control liposomes was explored using the encapsulation of the antagonist menadione. Within tissue culture, targeted liposomal menadione delivery was shown to inhibit cell viability (36.3 ± 12.4 %) whereas non-targeting exhibited minimal impact on viability (84.4 ± 11.7 %). This was the first exploration into menadione delivery and could potentially demonstrate a renewed application, given menadione has previously been reported as a chemotherapeutic agent. The study was replicated using a bioprinted tissue model, demonstrating the first application of liposomes to a bioprinted cardiac tissue model. Overall, this work achieved liposomal drug delivery within an academic setting using approaches that could be applied in scaled liposome validation studies.

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Abbreviations

AA – Amino acid

AM – Aceto methoxy

APTES – 3-Aminopropyltriethoxysilane

AR – Aspect ratio

CA – Contact angle

CFC - Chlorofluorocarbon

CHP – Cardiac homing peptide

CM – Cardiomyocyte

CMC – Critical micelle concentration

CNC – Computer numerical control

CRYO-EM – Cryo electron microscopy

COC – Cyclic olefin copolymer

COL-1 – Collagen (I)

DIC – Differential interference contrast microscopy

DIW – De-ionized water

DLS – Dynamic light scattering

DMEM – Dulbecco's modified eagle medium

DNA – Deoxyribonucleic acid

DOPC – 1,2-dioleoyl-sn-glycero-3-phosphocholine

ECM – Extracellular matrix

EM – Electron microscopy

EPR – Electron paramagnetic resonance

ETFE – Ethylene tetrafluorethylene

FAM – Carboxyfluorescein

FDM – Fused deposition modelling

FRR – Flow rate ratio

FWHM – Full width half maximum

GA – Glutaraldehyde

H9C2 – Proliferative cell line of rat cardiomyocyte cells isolated from myoblast tissue

ID – Inner diameter

IPSC – Induced pluripotent stem cells

IR – Infra-red spectroscopy

LUV – Large unilamellar vesicles

LNP – Lipid nanoparticles

MADLS – Multi-angle dynamic light scattering

MHF – Microfluidic hydrodynamic flow focusing

MI – Myocardial infarction

mRNA – messenger ribonucleic acid

MW – Molecular weight

NOVEC – NOVEC family of engineered fluid (3M)

OD – Outer diameter

OOC – Organ on a chip

OTS – 1H,1H,2H,2H-perfluorooctyldimethylchlorosilane, 1H,1H,2H,2H-perfluorooctyl dimethylchlorosilane

PDI – Polydispersity index

PDMS – Poly dimethyl siloxane

PEB – Post exposure bake

PEEK – Polyether ether ketone

PET – Polyethylene terephthalate

PIX – pixel

PMMA – Poly methyl methacrylate

POTS – 1H,1H,2H,2H-perfluorooctyldimethylchlorosilane, 1H,1H,2H,2H-perfluorooctyl trichlorosilane

PTFE – Polytetrafluoroethylene

PVA – Poly vinyl alcohol

ROS – Reactive oxygen species

SAXS – Small angle x-ray scattering

SD – Standard deviation

SLA – Stereolithography

SLB – Supported lipid bilayer

SUV – Small unilamellar vesicle

TC – Tissue culture

TFR – Total flow rate

TOC – Tissue on chip

UV-VIS – Ultraviolet - visible

VFF – Vertical flow focusing

WAXS – Wide angle x-ray scattering

XRD – X-ray diffraction

1 Liposomal facilitated drug delivery to the ischemic heart – the clinical challenge

1.1 Thesis aims

The primary aim of this thesis is to study *Developing artificial cells for delivering drug molecules to micro-engineered heart tissue*. To achieve the aim, this thesis works toward the following flow chart for the study of therapeutic liposomes (Figure 1.1). By outputting a high throughput method of liposome production to both conventional cardiac tissue culture and a micro-engineered cardiac model the liposomal performance can be assayed, and the results used to direct the liposomal design. Application of an accessible, high throughput liposome production could facilitate rapid formulation screening within academia. Employing both conventional tissue culture and a cardiac model enables study of therapeutic systems with increasing biomimicry. Finally, by using an appropriate assay the efficacy of drug delivery can be evaluated and used to direct the formulation.

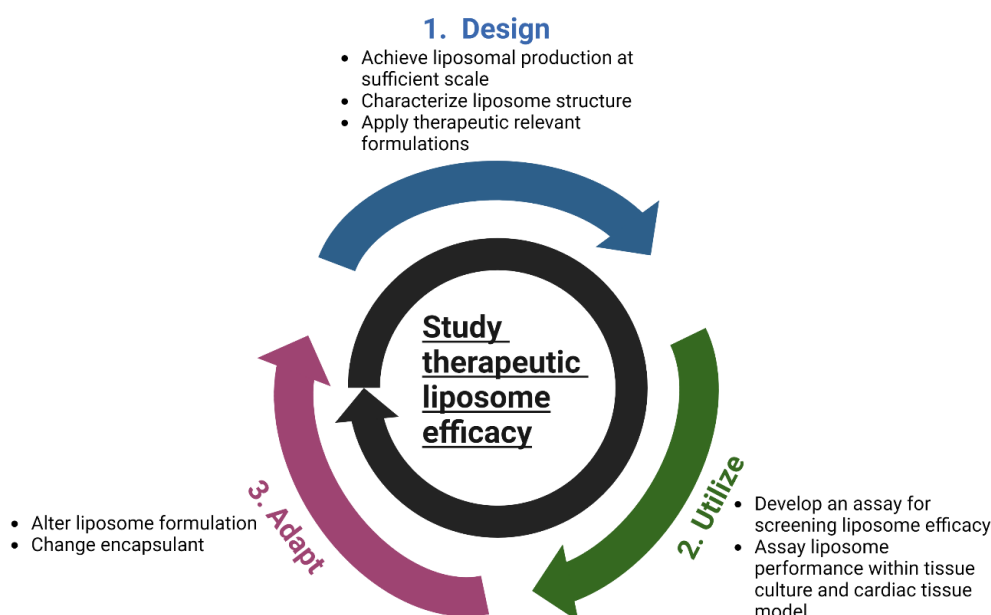


Figure 1.1 Paradigm for studying therapeutic liposomes within academia. First, the design and synthesis of liposomes employing scalable synthesis and characterising the output. Second, the cell-based testing utilizing both conventional tissue culture and engineered tissue *in-vitro* models. Finally, the adaptation of liposome formulation to improve efficacy

In the setup of the flow chart, four main aims are required.

- I. Achieve scalable production of therapeutically relevant liposomes through micro or millifluidics
- II. Further understanding and attempting to address the limitations of the millifluidic hydrodynamic flow focusing liposome synthesis method
- III. Achieve liposomal drug encapsulation and evaluate the drug delivery liposomes
- IV. Evaluate therapeutic liposomal systems on conventional cardiac (H9C2) cell culture and *in-vitro* tissue models using an effective assay

Each aim will be addressed within the following chapters and then the work will be concluded with reference to future work.

1.2 Understanding liposome facilitated drug delivery

1.2.1 Lipid-based drug delivery systems

Liposomes have widely been theorized as advantageous delivery systems for cytotoxic medicines such as doxorubicin as far back as 1990.^{1,2} The landmark therapeutic in the field was Doxil, doxorubicin loaded liposomes although as of 2013 only fifteen lipidic delivery systems have made market, despite over 600 clinical trials in progress.¹⁻⁴ Difficulty presenting success within the clinical environment has been a large setback for the technology, which may present from low stability, particle size control, poor encapsulation and sterilisation method.⁴ More recently, lipid nanoparticles (LNP) which are comprised of lipids and mRNA were approved in 2017 as influenza vaccines, during 2020 mRNA LNP were rapidly developed as COVID-19 vaccines.^{5,6} To further understand the significance of liposomal drug delivery it is best to cover liposome structure, formulation, and synthesis methods. Following this, the clinical background and MI pathology will be discussed with a description of recent cardio *in-vitro* models within academia.

1.2.2 Liposome self-assembly

The predominant constituent of liposomes are typically lipid molecules. Lipids are amphiphilic molecules comprised of a hydrophobic tail region and hydrophilic head group (Figure 1.2A).⁷⁻⁹ The amphiphilic structure drives lipid molecules to favour self-assembly into larger aggregates. Assembly can be illustrated by the tendency for lipids to associate at an oil-water interface, resulting in a lipid monolayer (Figure 1.2B).^{7,10} Self-assembly occurs when the concentration of lipid is above the critical micelle concentration (CMC). At the CMC, the surface energy of an interface is lowered by the partition of hydrophobic moieties from the aqueous phase. The driving energy of self-assembly is the hydrophobic effect, the same process that separates oil and water.^{11,12}

When in an aqueous phase lipid molecules can associate to form a bilayer, similarly to the monolayer example the driving effect is surface tension (γ_{phob}) (Figure 1.3).^{7-10,12,13} Surface tension is the tendency for stationary phases to remain separated, minimising the surface area for contact and avoiding disfavoured mixing interactions. Lipid molecules are held within the bilayer structure and lateral pressure (π_{tot}) is an effective way of describing the forces along a cross-section of the bilayer (Equation 1).¹⁴ Within a bilayer at mechanical equilibrium, net lateral pressure is zero ($\frac{\partial \Delta g_{tot}}{\partial A_l} = 0$) thus the net cohesive hydrophobic tension is balanced by other interactions. Along the profile of the bilayer repulsive values arise from; lipid-lipid interactions in both the tail and headgroup regions and interactions of the headgroup with surrounding water molecules.⁷⁻⁹ Within the tail region, steric repulsion occurs from the flexibility of the chains and thermal fluctuation, these effects are pronounced in the middle of the chain for aliphatic tails.^{7,10,12,13} Tail regions pack together tightly to avoid energetically disfavoured vacuum formation, often by the deviation from the favoured chain conformation.

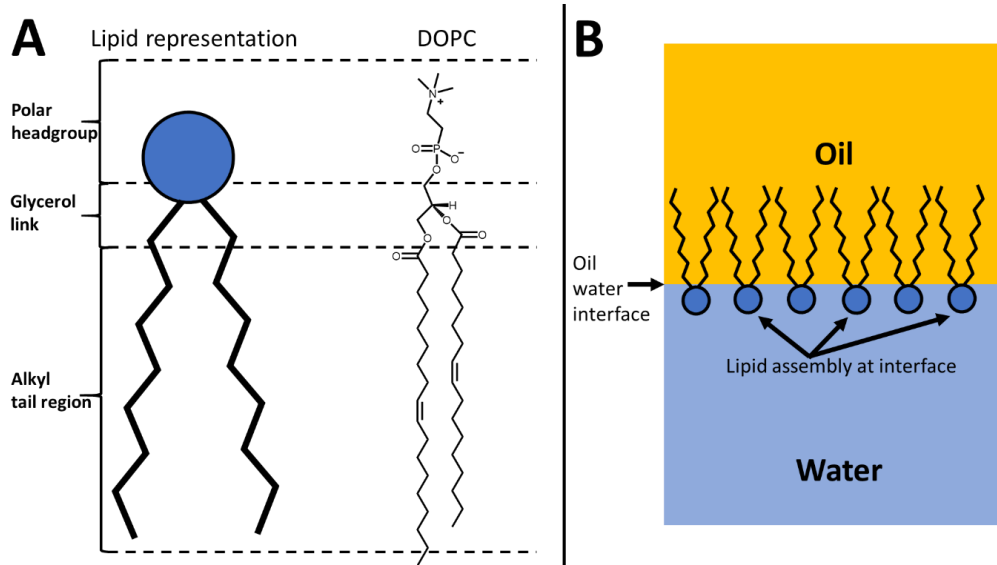


Figure 1.2) A. Cartoon representation of a lipid molecule compared with the structure of DOPC. B Depiction of lipid molecule monolayer assembly at an oil-water interface.

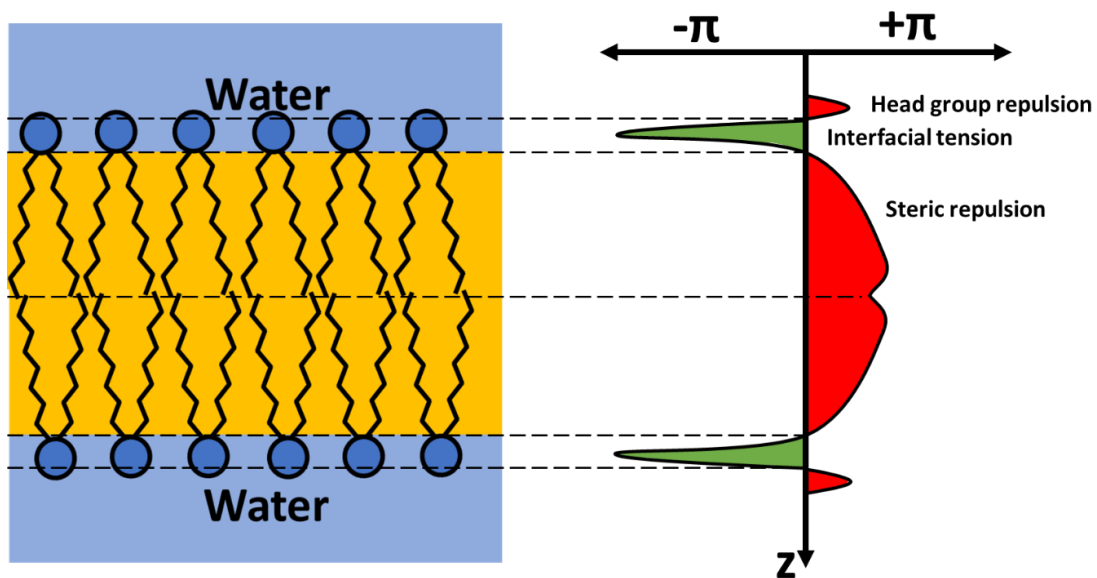


Figure 1.3) Cartoon representation of a lipid bilayer portraying the lipid headgroups as spheres at the aqueous interface (Blue) and lipid tail groups (Black) within the hydrophobic bilayer region (Yellow) [left] The right indicates the corresponding lateral pressure profile highlighting the effects of headgroup electrostatic repulsion, interfacial tension from the phase partitioning and steric repulsion between alkyl tail groups [right].

$$\pi_{tot} = -\left(\frac{\partial \Delta g_{tot}}{\partial A_l}\right)_t = -\left(\frac{\partial \Delta g_{int}}{\partial A_l}\right)_t - \gamma_{phob}$$

Equation 1. Lateral pressure is π_{tot} , Δg_{tot} is the total elastic free energy, A_l is the surface area per lipid molecule and γ_{phob} the surface tension. The $\frac{\partial \Delta g_{int}}{\partial A_l}$ term represents the isotropic lateral pressure; this accounts for both the tail and headgroup interactions.

To see application as a drug delivery liposome, the bilayer needs to adopt curvature. The ratio of volume occupied by the hydrophobic and hydrophilic elements can describe the accessible curvature of the bilayer interface.⁷⁻⁹ A useful metric to describe curvature is the critical packing parameter P (Equation 2).^{7,8} To form a curved bilayer, lipid molecules need to arrange within the positive and negative curvatures of outer and inner leaflets, $P < 0.5$ for the outer leaflet and $P > 1$ inner leaflet (Figure 1.4A). Categorization of lipid packing parameter can aid prediction of adopted curvature preference and by relation, lipid phase (Figure 1.4B).⁷ Liposomes are typically comprised of a single or concentric set of closed lipid bilayers (Figure 1.4C). Curvature is achieved using single lipid types or lipid mixtures, lipids can be categorized as type; zero, one and two. For type zero lipids the volume of the alkyl tail region is comparable to the volume of the headgroup. Whereas type 1 lipids have a larger headgroup when compared to the tail region and type 2 lipids have a larger tail region than headgroup.

$$P = \frac{v_{par}}{A \times l_{max}}$$

Equation 2. Where v_{par} is the alkyl chain volume per molecule, A is the interfacial area per molecule and l_{max} is the maximum chain length achievable in an all trans conformation.

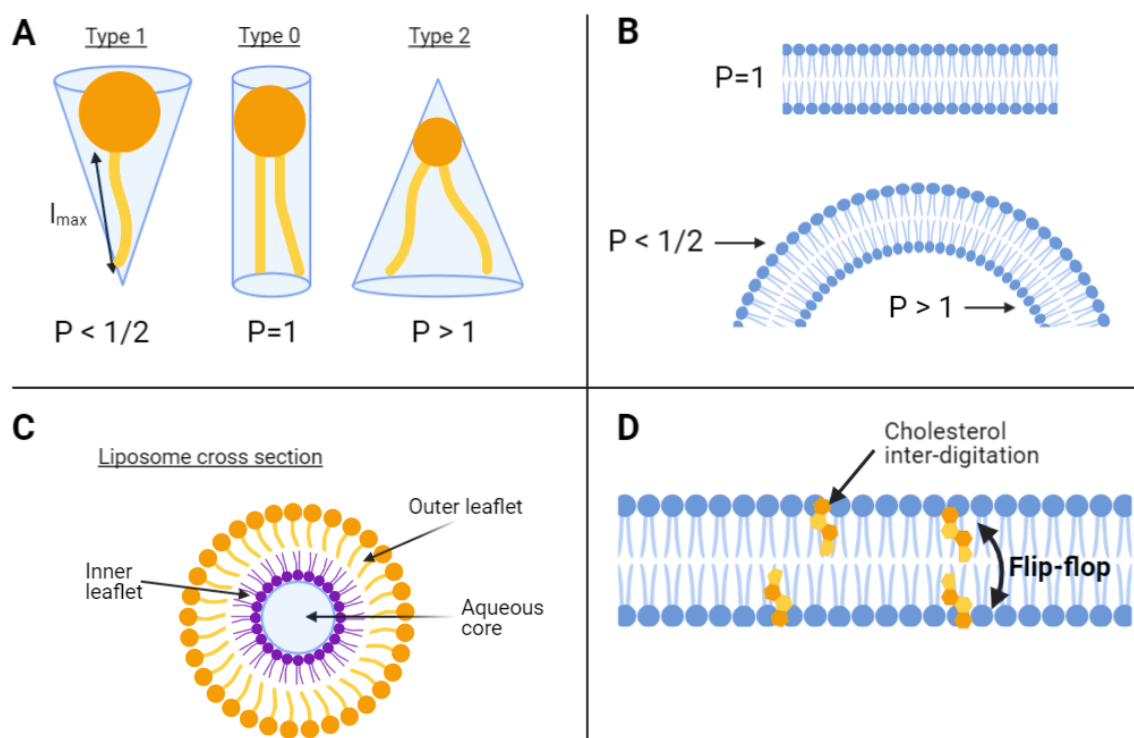


Figure 1.4) **A** Schematic of type 1, 0 and 2 lipids. **B** Flat and curved bilayer, highlighting the related packing parameters. **C** Cross sectional view of a liposome. **D** Cartoon representation of cholesterol insertion and flip-flop within a bilayer.

Liposomes often contain additives such as sterols, cholesterol is a popular liposome additive; its small rigid hydrophobic nature makes it ideal to stabilize fluidic chain regions within the bilayer increasing their rigidity in a dynamic manner through the 'flip-flop' mechanism (Figure 1.4D).¹⁵⁻¹⁷ The L_α and L_β lipid phases are the most applied liposome phases; they represent lamellar packing with fluid alkyl chain and rigid all trans alkyl chains, respectively.⁷ The behaviour of cholesterol is dependent on lipid phase, within the L_α phase, rigid cholesterol acts to reduce lipid fluctuations within the tail region. When the tail region is highly ordered such as in the L_β phase, cholesterol occupies randomly and disorders the alkyl chains. Application of cholesterol can be favourable under the right circumstances,

in the L_α phase cholesterol incorporation reduces diffusion of molecules through the bilayer (Figure 1.5). The ability to reduce leakiness makes cholesterol a constituent of most drug delivery liposomes.

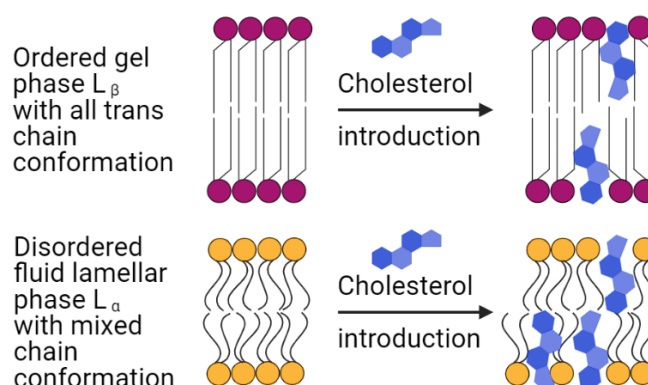


Figure 1.5) Schematic representation of cholesterol (blue) addition to the L_α and L_β phases.

After formation of a curved liposome, application in drug delivery can be achieved (Figure 1.6). Within the liposome structure is a compartmentalised aqueous core, where water-soluble drug molecules can be stored and transported *in-vivo*.^{4,18,19} Alternatively, hydrophobic molecules can be encapsulated within the lipid leaflet which can improve bioavailability of drugs with low water solubility *in-vivo*. Encapsulation allows for the delivery of toxic, sensitive or low efficacy drugs that would otherwise be ineffectual or harmful by conventional intravenous delivery.²⁰ There are a variety of modifications that can be made to the liposome structure to further enhance their efficacy as therapeutic systems for tailor made applications.²¹

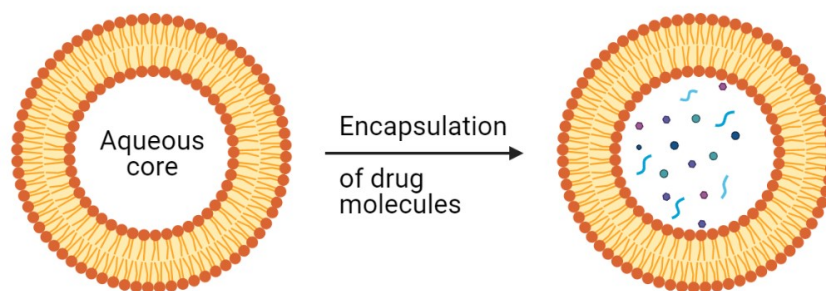


Figure 1.6) Schematic of liposomal encapsulation of hydrophilic drug molecules.

1.2.3 Liposome functionalization for drug delivery

Beyond the core structure of a liposome there are significant number of adaptations that can be made to achieve efficacious drug delivery, this often centres around increasing the longevity of liposomes *in-vivo* and enhancing the specificity of the carrier (Figure 1.7). One such adaptation is size, size is an important characteristic for liposomes and is navigated using nomenclature; small unilamellar vesicles (SUV) < 100 nm, large unilamellar vesicles (LUV) 100-1000 nm and giant unilamellar vesicles (GUV) >1000 nm. The larger length scale of a SUV carrier when compared to a free drug molecule,

necessitates functionalisation to prevent clearance by *in-vivo* immune response. SUV loss in circulation can occur from binding with proteins within blood plasma, resulting in premature cargo release and by sequestration by the liver and spleen.^{22–24} Through use of < 50 nm SUV, composed of high transition temperature lipids, increased circulation time was achieved for doxorubicin loaded SUV.²⁵ Application of < 50 nm SUV reduces the sequestration by the liver and spleen although it has minimal impact on the binding of proteins within the bloodstream.^{24,25}

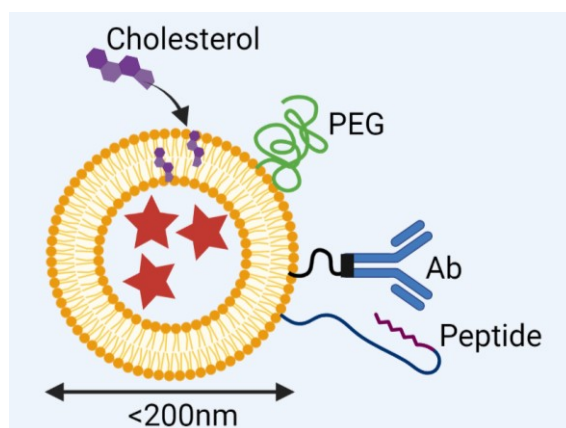


Figure 1.7) Common liposome adaptations for drug delivery; low <200 nm size, cholesterol incorporation, external PEGylation, antibody and peptide targeting. Highlighting lipid bilayer (Yellow), aqueous environment (Blue) and encapsulant (Red).

A common method to prevent proteins binding to SUV is the incorporation of PEGylated lipids, SUV functionalised with PEG, a long chain polyethylene glycol polymer are often termed ‘Stealth liposomes’.^{26–29} Addition of PEGylated lipids was found to improve circulation for doxorubicin SUV, achieving sustained drug delivery over a longer period, when compared to free doxorubicin.³⁰ PEG can be covalently tethered to lipid molecules, when applied within a SUV PEG creates a steric and hydrophilic barrier, preventing adhesion of plasma based proteins. When longevity of circulation is achieved, SUV are reported to achieve effective passive uptake in cancerous tumours by the EPR effect.^{24,31–33} The EPR effect describes the accumulation of circulating SUV within tissue around weakened vasculature, the EPR effect is reported within tumour and infarct tissues.^{32–36} The enhanced accumulation caused by EPR effect is reported to increase the efficacy of encapsulated therapeutic molecules, resulting from higher accumulation and longer retention of SUV within the tissue.^{32,33}

Beyond passive targeting effects, several active targeting methods have been reported in the field.^{37–41} Active targeting involves SUV functionalisation to achieve specific cell-based interaction or response to local environment.^{24,42,43} Cell-based interaction can include a ligand-based interaction to reduce immobilize the SUV at a target site which can also involve a receptor mediated endocytosis.^{24,42,43} Endocytosis is the process of cellular internalization of large molecular complexes, it can occur through a variety of mechanisms.⁴³ By utilising the endocytosis pathways, SUV could achieve drug delivery through the cell membrane, facilitating delivery of > 1 kDa drug molecules and improving efficacy of low water solubility drugs.⁴³

Antibodies and peptides are common ligand types used to facilitate delivery.^{24,42,44,45} Typically targeting moieties are linked to the PEG terminus of PEGylated SUV, this ensures the moiety is accessible for binding.²⁴ Inclusion of antibody targeting has been shown to improve drug delivery for specific cell types and disease states.^{38,41} Peptide targeting has been widely employed as many membrane interactive peptides have been discovered.^{39–41} Peptides can increase anchorage of SUV at a specific cell and facilitate SUV uptake, into cells.^{46,47} Similarly to antibody functionalized SUV, peptide

functionalized SUV have seen application to specific cell types and disease states.^{40,41,46,47} Dasa *et al*³⁹ and Vandergriff *et al*⁴⁰ have demonstrated the use of peptides to targeting SUV to the ischemic heart. Dasa *et al*³⁹ employed biopanning to establish the cardiomyocyte specific I-1 peptide, I-1 SUV exhibited increased accumulation within ischemic mouse models and human tissue sections. Vandergriff *et al*³⁹ employed the CSTSMLKAC 9-mer within therapeutic exosomes and reported therapeutic recovery within both *in-vivo* and *in-vitro* rat tissue. Ko *et al*⁴¹ demonstrate the cooperative use of anti-body and peptide functionalised SUV, to facilitate gene delivery. Beyond functionalisation, it is important to understand the synthetic methods for forming therapeutic liposomes.

1.2.4 Liposome formation and production

A significant number of approaches exist for the formation of SUV that are fit for therapeutic application, both outside and within the academic setting. (Figure 1.8).⁴⁸ Therapeutic SUV typically exhibit < 200 nm diameter, low dispersity and absent of cytotoxic contaminants.^{19,24} An early method of SUV production is organic phase injection, Batzri *et al*⁴⁹ reported this method in 1973 (Figure 1.8A). Injection of a lipid containing organic phase such as ethanol, into an aqueous phase dilutes the organic phase and facilitates self-assembly of SUVs. Bangham *et al*⁵⁰ employed the injection method using ether although subsequent ether evaporation was required to achieve SUVs. Alternative methods include, modified thin film hydration, ink jet printing, detergent dialysis, and a dense gas technique these are outlined in the review by Meure *et al*⁵¹.^{51–53}

The most applied method within academia is the extrusion method which was developed for bulk and batch production of SUVs and LUVs. The extrusion process works by cycling a multilamellar vesicle (MLV) suspension, a mixture of various vesicle sizes and lamellarity through a porous polycarbonate membrane (Figure 1.8B).⁵⁴ Size of the vesicles resulting from extrusion is determined by the pore size within the membrane, increasing cycles through the filter achieves more monodisperse suspensions.^{48,54,55} Size and sample quality is determined by the manufacturing specifications of the commercially produced membrane, which is a potential limitation, especially for the study of liposome size a critical aspect for therapeutic delivery. Other bulk methods employ controlled hydration of a lipid film from a substrate such as ITO electrodes, agarose, and silicon dioxide.⁵⁶ Bulk controlled hydration methods are mostly suited to produce GUVs, except in the work by Sundar *et al*⁵⁷ where prior absorption onto colloidal particles facilitates the production of SUV's once hydrated. Phase inversion is a bulk, droplet based method where aqueous droplets are passed through an oil phase containing lipid and then into an aqueous phase containing lipid.⁵⁶ Phase inversion can be applied to generate asymmetric bilayers by using different lipid components within the oil and aqueous phases.

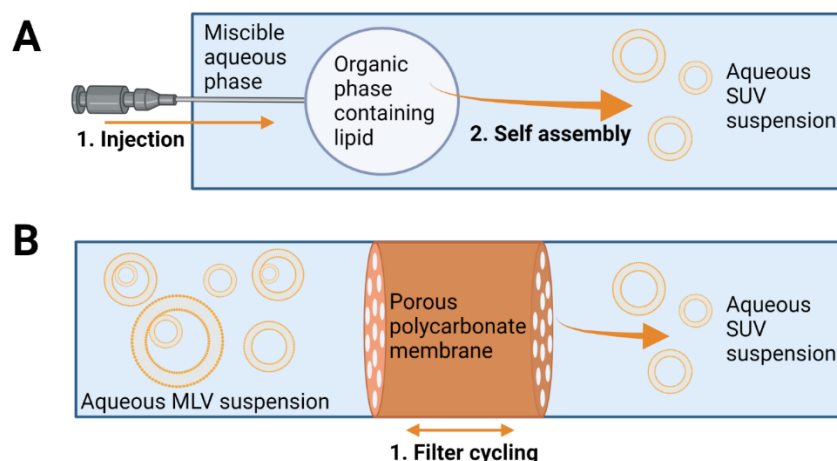


Figure 1.8) Schematic representation of liposome production methods **A** Injection and **B** Extrusion.

Within the field of liposome synthesis microfluidic methods are sought to achieve greater size control and more continuous production when compared to bulk methods. Using droplet microfluidics, the method of droplet emulsion has been applied to produce GUVs, this approach uses a similar principle as the bulk phase inversion method.⁵⁶ The microfluidic droplet emulsion method improves size control when compared to the bulk analogue. A more recent adaptation of droplet emulsion employs an octanol co-solvent to assist GUV formation and improve encapsulation efficiency.⁵⁸

To achieve SUV production with size control, the microfluidic hydrodynamic flow focusing (MHF) process, an adaptation of hydrodynamic flow focusing was first reported by Jahn *et al*^{59–61}. MHF employs a similar principle as the injection method although through miniaturisation, laminar flow predominates, and mixing is achieved through well-defined diffusion and convection.^{61,62} The background of fluids in flow is covered in greater depth within Chapter 1.3. MHF centres around a microfluidic crossflow junction through which a lipid in ethanol solution is co-flowed with an aqueous buffer solution (Figure 1.9). The phases within MHF meet to form a narrow, centralised fluid thread from which ethanol is depleted into the buffer phase. As the ethanol solution depletes, lipids within the solution approach the CMC, the concentration where lipid self-assembly occurs. Initially, in the ethanol solution lipids are fully solvated, as ethanol is depleted Jahn *et al*⁶¹ hypothesize lipid molecules assemble into disc like micelles which both recruit solvated lipid and undergo fusion with other micelles, when a critical size is reached, the structures are believed to undergo a rearrangement into liposomes. Size control within MHF is achieved through application of flow rate ratio (FRR), a dimensionless number, which relates to the rate of diffusional mixing with higher values undergoing more rapid mixing (Equation 3a).^{59,63} Another important value for MHF is the total flow rate (TFR), TFR is reported to exhibit minimal impact on liposomes size though it relates to both the liposome production rate and residence time, the time taken for sample to exit post flow focusing (Equation 3b). MHF provides a route to near continuous liposome synthesis as well as enabling liposome size control.

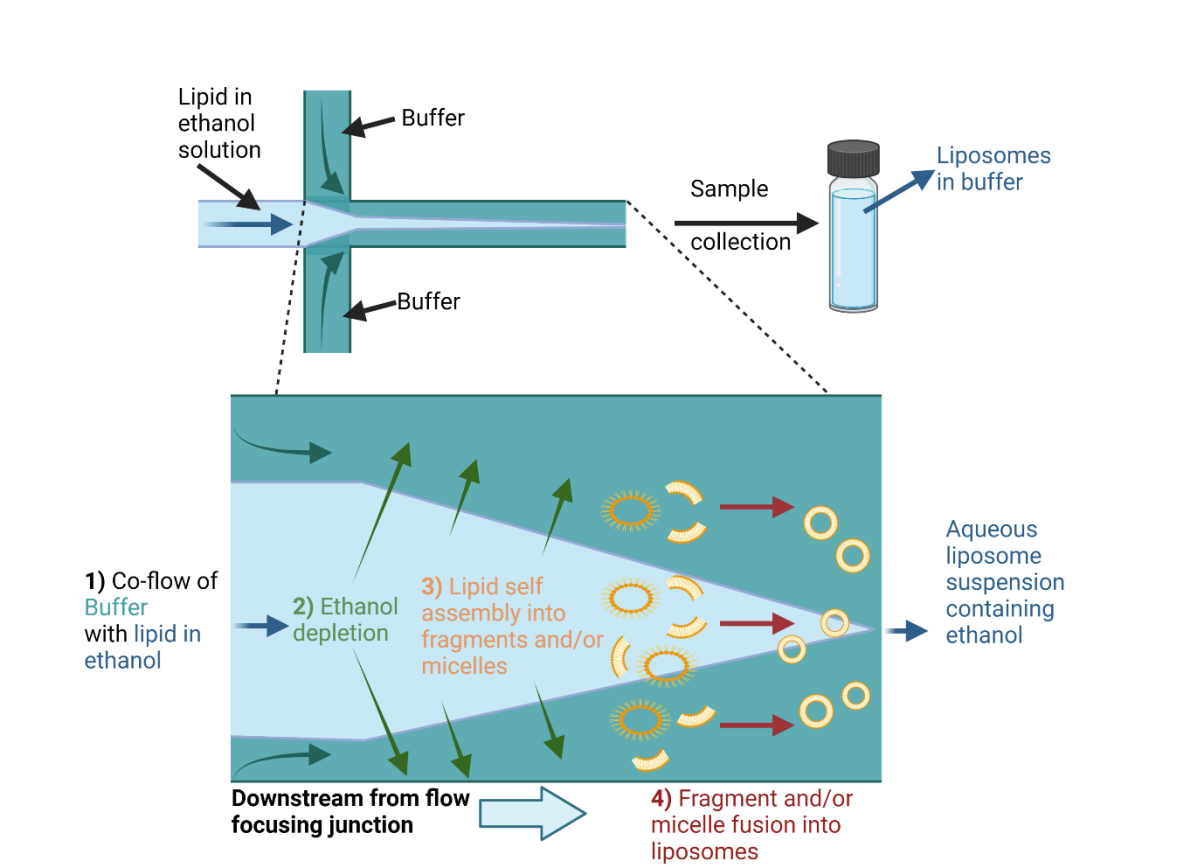


Figure 1.9) Schematic of a 2D MHF crossflow junction indicating the lipid in ethanol phase, buffer phase and liposome suspension output (Top) Enlarged schematic of the ethanol buffer phase boundary where the staged synthesis of liposomes is described (Bottom).

$$FRR = \frac{\text{Total aqueous phase flow rate}}{\text{Total ethanol phase flow rate}}$$

Equation 3a. Flow rate ratio equation

$$TFR = \text{Total aqueous phase flow rate} + \text{Total ethanol phase flow rate}$$

Equation 3b. Total flow rate equation

The vertical flow focusing (VFF) method was first published by Hood *et al*⁶⁴, VFF is an adaptation of MHF in which one dimension within the channel cross section is increased in size (Figure 1.10B). MHF channel dimensions are typically at the sub-millimetre length scale, VFF devices scale one dimension above the millimetre scale, yielding a millifluidic device. In MHF, the AR is typically around 1 with common application of square channels, the MHF device employed in this work has an AR of approximately 1.5 (Figure 1.10C). VFF devices have been reported with AR ranging from 5-100⁶⁴, the device within this work implemented an AR of 50 (Figure 10D). Hood *et al*⁶⁴ demonstrated increasing the AR resulted in higher throughput nanoliposome production, a favourable characteristic for therapeutic application.

The improvement in production is best illustrated by the velocity profiles across the respective channels.⁶⁴ In MHF, the channels are typically < 200 µm, the small width means the effects of friction from the channel edges are pronounced across the channel cross section (Figure 1.10E). The flow velocity decreases from the channel centre, as the fluid nears the channel edge, lower velocity ($v_c >$

v_e) is observed, from the non-slip boundary at the channel edge.^{61,65,66} With narrow flow-focusing within the MHF device, the mixing process is driven by convective-diffusive transport within the focusing region.^{61,65} When highly focused at high FRR, MHF achieves rapid mixing from the concentration gradient and high surface area to volume ratio of the stream cross-section.⁶¹ Rapid mixing typically results in smaller liposomes, this is evidenced by the effect of increasing FRR and by relation, diffusive mixing within MHF.^{59–61} Within this mixing regime, TFR is reported to exert minimal impact on liposome size and distribution.⁶⁰

VFF yields a larger length-scale central ethanol stream, and by relation lower surface area to volume ratio, when compared to MHF (Figure 1.10F). Within VFF a similar velocity trend is observed, as the channel edge is approached from the centre, fluid velocity decreases with friction. The scaling of one dimension within VFF, means the average stream velocity remains close to the value at the channel center (v_c). Remaining close to v_c ensures the impact of the non-slip boundary is minimal, retaining low sample PDI.⁶⁴ The increased scale of the central ethanol stream results in a mixing time that is determined by the molecular diffusion.^{61,65} Within the diffusive mixing regime, the concentration gradient is low, and the ethanol stream is gradually depleted. Conversely to MHF, this mixing process results in larger liposomes as lipid fragments assemble into larger structures before closure.⁶¹ Although the mixing regime is different within VFF, the fundamental lipid assembly process remains identical. VFF represents a means to higher throughput and more continuous liposomes production when compared to MHF and extrusion respectively.

Later adaptations of VFF have employed herringbone mixers, these models used chevron structures to induce chaotic advection.⁶⁷ Chaotic advection is a turbulent mixing regime intended to increase mixing efficacy, when compared to laminar mixing.⁶⁸ Another adaptation employs Dean flow to achieve greater mixing efficiency through generation of counterflowing vortices.⁶⁹ Although, when compared to VFF the entrapment of air bubbles may cause practical constraints. For this work, the method of VFF was thought to offer the most promise for production of SUVs for drug delivery at high throughput. When applying SUVs within drug efficacy screens, high throughput is favourable to achieve larger scale validation without sample variation. Well-defined laminar mixing regimes were thought to offer control with respect to mixing and drug encapsulation, enabling reproducible liposome production. Finally, the larger device scale is amenable to more accessible cleanroom-free fabrication methods and the incorporation of further functionality and device screening.

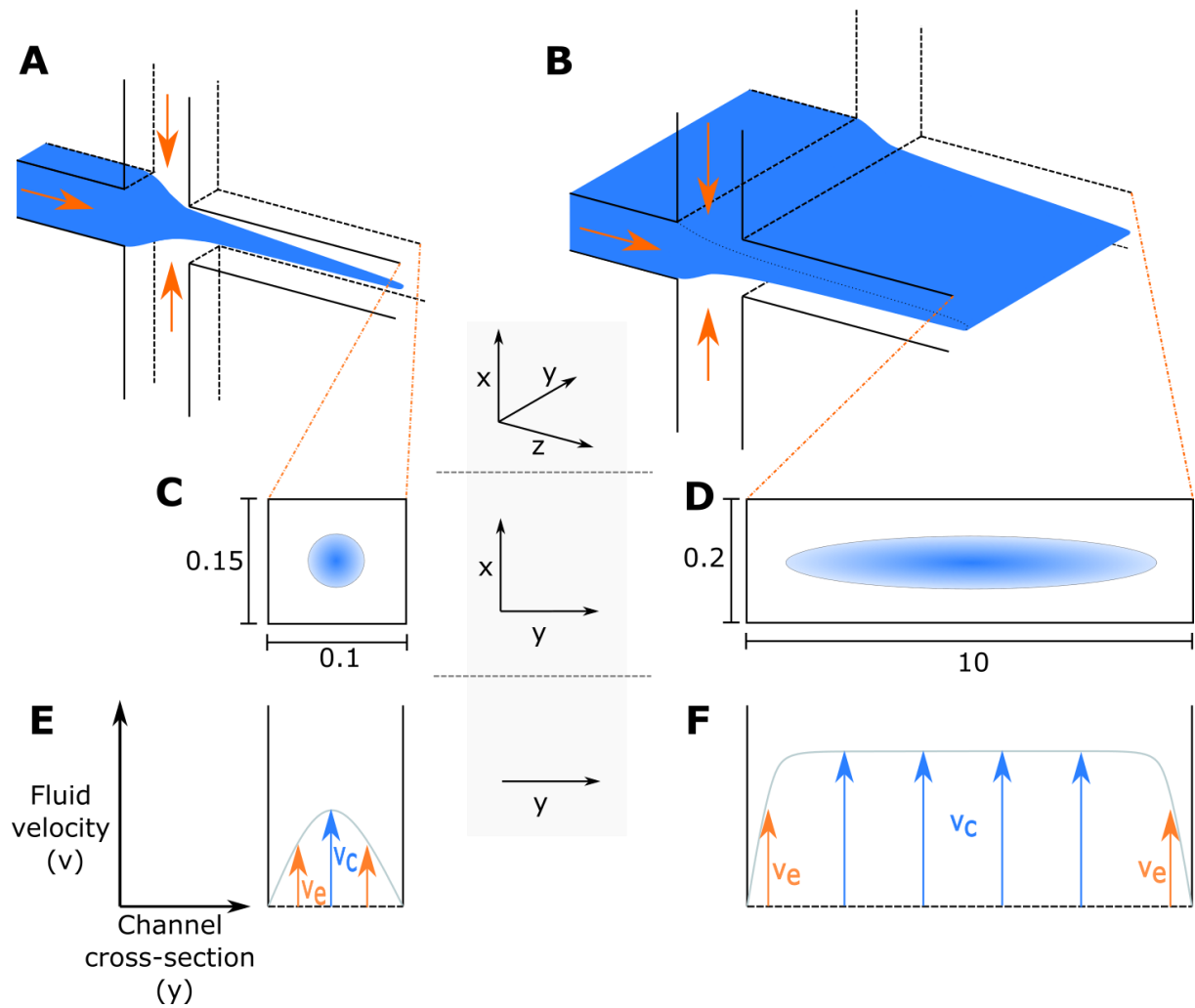


Figure 1.10) Diagrams of hydrodynamic focusing within MHF and VFF, representative channel cross-sections and velocity profiles are shown below. The reference axes are shown centre, all diagrams are not to scale. **A** Schematic representation of MHF in flow. The ethanol, lipid containing stream is represented in blue and the aqueous buffer stream is colourless, direction of flow for the inlets is indicated by the orange arrows. **B** Schematic representation of VFF in flow. The ethanol, lipid containing stream is represented in blue and the aqueous buffer stream is colourless, direction of flow for the inlets is indicated by the orange arrows. **C** Channel cross-section of the MHF device applied in Chapter 3, with the dimensions in mm. The cross section of the ethanol fluid stream is represented in blue. **D** Cross-section of VFF device applied within this chapter, with dimensions in mm. The cross section of the ethanol fluid stream is represented in blue. **E** Fluid velocity profile of the MHF channel, velocity increases vertically and displacement along the channel cross-section (y -axis) is shown horizontally. Representative fluid velocities are labelled; v_e is the velocity of fluid flow near the channel edge, v_c is the velocity of fluid flow near the channel centre. **F** Fluid velocity profile of the VFF channel, velocity increases vertically and displacement along the channel cross-section (y -axis) is shown horizontally. Representative fluid velocities are labelled; v_e is the velocity of fluid flow near the channel edge, v_c is the velocity of fluid flow near the channel centre.

1.3 Fluids under flow

1.3.1 Preface

To better understand microfluidic and millifluidic systems it is essential to have a grounding in the fundamental equations describing fluids under flow. Within in this work both microfluidic and millifluidic devices are encountered, the label microfluidic is applied to devices with < 1 mm width and height dimensions within the channel cross section. Devices with > 1 mm channel width or height are considered millifluidic as at this larger size the effects of surface tension, energy dissipation and fluidic resistance are more dominant.^{70,71} Flow in fluidic devices is often induced using pumps that can be either flow rate driven, or pressure driven such as syringe pumps and p-pumps respectively. Within this work, syringe pumps were used, and all devices comprised of rectangular channels, hence the focus of this introduction will be tailored accordingly. Systems of differing architecture are covered in-depth by Bruus⁷², though will not be discussed within this work.

1.3.2 Poiseuille flow and velocity profile

The volumetric flow rate (Q) is the appropriate place to start with fluidic devices, it is the fluid volume discharged from the channel per unit time.⁷² The volumetric flow rate is typically determined by the applied value when using a syringe pump, it is achieved by actuation of a stepper motor which drives the dispensation from a fluid filled syringe. The volumetric flow rate is defined as the integral of the velocity with respect to channel cross sectional width (y) and height (z) (Equation 4a). For a constant flow rate volumetric flow rate can be expressed in terms of the cross-sectional area (A) and average velocity (v_{avr}) (Equation 4b).

$$Q = \int_C dy dz v_x(y, z)$$

Equation 4a. Where Q is volumetric flow rate, dy and dz are axis in the plane of the channel cross-section and v_x is the velocity of fluid out of the plane of the channel cross section.

$$Q = A v_{avr}$$

Equation 4b. Where Q is volumetric flow rate, A is the cross-sectional area of the channel and v_{avr} is the average velocity of fluid.

The average velocity is an important concept for fluid flow within channels. As fluid flows through a channel the edges of the fluid are impacted by friction and this manifests as a non-linear velocity field along the channel dimensions (Figure 1.11). Within a rectangular pipe, the velocity maximum is at the channel center it retains an elliptical shape from the constraint that the channel width (w) exceeds the channel height (h) such that $w > h$. By applying Poiseuille flow, laminar flow of an incompressible Newtonian fluid which accounts for system pressure drop (ΔP) the volumetric flow rate can be approximately expressed in terms of the channel width, height, length (L) and viscosity of fluid (η) (Equation 5).⁷⁰⁻⁷² Poiseuille flow is one solution to the Navier-Stokes equation in a dynamic setting at steady state flow, the Navier-Stokes solutions are detailed further by Lukaszewicz *et al*⁷³, their full discussion is beyond the scope of this work.⁷² The exact solution for a rectangular channel has not yet been solved for a closed system, the approximation is least effective for when $w = h$ and effective for values where $w > h$. The terms from Equation 3 are often expressed in terms of the proportionality

factor, known as the hydraulic radius (R_{hyd}) (Equation 5a-c). The hydraulic resistance is central to characterising and describing velocity fields within fluidic devices.⁷²

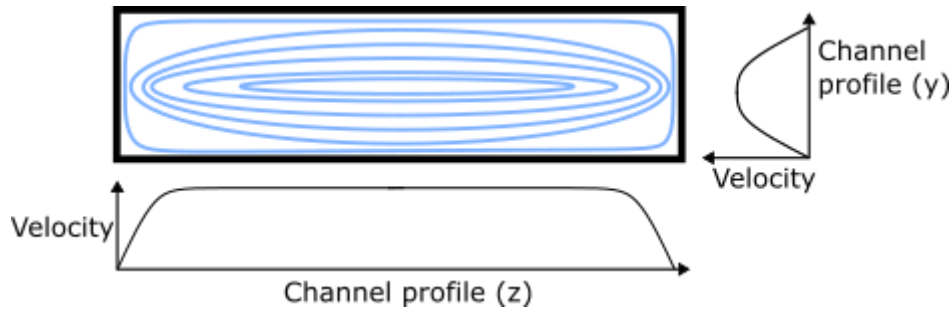


Figure 1.11) Cartoon representation of fluid flow through a rectangular cross section of a channel with the representative velocity fields for the y and z axes. Figure adapted from Bruus⁷².

$$Q \approx \left[1 - 0.63 \frac{h}{w} \right] \frac{h^3 w}{12\eta} \Delta P$$

Equation 5a. Where Q is volumetric flow rate, h and w are the height and width of the channel cross-section, respectively and ΔP is the pressure drop.

$$\Delta P = R_{hyd} Q$$

Equation 5b. Where ΔP is the pressure drop, R_{hyd} is the hydraulic radius and Q is the volumetric flow rate.

$$R_{hyd} \approx \frac{12\eta L}{1 - 0.63 \left(\frac{h}{w} \right)} \frac{1}{h^3 w}$$

Equation 5c. Where R_{hyd} is the hydraulic radius, η is the viscosity, L is the channel length, h and w are the height and width of the channel cross-section, respectively.

1.3.3 Reynold's number

Reynold's number (Re) is a dimensionless number that is derived from the ratio of inertial to viscous forces within a system (Equation 6a). Reynold's number relates to the fluid density (ρ) in one velocity scale (V_0) and channel length scale (L_0) for the viscosity (η) of the fluid in flow (Equation 6b). Viscosity relates the deformation of a fluid at a given applied force and describes how 'thick' a fluid is.⁷² At large values of $Re > 2000$, a turbulent flow regime is generally present and at low $Re < 10$ the system is typically laminar.^{70,74,75} Intermediate Re values typically exhibit features of both laminar and turbulent flow. Calculation of Re number can aid design of fluidic devices as flow regimes can impact the efficacy of processes such as mixing. Within the process of MHF and VFF a laminar flow regime is desired, Re number can inform what flow parameters can achieve this.

$$Re = \frac{\text{Inertial forces}}{\text{Viscous forces}}$$

Equation 6a. Reynold's number definition

$$Re = \frac{\rho V_0 L_0}{\eta}$$

Equation 6b. Where ρ fluid density, V_0 is fluid velocity in one direction, L_0 is channel length and η is the viscosity.

1.4 Myocardial infarction: a clinical and mechanistic background

Myocardial infarctions (MI) occur nearly twice every minute in the United States of America. Currently MI survival rate is higher than ever (85%⁷⁶) because of therapeutic intervention or by more invasive surgical procedures such as stent and bypass.⁷⁷ Ischemic reperfusion (IR) injury is a underlying ailment that typically presents after myocardial infarction or heart centric surgery, IR can result from an oxygen starved myocardium.⁷⁸ Ischemic injury was first documented in 1960 and the true extent of its implications were an area of discussion for years.⁷⁹ The IR injury is highly staged and complex, internal cell response as well as interactions with the local environment take place in its progression.

Ischemic reperfusion injury begins with ischemia, a marked decrease in blood flow, typically occurring in a localized region.⁸⁰ The magnitude of the decrease in flow has a large impact on the severity of the resulting ischemia. Following a low decrease in flow rate (>35% natural flow rate), cardiomyocytes can often recuperate, a moderate flow reduction (10-35% of natural flow rate) results in significant damage and cell death within 3-6 hours.⁸¹ The most severe reductions (less than 10% natural flow rate) yield vast cell death and often result in necrosis where cells subvert apoptosis, a controlled cell death.⁸²⁻⁸⁴ The impact of reduced blood flow marks the initial stage of ischemic reperfusion injury and this progresses into a highly heterogeneous injury.^{84,85}

During the initial stage of ischemia cardiomyocytes entirely change their energy production from aerobic to anaerobic glycolysis (Figure 1.12). During anaerobic glycolysis cardiomyocytes are sustained however insufficient energy is provided to support full cardiomyocyte activity. Anaerobic glycolysis results in cardiomyocytes accumulating a large amount of H⁺ ions and since it decouples from glucose, an overshoot in toxic fatty acid also occurs within the cell.⁸⁴ The large H⁺ accumulation can lead to rapid ion exchange as pH is corrected within the cell.^{78,84-86} This rapid ion exchange following ischemic injury is essential to normalize cell function but the process can be an added route of myocardium damage. Reperfusion, the second stage of IR injury, is the biological response that restores blood flow to the afflicted area. Following reperfusion, restoration of normal cardiomyocyte capacity can occur within a time lapse known as the myocardial stunning.⁷⁷ The reperfusion element of IR injury is composed of many compounded aetiologies from early to late stages.

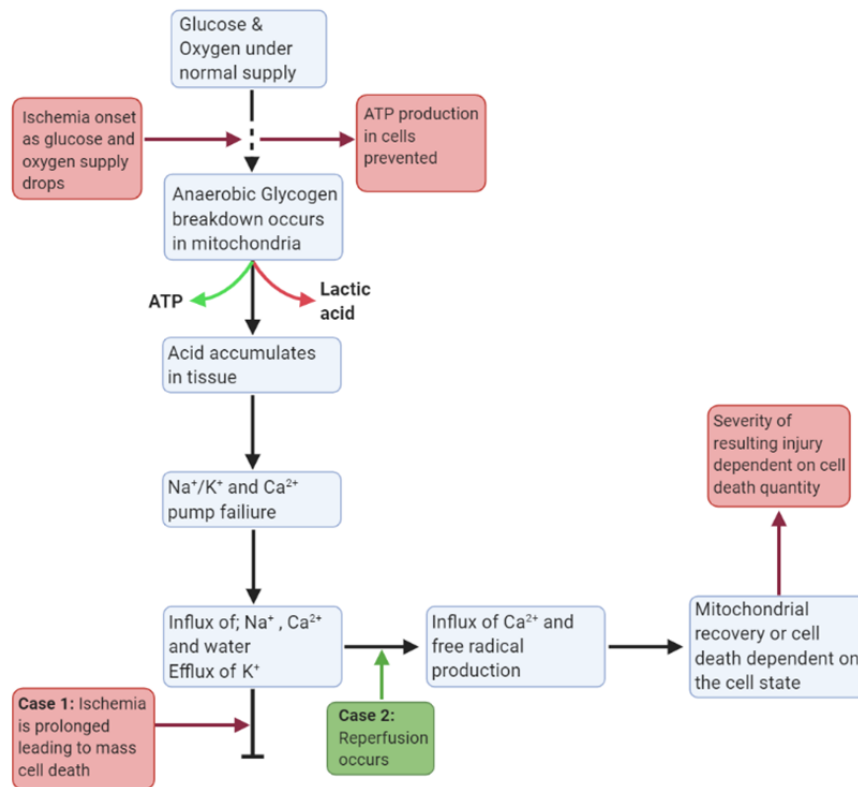


Figure 1.12) Flow chart diagram of onset of ischemic injury and reperfusion response and consequence.⁸⁷

Following reperfusion, a significant point is reached where therapeutic intervention is impactful. Currently there is still uncertainty around the progression of injury following MI which has impeded developments in therapeutic treatments.⁸⁸ With the mechanistic uncertainty and low rates of clinical success for cardiovascular drugs, further effective preclinical models are required.⁸⁹ This is highlighted best by the low phase 2 probability of success; phase 2 involves larger human trials and infers the success of translation from smaller trials used in phase 1 (Figure 1.13). The phase 2 success rates are below the average rate of phase 2 success and highlight the complexity of cardiology. The low phase success rates suggest the current *in-vitro* and animal models are unrepresentative for most drugs developed for cardiology. The fields of tissue-on-a-chip (TOC) and organ-on-a-chip (OOC) are currently in their infancy however they offer significant potential to address the short falls of conventional *in-vitro* and animal models.

Phase Success	Phase I to II		Phase II to III		Phase III to NDA/BLA		NDA/BLA to Approval	
	n	Phase POS	n	Phase POS	n	Phase POS	n	Phase POS
Hematology	92	69.6%	106	48.1%	82	76.8%	72	93.1%
Metabolic	136	61.8%	149	45.0%	66	63.6%	48	87.5%
Infectious disease	403	57.8%	414	38.4%	197	64.0%	156	92.9%
Others	154	63.6%	228	38.6%	90	60.0%	69	88.4%
Ophthalmology	88	71.6%	200	35.5%	82	51.2%	45	91.1%
Autoimmune	413	55.2%	471	31.4%	219	65.3%	202	94.1%
Allergy	55	56.4%	92	28.3%	34	64.7%	20	100.0%
Gastroenterology	45	46.7%	73	34.2%	35	57.1%	33	90.9%
All indications	4414	52.0%	4933	28.9%	1928	57.8%	1453	90.6%
Respiratory	179	55.9%	215	21.9%	62	64.5%	45	95.6%
Psychiatry	150	52.7%	164	26.8%	71	56.3%	57	91.2%
Endocrine	319	43.3%	293	26.6%	151	66.2%	124	86.3%
Neurology	516	47.7%	504	26.8%	226	53.1%	165	86.7%
Oncology	1628	48.8%	1732	24.6%	495	47.7%	324	92.0%
Cardiovascular	214	50.0%	252	21.0%	105	55.2%	80	82.5%
Urology	22	40.9%	40	15.0%	13	69.2%	13	84.6%

Figure 1.13) Clinical success rates for phased drug approval for each medicinal sector, taken from the 2021 report produced by PharmaIntelligence.⁹⁰

1.5 Tissue-on-chip as *in-vitro* models

1.5.1 An introduction to heart tissue-on-chip

Tissue on chip (TOC) systems are the fusion of bioengineering and conventional tissue culturing. TOC devices are often miniaturised tissue cultures that are maintained in a fabricated device. TOC aims to recapitulate elements of *in-vivo* topology, morphology or function which cannot be achieved by normal tissue culturing practice. TOC typically exploits miniaturisation to reduce cost, increase replication, and establish novel analysis methods.^{91–94} It is important to note the variety of terms used in this field, heart-on-a-chip encompasses related models of the heart which includes both TOC and organ-on-a-chip (OOC) models. OOC involves miniaturised devices containing multi cell types, aiming to recapitulate organ functionality. OOC differs from TOC, OOC uses multicellular culture which offers greater biomimicry however the design concepts and outputs are comparable to TOC.⁹⁵ This thesis focuses on TOC, as TOC is implemented in latter work. However, within the introduction OOC will be discussed to explore design concepts and applications. The intended use of a TOC can be anything from modelling the local environment, disease state or testing drug efficacy.^{96,97} OOC can be employed similarly to TOC, with the increased sophistication of exhibiting intercellular and intracellular response. TOC in contrast to OOC provides more reductionist models that present an equally important paradigm which can portray biomimetic features inaccessible by conventional cell culturing.^{96,98,99}

To understand the potential importance of TOC in cardiology it is best to explore the form of the human heart (Figure 1.14).⁹⁸ The human heart is marvel of natural engineering featuring a huge number of cell types within a cooperative complex.¹⁰⁰ Cells within the heart work to their role and

cross communicate to create essential blood flow.¹⁰¹ Within each region of the heart adversity is faced in the form of mechanical stress resulting from tissue contractility in the myocardium as well as the shear stress exerted on the endothelium by high velocity blood flow. Delving further into the tissue, complex electrical signals are present from synchronising Purkinje cells and ion transfer throughout the myocardium.¹⁰² Given the innate complexity of the heart, there are many different forms a heart-on-chip device can take.

This thesis is centred on the myocardium, the muscular tissue which drives the heart, which is of interest for therapeutic intervention following IR injury (Figure 1.14).⁷⁸ The myocardium is comprised of cardiomyocyte cells that align uniaxially to form myofibrils, these bundle together to create muscle tissue.¹⁰³ The bundled structure is facilitated by the extra cellular matrix which is comprised of proteins such as collagen, fibrin and laminin.^{98,100,104} Many examples in literature show how elements of this structure can be modelled in heart-on-a-chip devices using single or multicellular culture.

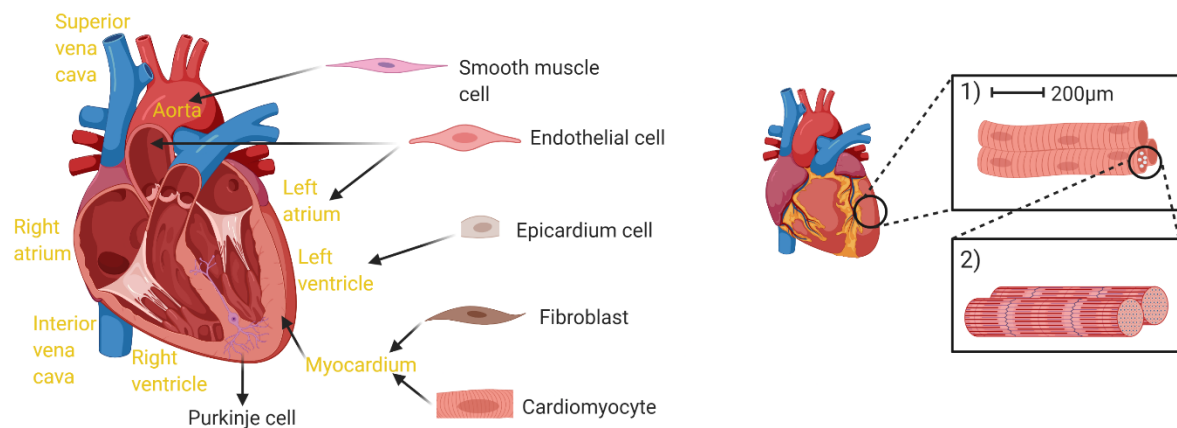


Figure 1.14) Cell localization within the heart (left) and the myofibril cellular structure within the myocardium (right).

Recent advancements have allowed for differentiation of pluripotent stem cells into cardiomyocytes.^{105,106} Cardiomyocytes derived from iPSC's often resemble foetal cardiomyocytes, however in the adult heart significant structural change occurs at a cellular level and within the myocardium.^{107,108} A major difference between immature and mature cells is the proliferative capacity, foetal cells readily proliferate to promote growth and formation of an adult heart. Adult cardiomyocytes in a healthy state are non-proliferative although proliferation can occur within certain disease states and is often lethal.¹⁰⁹ There are several structural differences in the cell that signify maturity. One such difference is present of sarcomeres, these long fibrous proteins of around 2.2 µm length align in parallel bands across the long axis of the cell.¹¹⁰ The next major component are t-tubules, these enable longitudinal transfer of Ca^{2+} enabling synchronous controlled beating within tissue.¹¹¹ Adult cardiomyocytes exhibit more mitochondria, and this is matched with a roughly 40% size increase compared to foetal CM.¹¹² Finally, the nucleus of an adult cardiomyocyte is elongated, and is often bi-nuclear.¹¹³ With the heart topography and cardiomyocyte cellular features covered, some examples of TOC and OCC in literature will be presented to contextualise the field.

1.5.2 Cantilever based tissue-on-chip

One approach to TOC is to produce CM coated cantilevers these can be used to directly measure contractility force. As the tissue pulls the substrate the cantilever is deflected, measuring deflection using microscopy determines the force and frequency of contraction. Many examples of this method exist in literature, Agarwal *et al*¹¹⁴ took the approach of high throughput manufacture of PDMS cantilevers (Figure 1.15A).^{115–117} Agarwal *et al*¹¹⁴ micro-contact printed cantilevers with fibronectin to facilitate cell adhesion in patterned lines. Patterned lines of cardiomyocytes replicate the fibre structure of heart muscle in 2D, reducing anisotropic alignment observed in conventional cell culture. Cantilevers were incorporated into a sealed chamber for seeding and media replenishment and the device was used to explore impact of therapeutic treatment on contractility, similarly to an earlier publication by Kim *et al*¹¹⁵. Agarwal *et al*¹¹⁴ improved scalability of the method by batch manufacturing and high seeding cell capacity although the method is limited to measuring contractility response. Morimoto *et al*⁹⁹ adapted the cantilever method with use of hydrogel supports (Figure 1.15B). Hydrogel supports enable formation of 3D CM fibres which more closely mimic myofibers. Fabrication by Morimoto *et al*⁹⁹ involves a series of manual steps and the implanting of the CM fibre into a chip containing cantilevers. Involved fabrication impacts the scalability of the process, though the implementation of an extracellular matrix allows enhanced biomimicry.

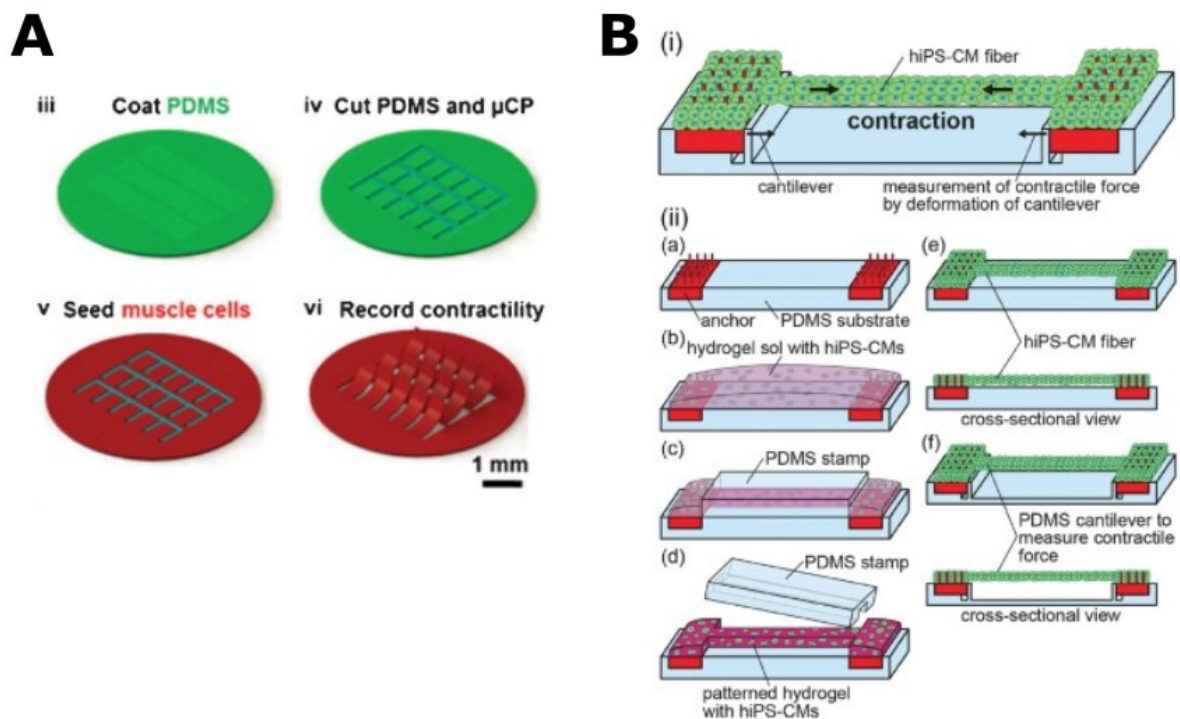


Figure 1.15) Cantilever cardiomyocyte TOC **A** Agarwal *et al*¹¹⁴ cantilever device production stages and scale **B** Morimoto *et al*⁹⁹ hydrogel CM fibre design (i) and fabrication (ii). Figures taken from the respective publications.

1.5.3 Paced cardiac tissue-on-chip devices

In a physiologically driven approach, Zhao *et al*⁹⁶ employed fabrication that contained bio wires which facilitated electrical pacing of stem cells, coaching them into cardiomyocytes (Figure 1.16A). Previously described examples employed immortalised cell lines which are considered favourable in terms of

reproducibility, cell lines are typically less genetically representative than primary cells.^{118,119} Electric pacing drives hydrogel seeded stem cells into cardiomyocytes and achieve a pulse at a biological frequency. Zhao *et al*⁹⁶ have yet to explore therapeutic study, they demonstrated acceleration of cardiomyocyte maturation and formation of working contractile tissue. Nguyen *et al*¹²⁰ employed an analogous driven approach using pressure driven fluctuations (Figure 1.16B). Nguyen *et al*¹²⁰ seeded cells in a PDMS well, a thin PDMS membrane with media contact was used to apply pressure to the culture by a peristaltic pump. The pump was part of a larger circuit designed to mimic the pulsatile behaviour of the left ventricle, using chick embryo left ventricular cardiomyocytes. The device was shown to produce more mature CM, which better react to applied voltage pacing. The device was amenable to confocal microscopy, which was used to image alignment of actin cytoskeleton, a signifier of CM maturity. Nguyen *et al*¹²⁰ primarily aimed for generating tissue transplants and therapeutic studies were not undertaken.

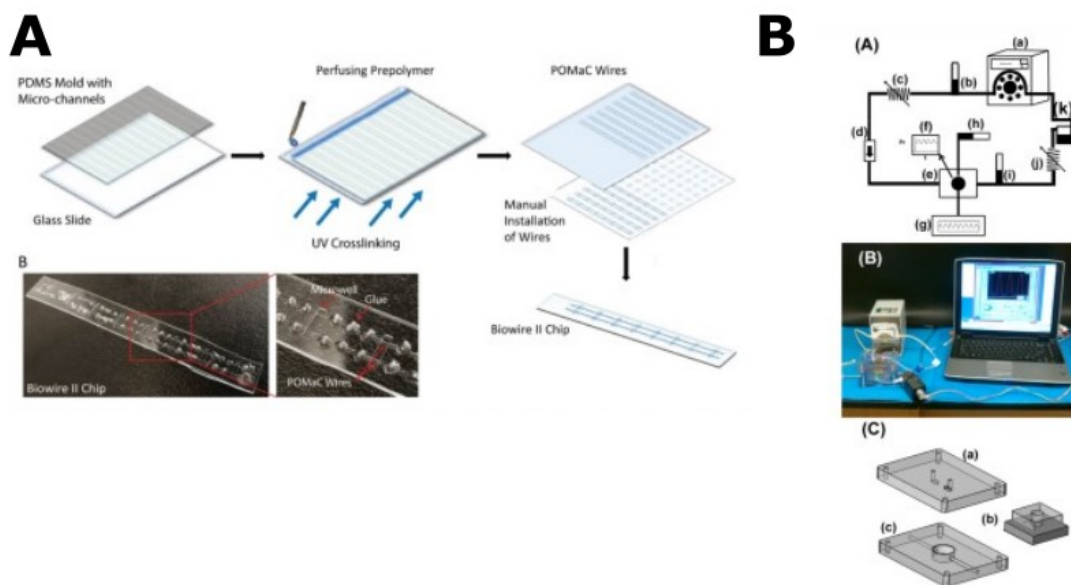


Figure 1.16) TOC devices employing electrical or pressure driven pulsation **A** Zhao *et al*⁹⁶ TOC device fabrication with bio wire implementation **B** Nguyen *et al*¹²⁰ pressure driven pacing TOC design and experimental setup. Figures taken from the respective publications.

1.4.4 Cardiac tissue-on-chip under flow

Kobuszewska *et al*⁹¹ explored the impact of culturing H9C2 cells under flow in different geometries (Figure 1.17). Controlled media perfusion, over the course of 96 hours, was shown to enhance cell proliferation of the H9C2 cells. Cardiomyocytes under perfusion were shown to have enhanced elongation, closely matching cells topology *in-vivo*. Kobuszewska *et al*⁹¹ observed that the linear flow chamber had the greatest proliferation rate. The circular and micropillar model demonstrated curved laminar flow fields, this was reported to reduce proliferation and alignment. The effect of Verapamil, a calcium channel blocker, was explored within perfusion chips by the flowing Verapamil media and exposing the cells to toxic amounts. Cells detached in response to Verapamil treatment and were removed by the media, providing an observable effect of the drug toxicity. Kobuszewska *et al*⁹¹ demonstrate how TOC can be used to explore drug toxicity, with enhanced biomimicry by delivering

drug molecules under flow. While free molecule therapeutic delivery has been investigated, there is still significant potential for exploration of encapsulating therapeutic delivery systems such as liposomes. Applying liposomal mediated drug delivery within the device could provide efficacy screening and the time dependent impact could be evaluated by both microscopy and analysis of collected media, providing a more dynamic readout. Alternatively, the liposomal application rate could be simulated to emulate *in-vivo* delivery and achieve a closer evaluation of the efficacy, versus static application.

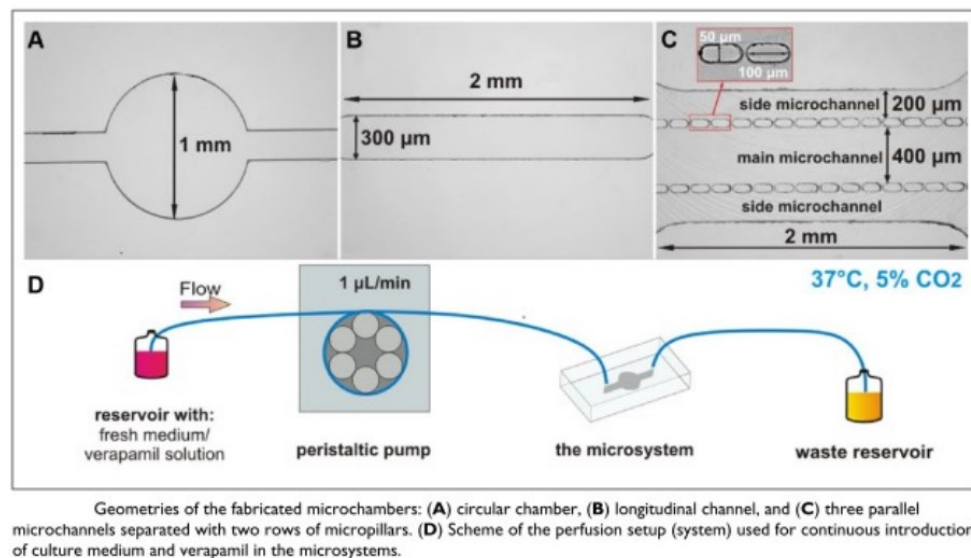


Figure 1.17) Kobuszewska *et al*⁹¹ device used to explore CM seeding within differing geometries, with a diagram of the experimental setup. Figure taken from the respective publication.

2 Materials and methods

This chapter summarises the commonly applied methods within the work. Further details and specific method adaptations are covered within the experimental chapters.

2.1 Measurement methods

2.1.1 Contact angle measurement

Contact angle measurements were undertaken on a Kruss Easydrop using a 100 μ L drop of DIW for the measurement. The contact angle was measured on six independently treated 10 x 10 mm PDMS squares at 6 different locations on the surface. Images were taken at 10X magnification using a camera and the baseline was manually fitted using Kruss DSA software. Drop edge fitting was achieved with the Kruss DSA software, contact angles were measured from both droplet edges and an average was taken. Contact angle data is produced from $n = 36$ replicates and error is reported as ± 1 SD.

2.1.2 DLS sizing, Zeta potential and MADLS sample preparation and measurement

DLS samples were measured using a Malvern Zetasizer Ultra (ZSU5700) and polystyrene sample cuvettes. Samples were diluted at 1:5 in PBS buffer, unless specified otherwise and total sample volume was 1 mL for DLS. Measurements were taken at 20°C following a 2-minute temperature equilibration, the sample sizing applied a minimum of 5 rounds of 20 measurement runs.

For MADLS, a larger volume (2 mL sample) 4 clear side cuvette was employed. A DLS size scan was first used to determine the average derived count rate (kcps, typically ≈ 200 -400), this value was then applied within the MADLS scan. MADLS measurements consisted of 5 rounds of 20 measurements for each scattering type (forward, back, side).

Zeta potential scans were taken using a Malvern zeta potential cell (DTS1070) using a 0.2 mL undiluted sample volume. A total of 30 scans were applied for all zeta measurements and the scans were processed in the Malvern software. Data fits for DLS/MADLS data sets were produced using Origin pro (2020), applying a gaussian peak fitting function to the raw data which was used to extract the maxima and FWHM values. The mean of sample replicates (mean diameter and FWHM) was taken, and error expressed as ± 1 SD for all plots.

2.1.3 SAXS/WAXS sample preparation and measurement

SAXS WAXS data was undertaken by the author and completed as part of an MRes in chemical biology, the first year of the 1 + 3 PhD programme centred around the same project as this thesis. Lipid samples were prepared for x-ray diffraction using the control liposome formulation, a 47:46:7 DOPC:CHOL:DSPEPEG₂₀₀₀ molar ratio. Lipid film samples for SAXS were prepared analogously to the liposomes except for the higher lipid content (10 mg mL⁻¹ once hydrated). When hydrated, 70wt%

water was used to ensure sufficient x-ray diffraction signal was obtained. The hydrated samples were freeze-thawed for 40 cycles using liquid nitrogen and air gun thawing. Samples were then loaded into a glass capillary and centrifuged to collect the sample at the bottom of the tube. SAXS data was collected at I22 facility at Diamond light source in Didcott using a 0.689Å wavelength beam at a detector distance of 112.41 mm. Detector calibration was achieved using a silver behenate ($D = 58.367$ Å) sample and DawnScience software, which was also used to subtract the capillary background and generate the processed data. Temperature sweeps used a Peltier heater waiting five minutes for each temperature step to equilibrate. Following DawnScience processing, the data was plotted in Origin 2020.

2.1.4 Electron microscopy and cryo electron microscopy

Transmission electron microscopy was kindly undertaken by Dr. Claudia Contini of Imperial College. Cryo electron microscopy measurement was kindly undertaken by Colin Pilkington of the Elani group, Imperial college. Samples were prepared for imaging and imaged at the Imperial College Centre for Cryo microscopy. Methodology was provided by the respective users.

Transmission electron microscopy samples were produced in DIW (to prevent salt aggregation) and used undiluted, within 5 hours of flow focusing. Electron microscopy images were taken with a uranyl acetate (2% solution in DIW) negative stain and a 5 μ L sample volume was deposited onto glow-discharged copper grids. After 1 min, the grids were blotted with filter paper and then immersed into the uranyl acetate staining solution for 20 s. Then, the grids were blotted again.

For cryo electron microscopy, samples were taken on an FEI Tecnai12 bio twin 120kV with a TVIPS XF416 4K CMOS detector. For imaging, 3 μ L samples were transferred onto a carbon grid (Quantifoil™ R 2/2 on 300 copper mesh; Jena Bioscience), in a Vitrobot (ThermoFisher) at 90% humidity. Sample grids were blotted (blot time: 5 s; blot force: -1; wait time: 35 s) then immediately plunged into liquid ethane bath contained within a liquid nitrogen reservoir. The grid was transferred to a grid holder at -180 °C. Grids were transferred under liquid nitrogen to an electron microscope sample holder (Cryo Transfer Tomography holder, Eden Instruments; Model 2550). Defocus values used were between -0.5 and -3 μ M, depending on the chosen magnification which ranged from 26000-52000x.

2.2 Fabrication methods

2.2.1 Photolithography wafer fabrication

Wafer fabrication was undertaken in an ISO 7/8 cleanroom using Inseto (100mm serial: 04/007) photolithography wafers and SU8 resins were purchased from Microchem™. The wafers were polished and pre-prepared by Inseto, further cleaning steps were not required. Briefly, the process involved spin coating SU8 onto the wafer surface, a soft bake was applied to harden the SU8. Next, a mask was then applied to the wafer surface before UV exposure and removal of uncured SU8 by developer (Figure 2.1). Spin, bake and exposure settings were optimised for each depth, settings were optimized from Microchem™ guidelines (Table 1). The Spin coating was undertaken on Laurell Model WS-400-6NPP-LITE, bakes were applied by a Stuart SD 300 hotplate. Exposure was undertaken on an Optical associate (Model 0130-044-03) using a 350W OSRAM mercury lamp, the power was measured using a OAI UV powermeter 306 prior to usage to determine exposure time. Development was

undertaken in propylene glycol monomethyl ether acetate >99.5% (Sigma Aldrich) on a Scilogex plate rocker for half the Microchem™ recommended time. At this point the development was quenched by removing the wafer from the developer and washing with IPA, if large amounts of white precipitate were observed the wafer was dried under nitrogen and placed in the developer solution. This process was then repeated until little or no white precipitate was observed, at this point quality was assessed using a Leica MZ12 microscope. After a good quality pattern was observed, a final wash in clean developing solution was used with rapid successive washing with IPA to ensure all undeveloped SU8 was removed.

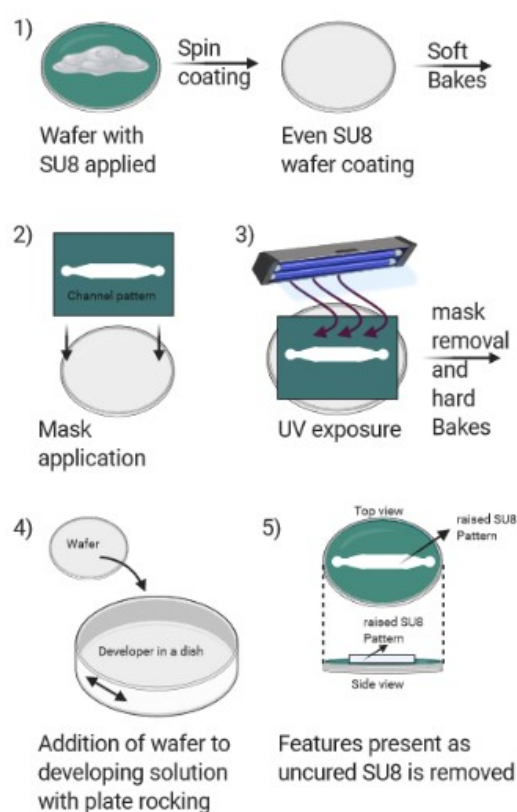


Figure 2.1) Diagram of the full wafer development procedure 1 Spin coating and soft baking process 2 Mask application 3 UV exposure followed by hard baking 4 Wafer development removing uncured SU8 5 Resultant wafer.

For < 50 μm depths, an alternative post exposure bake (PEB) bake, the curing after exposure, was adopted. The standard approach resulted in heat stresses which led to delamination and cracks within the SU8 structure. To avoid heat stress the PEB procedure was adapted to avoid sharp temperature changes. After exposure, the wafer was placed on a hotplate set to 20°C, the temperature was raised to 65°C achieving a slow heat climb. The wafer was left for 3 minutes to achieve temperature and then left for the appropriate first PEB time (Table 1). After the first PEB, the hotplate temperature was increased to 85°C and left for the second PEB duration (Table 1). Following the second PEB, the hotplate was switched off and the wafer was left on the plate to slowly reach room temperature before development.

SU8	Attempted depth (μm)	Spin coater rate (rpm)	Pre-Bake at 65°C (minutes)	Soft-Bake at 95°C (minutes)	UV lamp power (mJ/s)	Exposure time (seconds)	Post-exposure bake 1 65°C (minutes)	Post-exposure bake 2 95°C (minutes)
25	25	2000	3	8	10.44	19	1*	~3*
2100	150	2000	5	30	9.82	26	5	12
2100	200	1500	6	35	9.95	30.5	5	13

Table 1) Applied spin settings and timings for the bakes and exposure, for given SU8. *PEB for the 25 μm depth applied the adapted PEB method.

After development, wafers were silanized with Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (97% Sigma Aldrich) vapour deposition. This was achieved by placing 0.05 mL of the silane on a microscope slide within a vacuum desiccator, the wafer was placed with the desired functionalisation surface fully exposed. Vacuum was applied for five minutes before sealing the vacuum chamber and leaving overnight to deposit a thin film on the wafer surface.

2.2.2 PDMS preparation

PDMS was cast directly onto the face of the wafer within an ISO 7/8 cleanroom (Figure 2.2). All PDMS was purchased from Dow Corning and mixed in a 10:1 ratio of silicone prepolymer to crosslinking agent, mixing ratios were measured using scale. PDMS was mixed manually using a Pasteur pipette for 2 minutes before casting. PDMS was added directly onto the wafer contained within a petri dish, PDMS was weighed used scale to achieve specific thickness. After pouring, vacuum was applied for 20 - 40 minutes, dependent on thickness, to ensure all bubbles were removed. PDMS covered wafers were then baked at 70°C for three hours to achieve curing. PDMS was directly cut from the wafer using a scalpel and gently removed using tweezers. Excised PDMS was washed with IPA and DIW to degrease and dried under a nitrogen stream prior to plasma bonding.

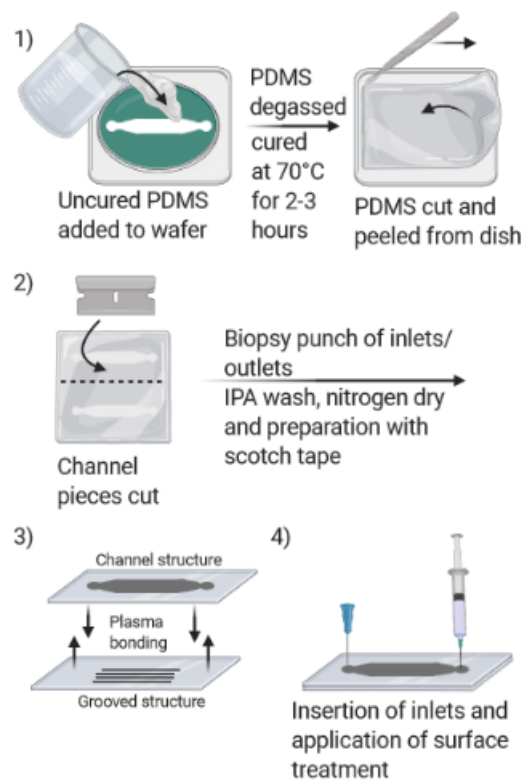


Figure 2.2) PDMS casting process 1 PDMS pouring, degassing and curing 2 PDMS cutting and preparation 3 PDMS plasma bonding 4 PDMS device surface treatment.

2.2.3 MHF chip preparation & surface coating

PDMS channel structures were cast from wafer designs and plasma bonded (Harrick, 18 W, air O₂, 2 minutes exposure) to a 1 mm thick PDMS base. Once cured, PDMS segments were removed from the wafer and plasma bonded, achieving a sealed microfluidic device. The device was comprised of a wafer-cast PDMS channel structure and 0.2 mm level-cast PDMS base; the sealed device was backed onto microscope slides to impart rigidity.

Following bonding, surface coatings were applied directly using a syringe and needle directly into the channel inlet. RainX coating was left to adhere for 20 minutes and washed with three 1mL portions of DIW. PVA and APTES coatings were applied using the solutions from Chapter 2.4.3. Fluorosilane treatments (OTS and POTS 1H,1H,2H,2H-perfluorooctyl trichlorosilane, 1H,1H,2H,2H-perfluorooctyldimethylchlorosilane Fluorochem) were applied as 5% solution in perfluorodecalin (Fluorochem) and washed with one 1 mL of absolute EtOH and then three 1 mL portions of DIW.

2.2.4 Dialysis on chip design

Preliminary work using a dialysis-on-chip (DOC) was achieved and the device comprised of two 10 mm wide and 80 mm long channels that were separated by a regenerated cellulose (RC) membrane

(Spectrum™ Spectra/Por™3 3.5 kd MWCO) (Figure 2.3A). The top (1) and base (2) were comprised of 1 mm PMMA which imparted structural stability. Channel sections (2,4) were made from 0.2 mm PMMA, this was to retain the same AR of 50 that was used within the VFF device. The AR 50 was chosen to increase the channel cross section and result in a lower velocity, for a given flow rate, increasing the dialysis time and reducing the pressure on the flexible dialysis membrane. Between each layer, a total of 4 channel cut-outs made from the 3M 7953MP membrane switch spacer used for a single device. Switch spacer was cut to match the channel shape, this was to minimise the exposed adhesive area once bonded. The adhesive from the spacers was required to bond the DOC design, our solvent bonding method could not be implemented as application of ethanol would damage the RC membrane. Adhesive PMMA bonding meant high temperatures could be avoided, ensuring the porous membrane retained structural integrity.

Alignment was undertaken using the heating block, applying a heating temperature and time of 40°C and 12 hours respectively. When applying adhesive layers, care was taken to minimise air bubble trapping. The RC membrane was manually cut to size and aligned; the membrane covered the rectangular channel part of the channel structure, leaving the inlet holes uncovered (Figure 2.3B). After bonding, the device appeared clear with minimal bubbles, the RC membrane can be seen as the rippled structure within the channel (Figure 2.3C). Post bonding, microfluidic adapters were adhered to the inlet holes and the device was tested for leaks.

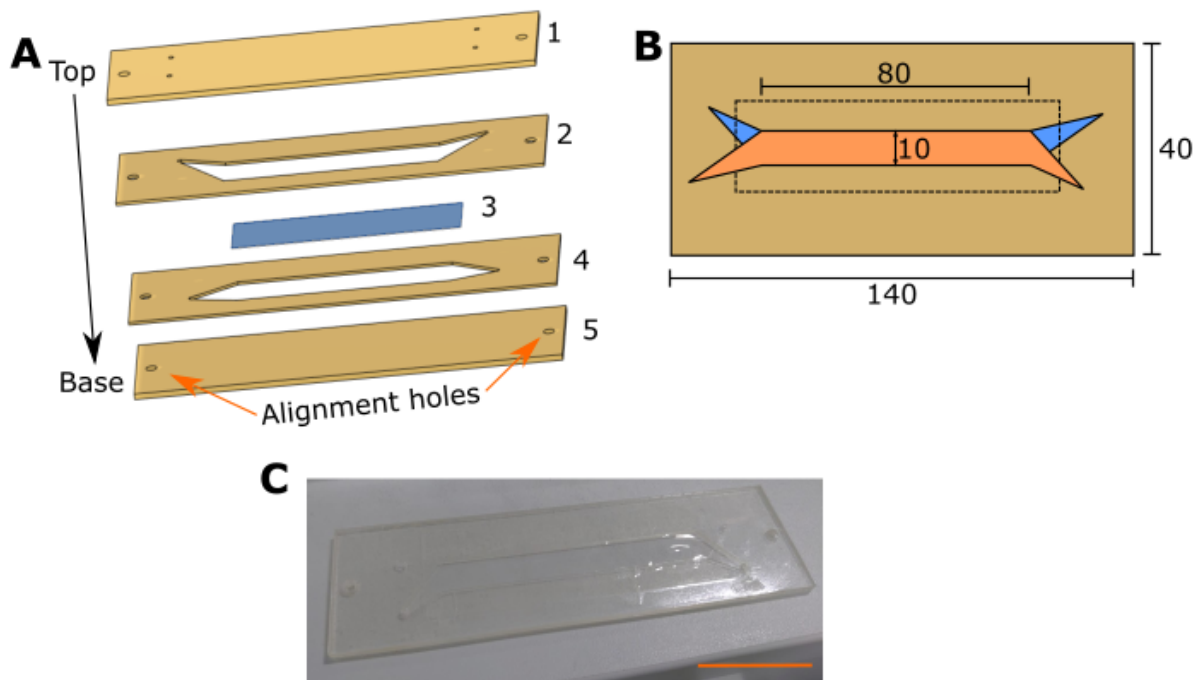


Figure 2.3) Diagrams of DOC assembly. **A** Exploded view of the DOC device indicating the; device top (1), top channel (2), RC membrane (3), base channel (4) and device base (5). Alignment holes used with the heating block are highlighted. **B** Bird's eye view of the DOC assembly showing dimensions. Approximate alignment of the RC membrane is shown with the dashed box in between the top channel (orange) and base channel (blue). **C** Image of the DOC device post-bonding (scale bar = 40 mm).

2.3 General lipid methods

2.3.1 Lipid extrusion and size exclusion

Lipid films were prepared by mixing the appropriate amounts of standard solutions of individual lipid in chloroform. Samples were vortexed for two minutes to ensure thorough mixing. After this time, a nitrogen stream was used to evaporate chloroform under manual vial rotation. The drying method ensured a thin film was produced around the inside of the vial, thus facilitating hydration in buffer and preventing lipid aggregation. Samples typically contained 1 mg of lipid mass prior to solvation, the dried samples were then lyophilised overnight to remove all residual chloroform.

For extrusion, a rough vesicle suspension was formed by hydration of the film using an appropriate fraction of solvent (0.2 - 1 mL) and solvent volume was chosen as a handleable amount for the extrusion process. Most samples were hydrated using filtered phosphate buffered saline (PBS) (pH 7.4, VWR). The rough suspension was then mixed by vortex for 2 minutes resulting in a cloudy solution. If residual lipid was observed on the side of the vessel the sample was heated to 40°C and vortexed until visibly homogenous. The sample was then drawn into a 1 mL gas tight syringe (Hamilton) for extrusion, it was noted which was the inlet syringe to minimise dust and contaminants in the final sample (Figure 2.4). Suspensions were cycled through the desired filter a total of 15 times to ensure a low polydispersity in the vesicle distribution.

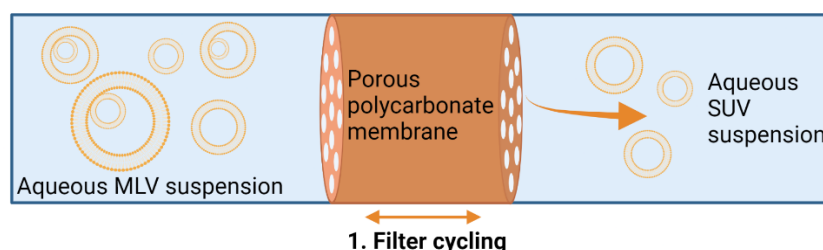


Figure 2.4) Diagram representation of the extrusion process where MLV are turned into SUV.

Samples containing the fluorescent encapsulants calcein and carboxyfluorescein were purified using a Sephadex™ size exclusion column. For this process, 0.4 g Sephadex™ in 12 ml PBS was left in an oven at 70°C to hydrate for at least one hour, it was then loaded into a 5 mL gel filtration column. After Sephadex™ loading, 2 mL of a 0.5 M sucrose (Sigma Aldrich) in PBS was loaded onto the column to osmotically balance in presence of encapsulating vesicles. The vesicle samples were then loaded, and 0.2 mL portions of PBS were washed through, collecting each aliquot. Two bands arose during the separation process, the first and pale band was the vesicle fraction the second and more intense band was unencapsulated calcein. Purified samples were taken as the most intense fraction from the vesicle band, this minimized sample dilution during purification.

2.3.2 Calcein leakage assay

Calcein was encapsulated through hydration of the lipid film using the 100 mM calcein solution and subsequent extrusion. Unencapsulated calcein was removed from the extruded sample using a sephadex column (Chapter 2.3.1). Samples were immediately run on a Cary Eclipse at 50-fold dilution in 0.5 M sucrose in PBS (pH 7.4), sampling applied a 'medium' photomultiplier tubes voltage and a low sample rate (> 5 minute) to minimize photobleaching. Sample lysis was achieved using 1 μ L of a 1 wt% Triton X-100 solution (Sigma Aldrich) in DIW. For long term storage, samples were stored in sealed tubes at 20°C. Calcein intensity was recorded at 517 nm for each sample, a mean was used for the graph and the error bars represent ± 1 SD.

2.3.3 Synthesis of lipid linked targeting peptide

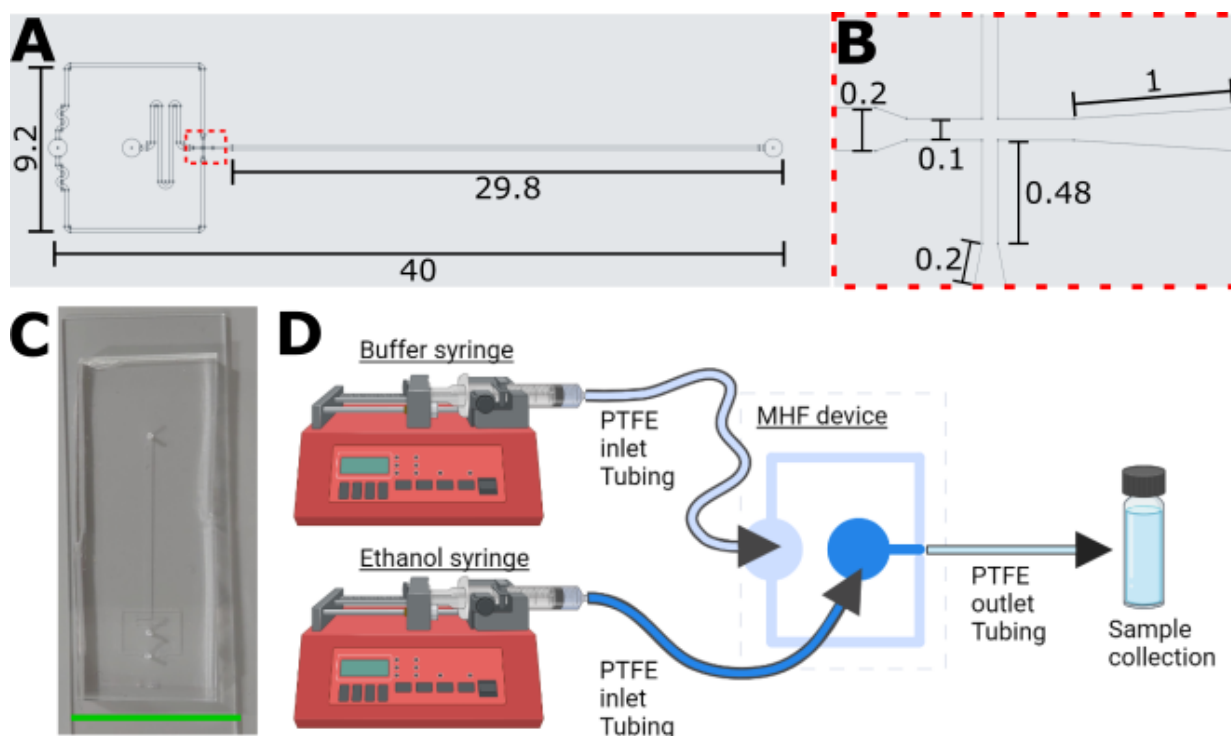
First DSPE-PEG₂₀₀₀-maleimide (Avanti, 9.5mg, 3.23 μ mol) was dissolved in 200 μ L methanol and added to a sealed round neck flask under N₂. The flask was kept under stirring within an ice bath for the duration of the process, next 800 μ L PBS was added to the lipid in methanol to result in a liposomal suspension. To the liposomes, peptide (Genscript, 4 mg, 4.24 mmol) was added in methanol at a 1000-fold excess and the mixture was left to stir overnight. Dialysis using a 3.5 kDa cut-off (Thermo Scientific, Slide-A-Lyzer) against DIW for 4 hours was undertaken to remove unreacted lipid, salt and methanol. The sample was then lyophilized to remove all solvent.

2.4 Microfluidic and millifluidic methods

2.4.1 MHF device manufacture and practical setup

The MHF design was adapted from the Jahn *et al*⁵⁹⁻⁶¹ publications which used cyclic olefin copolymer (COC) and less accessible, deep reactive ion etching fabrication. In this work, the MHF device design consisted of a crossflow junction where two phases are co-flowed, and a long exit channel that follows the junction.^{59,61} The device was designed with equal length inlet channels, to equalize the pressure drop for each stream (Figure 2.5A). Path length was equalized by adding serpentine and normalizing the quantity of bends across each inlet channel (Figure 2.5A). At the crossflow junction, a taper was used to reduce the channel width to 100 μ m, increasing the flow velocity within the section (Figure 2.5B). Tapers are an effective way to increase flow velocity in a localized area without significantly increasing the pressure drop of the device.^{121,122} Channels outside of the cross-junction were kept to 200 μ m width to minimize pressure drop. Increasing the velocity within the crossflow junction was hypothesized to aid formation of a stable ethanol and buffer interface. An exit channel length of 29.8 mm was chosen to provide a long residence time after the crossflow junction. A long residence time is favourable as liposome formation occurs within the exit channel.^{59,61} The channel depth was determined to be 175 μ m by profilometry of the wafer (Figure 2.6). PTFE tubing was used to link syringe pumps to the device, tubing was cut to the same length to equalize the pressure drop for the inlets (Figure 2.5D).

The model in this work was designed using wafer-based lithography, PDMS casting provided the channel structure, 1 mm outlet and inlets were punched into the PDMS using a biopsy punch (Figure 2.5C). The PDMS channel was then bonded to 0.2 mm thick PDMS, using plasma exposure (Harrick, 18 W, air O₂, 2 minutes exposure). After bonding a solution of RainX™ rain repellent was flushed into the device by manual injection. RainX™ was left for 20 minutes to adhere and then displaced using nitrogen gas. The model was washed with 1 mL DIW to remove residual RainX™. The model was fitted directly with PTFE tubing (1 mm OD, 0.5 mm ID Sigma Aldrich) connected to 5mL syringes on a syringe pump (AI-1000 Aladdin pump) (Figure 2.5D). Flow was visualized in real time using a Ximea xiQ camera through a Nikon eclipse TE2000-E under a 10x objective.



*Figure 2.5) Design and setup of MHF device, all dimensions are in mm. **A** CAD drawing of the MHF design highlighting the design size, exit channel length and pressure equalized inlets. **B** A magnified image of the crossflow junction highlighted in the red box, showing the critical dimensions and tapers around the cross-junction. **C** Image of PDMS MHF device backed onto a glass microscope slide (scale bar = 26 mm). **D** Schematic of MHF device setup depicting equipment and fluidic connections.*

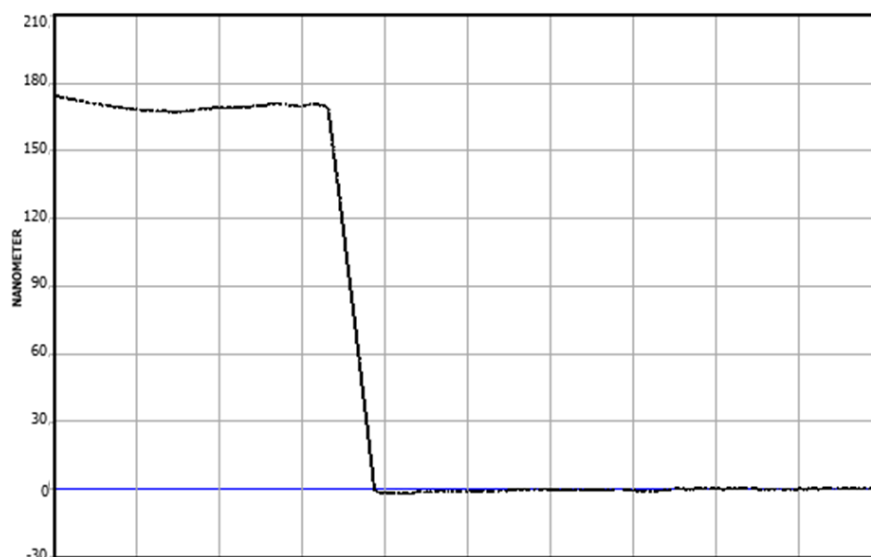


Figure 2.6) Profilometry of MHF device measured at the exit channel.

2.4.2 VFF setup and data acquisition

Within this chapter, two syringe pumps were used to apply flow to the VFF device (Figure 2.7). One pump displaced ethanol and the other was for the buffer from two syringes, this avoided need for a microfluidic splitter. Microfluidic splitter usage was avoided to prevent potential asymmetric flow splitting and increase the volume of buffer in reserve. Syringe pumps were loaded with a 6 mL and two 12 mL Henke-Ject syringes, for the ethanol and buffer streams respectively. To connect syringes, PTFE tubing with a 0.5 mm ID and 1/16" OD was used in conjunction with Luer lock to 1/16" head fitting F/F adapters, PEEK 1/16" head fittings and PTFE 1/16" ferrules. PTFE thread tape was applied to the head fitting thread to provide improved connection seals. The VFF device was purged with PBS buffer and a pulsatile flow was used to remove trapped air, prior to usage.

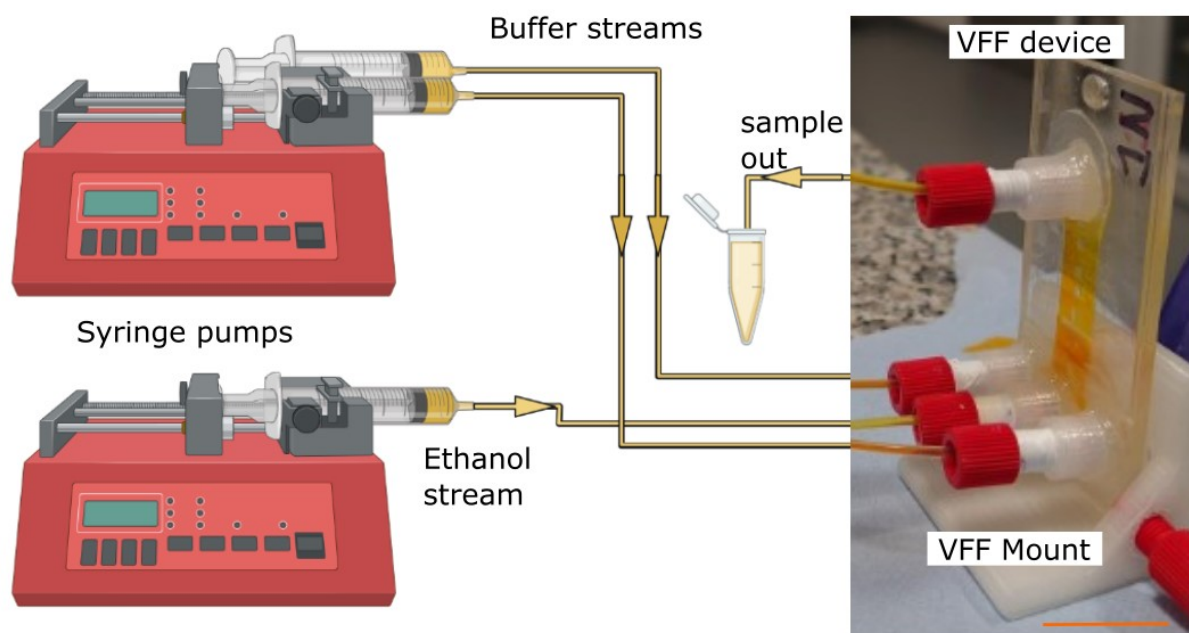


Figure 2.7) Schematic of the experimental setup for VFF, syringe pumps were used to apply flow to the device which was secured in a 3D printed mount (white). The head fittings (red) are shown with the PTFE thread tape and secured in the microfluidic adapters (white) adhered onto the PMMA device. PTFE tubing runs directly through the headspace fittings and samples were collected directly from the tubing into an Eppendorf vial. Cartoons are not to scale and the image scale bar = 36 mm.

For preliminary studies a 0.1 mg mL^{-1} DOPC solution in absolute ethanol was used as the central phase and filtered PBS was used as the outer buffer phase. For FRR sweep data sets, an applied ethanol flow rate of 0.1 mL min^{-1} was used, and the outer PBS flow rate was gradually increased from 0.1 mL min^{-1} to 2.5 mL min^{-1} . Within the FRR sweep, internal flow rate was not decreased proportionally, this meant TFR was also increased from 0.2 mL min^{-1} to 2.6 mL min^{-1} within the FRR sweeps.

TFR sweep data sets were explored using a fixed FRR of 5, this was applied to decouple impact of FRR from the data. Within the TFR sweep, ethanol flow rate was increased from 0.05 mL min^{-1} to 0.35 mL min^{-1} and PBS flow rate was increased from 0.25 mL min^{-1} to 1.7 mL min^{-1} , proportionally. Sample replicates within this work are derived from independently made lipid films, for instance an $n = 3$ is produced from 3 samples, each from an independently produced ethanol and lipid solution. DLS was used to characterise the resultant distributions at a 1:5 dilution in PBS, samples were not dialysed before measurement. For dialysed samples a 3.5 kDa cut-off (Thermo Scientific, Slide-A-Lyzer) was employed against PBS for 12 hours to remove ethanol.

2.4.3 MHF and VFF encapsulation of CBF

To encapsulate CBF (Sigma Aldrich) a 100 mM solution was made in PBS and corrected to pH 10 using NaOH (1 M). The ethanol stream was loaded with lipids for liposome formation. The other process employed were analogous to Chapter 2.3.2 except when running the Sephadex™ column, the sucrose solution and washing buffer were corrected to the pH of the liposome resultant suspension ($\approx \text{pH } 10$).

2.4.4 VFF encapsulation of menadione for UV-vis spectroscopy

Menadione was added to the ethanol stream prior to VFF at a concentration of 0.4 mg mL⁻¹, encapsulation was applied during flow. The high concentration of menadione was required to achieve a signal difference when lysed, this was likely a result of menadione lipophilicity. A biochrom UV-Vis spectrophotometer was used to read the absorption at 331 nm for the 1 mL samples, in cuvettes. Each data point was comprised of 3 film replicates and lysis was achieved with 150 µL of 1wt% Triton X-100 solution.

2.4.5 Encapsulation of menadione and Texas red stain for cell study

Menadione was encapsulated directly using MHF and VFF. Menadione was prepared in a 20 mg mL⁻¹ stock in chloroform and the appropriate aliquot was mixed with the lipid in chloroform mixture, prior to drying. Lipid and menadione films were then dissolved in anhydrous ethanol and used directly in VFF. Texas red liposomes were produced with 3 mol% DHPE-Texas red. DHPE-Texas red was added from a chloroform stock solution and mixed with the other lipid in chloroform solutions by vortexing. Lipid and DHPE-Texas red films were then dissolved in anhydrous ethanol and used directly in VFF.

2.4.6 Dialysis on chip application

The dialysis on chip (DOC) device was tested by flowing a 100 mM calcein solution through the top channel and PBS through the base channel (Figure 2.8). The flow directions were opposed, this was determined by Hood *et al*¹²³ to be the most effective setup. A flow rate of 0.1 mL min⁻¹ was applied to each channel, this ensured the pressure on the membrane was even from each side. During application, the top channel was expected to appear filled with the yellow calcein solution. Despite balancing the flow rates, the RC membrane was observed to deform under flow (Figure 2.8B). The buffer stream displaced the membrane upward in the channel center, this led to an apparent clear and colourless central stream. As a result of the RC flexibility, a flow rate balance where the RC membrane remained level could not be established.

Application of a serpentine structure with a narrower channel dimension is likely required, such as the one applied by Hood *et al*¹²³. The design in this device was intended to integrate with 10 mm wide channels applied within the VFF device. Adding a serpentine dialysis channel to the VFF device could lead to potential disturbance in the flow focusing region. Spectrum™ operating specifications suggested exposure to ethanol should be avoided, this meant long-term application was unfeasible. Post flow-focusing, the ethanol fraction should be low ≤ 20 vol% for FRR 5, application over sustained periods may deteriorate the membrane integrity. The application of DOC could be more feasible for high FRR (>25) as reduced ethanol content in the sample would result in lower membrane degradation and a sample close to the micro viscosity of an extrusion sample. Commercially available alternatives with greater solvent resistance and sheet form to facilitate implementation into PMMA device were not found. An alternative RC treatment for greater solvent resistance or new dialysis membrane material would vastly improve the feasibility of DOC for VFF. Overall, the DOC chip was found to be ineffective for the channel geometry and therefore excluded from the main body of work, the

methodology is however presented in this section. When under flow, within a wide channel, the membrane was shown to bend effecting the effective channel geometry (Figure 2.8B).

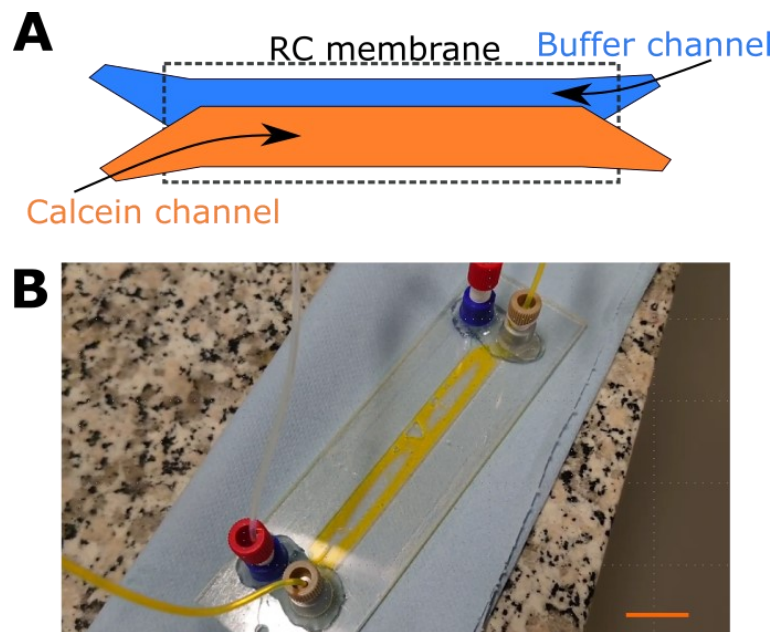


Figure 2.8) **A** Diagram of DOC device in use, directions of flow and location of dialysis membrane indicated. **B** Image of DOC device in use, demonstrating the bending of the membrane along the channel center by the displacement of the top calcein solution (Scale bar = 20 mm).

2.4.7 TOC device experimental setup

Media was perfused through the TOC device using a syringe pump loaded with sterile plastic syringes (BD biosciences) containing DMEM H9C2 culturing medium (Figure 2.9). A sterile, blunt steel 19 Ga needle (1.067 mm OD, 0.686 mm ID) was used to connect the syringes to the silicone tubing (Watson Pt cured, 0.5 mm ID, 5 mm OD). Silicone tubing is resistant to high temperature ($> 200^{\circ}\text{C}$) which meant it could be sterilised by autoclaving prior to use.¹²⁴ A metal hub comprised of a 20 mm long metal tube (1 mm OD, 0.5 mm ID, Coopers needle works Ltd.) was used to interface the tubing with the TOC device, hubs were autoclaved prior to insertion within a TC hood. Dimensions of the tubing and metal hub were chosen to result in fluidically tight connections, preventing media leakage and air bubble formation within the device.

The setup was placed within an incubator (37°C , 5% CO_2) to ensure optimal growth conditions. PMMA mounts were designed to secure devices, this ensured reliable perfusion with no observed leakage over periods of several days. Parallelization of devices was limited by the syringe capacity of the pump, this meant three devices could be maintained simultaneously from one pump use of splitters was avoided to ensure accuracy in the applied flow rates. TOC devices could be connected in series although this would lead to a significant pressure drop, increase risk of contamination and leakage. After confirming that a suitable setup had been developed, a biocompatible coating for PDMS was developed to facilitate adhesion (Chapter 2.5.3).

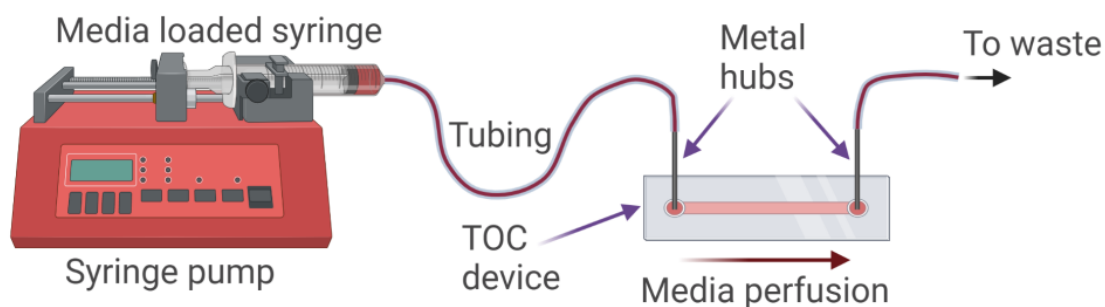


Figure 2.9) Schematic of TOC device setup with perfusion achieved from a syringe pump.

2.5 Cell methods

2.5.1 H9C2 cell culturing

H9C2 rat cardiomyocytes were purchased from ATCC and plated in tissue culture treated T25-T75 flasks (Falcon). Cell aliquots were made and frozen at -80°C in DMEM containing 1 vol% DMSO, cell concentration was 1×10^6 cells mL^{-1} . Thawing was achieved with gentle warming within a 37°C water bath, resuspension in 8 mL DMEM and pelleting, followed by a second resuspension. Specific cell concentrations were measured using a Beckman Coulter VicellXR cell counter. Pelleting cells (Eppendorf 5810R centrifuge at 4°C , 300RCF, for 3 minutes) and resuspension in media was used to achieve specific seeding concentrations. Cells were seeded at 10,000 cells per cm^2 in DMEM (ATCC high glucose DMEM) supplemented with 10 vol% FBS (Gibco), 1 vol% L-glutamine (Gibco, 10mM) and 1 vol% antibiotic-antimycotic (Gibco, 100X). Cells were grown in a Thermo Scientific incubator (37°C , 5% CO_2). Replating was achieved by first aspirating all media, avoiding direct contact with the cells, cells were then washed with 5 – 10 mL of sterile PBS and 250 μL trypsin solution (Gibco, 0.25%) in EDTA and DMEM was added to the flasks. Flasks were incubated for 5 minutes to ensure full detachment occurred, trypsin was quenched with the supplemented DMEM, and the cells were replated.

2.5.2 H9C2 menadione assay

Cells were seeded in a half area (0.5 cm^2) Greiner 96 well plate, 100 μL of a 2.5×10^4 cells mL^{-1} suspension was added to each well. Cells were left 12 hours to reach $> 80\%$ confluence, confluence was measured by a Cloneselect imager. Once confluent, the cells were aspirated and 80 μL of fresh DMEM was added to each well. At each time point within the study, fresh menadione (Sigma Aldrich) stocks were made in DMEM and added to each well achieving the desired concentrations. Gentle mixing using pipette aspiration was used after stock addition to ensure homogeneity. After each timepoint had been reached, wells were aspirated and 10 μL of a 0.05 mg mL^{-1} Hoechst 33342 (Thermo Fisher) solution in DMEM was added to each well, the plate was incubated for ten minutes. Hoechst solutions were then aspirated and 10 μL of a 0.03 mM DRAQ7 (Biolegend) solution in DMEM was

added to each well, followed by a further ten-minute incubation. The DRAQ7 solution was aspirated and fresh DMEM was added to each well. Samples were then analysed using a Cellomics™ array scan vti, with two fluorescent channels for Hoechst and DRAQ7 respectively. Images were comprised of 7-tiled regions within the well, excluding the bottom and left tiles, as this is where pipetting was undertaken and could result in lower cell confluency. Data was analysed using HCS Studio™ Cell Analysis Software, sample quantity identification and parameters were assessed for all sample batches to ensure representation of the sample. All cell based assay results are displayed with n and n_{bio} values where n is the measurement replicates and n_{bio} is the number of biological replicates and the total number of measurements is $n_{\text{tot}} = n \times n_{\text{bio}}$.

2.5.3 Biocompatible PDMS surface treatment

The initial surface treatment tested was a direct application of type 1 collagen (col-1) and fibronectin, respectively. A surface treatment of $50 \mu\text{g mL}^{-1}$ col-1 was prepared by dilution with sterile de-ionized water (Invitrogen) and correction to pH 7.2 with glacial acetic acid (Sigma Aldrich). Col-1 treatment was applied directly after plasma treatment (Harrick, 18 W, air O_2 , 2 minutes exposure) and the surface was incubated for 12 hours at 4°C . After incubation, the surface was washed with sterile DIW and left in a Biomat2 cell hood to dry. When dry, UV-C sterilisation was applied for 45 minutes prior to cell seeding. The process for direct fibronectin coating was analogous the col-1 process, a lower fibronectin concentration of $25 \mu\text{g mL}^{-1}$ was used.

The PVA (Sigma 87-90% hydrolysed 30-70 kDa) treatment was a 1 wt% solution in DIW. PVA solutions were produced by weighing the appropriate amount into a beaker containing DIW, the beaker was sealed with parafilm and left for 12 hours at 60°C with stirring. After the stirring stage, extra DIW was added to account for evaporation loss. The solution was applied directly to the PDMS surface and baked at 60°C on a hotplate. This treatment was found to produce surface voids and layers visible by eye and microscopy (Figure 2.10 & 2.11). To reduce formation of voids the PVA solution was mixed with 50% IPA (Sigma Aldrich) to produce a solution of 0.5wt% PVA in 50:50 IPA:DIW. The 0.5 wt% coating was applied and left in a fume hood to evaporate overnight. After evaporation, the surface was washed with DIW and dried under nitrogen.

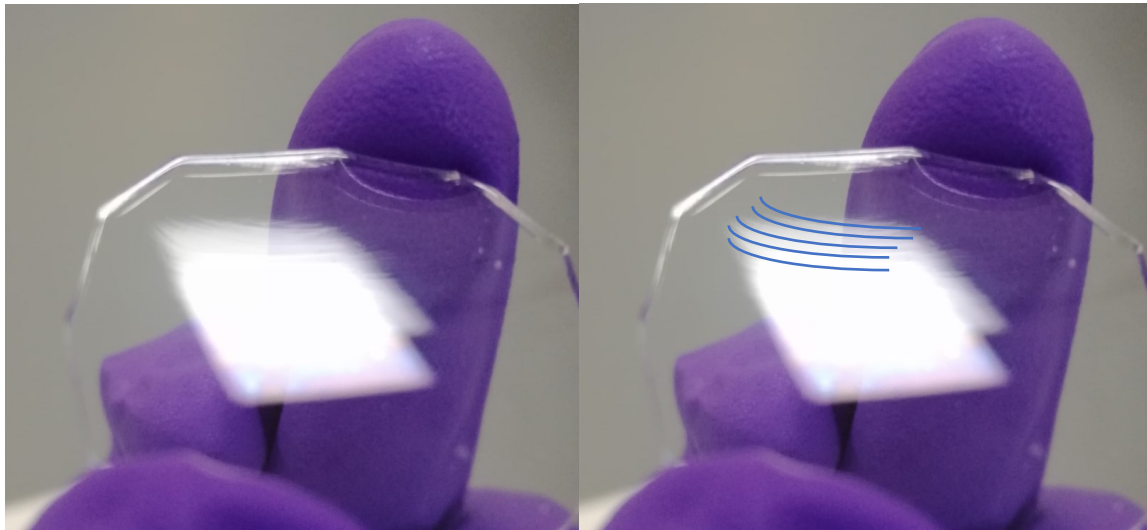


Figure 2.10) PDMS segment treated with 1 wt% PVA in DIW (**left**) Direct surface image (**Right**) Surface image with voids highlighted.

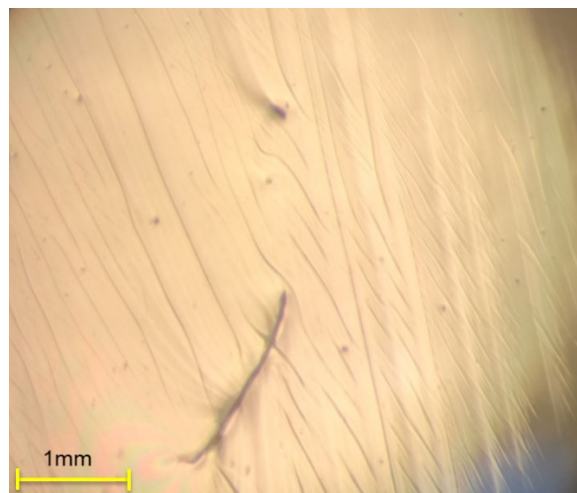


Figure 2.11) Brightfield microscopy image of PDMS segment treated with 1 wt% PVA in DIW (scale bar = 1 mm).

In place of PVA, covalently linked col-1 was explored with adaptation of work by Gunda *et al*¹²⁵. After plasma treatment (Harrick, 18 W, air O₂, 2 minutes exposure) a 5% (3-Aminopropyl)triethoxysilane (APTES) solution (> 98%, Sigma Aldrich) in absolute ethanol (VWR) was applied by direct immersion for 30 minutes. After immersion, the surface was washed with DIW to remove unbound APTES and dried with nitrogen. Samples were left overnight to dry, reducing the swelling effect of ethanol. At this point, some surfaces were treated with GA (50% in water, Sigma Aldrich), GA treatment applied a 2.5 vol% GA in DIW solution for 30 minutes. After GA application, the surface was washed in DIW and left to dried with nitrogen. In the next step, covalently linked col-1 to either the PDMS-APTES surface or the PDMS-APTES-GA surface. This was achieved by incubation of the surface with 0.1 mg mL⁻¹ col-1 solution at 4°C. The col-1 solution was made in an analogous fashion as above. Col-1 linked surfaces were washed with tissue culture grade water (VWR) and DMEM before usage

2.5.4 H9C2 surface adhesion assay

To assess surface adhesion standard solutions were applied, as previously described (Chapter 2.4.3). Treated 10 mm diameter round PDMS inserts were then fixed at the bottom of Falcon 12-well plates using uncured PDMS as glue. After sterilisation by UV-C, the cell standards were applied using a stripette and applying the 'g' setting for slow application of 2 mL of the standard per well. Cells were left to adhere for the allotted time and then the media was aspirated and washed with two aliquots of 2 mL of PBS. After washing, a 4% paraformaline (Sigma Aldrich) was applied to each well and left for 20 minutes to fix the cells. Paraformaline was then removed, and cells were washed with two aliquots of PBS before adding 0.5% Triton x-100 in PBS (Sigma Aldrich) for 15 minutes. Tx-100 was then removed, and cells were washed again with two aliquots before adding 0.1 mg mL⁻¹ Coomassie blue R-250 (Thermo Scientific). The Coomassie blue stain was left for a further 15 minutes before removal by aspiration and washing with 3 aliquots of PBS and finally storage under 2 mL of PBS. Cells were imaged on a Zeiss axio observer microscope, each well was manually imaged at 5 - 6 different points at 10x magnification. Image analysis was undertaken on a user-made pipeline in Cell Profiler software.

2.5.5 Liposome dose response

VFF produced liposomes were diluted at 10 – 90 vol% in cell media, representing a menadione concentration of 7 – 63 µM within the samples. When diluted, both the menadione concentration and liposome concentration are reduced. Samples were added to confluent TC within a 96 well plate, using a Gilson multipipette and incubated for 3 hours, viability was assessed using Hoechst and DRAQ7 stains following the procedure from Chapter 2.4.2. Within each plate 6 measurement replicates were made and a total of 3 plates were read for $n_{\text{bio}} = 3$ and a total of 18 measurements per data point. A row ($n = 6$) was used for a media and 70 µM menadione control to assess low and high cell death, ensuring the cells were responsive.

2.5.6 Bioink preparation

H9C2 cells were first collected as a pellet within a centrifuge at 2000 rpm and 4°C, 1×10^6 cells were pelleted per mL of Cellink. Working in a sterile hood, the pellet was then resuspended in 100 µL cell media and loaded into a 6 mL sterile syringe, the syringe was manually degassed. Cellink alginate-based ink (IKC200000305) was loaded into a 6 mL sterile syringe, taking care to remove all air bubbles, the Cellink syringe was then connected to the cell suspension syringe by a female-to-female Luer adapter. When connected the syringes were cycled through the Luer adapter 10 times, at a low pressure, to gently mix the two parts. The partially mixing suspension was split between each syringe, syringes were then disconnected and attached to a Cellink CELLMIXER. Displacing the syringes through the CELLMIXER achieved a greater degree of mixing and loaded the cell laden hydrogel into a printing cartridge. Once loaded, the cartridge was assessed to ensure minimal air bubbles were trapped and then loaded into the Inkredible+.

2.5.7 3D bioprinting method

Bioprinting was undertaken using a Cellink Inkredible+, prints were designed in Autodesk fusion 360 and prepared for print using Cellink Heartware and SLIC3R. Manual edits to the GCODE and printer setup were made to achieve prints into structures within Heartware. Designs employed a round border to contain the parallel line infill, the thickness of the print was determined by the 22G pipette nozzle employed (Cellink).

For printing, a 22G pipette nozzle was used for extrusion at a 5 mm s^{-1} travel speed. Cellink alginate-based bioink (IKC200000305) was used for cell prints it was mixed with cell pellet suspended in media using a Cellink mixer at a 10:1 ratio Ink:Cell suspension (approx. $1 \times 10^5 \text{ cells mL}^{-1}$). Print pressure was calibrated by eye, ensuring sufficient dispensation rate, prior to bioprinting. The applied print pressure varied batch to batch though typically was in the range 22-30 kPa. Prints were undertaken on a benchtop employing the Inkredible HEPA filtered system, prints were covered and transferred to cell hood for curing and media application. Post curing was achieved by application of CaCl_2 curing solution (Cellink) for 30-120 seconds, depending on print size. After aspiration of curing solution, media was added to fill the wells employed and ranged from 100 μL (0.5 cm^2 , 96 well plate) to 2 mL (6 well plate) per well. Subsequent analysis was achieved using the Cellomics analogously to Chapter 2.4.2.

3 Scalable production of drug delivery liposomes

3.1 Overview

This chapter demonstrates the capability of microfluidic hydrodynamic focusing (MHF) to produce therapeutically relevant, nano-sized liposomes. The MHF process was first documented by Jahn *et al*⁵⁹. MHF is used to produce SUVs in a controlled fashion using microfluidic laminar mixing. In this work, microfluidic production was applied to generate therapeutically relevant liposomes at a continuous rate, with the aim to facilitate future liposome efficacy testing within cell studies. Initial chip design and fabrication are first described, device optimisation was then explored through surface chemistry modifications and characterisation with CA measurement. After applying select surface modifications, liposome distributions were profiled by DLS, and efficacy of modifications were compared. The capability of flow conditions to impact liposome size and distribution were explored within the device; control over liposome size and distribution is critical for therapeutic application. Varied lipid formulations were then applied within MHF, to establish potential trends and limitations of the MHF device for producing therapeutically relevant liposomes.

3.2 MHF development

3.2.1 Introduction to soft lithography and polydimethylsiloxane

The first designs for MHF were produced in polydimethylsiloxane (PDMS), a silicone polymer that sees significant application within microfluidic device fabrication (Figure 3.1).^{126,127} Uncured PDMS is a viscous silicone oil, when mixed with a curing agent it crosslinks becoming a rubbery plastic. Crosslinking using Pt catalysts occurs with the addition of siloxanes to carbon double bonds.¹²⁸ Crosslinked PDMS is widely applied as it offers optical clarity and suitability as a stamp resin.^{126,129} In the generation of microfluidic devices, photolithography is first used to generate wafer-based patterns comprised of micron scaled features (Chapter 2.2.2). When PDMS is cast on a patterned wafer, crosslinking of PDMS results in a cured plastic imparted with the wafer features.

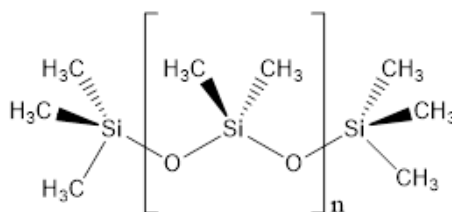


Figure 3.1) PDMS chemical structure, it is comprised of a siloxane back bone typically of around $n \approx 930$ that is terminated by methyl groups. The chain has an average MW of 34.5 kDa.¹³⁰

Photolithography contact printing allows for generation of structures with features ranging from the hypothetical minimum of 30 nm to a maximum of around 100 μm .¹³¹ An effective summary of the photolithography and present adaptations are discussed by Xia *et al*.¹³¹ The size limit is a result of the applied UV wavelength (λ_s) and photoresist thickness (d_{pr}); the latter being determined by the spin coating process of SU8 onto the wafer substrate (Equation 7).¹³² In this work, features were designed to be above 7.85 μm (365 nm light source, 150 μm deep SU8-2100) to ensure feature development (Chapter 2.4.1). The pattern of features is determined by the mask, which was commercially generated by SUSS Microtech, using laser ablation. The control over each dimension enables the production of microfluidic channels, which typically exhibit sub millimetre feature sizes and microliter volume.

$$\text{Minimum Feature Size} = \frac{3}{2} \sqrt{\lambda_s \frac{d_{pr}}{2}}$$

Equation 7. Where λ_s is applied UV wavelength and photoresist thickness is d_{pr}

3.2.2 An introduction to the dynamic light scattering measurement

Dynamic light scattering (DLS) is a method used to determine particle size within solution.¹³³ Fundamentally DLS operates by measuring incident laser intensity and scattered light intensity from a sample. Particles within solution scatter light, mobile particles create varying areas of constructive and destructive interference which results in intensity fluctuations. DLS first correlates intensity fluctuation ($G(\tau)$) against the short decay intervals (τ) and the autocorrelation function (ACF) (Equation 8a). The scattering vector q relates to the refractive index (n_0), wavelength in vacuum (λ_0) and scattering angle (θ) (Equation 8b).

$$G(\tau) = 1 + b e^{-2D_t q^2 \tau}$$

Equation 8a. Where D_t is the translational diffusion coefficient and q is the scattering vector.

$$|q| = \frac{4\pi n_0}{\lambda_0 \sin(\frac{\theta}{2})}$$

Equation 8b. Where scattering vector q relates to the refractive index n_0 , wavelength in vacuum λ_0 and scattering angle θ .

DLS instruments then generate raw correlation function (RCF) plots against the decay time (τ) and the output is used to quantify D_t and by relation to the Stokes-Einstein equation, the hydrodynamic radius (R_h) (Equation 9). The hydrodynamic radius is an approximation within this work, given measurements were taken of soft matter, a deviation from hard sphere topology.

$$D_t = \frac{k_b T}{6\pi\eta R_h}$$

Equation 9. Where k_b is the Boltzmann constant, T is temperature and η is viscosity

3.2.3 Initial MHF application

The MHF device is operated with a central anhydrous ethanol stream co-flowed with a PBS outer stream, resulting in a thread-like ethanol stream (Figure 3.2A). To produce liposomes, lipid is first dissolved within the ethanol phase before co-flow and control over the diffusive mixing of the two phases is predominantly used to control formation.^{59,134,135} The mechanism for liposome formation is currently hypothesized by Jahn *et al*⁵⁹ to occur by the formation and subsequent fusion or rearrangement of disc like micelle intermediate structures.⁵⁹⁻⁶¹ To establish our model efficacy, a stream of anhydrous ethanol was co-flowed with PBS and the crossflow junction was imaged. A crossflow junction is a meeting point of two or more phases flowing through 90° displaced channels.

Images of the central ethanol stream indicated wetting of the untreated PDMS surface (Figure 3.2B). Wetting is implied by the deviation from a well-defined fluid thread, as the stream exits the crossflow junction. Surface wetting results in a broad ethanol and PBS interface, as it deviates from a fine centralised fluid thread. Within the channel, a non-uniform velocity profile is present across the channel cross section, this results from an increasing shear rate as the channel edge is approached.⁷² When the ethanol stream is centralized and narrow, ethanol flows at a uniform velocity close to value at the channel center (Figure 3.2C). In a broad stream, the ethanol phase sees a range of velocities and by association, diffusive mixing rates (Figure 3.2B).^{59,61} Exhibiting a range of diffusive mixing rates will lead to an unfavourable, disperse distribution of the resultant liposomes.⁵⁹ The impact of fluid flow on the end liposome distribution is explored in depth by Jahn *et al*⁶¹.

The surface wetting was hypothesized to be driven by the PDMS surface chemistry. The process of plasma bonding results in PDMS becoming more hydrophilic as surface silanol groups are exposed.¹³⁶ Plasma hydrophilization of PDMS is labile, Trantidou *et al*¹³⁷ report an initial water contact angle (CA) of $7.2 \pm 0.5^\circ$ shifting to $92.6 \pm 0.3^\circ$ after 24 hours.¹³⁷ Water contact angle is the angle at the edges of a water droplet on a surface, lower values suggest a greater interaction between the droplet and surface. This shift in hydrophilicity could be the cause of the observed surface wetting, as devices were used within 2 hours of manufacture. To reduce wetting within the device, a variety of PDMS surface modifications were explored.

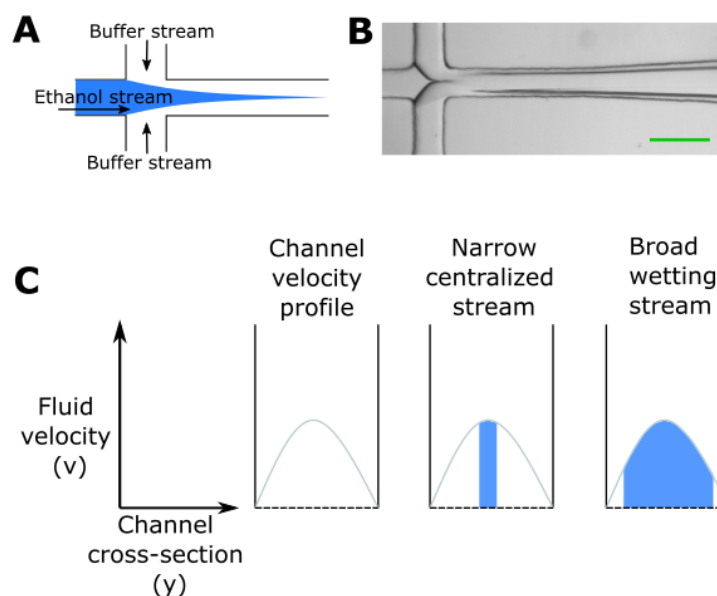


Figure 3.2) **A** Diagram of an ideal MHF crossflow junction showing formation of an ethanol fluid thread, directions of flow are indicated using arrows. **B** Bright field image of the cross-section with co-flow of anhydrous ethanol with 1X PBS buffer $FRR = 5$, $TFR = 0.24 \text{ mL min}^{-1}$ (scale bar = $200 \text{ }\mu\text{m}$). **C** Diagram of the velocity profile across a microfluidic channel and representative velocity distributions for a narrow-centralized stream and broad wetting stream.

3.2.4 MHF surface modification

Oxygen plasma treatment is a key component of PDMS modification. Exposure to oxygen plasma removes hydrogen radicals from the PDMS surface, forming silicon radicals which react with oxygen radicals.¹³⁶ The reaction with oxygen radicals results in terminal hydroxyl groups at the PDMS surface. These hydroxyl groups can participate in hydrogen bonding and increase hydrophilicity of the surface.^{137,138} Oxygen plasma hydrophilic modification is widely known to undergo hydrophobic recovery with storage.^{136,138–140} Re-orientation of the surface hydroxyl groups is said to be a thermodynamically driven process; aided by the ability of low MW chains to diffuse from the bulk material to the surface.¹³⁶

Midwoud *et al*¹⁴¹ measure the water CA on untreated PDMS to be 110° . In this work, PDMS was treated with oxygen plasma for 1 minute and measured within 120 minutes to mimic the internal surface of the bonded MHF device. The CA of de-ionized water (DIW), water with a low ion content, on the air plasma treated PDMS was measured as $84.1 \pm 1.8^\circ$ (18 W, air O_2 , 2 minutes exposure, $n=15$, from 5 functionalized surfaces, error = 1 SD). Our value was close to the value of $84 \pm 6^\circ$ (600 W, 98% O_2 , 30 minutes exposure) determined by Mata *et al*¹³⁸, using higher power and oxygen concentration. The water CA on PDMS can be impacted by a great number of factors such as batch variation of silane chain length, vinyl prevalence, mixing procedure and curing method.¹³⁹ A higher ratio of silicon hydride curing agent to silane polymer leads to a greater extent of crosslinking in the cured PDMS, crosslinking increases the mechanical rigidity of the cured PDMS (Figure 3.3).¹³⁸ Excess quantities of curing agent can contribute to the hydrophobic recovery following oxygen plasma treatment, inherent presence of low molecular weight moieties also contributes to this.^{142,143} Other factors affecting CA include plasma power, oxygen concentration, exposure time and surface age post-exposure.^{136,141}

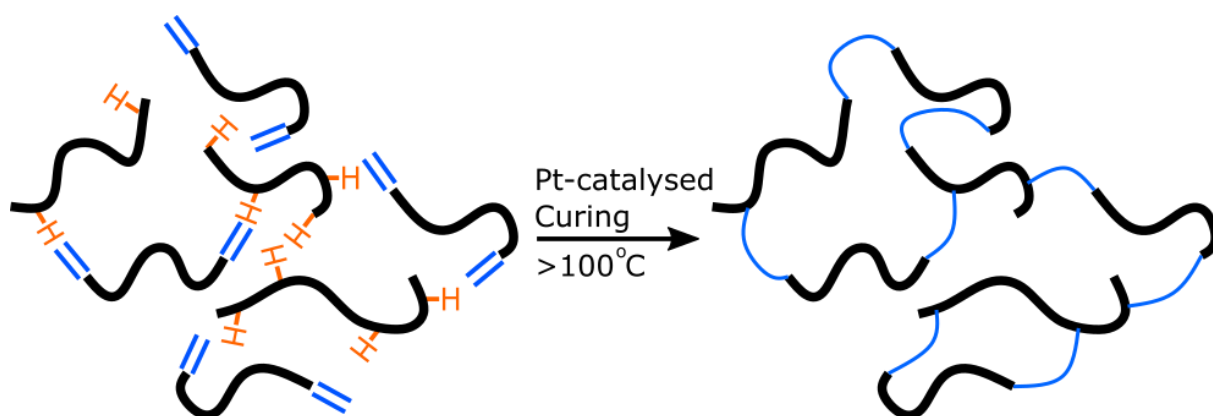


Figure 3.3) Schematic representation of platinum catalysed crosslinking of silicon hydride and vinyl groups. Adapted from Wacker Chemie AG datasheet <https://www.wacker.com/h/medias/6709-EN.pdf> accessed: 01/12/2021.

3.2.5 Modified PDMS water contact angle

Modifying PDMS surface chemistry is a crucial step in optimizing application. In this work, MHF surface modifications were chosen to range from hydrophilic to hydrophobic and encompass a variety of surface moieties. PDMS was prepared with the same ratio of curing agent and surfaces were stored in DIW and dried prior to measurement. Surface samples were comprised of 10 mm diameter discs of PDMS treated using procedures described in Chapter 2.1.1, after drying surfaces were measured within 120 minutes. CA values were determined using the Sessile drop method, applying a 200 μ L DIW droplet on the functionalized surfaces.¹⁴⁴ The value from for native PDMS and DIW CA is shown first (Figure 3.4).

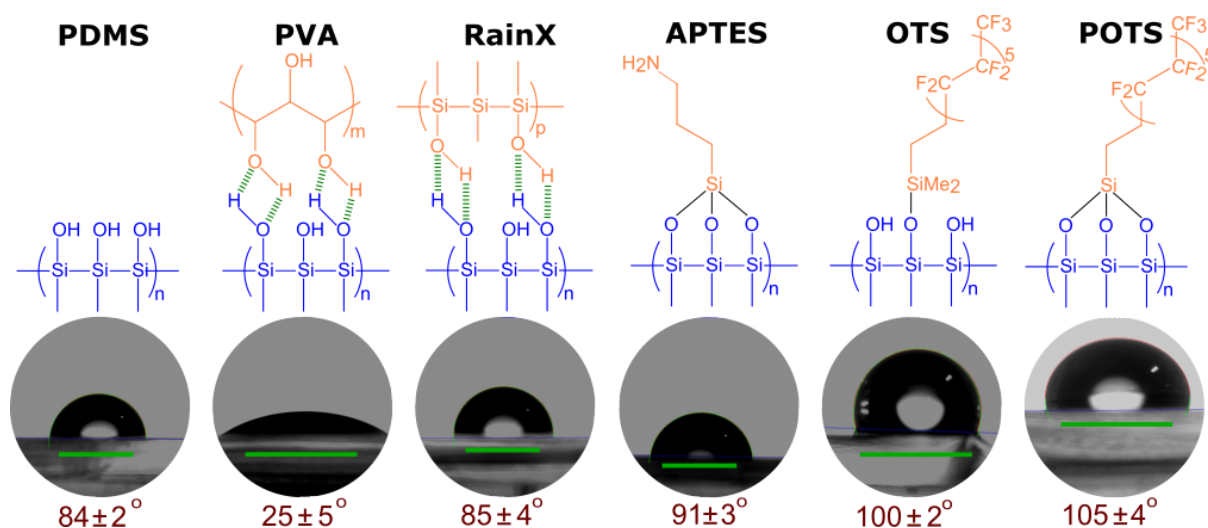


Figure 3.4) The different applied surface treatments are shown left to right, below are the schematic chemical representations of the PDMS surface (blue), surface treatment moieties (orange), interactions (green) and formed covalent bonds (black). Below the chemical representations are bright field images of the DIW droplet on the respective surfaces (Imaged using 5x and 10x magnification scale bar = 4mm) and measured contact angle ($n = 15$ Error $\pm$ SD).

PVA, a polymeric molecule with alcohol side groups, was explored as a hydrophilic PDMS treatment ($25 \pm 5^\circ$). Treatment was achieved with the direct application of 87-90% hydrolysed 30-70 kDa PVA solution. Briefly, the coating was directly applied and left to adhere and followed by washing using DIW, Chapter 2.5.3 describes this in more detail. The extent of hydrolysis and the backbone chain length determines the coating impact on hydrophilicity.¹³⁷ The significant degree of hydrolysis within the 87-90% hydrolysed 30-70 kDa PVA meant the coating resulted in a hydrophilic surface. Trantidou *et al*¹³⁷ measured the CA of DIW on the surface to be $37.0 \pm 19.2^\circ$, in this work a value of $25 \pm 5^\circ$ was determined, as steps were taken to reduce formation of surface voids (Chapter 2.5.3). Trantidou *et al*¹⁴⁵ used PVA treatment to impart long term modification for an oil/water co-flow system, as PVA has limited solubility in the flowing phases. When applied to MHF, absence of covalent surface bonding could lead to surface degradation over time from ethanol contact.

RainX is a coating designed for hydrophobic modification of car windscreens, ensuring rain droplets bead, and are more easily removed by windscreen wipers. It has seen wide application within microfluidic devices as cost-effective hydrophobic treatment for PDMS and glass surfaces. RainX is comprised of solvated polysiloxanes which interact with surface hydroxyl groups on glass or PDMS surfaces, this process screens the hydroxyl groups resulting in a more hydrophobic surface.¹⁴⁶ In this work, the CA was found to be $85 \pm 4^\circ$ showing minimal impact for the treatment. As with PVA, the non-covalent attachment could result in modification removal during MHF application.

APTES is another silicon-based modification, it is typically used for immobilizing proteins on PDMS.¹⁴⁷ It differs from RainX in structure and by the covalent attachment of the silicon moiety to the PDMS surface. The covalent linking occurs at the exposed hydroxyl groups by a nucleophilic substitution reaction, APTES has three ethoxy groups that are removed during reaction with surface hydroxyl groups.¹⁴⁸ Covalent adhesion provides a surface with greater longevity at the cost of a typically more involved treatment procedure.¹⁴⁷ APTES treatment was optimised using guidelines reported by Gunda *et al*¹²⁵ to reduce the competing polymerisation of APTES and favour monolayer surface modification. The treatment led to an overall increase in CA to $91 \pm 4^\circ$, demonstrating greater hydrophobicity than RainX. Despite the ability of the primary amine group to participate in hydrogen bonding ($-14.1 \text{ kJ mol}^{-1}$ ¹⁴⁹), an increase in hydrophobicity was observed. Hydrophobicity likely increases from the covalent bonding capping silanol groups which form stronger hydrogen bonds ($-30.1 \text{ kJ mol}^{-1}$ ¹⁵⁰).

OTS and POTS are fluorinated silanes with reactive silicon chloride bonds capable of facilitating covalent bonding to the PDMS surface.¹⁴⁸ Both OTS and POTS reduce presence of surface hydroxyl groups and link a fluoroalkane chain, which further acts to increase hydrophobicity (OTS $100 \pm 2^\circ$ and POTS $105 \pm 4^\circ$). The fluorosilane treatments led to the highest DIW CA within the study (Figure 3.4). The key difference between OTS and POTS is the number of Si-Cl bonds, POTS has two more Si-Cl bonds enabling it to react with two more hydroxyl groups at the PDMS surface.¹⁴⁸ This increase in POTS reactivity could have contributed to the increased hydrophobicity of the POTS surface. The standard deviation in the POTS surface was larger than OTS, the enhanced reactivity may make the silicon chloride bonds within POTS more susceptible to a competing hydrolysis (Figure 3.5). An increased number of chloride groups enhances electrophilicity of the central silicon atom, making it more susceptible to nucleophilic attack. Hydrolysis would remove the Si-Cl groups and reduce reactivity with the PDMS substrate. Careful storage and handling of the fluorinated silanes was applied to minimize potential hydrolysis.

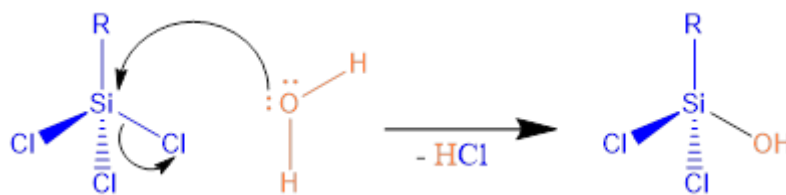


Figure 3.5) Hydrolysis of the silicon chloride bond in POTS where R = 1H,1H,2H,2H-perfluorooctane.

To explore a range of contact angles the PVA, RainX and POTS modifications were chosen. PVA was applied as a hydrophilic surface ($25 \pm 5^\circ$) with reported longevity within microfluidic devices.¹³⁷ The RainX surface ($85 \pm 4^\circ$) acted as a treated surface with a similar hydrophilic CA to the plasma treated PDMS ($84 \pm 2^\circ$). POTS treatment led to a hydrophobic surface ($105 \pm 4^\circ$) with the highest CA within the study. The selected PVA, RainX and POTS treatments were explored within the MHF device as they present the greatest range in CA values.

3.2.6 Surface modified MHF application

When applied to the MHF chip, RainX treatment led to a uniform stream, this was unexpected as RainX treatment yields a similar DIW CA to untreated PDMS, which exhibited a broad stream within the previous study (Figure 3.6A). A slight wetting of the stream to the RainX surface is indicated by the presence of the shadows parallel to the ethanol stream. Streaking was observed in the aqueous channels, this may be a result of the hydrophilic DIW CA ($< 90^\circ$).

In the PVA modified device, central stream wetting was observed, this is implied by the broadening ethanol stream (Figure 3.6B). The imaging suggests PVA hydrophilic treatment is disfavoured for MHF application. When the hydrophobic POTS treatment was applied, minimal stream wetting was observed (Figure 3.6C). Within the POTS chip, a well-defined centralized ethanol stream resulted, the stream remained narrow along the channel section. To explore the impact of the coatings on the formulation, DLS analysis was undertaken on the samples produced.

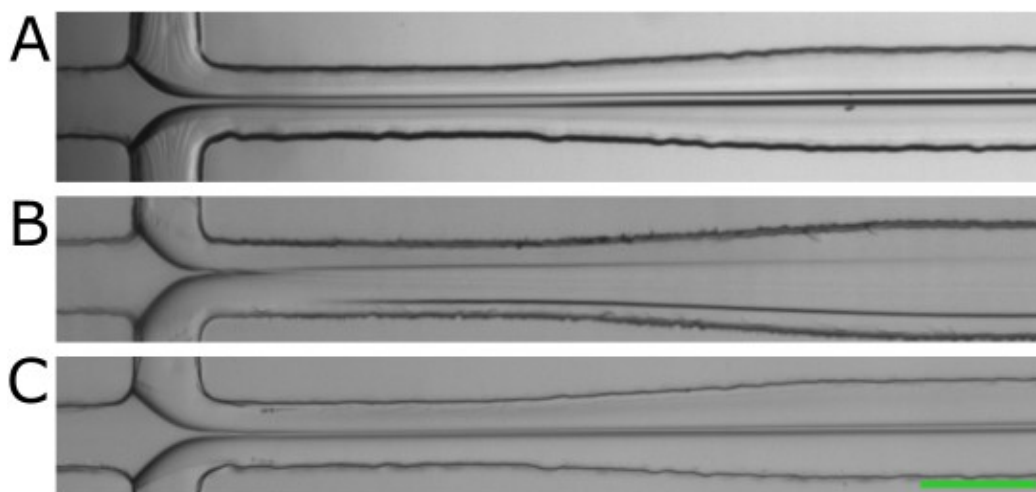


Figure 3.6) Bright field images of the crossflow junctions of **A** RainX, **B** PVA and **C** POTS surface modifications with of co-flow of anhydrous ethanol and PBS buffer FRR = 5, TFR = 0.24 mL min^{-1} (scale bar = $200 \mu\text{m}$).

3.2.7 Surface modified MHF distribution data

The following preliminary study of liposome distribution sets was produced using the surface modified, PDMS based MHF devices, DLS was undertaken as described in Chapter 2.1.2 (Figure 3.7). Identical flow conditions ($FRR = 5$, $TFR = 0.2 \text{ mL min}^{-1}$) were applied for each sample and the lipid phase was comprised of the same 0.1 mg/ml DOPC in EtOH anhydrous stock solution. DOPC was chosen as it is a well-documented lipid, capable of forming stable fluid lamellar liposomes.¹⁵¹ A second sample rerun was undertaken after the first sample and a cleaning step; this was used to explore both the surface stability and sample reproducibility. The devices were washed with 0.5 mL 70% ethanol and 0.5 mL 1x PBS before being dried with compressed air.

Distribution data was evaluated with the end application of therapeutic liposomes in mind. Therapeutic liposomes are typically made to be $< 250 \text{ nm}$ in diameter, this is partially to minimize clearance by the immune system.^{19,20,24,68} Monodisperse, low polydispersity samples ($PDI < 0.2$) are favoured to meet guidelines for quality and control behaviour during application.

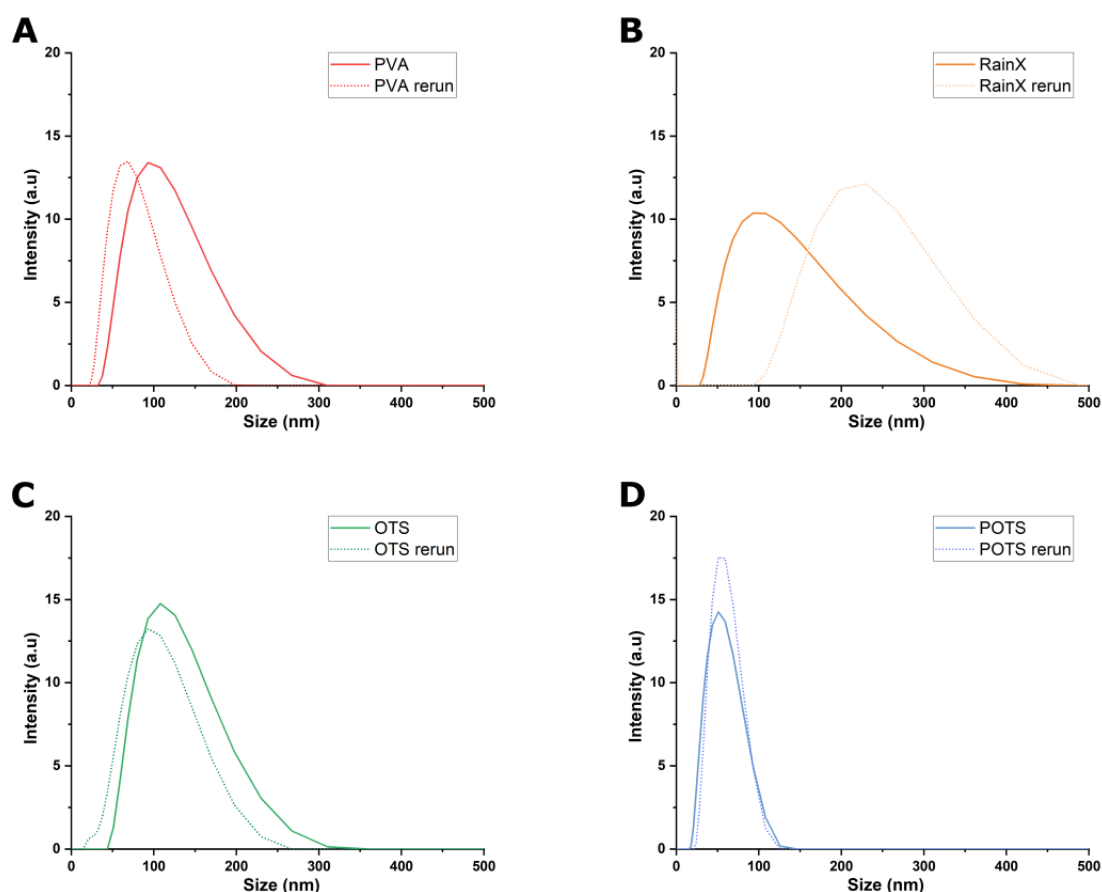


Figure 3.7) DLS distribution data for; **A** PVA, **B** RainX, **C** OTS and **D** POTS surface modifications with of co-flow of anhydrous ethanol containing 0.1 mg mL^{-1} DOPC and PBS buffer $FRR = 5$, $TFR = 0.24 \text{ mL min}^{-1}$.

To aid comparison, the polydispersity index (PDI) was calculated for samples using standard deviation (σ) and peak maxima from the gaussian data fit (Equation 10a). Standard deviation was calculated from the full width half maximum (FWHM) of the gaussian fit (Equation 10b). Gaussian fitting was an approximate fit, this was used to approximate FWHM and all fits were assessed to ensure effective representation of the peak maxima and FWHM. For asymmetric distributions a gaussian fit is unrepresentative at low intensity values, here a log normal fit would provide a better representation. To describe distributions, the PDI value is often applied as a dimensionless number where lower values indicate more monodisperse distributions. PDI assumes samples are monodisperse and gaussian so is often an approximation. Peak maxima centres and FWHM values were extracted using Origin pro peak fitting and tabulated (Table 2). The reported peak maxima are also referred to as the mean diameter of the liposomes. PDI is a commonly applied metric for gaussian distributions, as not all the distributions are symmetric gaussians it can underrepresent samples. A PDI value of < 0.2 is considered favourable for therapeutic application *in-vivo*.^{23,152}

$$PDI = \frac{\sigma^2}{Peak\ maxima^2}$$

Equation 10a. Polydispersity index (PDI) calculated using standard deviation (σ) and peak maxima

$$\sigma = \frac{FWHM}{2\sqrt{2\ln(2)}} \approx \frac{FWHM}{2.3}$$

Equation 10b. calculation of standard deviation (σ) using the full width half maximum (FWHM)

Surface coating	Replicate	Peak maxima (nm)	FWHM (nm)	PDI
PVA	1	111.54	92.78	0.240
	2	68.83	22.87	0.038
RainX	1	123.54	114.97	0.300
	2	161.10	284.51	1.081
OTS	1	123.99	96.72	0.211
	2	106.14	89.59	0.247
POTS	1	56.71	45.92	0.227
	2	58.73	40.93	0.168

Table 2) MHF surface coating impact on liposome mean diameter, FWHM and PDI. Replicate 1 is the first use of the device and replicate 2 is the second use after a washing step.

The initial mean diameter of liposomes produced using the PVA surface was 111.54 nm, after washing mean diameter decreased to 68.83 nm. The PVA surface exhibited better performance than expected, as the central stream was likely wetting the channel (Figure 3.6). The initial value for the mean diameter is within the size requirement for therapeutic liposomes < 200 nm, the PDI was greater than the suggested threshold of < 0.2 for therapeutic application.^{23,153} After washing the device, the liposomal peak mean shifted by 42.71 nm, indicating the ethanol wash may act to remove the PVA coating. The mean diameter and PDI value after the washing step were both favourable for

therapeutic application, the PDI value was the lowest within the study. The shift in size and distribution, following the washing step was thought to impact sample reproducibility and the PVA surface was considered unfavourable.

RainX surface modification gave liposome mean diameter values of 123.54 nm initially and 161.10 nm after washing, the surface exhibited the largest peak mean and PDI values. The DLS distribution profiles were asymmetric, with a bias toward larger particle size. The asymmetric profile of the distributions indicates a significant fraction of liposomes were above the 200 nm threshold, which is imposed for therapeutic application. Samples with high > 200 nm populations are liable to be ineffective *in-vivo*, since they are cleared by immune response.¹⁵³ It is possible the RainX samples are multimodal distributions this would disfavour the application in therapeutic delivery. Additionally, PDI values for multimodal samples are unrepresentative, this means the dispersity of the RainX samples are potentially underrepresented. During application, a precipitate was observed within RainX treated devices, this could indicate the removal of RainX. Precipitate formation led to channel blockage and flow disturbances which could impact the PDI values measured. After application of a wash, the device yielded a larger PDI, and mean diameter increased by 37.56 nm. Broad asymmetric distribution, mean diameter shift and precipitate formation, meant RainX use was discontinued.

The OTS modification yielded mean diameters of 123.99 nm initially and 106.14 nm after washing, POTS modification gave 56.71 nm initially and 58.73 nm after washing. Each fluorosilane surface gave < 200 nm mean diameter and demonstrated consistent distributions after washing. The PDI values for the surface were mostly outside the < 0.2 threshold though the second replicate for POTS met this criterion. The difference in mean diameter was unexpected as both POTS and OTS result in similar surface chemistry. The difference in the DIW CA of each fluorosilane was comparably small, indicating the DIW CA is an ineffective predictor of MHF performance. POTS gave the lowest recorded mean diameters and consistent performance after washing, practical considerations involving the storage and shelf life of POTS, made it less favourable. OTS was chosen for application in future MHF studies because of its stability, reproducibility in the CA study and narrow distribution of resultant liposomes.

3.3 MHF application

3.3.1 FRR and TFR impact within MHF

Different flow parameters were explored within MHF with the aim to reduce dispersity. The FRR is documented to change the resultant liposomes size, TFR is reported to minimally impact the resultant liposomes.^{59,61,62,134,135} In this work, FRR and TFR were explored within an OTS modified MHF device. During the study, the central ethanol flow rate was fixed at 0.04 mL min⁻¹ and the buffer flow rate was incremented by 0.2, 0.4, 0.8 mL min⁻¹ to explore both FRR and TFR.

Overall, increasing FRR was shown to decrease the mean liposome diameter, this observation is supported in work by Jahn *et al*^{59,61} (Figure 3.8A). Increasing FRR beyond 5 resulted in a more symmetric distribution profile and reduced prevalence of > 200 nm liposomes. PDI was observed to decrease from TFR 0.24 mL min⁻¹ to 0.44 mL min⁻¹ and increase at the highest TFR of 0.84 mL min⁻¹ (Figure 3.8B). The FRR behaviour is reported, Jahn *et al*⁶¹ who demonstrate increasing FRR leading to a decrease the mean diameter. Golden *et al*¹³⁴ report the effect of cusping at high TFR, cusping is the induction of curvature at the interface of the flow focused stream and sheath fluid, Golden *et al*¹³⁴ report cusping at higher Reynold's (Re > 5) number at FRR 10. Cusping is reported to increase PDI at

low Reynold's number ($Re < 100$) and may be the reason for the increased PDI at TFR 0.84 mL min^{-1} . At the maximum applied TFR (0.84 mL min^{-1}) the Reynold's number was determined to be 80, this Reynold's number is within the threshold for observed cusping within the study by Golden et al¹³⁴.

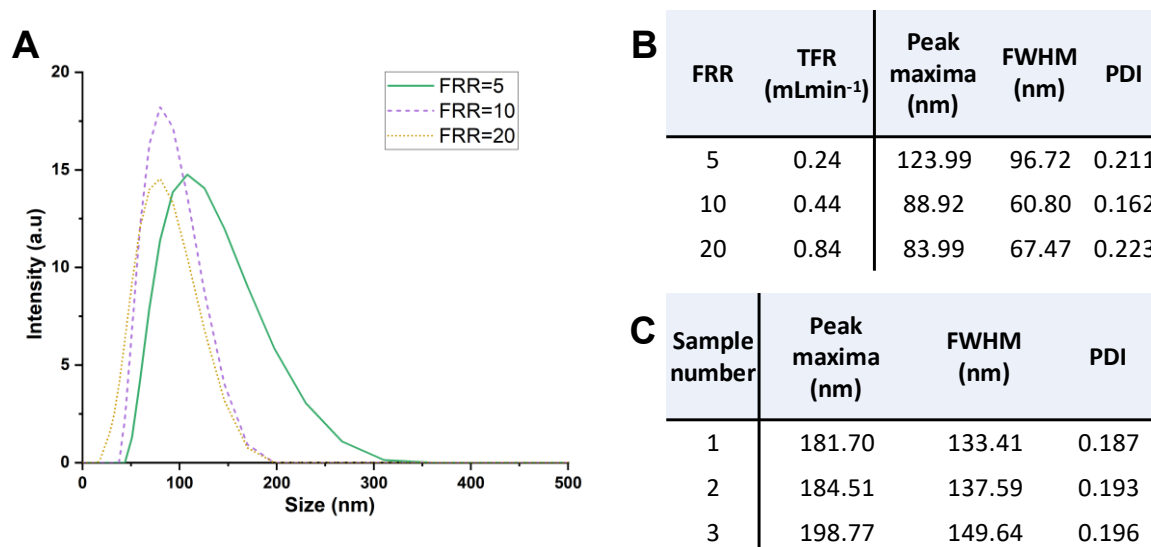


Figure 3.8) MHF FRR sweep data within an OTS treated device **A** Resultant distribution of liposomes determined by DLS for FRR values 5, 10 and 20 $n=1$ samples. **B** Tabulated peak maxima and FWHM data extracted from the DLS distribution data. **C** Tabulated distribution data achieved from three independent samples of 0.1 mg mL^{-1} DOPC extruded using a 200 nm filter for 15 cycles.

Based on the applied values within the preliminary study, an FRR of 10 and TFR of 0.44 mL min^{-1} were the most suitable for application since the PDI was the lowest observed (0.162). A comparative data set generated from three independent lipid films and extrusion, using a 200 nm filter was generated (Figure 3.8C). When compared, the MHF device offers potential for size control and lower resultant PDI. To further explore the potential and limitations of MHF production, a formulation study was undertaken.

3.3.2 MHF applied lipid introduction

Therapeutic liposome formulations are typically comprised of multiple lipids and other molecular components.^{19,20} Components are chosen to prevent encapsulant leakage, reduce clearance *in-vivo*, and add organ specificity.^{20,39,68} It is essential that the MHF platform remains effective at producing liposomes with multicomponent formulations. A study was undertaken into a variety of lipid formulations within the OTS functionalised MHF device, to establish potential effects of differing lipid compositions and evaluate whether resultant liposomes were $< 200 \text{ nm}$ and low PDI < 0.2 .

In the study, formulations based on DOPC, DOPG and cholesterol were explored (Figure 3.9). With the fundamentals of lipid assembly covered in Chapter 1.2, this section discusses characteristic differences in the applied lipids. DOPC is a commonly used lipid that typically forms fluid lamellar lipid structures, its headgroup is a zwitterionic choline group (Figure 3.9A).^{151,154} The DOPG tail region is identical to DOPC, the head group is comprised of a glycerol head group (Figure 3.9B). The glycerol head group on DOPG results in an anionic headgroup charge. Cholesterol is a rigid sterol which readily incorporates into lipid bilayers, it is commonly applied to drug delivery systems to reduce encapsulant leakage

(Figure 3.9C).¹⁵¹ Cholesterol undergoes by a process called ‘flip flopping’, this acts to reduce membrane permeability and is described in work by Bennet *et al*¹⁵⁵.

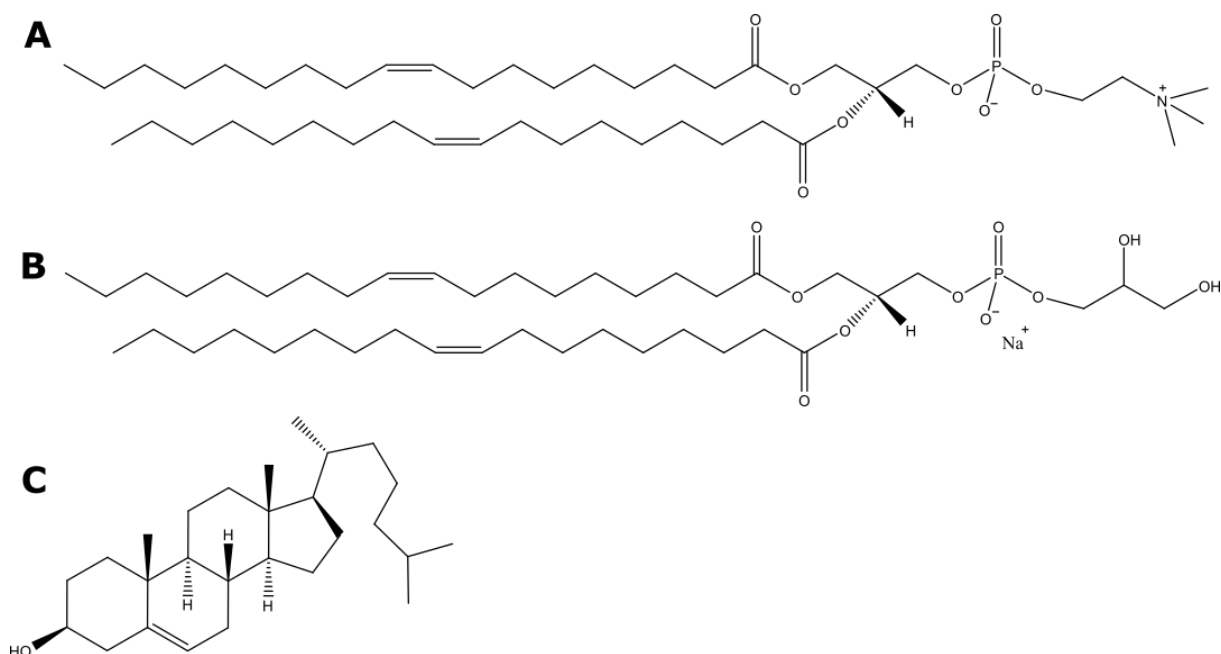


Figure 3.9) Molecular structures of **A** 1,2-dioleoyl-sn-glycero-3-phosphocholine **B** 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) **C** cholesterol.

3.3.3 MHF formulation study

The impact of increasing CHOL fraction was first explored, CHOL was increased up to 40 wt% (58 mol%), within this range CHOL is typically applied in liposome formulations (Figure 3.10A).^{19,20,39} Adding 10 wt% cholesterol was shown to increase mean diameter and decrease PDI relative to the pure DOPC values. Jahn *et al*⁵⁹ propose micelle formation is the first stage in the lipid MHF mechanism, followed by fusion to yield liposomes. At low concentration, CHOL could aid fusion of micelles which may yield the larger mean diameter values and the reduced dispersity. At higher CHOL fractions, CHOL decreased mean diameter and increased sample PDI. This data set demonstrates addition of CHOL to a DOPC formulation gives rise to typically larger mean diameter and lower PDI liposomes. The data set highlights the importance of characterizing resultant liposomes after formulation change, within MHF, before application of liposomes.

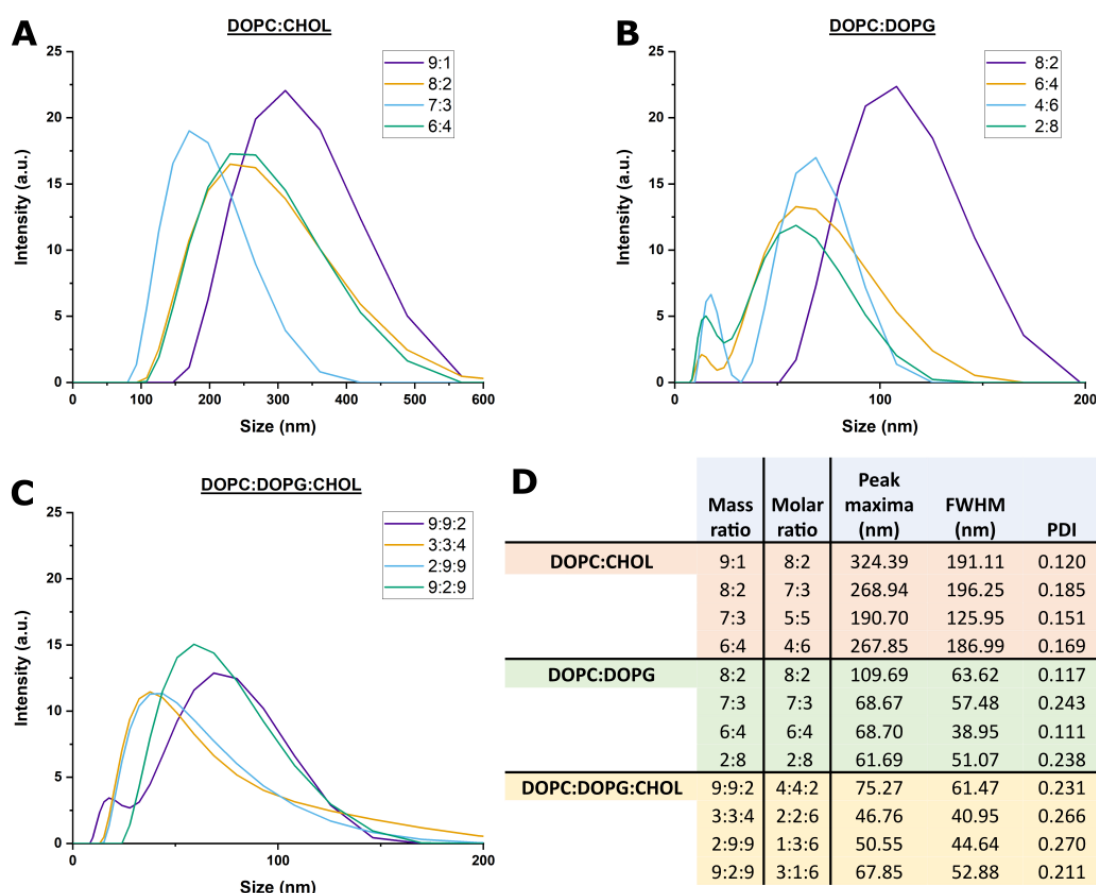


Figure 3.10) Resultant distributions of DOPC based formulations determined by DLS, samples were produced at FRR = 5, TFR = 0.24 mL min⁻¹ and using n = 1 film replicates. **A** Resultant liposomal distribution from increasing cholesterol mass ratio in the sample from 10 - 40 wt% **B** Resultant liposomal distributions from increasing DOPG mass ratio in the sample from 20 - 80 wt% **C** Resultant liposomal distributions from a varied ternary mixture of DOPC, DOPG and CHOL **D** Tabulated peak maxima and FWHM values for all samples, in the case of bimodal distributions the larger peak mean value and FWHM are reported.

DOPG addition to DOPC was explored up to 80 wt% (76 mol%) (Figure 3.10B). Overall, addition of DOPG lead to a decrease in the mean diameter of liposomes. This may be a result of the preferred curvature for this formulation, given that DOPG molecules within a bilayer would exert an electrostatic repulsion within the head group region. At 10 wt% DOPG the distribution was monomodal and exhibited a lower PDI of 0.120 than the pure DOPC value of 0.211. Above 10 wt% bimodal distributions were observed, a population of particles around 25 nm in diameter were observed; a 25 nm diameter suggests particles in this population were micellar. Presence of micelles is unfavourable as it increases sample dispersity, indicating a binary mixture of DOPC and DOPG above 10 wt% DOPG is unsuitable for therapeutic application. When considering the proposed mechanism for MHF, the presence of anionic DOPG in intermediate micelles could act as an electrostatic barrier for micelle fusion, yielding a sub population of DOPG rich micelles. The PDI values for the >30 nm diameter distribution was the lowest observed, a comparison to monomodal samples is unrepresentative as PDI is more valid for monomodal samples. This data complements the CHOL fraction data by demonstrating how formulation can impact resultant liposome distributions.

To further explore MHF, ternary mixtures of DOPC, DOPG and CHOL were applied (Figure 3.10C). All samples within the ternary mixture were within the < 250 nm in size, within the mixture CHOL ability to result in larger mean diameter appeared reduced. The PDI values for the ternary mixture were >

0.2, this may result from the stochastic liposome formation process being exacerbated by multiple components. CHOL rich ternary samples (2:2:6 and 1:3:6 mol%) both gave highly asymmetric distributions, that were weighted toward large particle size. The CHOL rich samples support the observation that CHOL may act to increase the average particle size. Within the ternary mixtures, particle mean diameter was below 75 nm suggesting DOPG has a dominant effect on particle size, CHOL within the binary system produced > 190 nm mean diameter particles. The DOPG rich sample (4:4:2 mol%) gave rise to a bimodal distribution similarly to those observed in the binary DOPC:DOPG system. This set demonstrates that multi-component formulations may require optimization for MHF application, to facilitate application as therapeutic liposomes.

3.4 Conclusion and future work

3.4.1 Conclusion

The results in this chapter show MHF can be successfully implemented within a PDMS microfluidic device. Surface modifications were shown to be impactful on the liposomal distributions, this is likely a result of the small 100 μm channel width since the ethanol fluid thread is in close vicinity to the channel edge. For a balance of functional and practical reasons OTS was determined to be the most effective MHF surface modification, from the studied surface modifications. The covalently linked fluorosilanes were considered to exhibit the best overall performance within the study.

Impact of FRR and TFR were explored within MHF, and resultant distributions were characterized. MHF demonstrated capability to decrease mean liposome size by 40 nm, between FRR 5 and FRR 20. Our device exhibited similar behaviour with FRR increase, as in work by Jahn *et al*^{59,61}. The ability of FRR to decrease the mean size is important for producing < 250 nm liposomes for therapeutic application. TFR was shown to have a potential threshold, above which TFR increased PDI values which could be a result from cusping. Lowering TFR is a potential way to reduce resultant PDI values and remain in a suitable range for therapeutic application, although at the cost of liposome production rate.

Liposome formulation applied within MHF was shown to greatly impact resultant sample distributions. Addition of CHOL typically yielded larger liposomes within the DOPC:CHOL mixture. In the DOPC:DOPG mixture, bimodal distributions were prevalent above 20 mol% DOPG. Above 20 wt% DOPG, a potentially micellar sub-population was present to avoid this within MHF a < 20 mol% DOPG fraction is required. Application of method to selectively remove of micelles using ultra-centrifugation and low MW cut-off filters could improve the sample quality of > 20 mol% DOPG samples, at the cost of increased processing times and lipid loss.¹⁵⁶ Characterisation of the impact of lipid constituents on resultant distributions is currently uncovered within literature through establishing trends with different components, further guidelines for application of MHF could be developed. In the next chapter an adaptation of MHF is explored, to further decrease polydispersity and increase production rates.

3.4.2 Future work

To support screening the flow-based parameters simulation of the device using COMSOL Multiphysics could shed light on some of the underlying issue with surface modification and support characterising the mixing regime. In addition to this, a significant number of design variations exist for MHF and a comparative study could aid design within the field.

4 High throughput production of therapeutic nanoliposomes

4.1 Overview

A present challenge for the study and exploration liposomal formulation, is the high throughput production of therapeutic liposomes. Producing nanoliposomes with continuous flow-based methods facilitates larger scale efficacy testing within cell culture which avoids sample batch variation from methods such as extrusion. The method of VFF was first developed by Hood *et al*⁶⁴, VFF operates analogously to MHF whilst offering higher throughput production. In this chapter, VFF device manufacture from PMMA is presented, demonstrating the accessibility of the cleanroom free fabrication method. PMMA manufacture enables generation of complex 3D millifluidic architectures without need of specialist equipment. Devices made from PMMA feature a low cost and manufacture time, making them highly accessible and suitable for rapid prototyping. The VFF design was explored to establish the practical working procedures and the performance is compared to the MHF device within Chapter 3. Using the design further, an exploration into the lipid formulation impact on resultant liposome distribution is covered. By understanding formulation impact, VFF production can be optimised to produce therapeutic liposomes.

4.2 VFF development

4.2.1 PMMA device accessibility

The full device used within this chapter was fabricated using cost effective materials without need of a specialist clean room (Chapter 2.2.4). For each device the estimated total material cost was £1.83, fabrication time was thirteen hours start to finish. With parallel production, 4-5 models could be produced in the thirteen-hour period using a single heating block. Typical fabrication time for a PDMS device is 24 hours, provided access to a lithography mask. The VFF fabrication method was made accessible, subverting the need for specialist cleanroom and soft lithography training. The method offers significantly faster prototyping time when compared to soft lithography. At extra cost, laser cutting, and 3D printing services could be employed to subvert the respective high equipment costs and ensuring fabrication can be undertaken in non-specialist institutions. Through application of PMMA layered assembly, complex design features and component integration can be achieved, without the need of specialist equipment. In summary, the accessibility, scalability, speed, and scope of PMMA manufacture for academia is highly promising.

4.2.2 Material properties of polymethylmethacrylate

To produce a VFF device with a millimetre scale width, soft lithography fabrication was deemed inappropriate, as it is typically used for micron length scales.¹³¹ To achieve wide channels, a rigid material is required, PDMS is generally flexible and could lead to channel deformation. Hood *et al*¹²³ manufactured the device using COC, this work used polymethylmethacrylate (PMMA), chosen for the broad literature background and accessible cost.^{141,157-171} PMMA is a polymer formed from the methyl methacrylate monomer and is favoured for its mechanical rigidity, optical clarity, and low cost (Figure 4.1).^{167,172} Extrusion and continuous casting are the two commonly used production methods of PMMA, and each imparts different capabilities.^{157,172} Extrusion produces a wider range of sheet thickness, as extrusion is mechanically driven it reduces PMMA melting point and optical clarity. Clarex-001 produced by Weatherall was exclusively used within this work, it is formed by continuous casting. The continuous casting process imparts Clarex-001 with a high optical clarity and melting point. The current available thickness for Clarex-001 are 0.2 - 5 mm in 0.1 mm increments, a result of the manufacturing method.

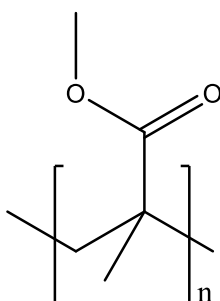


Figure 4.1) Polymethylmethacrylate chemical structure with n repeating units.

PMMA is amenable to the production of cost-effective micro and milli-fluidic devices. The first step in this application is the patterning of the PMMA substrate. PMMA can be patterned using various methods including laser ablation, laser cutting, CNC milling and embossing.^{161,163,164,170} Once a pattern or channel structure is generated, PMMA can be bonded to other PMMA sheets. The bonding process can act to fluidically seal channels providing a confined environment for millifluidic processes. PMMA bonding is conventionally achieved by heating PMMA above the glass transition temperature ($T_g > 100^\circ\text{C}$ ¹⁷⁰) and applying pressure.^{167,170} Above the T_g molecules become flexible at the PMMA surface, they are then able to disentangle from the bulk material. Following heating, pressure causes molecules from opposing PMMA layers entangle and as the PMMA is cooled, the entanglement becomes irreversible leading to sheet bonding. During the bonding process sharp temperature change is avoided to prevent defects forming in the PMMA.¹⁶⁷

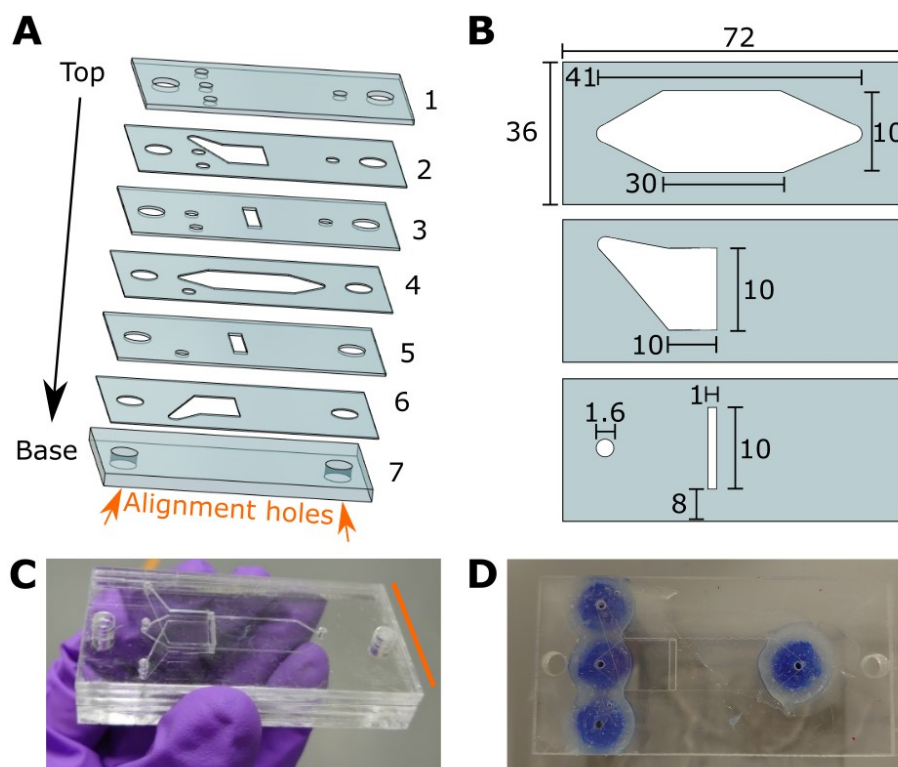
4.2.3 VFF design and fabrication

Initially SLA printing was explored as a method of VFF fabrication, channels often became obstructed in the curing process and uncertainty of proprietary resin formulation (Formlabs), PMMA based manufacture was explored as an alternative. The PMMA VFF device was created from a stack of laser cut PMMA sheets of varying thickness (Figure 4.2A). Alignment was achieved from alignment holes throughout the structure, these are highlighted in the base. When aligning, the sheets were fed onto pins within the heating block (Figure 4.3). The base was made 5 mm thick; the 5 mm base functioned as a heat sink preventing heat shock to the PMMA channel structures, during base heating in the bonding stage. The channel segments (Parts 2,4,6) were made from 0.2 mm PMMA, this is the thinnest available Clarex-001, its use maximised channel AR. The channel connecting segments (Parts 3,5) were made from 1 mm PMMA, the connecting segments are responsible for connecting the aqueous flow to the central channel by a right-angle bend. Channel connecting segments ensure the aqueous flow enters at 90° , perpendicular to the central channel flow direction. A 1 mm PMMA depth was chosen to distance the right-angle bend from the central channel, increasing time before the fluid met the central channel, resulting in stable flow focusing. The top segment (Part 1) was made of 1 mm PMMA, the depth was required for the alignment process when microfluidic adapters were adhered, this process is described in Chapter 2.2.6.

Channels were designed to have a 10 mm width and with the 0.2 mm PMMA depth, an aspect ratio ($AR = \text{width} / \text{depth}$) of 50 resulted (Figure 4.2B). The exit channel was made 25 mm in length, this ensured the ethanol stream had a 1.5 - 10 s residence time before exiting the device, within the experimental fluid velocity range ($v = 2.50 - 16.7 \times 10^{-3} \text{ m s}^{-1}$). Within the experimentally applied TFR, the Reynold's number was between 0.980 - 6.54, this implies fully laminar flow ($Re < 10$). Inlet segments were designed to have a path length greater than 15 mm before the cross-junction. Inlet path length was chosen to aid the transition from the 1.6 mm diameter round inlets to the 10 mm wide rectangular channels. Connection of the aqueous inlets (Parts 2,6) was achieved using a 1×10 mm cross-section rectangular channel (Parts 3,5), which meant the aqueous cross section was increased upon entry into the cross junction (Part 4). Rectangular cut channels (Parts 2,6) were required to be above the 0.2 mm minimum resolution of the laser cutter, this avoided edge defects during the cutting process. For each PMMA piece, a minimum margin of 4 mm was used between cut structures and the PMMA edge, this surface area facilitated effective fluidic sealing during the bonding process.

Post bonding, models were assessed for warping within the channel structures (Figure 4.2C). The surface areas around the inlet holes were roughened using a file, this was to increase stability of microfluidic adapter adhesion. Microfluidic adapters were made compatible with standard microfluidic fittings by applying a M7 internal thread, adapters enabled attachment of microfluidic tubing to the VFF device. Microfluidic adapters were 3D printed in PLA using high infill for mechanical strength and adhered using quick set epoxy (Figure 4.2D). Inlet holes were aligned to spatially separate microfluidic adapters. Alignment of the adapter's during adhesion was achieved using 1/16" (1.59 mm) diameter PTFE capillaries, insertion of the capillaries into the inlet holes prevented epoxy from entering the channel structures and centred the adapter's, this was confirmed by visual inspection. Once adhered, a 12-hour curing time was applied for the epoxy glue, models were perfused and checked for leaks.

Narrow channel devices (AR 161) were made by laser cutting the channel structures (Parts 2,4,6 in Figure 4.2A) in 3M™ 7953MP Membrane Switch Spacer (38 µm PET film double backed with 12 µm thick acrylic adhesive, total thickness was 62 µm). Layers were carefully placed, adhered and aligned using the heat block whilst minimising air bubbles trapped. Once layered, the chips were put under 0.5 N m⁻¹ torsion per bolt within the heat block and heated at 50°C overnight to laminate.



*Figure 4.2) Depictions of the VFF device at various stages of the manufacturing process, highlighting critical dimensions. **A** Exploded view of the VFF device, PMMA layers were used for the top (Part 1), base (Part 7), channel structures (Parts 2,4,6) and aqueous connection channels (Parts 3,5). The respective PMMA thicknesses were 5 mm (Part 7), 1 mm (Parts 1,3,5) and 0.2 mm (Parts 2,4,6). Alignment holes are highlighted on the base. **B** Dimensions of the central channel structure (top), aqueous inlet channel (middle) and aqueous connection channel (bottom). **C** Top view of VFF model, post bonding (scale bar = 36 mm) **D** View from the base of the VFF model post adapter (blue) adhesion (scale bar = 36 mm).*

4.2.4 Adapting multilayer PMMA bonding

The initial bonding method attempted heating at 110°C, a temperature above the T_g of PMMA, for two hours. An aluminium heating block was used to apply pressure to the PMMA sheets, and the block was heated with the base on a hotplate (Figure 4.3). Each block was comprised of a 90 x 50 mm rectangle of 15 mm depth, alignment pins were 3 mm OD and centred and displaced by 60 mm. The applied pressure within the heat block was optimised to prevent mechanical deformation (0.5 N/m torque per M6 bolt). Despite slow heating (>40 minutes) and cooling steps (>120 minutes) the high temperature warped the 0.2 mm and 1 mm PMMA, this led to visible channel deformation. Devices produced at 100°C were non-perfusible and exhibited reduced optical clarity. To avoid heat application, a solvent-based bonding method was attempted applying acetone between PMMA layers. Acetone application visibly roughened the internal channel structures and resultant bonding was unreliable. The next approach involved using less harsh solvents in conjunction with lower temperature heating.

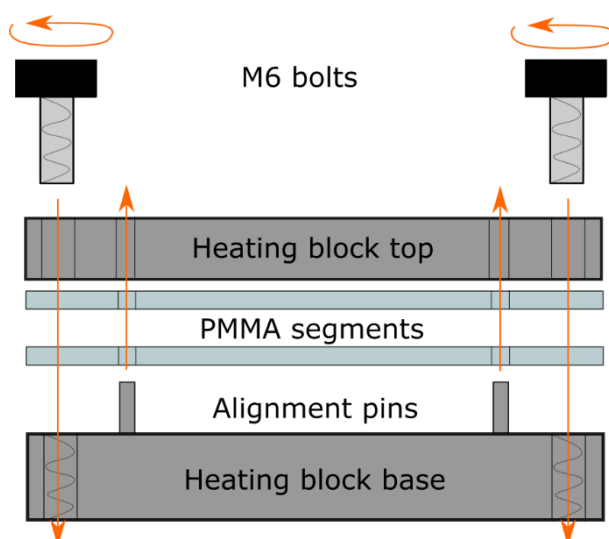


Figure 4.3) Cross sectional view of the aluminium heating block assembly used to bond PMMA sheets. PMMA segments were first placed onto the alignment pins, the top was secured with four M6 bolts. Each M6 bolt was tightened using a torque wrench, applying 0.5 N/m.

The heat bonding method was adapted with an ethanol pre-treatment and lower heating temperature (Figure 4.4). By aerosol application of ethanol to the PMMA substrate prior to bonding, a lower heat could be applied in the bonding stage, this method yielded high clarity fluidically sealed devices with no observable warping (Figure 4.2C). This method is published by Liga *et al*¹⁷⁰ who applied ethanol and a temperature of 70°C for 5-minutes. Ethanol contact reduces the T_g at the surface to 11°C¹⁵⁸, this activation means bonding can occur far below the T_g of the bulk material, minimizing warping of PMMA sheets. Liga *et al*¹⁷⁰ documented less effective bonding above 80°C, this is near the atmospheric boiling temperature of ethanol where evaporation of ethanol prevents bond facilitation.

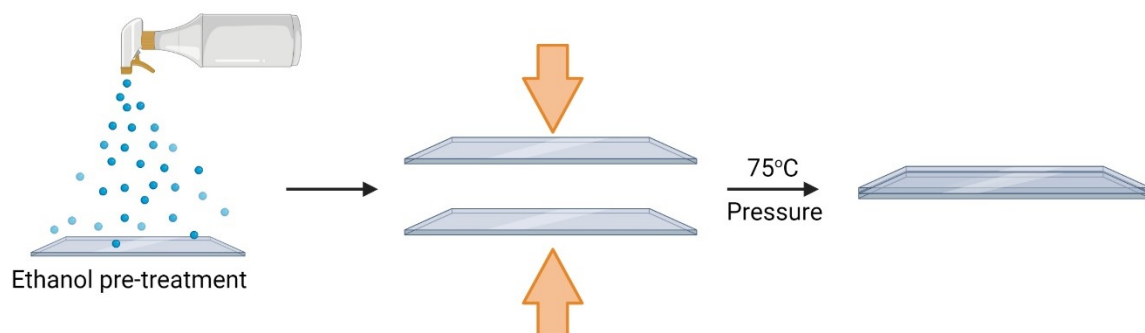


Figure 4.4) Schematic of ethanol pre-treatment, where 70% ethanol is applied to the surface of laser cut PMMA, where bonding is desired. Pieces were aligned and heated within the heating block at 75°C for two hours.

Alternative methods for bonding include the use of plasma, UV, microwaves, and plasticizers. Plasma use by Cheng *et al*¹⁶⁴ provides a low temperature method of bonding. Plasma bonding requires successive treatments for each layer within the device, building of multi-layer structures is more time consuming compared to heat bonding. UV treatment by Tran *et al*¹⁶⁶ necessitates specialist UV treatment equipment, making the process less accessible. Microwave treatment by Toossi *et al*¹⁶⁹ requires application of a thin metallic microwave susceptor layer, an additional stage within the fabrication. Finally, the plasticiser dibutyl phthalate was used by Duan *et al*¹⁷³, dibutyl phthalate is considered a teratogen and requires specialist handling. A combination of speed and accessibility meant ethanol assisted heat bonding was considered the favourable method for our design.

4.2.5 VFF chip microfluidic adapters

Microfluidic adapters were designed on Autodesk fusion 360 and printed in PLA by an Ultimaker 2, 3D Prints were prepared using CURA slicer with raft adhesion and 50% infill using a gyroid pattern (Figure 4.5). Adhesion was achieved using quick set epoxy (RS components) and PTFE (1/8") capillaries (Darwin) to guide alignment and prevent glue entering the VFF device.

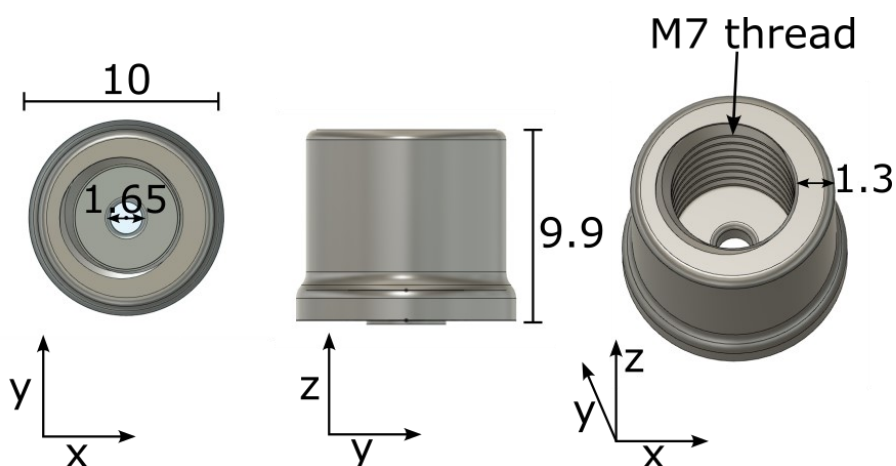


Figure 4.5) Design of 3D printed microfluidic adapters used to connect M7 microfluidic fittings into the PMMA chip (dimensions in mm).

4.3 VFF initial results

4.3.1 Preliminary data acquisition

The large dimensions within the VFF device meant an equilibration time for the flow regime to become steady was expected. To explore this, a central ethanol phase with 0.1 mg mL^{-1} DOPC was co-flowed with PBS buffer at FRR 5 and TFR = 0.6 mL min^{-1} . Samples were collected into Eppendorf tubes at one-minute intervals and the resultant liposome populations were characterised by DLS (Figure 4.6). Four independently produced lipid in ethanol samples ($n = 4$) were used to explore the reproducibility, the sample variation is representation by the ± 1 standard deviation, plotted within the error bars.

Initially ($< 1 \text{ min}$), liposomes produced were centred around 183 nm whilst, later aliquots remained within error. The values are close to the MHF value of 124 nm , determined from our device at FRR 5, using DOPC. With the molecular diffusion driven mixing regime within VFF, larger resultant liposomes were expected when compared with the MHF device. However, the higher TFR applied within VFF may result in a reduced mean diameter. Later aliquots exhibited variable FWHM and by relation PDI values although remained within experimental error. Overall, samples exhibited lower PDI than the equivalent FRR 5 MHF value of 0.211 , from the OTS modified MHF device.

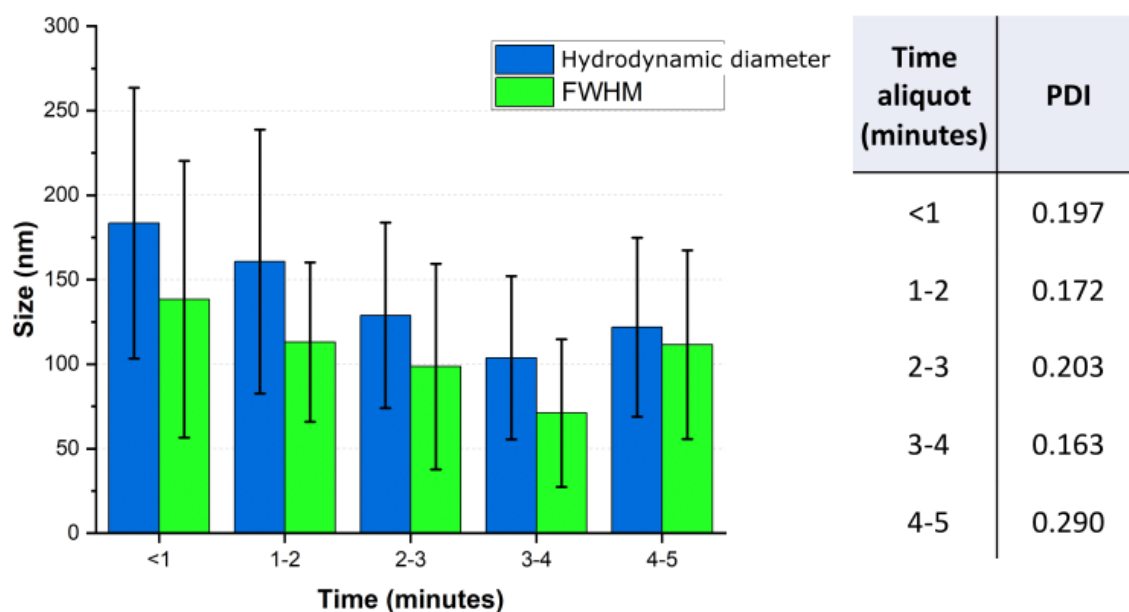


Figure 4.6) Size distributions resulting from time aliquot samples produced from 0.1 mg mL^{-1} DOPC in ethanol co-flowed with PBS at FRR = 5, TFR = 0.6 mL min^{-1} using $n = 4$ samples. Shown to the right are the calculated PDI values for each time point.

4.3.2 Case study of millifluidic device orientation

After initial PDI values were found to be close to MHF, a study was undertaken to improve the efficacy of the device. Giorello *et al*¹⁷⁴ explored the impact of VFF orientation relative to gravity and were able to model observed results. As a result of the scale and different fluid densities within the co-flow, Giorello *et al*¹⁷⁴ demonstrate that with ethanol and buffer co-flow, the VFF exit channel flow direction should be orientated in the opposing direction to gravity. At room temperature, absolute ethanol is approximately 20% less dense than water, it will naturally rise within an ethanol water mixture.¹⁷⁵ Orientation of VFF flow to oppose gravity will lead to a narrowing of the observed stream, when compared to aligning with gravity. Giorello *et al*¹⁷⁴ demonstrate orientation of flow perpendicular to gravity led to a deviation of the ethanol stream, from the channel center. Our preliminary VFF start-up data used the level orientation, the stream deviation from gravity may have produced the large PDI values observed. As the ethanol stream is deflected from the center the fluid velocity varies and by relation the diffusive mixing rate, which would likely generate larger PDI values.

To explore orientation impact with our device, absolute ethanol containing 0.1 mg mL⁻¹ DOPC was co-flowed with PBS at FRR 5, TFR = 0.6 mL min⁻¹. Using a 3D printed mount to secure vertical orientations of the VFF device, three orientations were explored; level, downward, and upward (Figure 4.7A). The level orientation was perpendicular to gravity, upward and downward orientations were parallel to gravity. The mean liposome diameter and FWHM achieved from DLS sizing and then plotted (Figure 4.7B). The level and downward orientations gave the highest observed mean diameter and FWHM values. In the upward orientation, the mean diameter and FWHM were 108 nm and 73 nm respectively, these were the lowest values within the study. The reduced mean diameter and PDI for the upward orientation within our study supports the observations made by Giorello *et al*¹⁷⁴. The smaller mean diameter, resulting from the upward orientation, implies more effective flow focusing within the channel cross-section.⁶¹ The level and downward orientations should present the most diffuse streams, based on the study by Giorello *et al*¹⁷⁴. Stream deflection appeared to be the greatest determinant for sample quality, since the largest PDI shown in the level orientation. Based on this study, to achieve lower dispersity and smaller liposomes the favoured orientation is upward, this yielded the lowest observed PDI value and mean diameter. In the upward orientation, the resultant distribution met the requirements of < 200 nm diameter and < 0.2 PDI for potential therapeutic application. This set demonstrates the importance of VFF orientation when producing liposomes, future samples were generated in the upward orientation, unless specified otherwise.

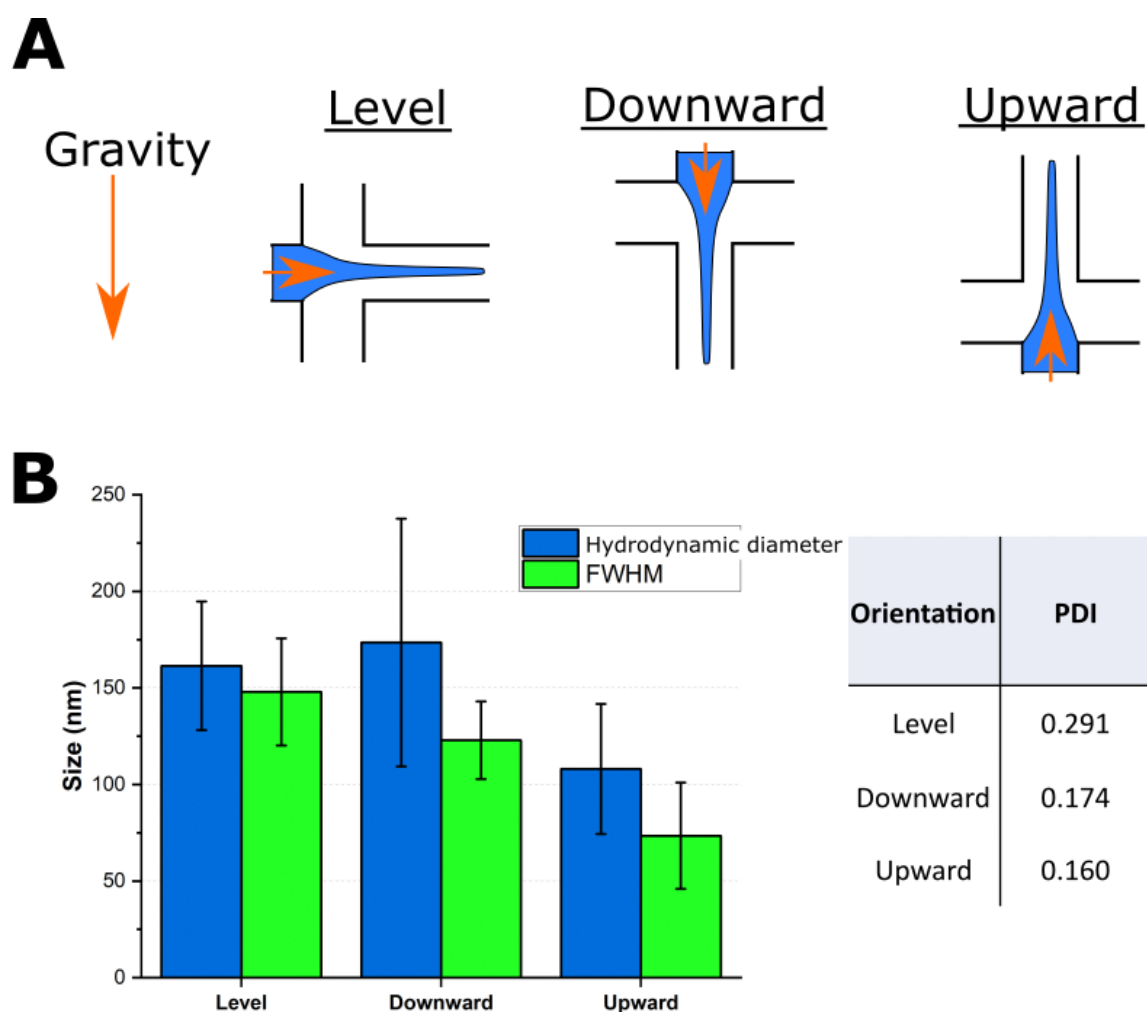


Figure 4.7) Impact of VFF device orientation on the resultant liposome distributions. **A** Depicts the gravity (orange arrow shown left) orientation, relative to the applied flow (orange arrow within blue ethanol phase). **B** Size distributions resulting from the orientations, generated using an ethanol phase containing 0.1 mg mL^{-1} DOPC and co-flow with PBS at $\text{FRR} = 5$, $\text{TFR} = 0.6 \text{ mL min}^{-1}$. Distributions were from $n = 4$ samples, error bars = 1 SD. The table to the right shows the calculated PDI values for each orientation.

4.4 Investigating flow parameters within vertical flow focusing

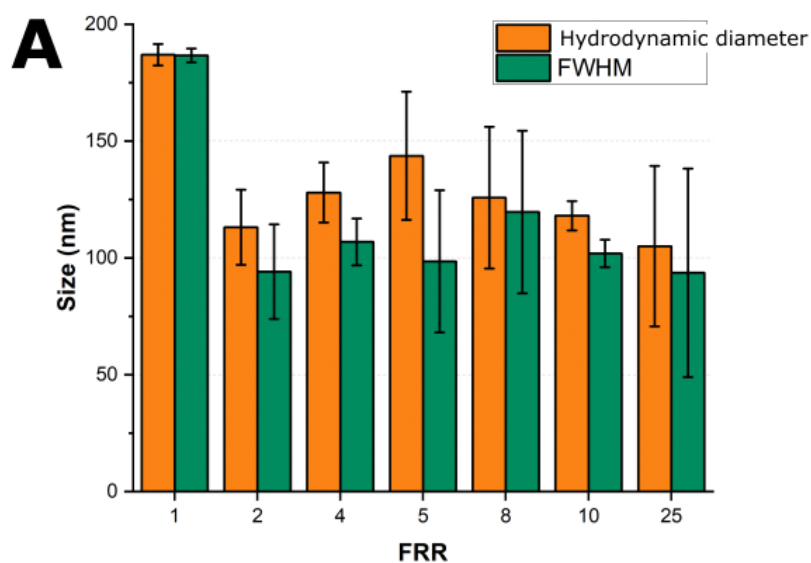
4.4.1 FRR and TFR impact on DOPC

Size control is an important facet of VFF, the size of resultant liposomes can determine both application and efficacy.¹⁷⁶ The ability of VFF to scale resultant liposome size was explored through applying different FRR and TFR within our device. FRR was increased from 1 to 25, at FRR 1 a 187 nm mean diameter and 187 nm FWHM were observed (Figure 4.7A). The FRR 1 sample contained 50 vol% ethanol and 0.05 mg mL^{-1} DOPC, these were the highest values within the study, and it is possible ethanol interaction with the bilayer resulted in larger liposomes. Ethanol impact within VFF is currently

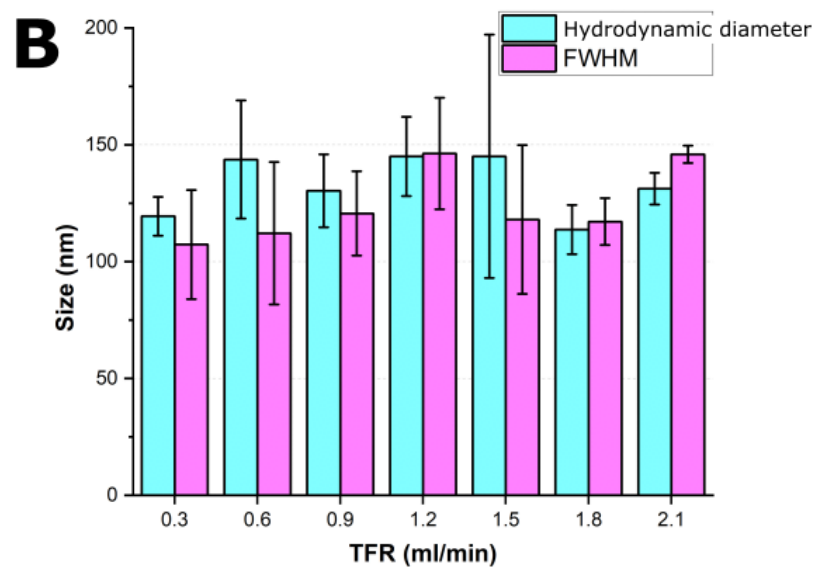
unexplored and marks a limitation of VFF, ethanol impact is explored in Chapter 5.4. At FRR 2, which is 33 vol% ethanol, mean diameter decreased to 113 nm, this was the only size shift observed with FRR increase. Overall, when considering the error within the replicates, FRR 2 – 25 demonstrated undistinguishable impact on mean diameter.

As mean diameter remained within error above FRR > 2, the back pressure was calculated to ensure it was below the syringe pump limit. High back pressure could impede perfusion, this would impact the validity of the data set (Figure 4.7A). The back pressure was calculated from Equation 5b for cylindrical tubing and rectangular channels, respectively. The tubing back pressure was 3.25 mbar and 14.2 mbar at FRR 5 and 25. The back pressure within the device was calculated as 0.304 mbar at FRR 5 and 1.32 mbar at FRR 25, for the central channel. The combined back pressure of the device and tubing is significantly less than the maximum pump operating pressure of 2 bar, therefore it is unlikely the back pressure is impacting the FRR data set. It could possible 125 nm is the minimum diameter producible by the VFF design or an intrinsic limit from using DOPC. This FRR data set demonstrates size control occurs within the device between FRR 1 - 2 resulting in production of 187 - 125 nm diameter liposomes.

Across the TFR sweep, mean diameter values remained within error (Figure 4.7B). In work by Jahn *et al*^{59,61} and hood *et al*¹²³ TFR was shown to have minimal impact on mean diameter at high flow focusing. In this work, no trend was observed with PDI and as TFR was increased PDI remained within error (Figure 4.8). Jahn *et al*⁶¹ reports high TFR can result in a sheaving effect, sheaving is the process by which curvature is induced on a flowing central stream by a sheaving fluid with differing viscosity. When sheaved the surface to volume ratio of the ethanol stream increases, this shifts the mixing process along the channel increasing mixing time and the observed PDI.⁶¹ Within millifluidic devices, Golden *et al*¹³⁴ report the effect of cusping, where at higher Re ($Re > 10$) curvature is induced at the ethanol-buffer interface which could increase the sample PDI at high TFR. The TFR sweep has important connotations for the choice of TFR when producing therapeutic liposomes. When considering therapeutic liposome specifications, FRR 5 and TFR 0.6 mL min^{-1} could be considered the optimal conditions of those explored for DOPC. Having explored the impact of flow parameters FRR and TFR within VFF, the impact of lipid constituents on liposome formation was explored within VFF.



FRR	PDI
1	0.345
2	0.240
4	0.242
5	0.163
8	0.314
10	0.258
25	0.276



TFR (ml/min)	PDI
0.3	0.280
0.6	0.211
0.9	0.297
1.2	0.352
1.5	0.229
1.8	0.368
2.1	0.429

Figure 4.7) Impact of FRR and TFR on the resultant liposome distributions of an ethanol phase containing 0.1 mg mL^{-1} DOPC. **A** Size distribution resulting from FRR in the range 1 - 25, achieved from $n = 3$ samples. Shown to the right are the calculated PDI values for each point. **B** Size distributions resulting from TFR in the range $0.3 - 2.1 \text{ mL min}^{-1}$ and FRR 5, using ethanol co-flow with PBS and achieved from $n = 3$ samples. Shown to the right are the calculated PDI values for each point.

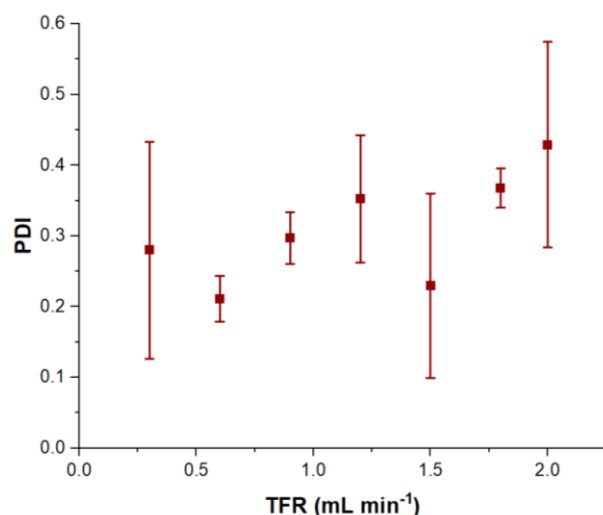


Figure 4.8) TFR against PDI plot for 0.1 mg mL⁻¹ DOPC in ethanol.

4.4.2 FRR and TFR impact on DOPC and CHOL binary mixtures

VFF liposome production was explored with increasing CHOL fraction in a DOPC:CHOL binary mixture, at varied FRR and TFR. CHOL is a rigid sterol that sees significant use in therapeutic liposomes, its use is discussed in Chapter 1.2.2. To a 0.1 mg mL⁻¹ DOPC in absolute ethanol solution, 0.02 mg mL⁻¹ and 0.04 mg mL⁻¹ CHOL were added for the 20 wt% and 40 wt% samples. The equivalent molar fraction of CHOL in each sample was 24 mol% and 58 mol%. The utilised CHOL fractions represent the lower and higher concentrations applied within therapeutic liposome formulations in literature.^{39,177}

At FRR 1, inclusion of 20 wt% CHOL was shown to decrease the approximate observed liposome mean diameter to 139 nm, relative to the value observed in the DOPC data set (187 nm) (Figure 4.9A). For the DOPC sample previously discussed, the large mean diameter at FRR 1 was hypothesized to be a result of the high ethanol content, the impact of ethanol is covered in more detail within Chapter 5.4. For the 20 wt% CHOL sample, it is possible CHOL incorporation within the bilayer reduces the ethanol retention within the bilayer, resulting in smaller liposomes at low FRR. A study on the impact of ethanol within model unsaturated membranes and yeast bio membranes is published by Terama *et al*¹⁷⁸. Terama *et al*¹⁷⁸ Quantified ethanol partitioning into single lipid bilayers and characterized the impact on lateral diffusion of saturated and unsaturated lipid molecules. Work by Terama *et al*¹⁷⁸ indicated bilayers containing unsaturated bilayers are most impacted by ethanol and that sterols at low concentration, can act to 'protect' bilayers from ethanol. When FRR was increased from FRR 1 to 5, mean diameter remained within error for the 20 wt% CHOL sample.

Adding a large fraction of 40 wt% CHOL resulted in liposome mean diameters centred around 150 nm, this was comparable to MHF data suggesting CHOL increased the mean diameter of liposomes (Figure 4.9B). When FRR was increased, minimal impact on mean diameter was observed. It is possible higher CHOL fractions increase the rate of liposome formation within VFF, by facilitating fusion of intermediate lipid fragments and micelles. FWHM was similar between 20 wt% and 40 wt% CHOL although as the mean diameter was larger for 40 wt% CHOL, it follows that PDI was lower from the inverse proportionality. Therapeutic liposomes typically use < 20 wt%^{3,39,40,179} CHOL, when applying these formulations to VFF, higher CHOL fractions can be considered favourable to reduce PDI.

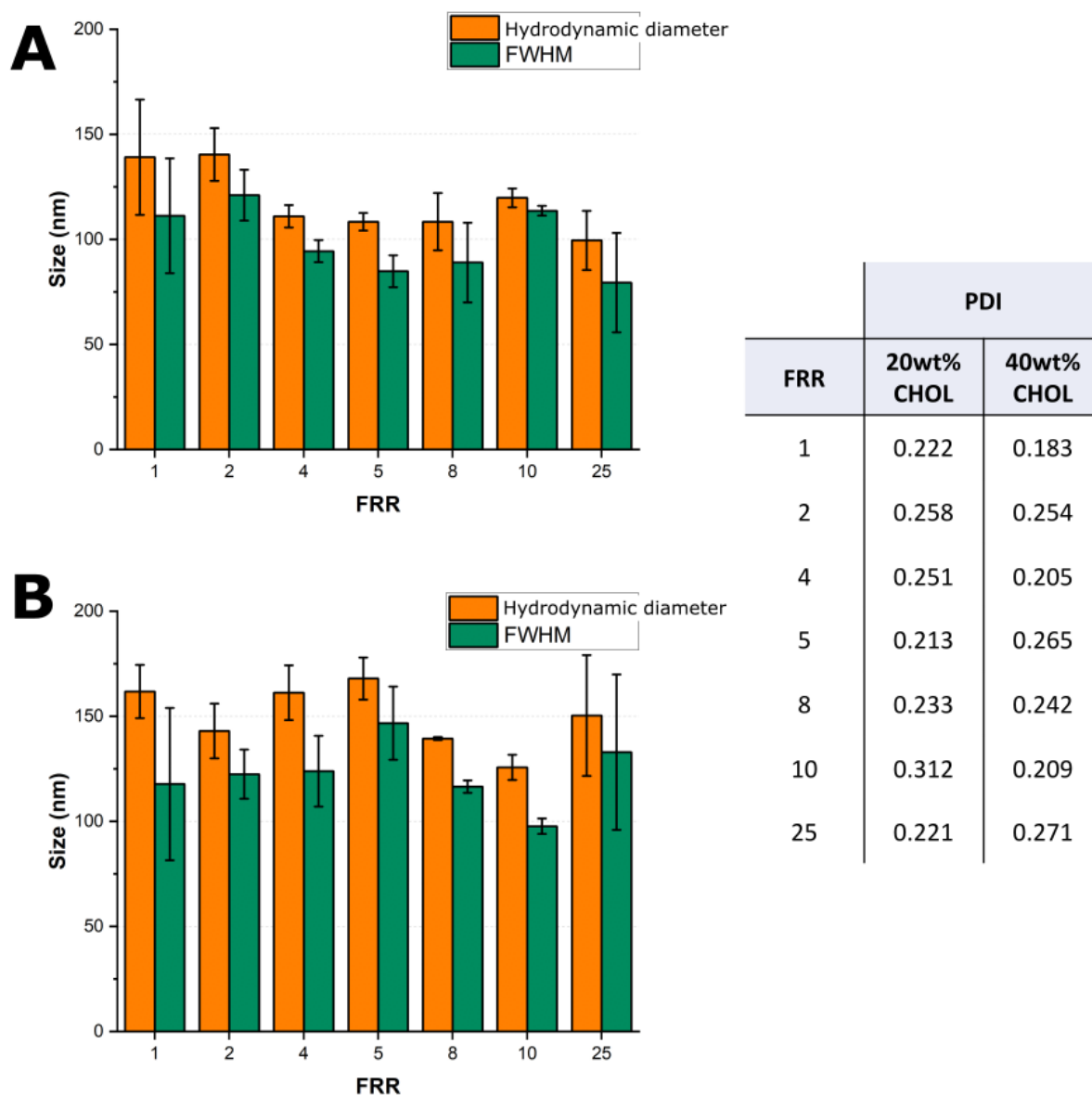
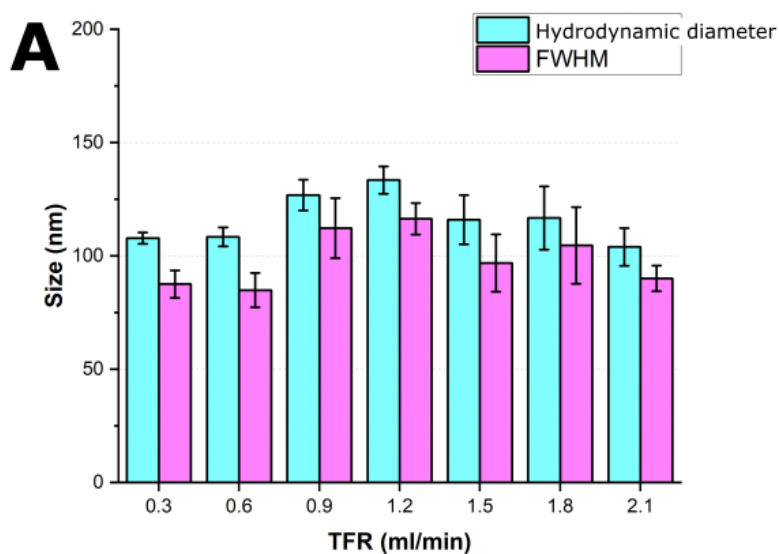


Figure 4.9) Impact of FRR on the resultant liposome distributions of an ethanol phase containing 0.1 mg mL^{-1} DOPC and 20-40 wt% CHOL. **A** Size distribution resulting from 20 wt% CHOL and FRR 1-25 using $n = 3$ samples. Shown to the right are the calculated PDI values for each point. **B** Size distribution resulting from 40 wt% CHOL FRR 1-25 using $n = 3$ samples. Shown to the right are the calculated PDI values for each point.

Within the TFR sweep, 20 wt% CHOL yielded mean diameters around 120 nm and remained within error with increasing TFR (Figure 4.10A). Adding 40 wt% CHOL led to formation of liposomes initially centred around 140 nm, the diameters observed were larger than those produced using 20 wt% CHOL (Figure 4.10B). Increasing TFR with 40wt% CHOL did not affect PDI, similarly to the DOPC TFR sweep (Figure 4.11). The CHOL TFR sweep demonstrates lipid formulation can impact PDI with differing applied TFR.



TFR (ml/min)	PDI	
	20wt% CHOL	40wt% CHOL
0.3	0.228	0.225
0.6	0.213	0.265
0.9	0.272	0.207
1.2	0.264	0.241
1.5	0.242	0.233
1.8	0.279	0.269
2.1	0.260	0.262

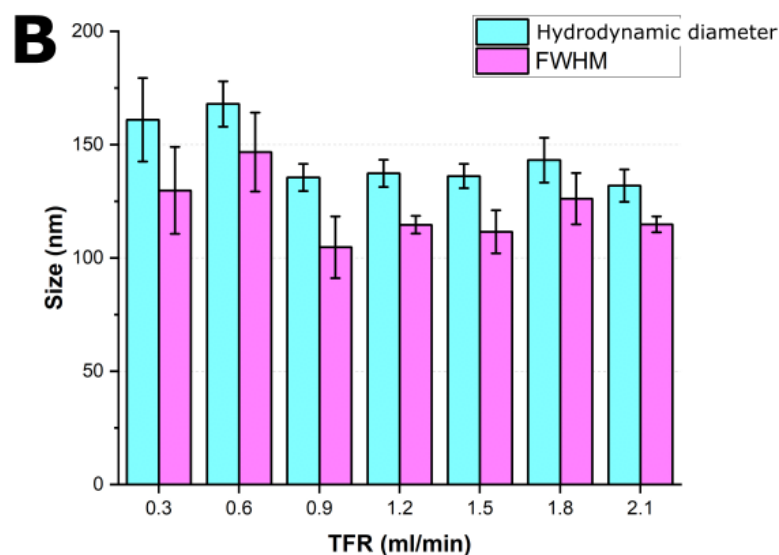


Figure 4.10) Impact of increasing TFR from 0.3 – 2.1 mL min⁻¹ on the resultant liposome distributions of an ethanol phase containing 0.1 mg mL⁻¹ DOPC and 20 - 40 wt% CHOL. **A** Size distribution resulting from 20 wt% CHOL and increasing TFR using $n = 3$ samples. Shown to the right are the calculated PDI values for each point. **B** Size distribution resulting from 40 wt% CHOL and increasing TFR using $n = 3$ samples. Shown to the right are the calculated PDI values for each point.

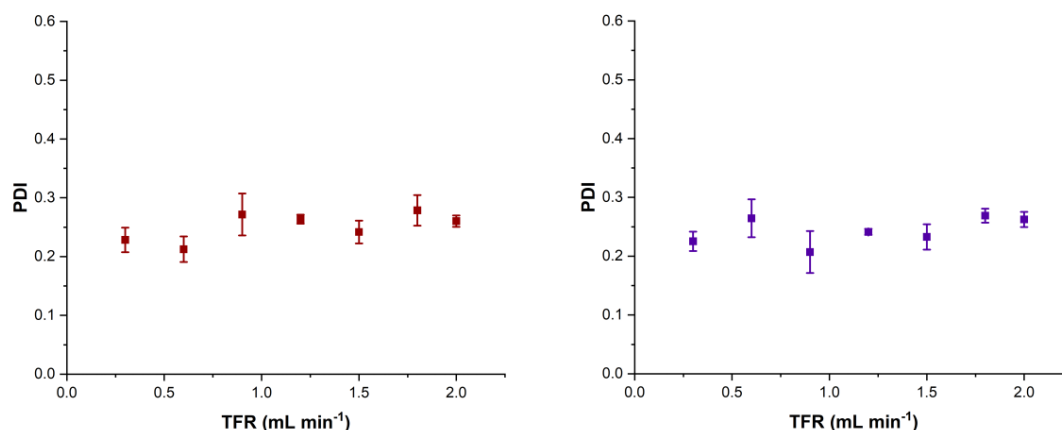


Figure 4.11) TFR against PDI plot for 0.1 mg mL⁻¹ DOPC in ethanol with (left) 20 wt% and (right) 40 wt% CHOL.

4.4.3 FRR and TFR impact on DOPC and DOPG binary mixtures

The potential of VFF to produce liposomes with differing DOPG content was explored with application of different FRR and TFR. DOPG is an anionic charged lipid with a similar MW and identical alkyl tail structure to DOPC, it is discussed in within Chapter 3.3.2. To a 0.1 mg mL⁻¹ DOPC in absolute ethanol solution, 0.02 mg mL⁻¹ and 0.04 mg mL⁻¹ DOPG were added for the 20 wt% and 40 wt% samples. The equivalent molar fraction of DOPG in each sample was 19 mol% and 38 mol%.

Within VFF, DOPG was shown to produce predominantly monodisperse samples at 10wt%, which was like MHF at comparable FRR conditions (Figure 4.12). At FRR 25 a sub population was observed within the samples, at around 37 nm, indicating high FRR is unsuitable for producing therapeutic liposomes. It is possible lipid streaking onto the PDMS channel within MHF led to production of small diameter particles.^{46,180} PMMA offers reduced solvent adsorption compared to PDMS, when combined with the VFF channel size, this may reduce streaking of lipid.¹⁴¹ An alternative explanation for DOPG micelle formation could be that the short MHF mixing times provided were insufficient for DOPG micelle intermediates or lipid fragments to undergo fusion into liposomes during flow focusing. Within VFF, the slower molecular diffusion driven mixing regime and long exit channel may favour DOPG rich micelles fusion, yielding liposomes. DOPG inclusion could reduce fusion rate of micelle intermediates or lipid fragments by electrostatic repulsion, resulting from the anionic headgroup.

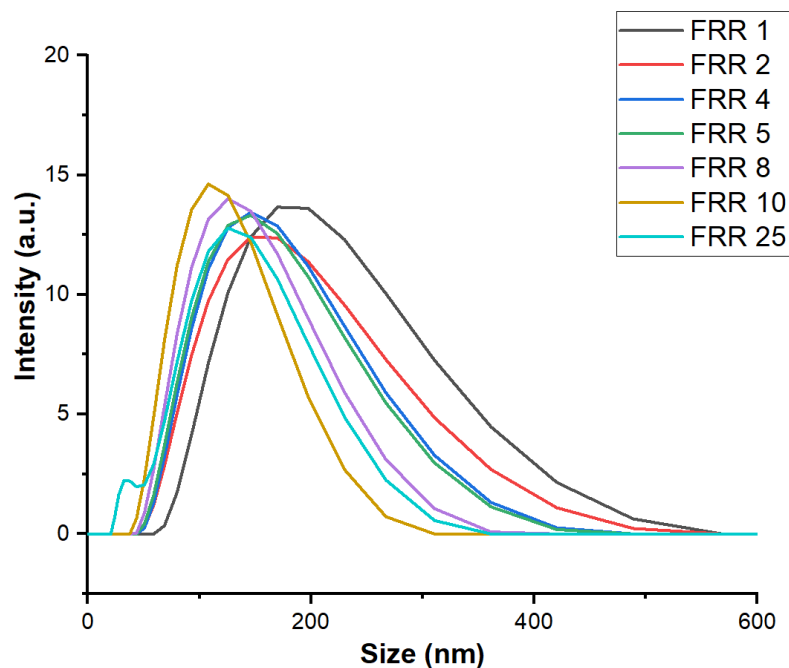


Figure 4.12) Size distributions from FRR 1, 2, 10 taken from one 10wt% DOPG sample.

With 20 wt% DOPG, bimodal distributions frequently presented, to aid comparison the value for the larger peak is reported (Supplementary S12.1). FRR increase 1 – 25 remained similar in mean diameter values (Figure 4.13A). DOPG formulations resulted in larger liposomes when compared to the pure DOPC set, this was the opposite trend observed in MHF and likely a result of the bimodal MHF distributions. With 20 wt% DOPG the mean diameter range of 204-150 nm was comparable to the range of 40 wt% sample (Figure 4.13B). The FRR data sets suggest increasing DOPG content results in better size tunability between FRR 1 and 2. This data set reinforces the idea that both lipid constituents and VFF flow conditions influence liposome size and distribution. For therapeutic formulations higher fractions (>10 wt%) should be avoided as they promote bimodal distributions. PDI values of the DOPG samples were similar to the CHOL samples and remained above the 0.2 threshold for therapeutic application.

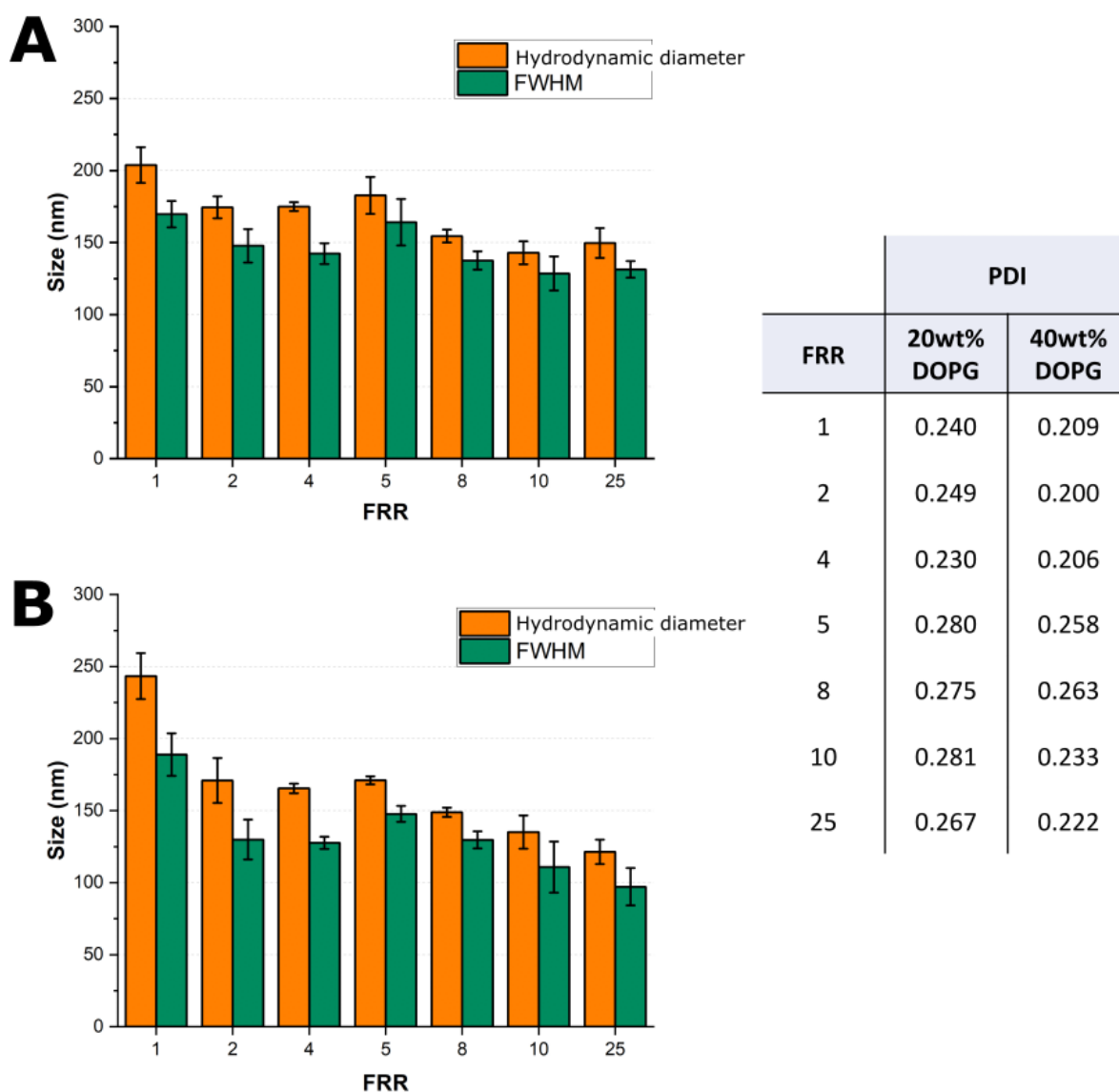


Figure 4.13) Impact of FRR on the resultant liposome distributions of an ethanol phase containing 0.1 mg mL^{-1} DOPC and 20-40 wt% DOPG. **A** Size distribution resulting from 20 wt% DOPG and FRR 1 - 25 using $n = 3$ samples. Shown to the right are the calculated PDI values for each point. **B** Size distribution resulting from 40 wt% DOPG FRR 1 - 25 using $n = 3$ samples. Shown to the right are the calculated PDI values for each point.

Within the 20 wt% DOPG TFR sweep, increasing TFR had no impact on the mean diameter, within experimental error (Figure 4.14A). PDI was shown to increase above TFR 0.6 mL min^{-1} and below 0.6 mL min^{-1} , TFR yielded PDI values closer to 0.2 (Figure 4.15). The 40wt% DOPG samples demonstrated similar TFR trends and PDI was unchanged with TFR (Figure 4.14B).

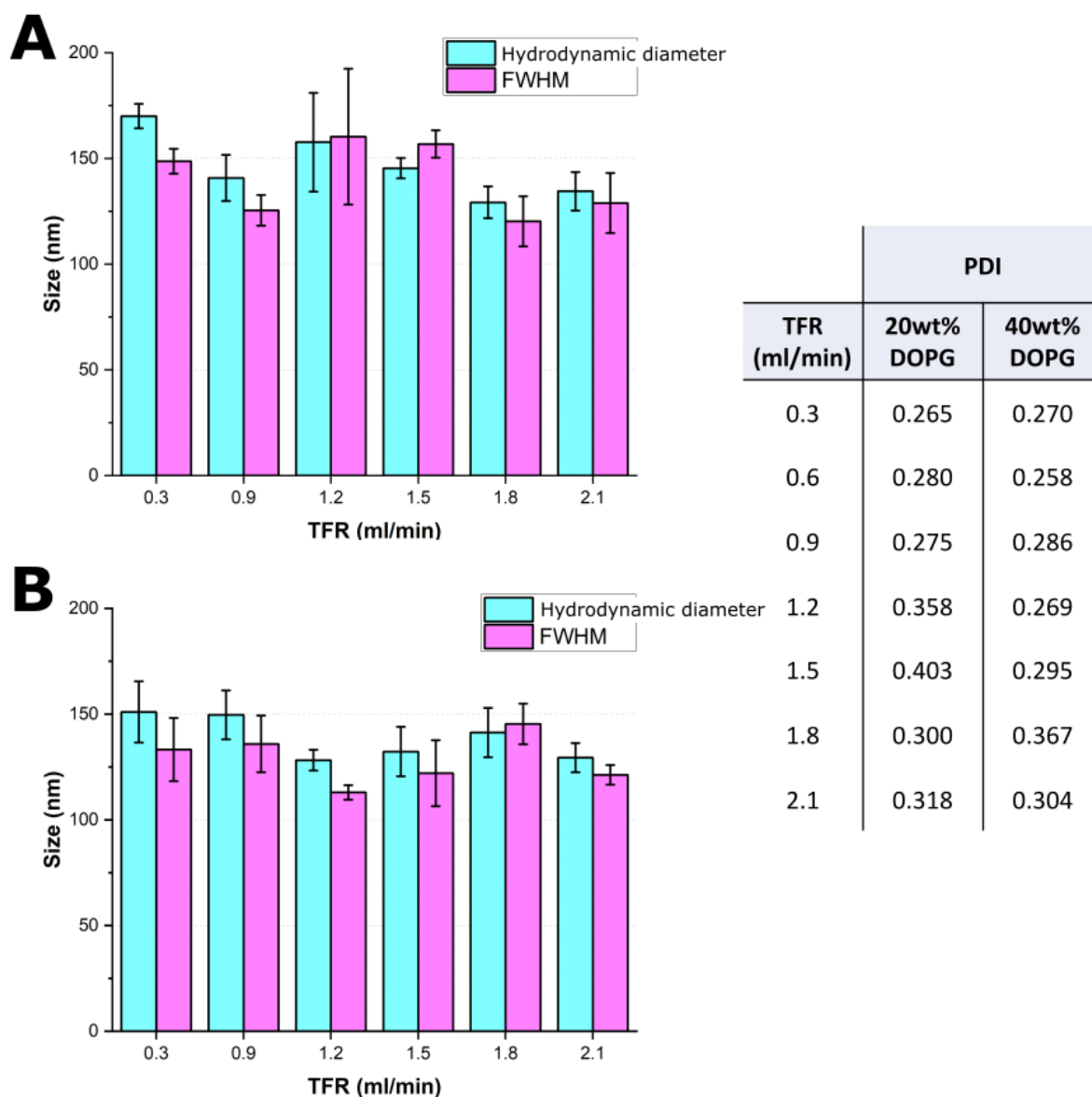


Figure 4.14) Impact of increasing TFR from 0.3 – 2.1 mL min⁻¹ on the resultant liposome distributions of an ethanol phase containing 0.1 mg mL⁻¹ DOPC and 20-40 wt% DOPG. **A** Size distribution resulting from 20 wt% DOPG and increasing TFR using n=3 samples. Shown to the right are the calculated PDI values for each point. **B** Size distribution resulting from 40 wt% DOPG and increasing TFR using n=3 samples. Shown to the right are the calculated PDI values for each point.

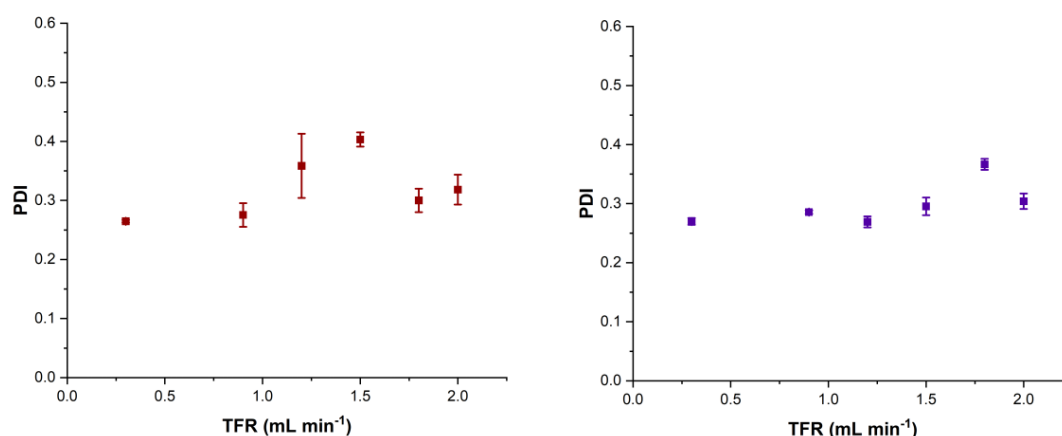


Figure 4.15) TFR against PDI plot for 0.1 mg mL⁻¹ DOPC in ethanol with (left) 20 wt% and (right) 40 wt% DOPG.

4.4.4 FRR and TFR impact on DOPC and DSPE-PEG binary mixtures

The potential of VFF to produce liposomes with differing DSPE-PEG₂₀₀₀ content was explored with application of different FRR and TFR. DSPE-PEG₂₀₀₀ is a sterically bulky, pegylated lipid with a high MW, it is commonly used in therapeutic formulations to increase formulation half-life *in-vivo* as discussed within Chapter 1.2.3 (Figure 4.16).⁶⁸ To a 0.1 mg mL⁻¹ DOPC in absolute ethanol solution, 0.02 mg mL⁻¹ and 0.04 mg mL⁻¹ DSPE-PEG₂₀₀₀ were added, for the 20 wt% and 40 wt% samples. The approximate molar fraction of DSPE-PEG₂₀₀₀ was based on an average MW provided by Avanti, for each sample this was estimated as 5.4 mol% and 11 mol%.

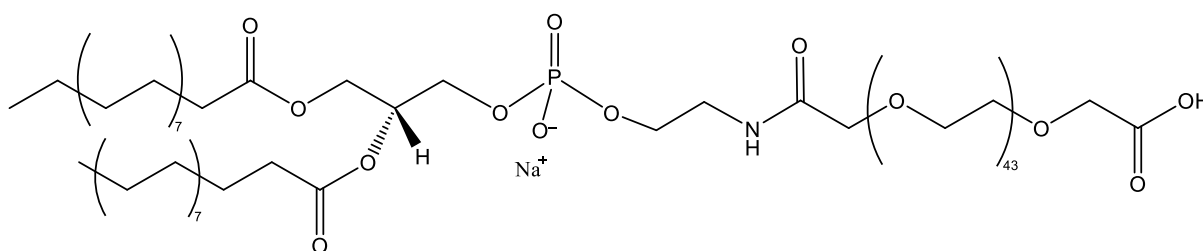


Figure 4.16) DSPE-PEG₂₀₀₀ average chemical structure, MW is approximate due to the polymerization process.

Adding 20 wt% DSPE-PEG₂₀₀₀ resulted in a pronounced decrease in mean diameter with increasing FRR (Figure 4.17A). At FRR 1, a diameter of 113 nm was measured, this decreased to 50.3 nm at FRR 25. This set demonstrated size control over a range of 60 nm, this exceeded the 20 wt% DOPG range of 46 nm. Within a molecular diffusion driven mixing regime, large molecules are expected to exhibit slower assembly of liposome intermediates.⁶¹ Within distinct samples at higher FRR (>8) bimodal distributions were observed, this suggests more rapid mixing can produce larger particles (>200 nm) although this results in unfavourable bimodal distributions. The steric bulk of the PEG group may act to reduce the rate of fusion of micellar and lipid fragment intermediates, based on the hypothesized mechanism by Jahn *et al*⁵⁹⁻⁶¹. If DSPE-PEG₂₀₀₀ acts in this fashion, it could result in typically smaller liposomes. The increased diffusion timescale resulting from PEG steric bulk could be the cause of low

mean diameters observed, especially at high FRR and TFR.^{181,182} It is possible to see from this data set that study of specialised lipids could aid determination of the mechanism of MHF/VFF liposome formation through stabilisation of intermediate structures. Within our data set, a small diameter at high FRR was observed, this may indicate preferential formation of micelles. Increasing FRR reduced PDI for the 20 wt% DSPE-PEG₂₀₀₀ sample, this is the opposite of the behaviour observed in previous lipid formulations. At FRR 25, a PDI of 0.09 was observed for the 50 nm particles although the high FRR was prone to forming bimodal distributions resulting in unfavourable sample quality.

Similar trends were observed for 40 wt% DSPE-PEG₂₀₀₀, addition resulted in smaller liposomes (Figure 4.17B). A decrease in size from 110 - 39.2 nm was observed with increasing FRR from 1 to 25. At FRR 25, 40 wt% DSPE-PEG₂₀₀₀ led to the lowest observed PDI value of 0.06, this far exceeds the 0.2 guideline for application as therapeutic liposomes. DSPE-PEG₂₀₀₀ is a common additive to therapeutic liposomes and its impact within VFF is highly favourable for the formation of monodisperse small liposomes. These results are in-line with a study on thermodynamically stable phase behaviour within binary DPPC: DSPE-PEG₂₀₀₀ mixtures.¹⁸³ Johnsson *et al*¹⁸³ report predominantly micellar distributions above 5 mol% DSPE-PEG₂₀₀₀ and bimodal liposome and micellar distributions at < 5 mol% DSPE-PEG₂₀₀₀. In this work, monomodal distributions were observed by DLS, characterisation using cryo-TEM would indicate the nature of the particles and whether the sample was truly monomodal.

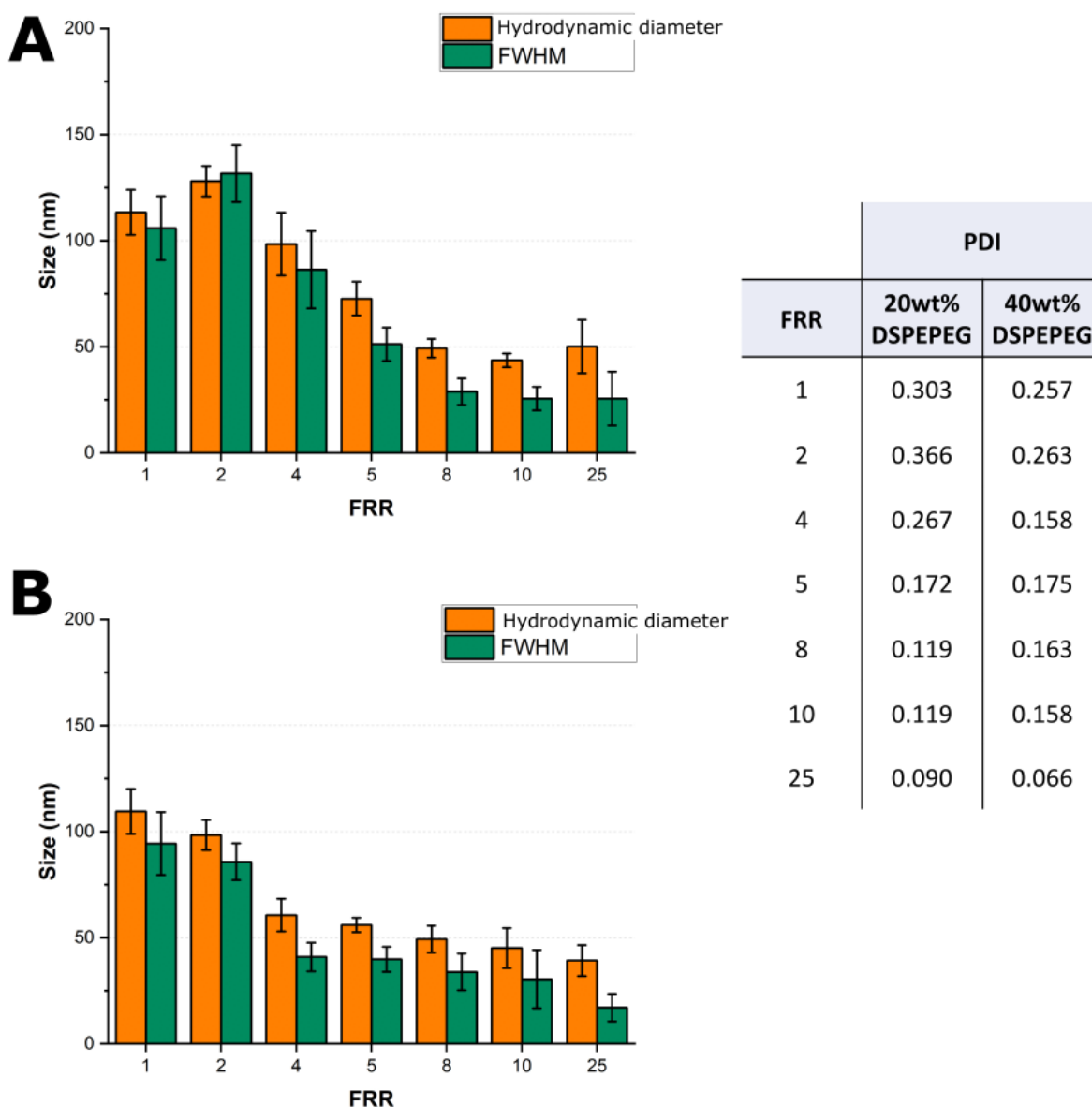


Figure 4.17) Impact of FRR on the resultant liposome distributions of an ethanol phase containing 0.1 mg mL^{-1} DOPC and 20 - 40 wt% DSPE-PEG₂₀₀₀. **A** Size distribution resulting from 20 wt% DSPE-PEG₂₀₀₀ and FRR 1 - 25 using $n = 3$ samples. Shown to the right are the calculated PDI values for each point. **B** Size distribution resulting from 40 wt% DSPE-PEG₂₀₀₀ FRR 1 - 25 using $n = 3$ samples. Shown to the right are the calculated PDI values for each point.

Within the 20 wt% DSPE-PEG₂₀₀₀ TFR sweep, mean diameter was initially 81.9 nm (Figure 4.18A). Above TFR 0.9 mL min^{-1} , a decrease in mean diameter was observed reaching a value of 60.2 nm at TFR 1.2 mL min^{-1} ; at TFR 2.1 mL min^{-1} mean diameter decreased further to 49.9 nm. DSPE-PEG₂₀₀₀ was the only moiety which demonstrated a trend between mean diameter and TFR, this may be a result of the steric bulk and slow molecular diffusion. Increasing TFR can impact mixing characteristics such as sheathing⁶¹ and cusping¹³⁴ whilst also impacting the residence time of the stream after flow focusing within the device. At TFR 2.1 mL min^{-1} , the residence time was approximately 1.5 s along the exit channel, this time may be close to the formation time of pegylated liposomes. With 40 wt% DSPE-PEG₂₀₀₀ a similar trend was observed, at TFR 0.3 mL min^{-1} mean diameter was 80.9 nm and at TFR 2.1 mL min^{-1} mean diameter decreased to 35.9 nm (Figure 4.18B). Addition of the higher fraction of DSPE-PEG₂₀₀₀ resulted in a less steep gradient in the TFR against PDI plot, suggesting the impact of DSPE-

PEG₂₀₀₀ on TFR is greater at higher molar fractions (Figure 4.19). Within the study, 40 wt% DSPE-PEG₂₀₀₀ exhibited fewer bimodal distributions indicating greater concentrations of PEGylated lipids can favour production of smaller (< 100nm) particles.

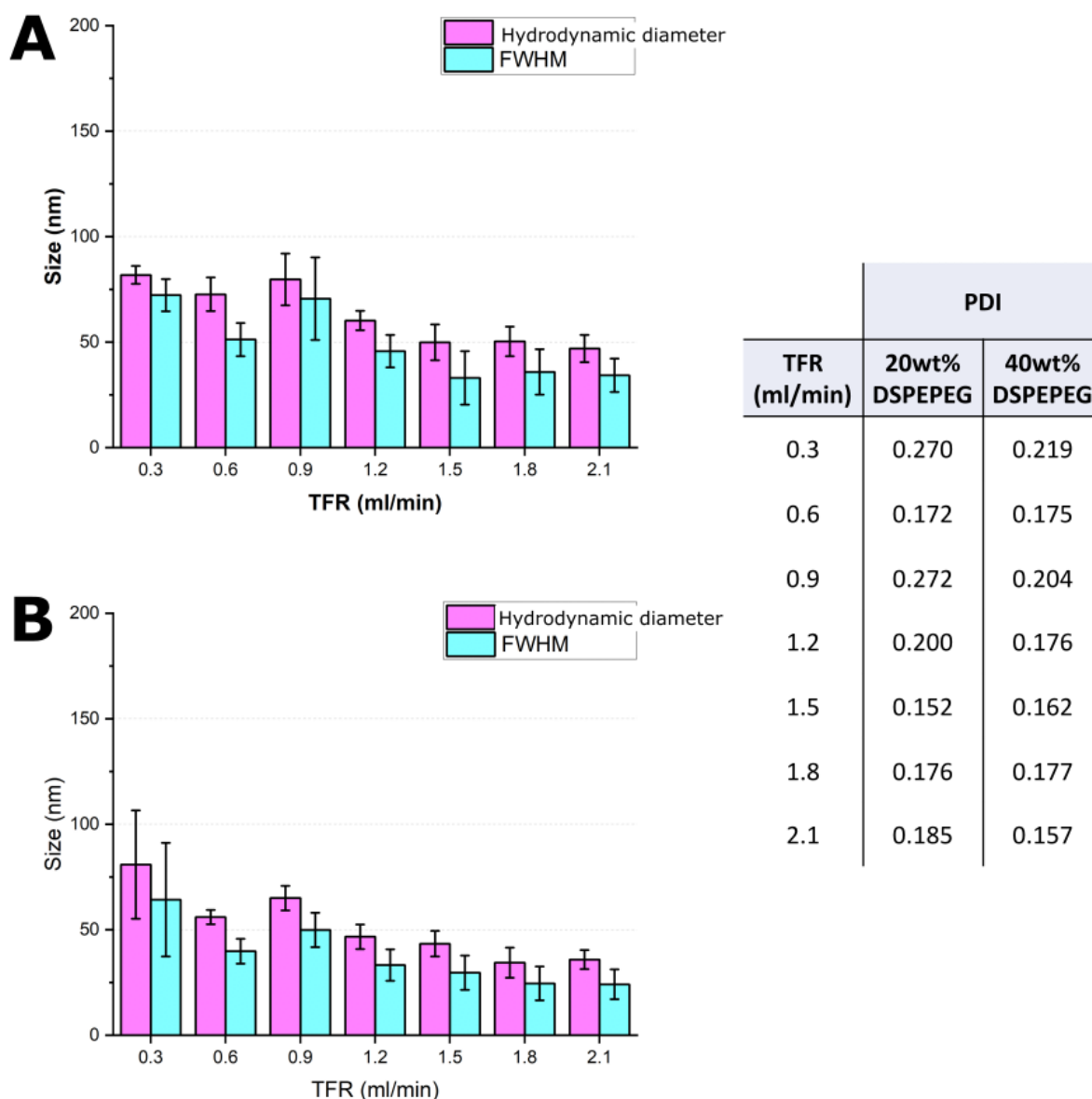


Figure 4.18) Impact of increasing TFR from 0.3 – 2.1 mL min⁻¹ on the resultant liposome distributions of an ethanol phase containing 0.1 mg mL⁻¹ DOPC and 20 - 40 wt% DSPE-PEG₂₀₀₀. **A** Size distribution resulting from 20 wt% DSPE-PEG₂₀₀₀ and increasing TFR using n=3 samples. Shown to the right are the calculated PDI values for each point. **B** Size distribution resulting from 40 wt% DSPE-PEG₂₀₀₀ and increasing TFR using n = 3 samples. Shown to the right are the calculated PDI values for each point.

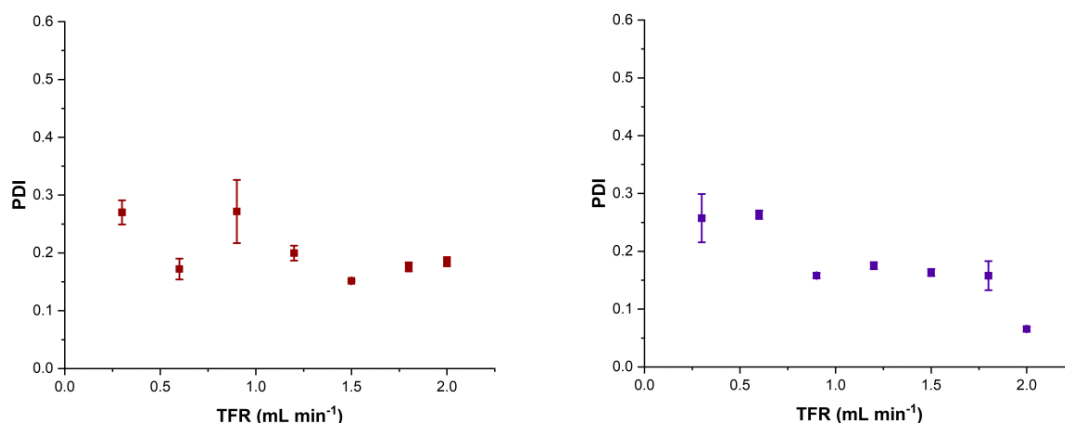


Figure 4.19) TFR against PDI plot for 0.1 mg mL⁻¹ DOPC in ethanol with (left) 20 wt% and (right) 40 wt% DSPE-PEG₂₀₀₀.

4.5 Conclusion and future work

4.5.1 Conclusion

In this chapter a cleanroom-free, PMMA based fabrication method was optimised to produce a VFF device. The device employed a low material cost and non-specialist fabrication method which demonstrates an accessible means to study liposomes within academia. Additionally, the multilayer PMMA assembly is highly adaptable with the potential to add further functionality such as fluid mixing and dialysis. The device was used to produce liposomes that were < 500 nm in size. Start-up and orientation data sets were produced that determined the effective sampling range of > 3 minutes and favourable vertical device orientation. FRR and TFR data was used to explore the impact of flow conditions on resultant DOPC liposomes, produced by our device (Figure 4.19). Size and distribution control is an important facet of VFF as for therapeutic delivery liposomes of < 250 nm diameter and low PDI are favoured. For DOPC, size control was found to occur between FRR 1 and FRR 2 whilst PDI was found to increase with increasing TFR.

Addition of CHOL, DOPG and DSPE-PEG₂₀₀₀ to a DOPC solution were explored using FRR and TFR sweeps (Figure 4.19). CHOL addition was shown to have minimal impact on mean diameter and reduced size control within the applied FRR range. Addition of DOPG led to larger mean diameter liposomes whilst increasing FRR gave a gradual decrease in size. The VFF DOPG data sets were monomodal for 10 wt%, and at 20 wt% yielded bimodal distributions, similarly to MHF. Therefore, for DOPG formulations, behave comparably in MHF and VFF a consideration for producing therapeutic liposomes. Adding DSPE-PEG₂₀₀₀ led to smaller mean diameter and a large degree of size control was shown within the FRR data set.

Increasing TFR with a fixed FRR was also explored across all lipid constituents to establish present trends (Figure 4.19). Increasing TFR was found to have minimal impact on mean diameters, except for formulations containing DSPE-PEG₂₀₀₀ (Figure 4.19). Addition of DSPE-PEG₂₀₀₀ led to a decrease of mean diameter with increasing TFR. This was unexpected, and it highlights a dependence of lipid structure on liposome formation within VFF. As a commonly used lipid in therapeutic formulations,

the impact of DSPE-PEG₂₀₀₀ is especially critical for high throughput liposome production. DSPE-PEG₂₀₀₀ demonstrates the potential of specialised lipids to entrap VFF intermediates and elucidate the mechanism of liposome formation. Overall, addition of DOPG and CHOL both reduced the rate of PDI increase with incremented TFR. CHOL and DOPG addition could be considered advantageous as at higher TFR a greater rate of vesicle production is achieved.

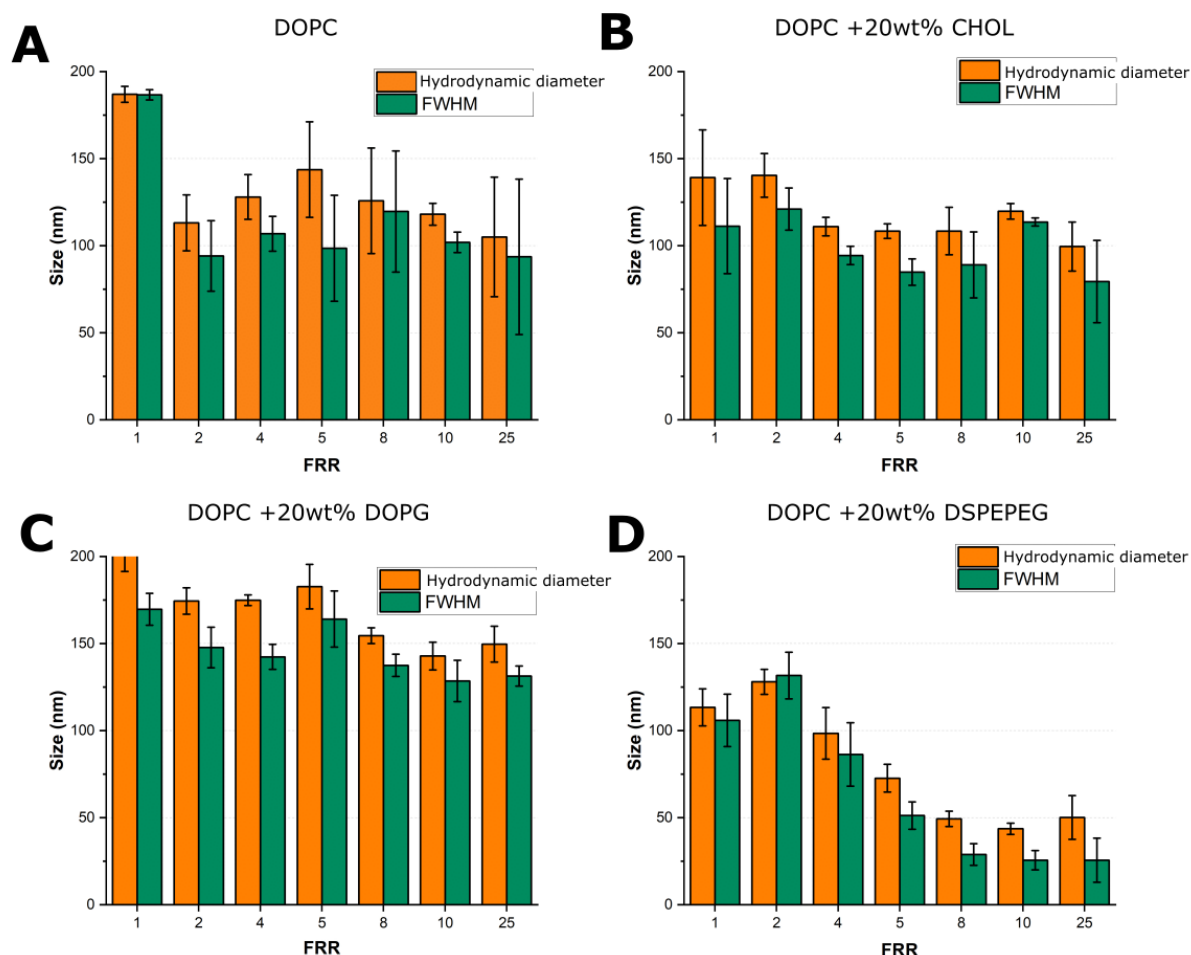


Figure 4.19) Impact of FRR on mean diameter and FWHM of liposomal distribution for **A** DOPC **B** DOPC + 20 wt% CHOL **C** DOPC + 20 wt% DOPG **D** DOPC + 20 wt% DSPE-PEG

4.5.2 Future work

Future work could aim to characterize DSPE-PEG₂₀₀₀ particles using cryo-TEM, to ensure they are liposomal and not micellar. Additionally, the impact of differing channel lengths within VFF could be explored to see if larger liposomes can be formed with DSPE-PEG₂₀₀₀ formulations. Finally, exploration into the 'fusogenic' lipid DOPE could potentially aid studying the underpinning mechanism of MHF/VFF through its relation to intermediate fusogenicity.

5 Exploration of VFF limitations and adaptations

5.1 Overview

This chapter covers the potential limitations of applying VFF for the production and study of therapeutic liposomes. The limitations covered include device geometry, resultant liposome concentration and residual ethanol content. The chapter begins with a method for increasing the aspect ratio of the VFF device applied in Chapter 4, the increased AR device was explored to establish if the size decrease impacted the mixing-regime and resultant liposome distribution. The higher AR device was found to produce bimodal particle distributions and exhibited a TFR size dependence, an unusual characteristic. Liposome concentration is a known limitation for VFF, the co-flow of miscible phases results in a dilution of the central lipid containing phase, this results in a flow regime concentration dependence for the resultant liposomes. Here the method of MADLS is applied to quantify liposome concentration this gave values around $3.121 \times 10^{11} \pm 1.841 \times 10^{11}$ particles mL^{-1} (FRR = 5, TFR = 0.6 mL min^{-1}). The MADLS value was compared to a calculated yield ($1.27 \times 10^{11} \pm 0.26 \times 10^{11}$ particles mL^{-1}) based on lipid content, and minimal lipid loss was thought to occur within the device. A further limitation of VFF is the ethanol content within samples as ethanol could result in complications within cell-based studies from the potential cytotoxicity and influence on liposomal drug delivery. The ethanol impact within samples was evaluated by DLS, TEM and time resolved fluorescence spectroscopy. DLS serial dilution data indicated liposomes were stable which was inferred from dilution at the 5-20 times, this range had minimal impact on mean liposome diameter. TEM indicated multiple particle populations within samples, although primarily SUV ($< 100 \text{ nm}$) were observed. At low FRR, TEM showed presence of large potentially non-liposomal structures suggesting low FRR is unsuitable for therapeutic application. Finally, time resolved fluorescent BODIPY molecular rotor spectroscopy was employed and gave novel insight into the ethanol impact within the lipid bilayer for VFF samples. Based on the molecular rotor measurements, a combination of high FRR (FRR > 10) and sample dialysis was required to achieve sample micro-viscosity comparable to extruded samples.

5.2 Aspect ratio impact within VFF

5.2.1 Higher aspect ratio VFF design

To explore the impact of increased AR on liposome distribution the VFF device was re-designed with a higher AR of 161 (Figure 5.1A). By increasing AR, the device channel cross section is reduced which could result in differing mixing regimes and resultant liposomal distributions (Figure 5.1B). To increase AR, channel segments (2,4,6) were replaced with 3M 7953MP membrane switch spacer (Figure 5.1A). The membrane switch spacer is comprised of a 38 μm PET film double backed with 12 μm thick acrylic adhesive, the total thickness was 62 μm . PET can be manufactured into < 0.2 mm films, this allows further depth reduction from Clarex-001 which is limited to a 0.2 mm minimum thickness. The non-channel segments of the device were comprised of 1 mm PMMA, this imparted structural rigidity to the device. When adhering the channel structures, the film was carefully applied to reduce formation of air bubbles, after adhesion a clouded device resulted (Figure 5.1C). Alignment of the film was undertaken using the alignment pins within the heating block used in Chapter 2.2.4.

Once adhered, the device was heated at 40°C for 12 hours, this ensured fluidic sealing improving adhesion achieved between layers. The improvement in sealing was implied from increased clarity between the PMMA sheets, following the heating stage. The heating temperature was 30°C less than the ethanol bonding applied in Chapter 2.2.4, this meant the 5 mm thick PMMA design feature was not required for action as a heat sink. Microfluidic adapters were prepared and adhered as in Chapter 2.2.4. Following adapter adhesion, the device was flushed with 4 mL 70% ethanol aliquots and one 4 mL DIW to remove uncured adhesive near the channel edge. During application, adhesive at the channel edge may be solvated, this potential impact was reduced by the low surface area of adhesive, long curing times and cleaning post-fabrication. Models produced by this method remained fluidically sealed over the course of several months, indicating minimal erosion of the adhesive.

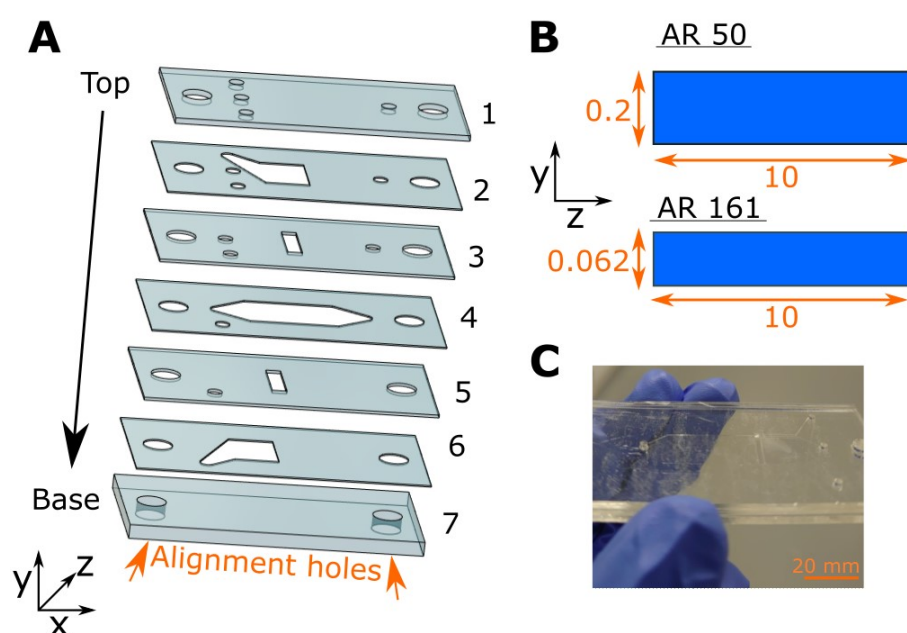


Figure 5.1) Overview of the AR 161 VFF device. **A** Exploded view of the VFF device with the PMMA top (1), channel connectors (3,5) and base (7). Adhesive backed PET spacer was used for the channel structures (2,4,6). Alignment holes are highlighted on the base. **B** Exit channel cross sectional area for AR 50 and AR 161 devices with reference axes. **C** Image of the AR 161 device post-assembly, before the heating step (scale bar = 20 mm).

5.2.2 Limitations of application for the AR 161 device

Within the following chapter, resultant liposome distributions collected within the AR 161 device frequently contained bimodal or asymmetric distributions. This is best highlighted by the DLS distribution data for the FRR and TFR sweeps, produced from one sample (Figure 5.2). The FRR sweep demonstrates the effect of increasing FRR and TFR between FRR 1 - 4 and TFR 0.2 - 0.5 mL min⁻¹ (Figure 5.2A). At FRR 1 the distribution is centred around 200 nm with a shoulder peak at 38 nm. At FRR 4 and TFR 0.5 mL min⁻¹, the 38 nm peak became the dominant distribution, by relative intensity. The TFR sweep explored the impact of increasing TFR from 0.1 - 0.5 mL min⁻¹ with a fixed FRR (Figure 5.2B). At TFR 0.1 mL min⁻¹ a peak centred around 200 nm was observed with a shoulder peak at 51 nm. Increasing TFR to 0.5 mL min⁻¹ resulted in a distribution centred around 51 nm. The discrete populations could suggest a potential shift in the mixing regime occurs at higher TFR, this could be the shift from a diffusion to a convection-diffusion mixing regime.^{61,65,66} Molecular diffusion is reported to result in typically larger liposomes, conversely a convection-diffusion mixing regime would result in smaller liposomes.⁶¹ Alternatively, within the AR 161 device the onset for cusping, reported by Golden *et al*¹³⁴, could onset at lower TFR resulting in the higher sample dispersity for most samples.

The prevalence of bimodal distributions meant the AR 161 device was unfavourable for producing therapeutic liposomes at low FRR and TFR. To aid comparison with AR 50 data, the larger peak was used for the mean diameter and FWHM value for bimodal distributions within this chapter. This meant samples with FRR ≤ 3 and TFR ≤ 0.3 mL min⁻¹ are unrepresentative of the true sample dispersity. With the focus of this thesis on therapeutic application, samples containing > 100 nm liposomes were disfavoured regardless additionally, all samples containing > 100 nm liposomes were bimodal another unfavourable characteristic. Above FRR 3 and TFR 0.3 mL min⁻¹, the peak maxima and FWHM are monomodal and present a better comparison to liposomes produced with the AR 50 device.

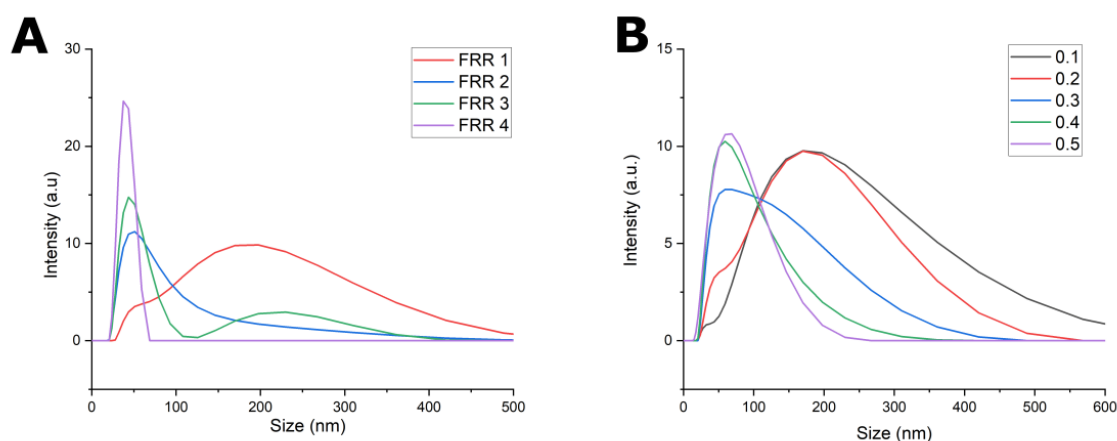


Figure 5.2) DLS distributions produced from one 0.1 mg mL⁻¹ DOPC in ethanol sample applied within the AR 161 device. **A** FRR sweep data between FRR 1 - 4 and TFR 0.2 - 0.5 mL min⁻¹. **B** TFR sweep data between TFR 0.1 - 0.5 mL min⁻¹ at FRR 1.

5.2.3 AR161 preliminary data acquisition

To establish the working principles of the AR 161 VFF device, an investigation was undertaken as to whether an equilibration time for the resultant distributions to reach steady state was present. Within this chapter experimental setup, sampling and DLS procedure was applied from Chapter 2.1.2. For this start-up data set, the device was kept in the vertical orientation as this was deemed the most effective in Chapter 4.3.2. To explore start-up distributions, a central ethanol phase with 0.1 mg mL^{-1} DOPC was co-flowed with PBS buffer at FRR 5 and TFR of 0.6 mL min^{-1} (Figure 5.3A). At the TFR of 0.6 mL min^{-1} , the flow velocity is $16.12 \times 10^{-3} \text{ m s}^{-1}$, a 3-fold increase compared to the value of $5 \times 10^{-3} \text{ m s}^{-1}$ within the AR 50 device, at equivalent TFR. Reynold's number at the TFR of 0.6 mL min^{-1} was 1.99, at this value laminar flow is expected, whilst the back pressure was calculated as 9.94 mBar. Prior to the study, the VFF device was purged with PBS to ensure the breakthrough volume was achieved and trapped air within the device was displaced.

Initially, the mean diameter of the resultant liposomes was 191.7 nm this decreased to a value of 152.9 nm at 2-3 minutes, and to 112.7 nm at 4-5 minutes. Mean diameter appeared to steady above 3 minutes where minimal change was observed between 3-4 and 4-5 minutes. Despite the reduction in channel depth, time was still required for the distributions to steady, future samples applied a three-minute equilibration time before sampling. Equilibration time for the AR 161 device was akin to the AR 50 device, this suggests equilibration time may be independent of device geometry within this range.

Following the equilibration time, the sample PDI was 0.190 – 0.319, these values are too high for the sample to see therapeutic application (Figure 5.3B). Samples produced by the AR 161 device yielded similar PDI (0.190 - 0.319) and mean diameter values as the AR 50 device (0.163 - 0.290) in Chapter 4.3.2. The standard deviation of repeat samples was lower ($\pm 25 \text{ nm}$) when compared to the AR 50 device ($\pm 100 \text{ nm}$), this was attributed to the upward orientation applied in this experiment, a level orientation was used in Chapter 4.3.1. The AR data suggests an eight-fold increase in AR may not be sufficient to impact mean diameter and PDI.

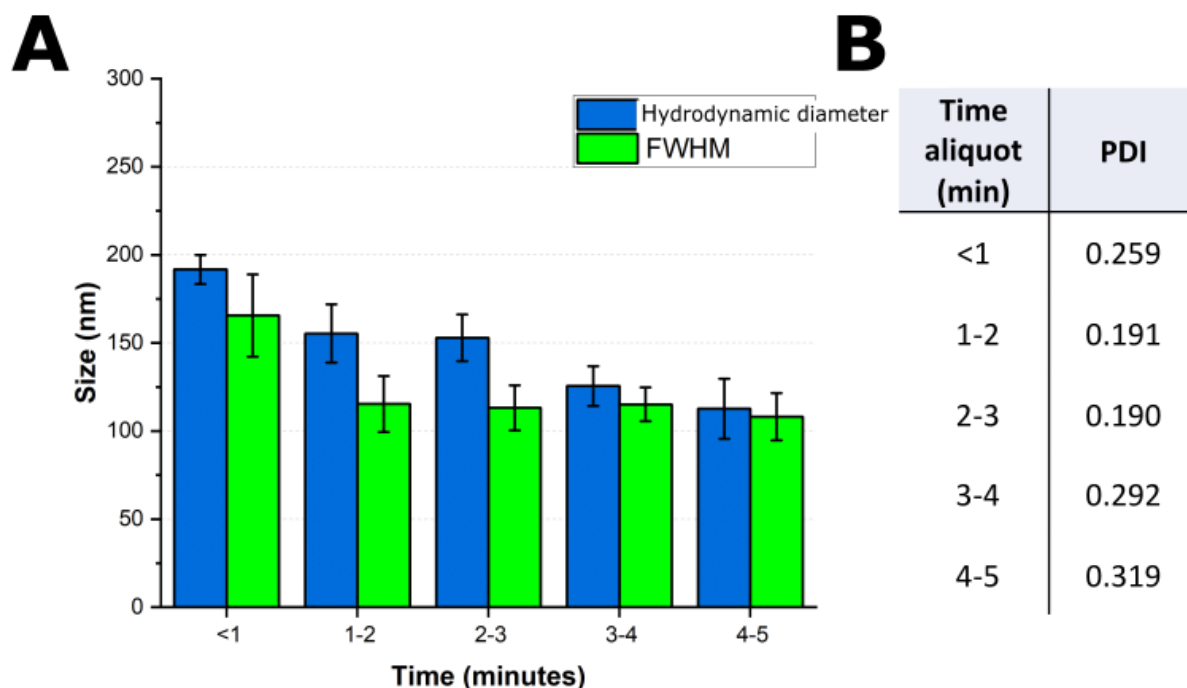


Figure 5.3) The resultant distributions from different sampling periods after flow initiation. **A** Size distribution resulting from time aliquots produced from 0.1 mg mL⁻¹ DOPC in ethanol co-flow with PBS at FRR 5 and TFR of 0.6 mL min⁻¹, achieved from *n* = 3 samples. **B** Tabulated PDI values at each aliquot.

5.2.4 Impact of FRR and TFR within the AR 161 device

Size control is critical for the application of the resultant liposomes as reduction in size is typically favoured to yield < 200 nm vesicles for liposomal drug delivery.^{19,153} In Chapter 4.4.1, size control within the AR 50 device was found to predominantly occur between FRR 1-2, for a 0.1 mg mL⁻¹ DOPC in ethanol central phase. To explore size control within the AR 161 device, an analogous FRR sweep data set was collected (Figure 5.4A). A lower TFR range was explored within the AR 161 device, the flow velocity range applied was 4.03 - 16.1 × 10⁻³ m s⁻¹, to enable comparison with the range of 2.50 - 16.7 × 10⁻³ m s⁻¹ explored within the AR 50 device. Exploring a similar flow velocity range ensures that residence times within the devices were comparable between data sets.

FRR was explored between 0.5 - 5 for the AR 161 device, decreasing from the range explored within the AR 50 device (1-25). At FRR 0.5, large 528 nm particles were formed with lowest PDI within the study, this sample is explored further in Chapter 5.4 (Figure 5.4B). Size control was shown in the decrease in mean liposome diameter of 427 nm from FRR 0.5 to FRR 2. Samples produced at FRR 1 and FRR 2 exhibited high PDI, which exceeded the 0.2 threshold value. Above FRR 2, mean diameter and PDI were largely unchanged within error. The ethanol fraction within the sample is dependent on the FRR, with larger FRR reducing the ethanol content and FRR 1-25 exhibiting 50 – 4 vol% ethanol content. Given the low mean diameter, PDI, residual ethanol content and monomodal distributions, FRR > 5 offers the most suitable distributions for a pure DOPC composition within the device.

TFR was explored between 0.1 - 0.6 mL min⁻¹ at the fixed FRR of 1 within the AR 161 device (Figure 5.5A). A low fixed FRR of 1 was chosen to establish whether TFR could impact the larger 200 nm particles. Between TFR of 0.1 - 0.2 mL min⁻¹, similar mean diameter values were shown, above TFR 0.2

mL min⁻¹ mean diameter decreased. Decreasing mean diameter was unexpected as Jahn *et al*^{59,61} reported minimal size change with TFR. PDI exceeded 0.2 for all TFR values explored, indicating FRR 1 is unsuitable for producing therapeutic liposomes, within the explored range (Figure 5.5B). When TFR was plotted against PDI, the trend was like previous PDI plots (Figure 5.6).

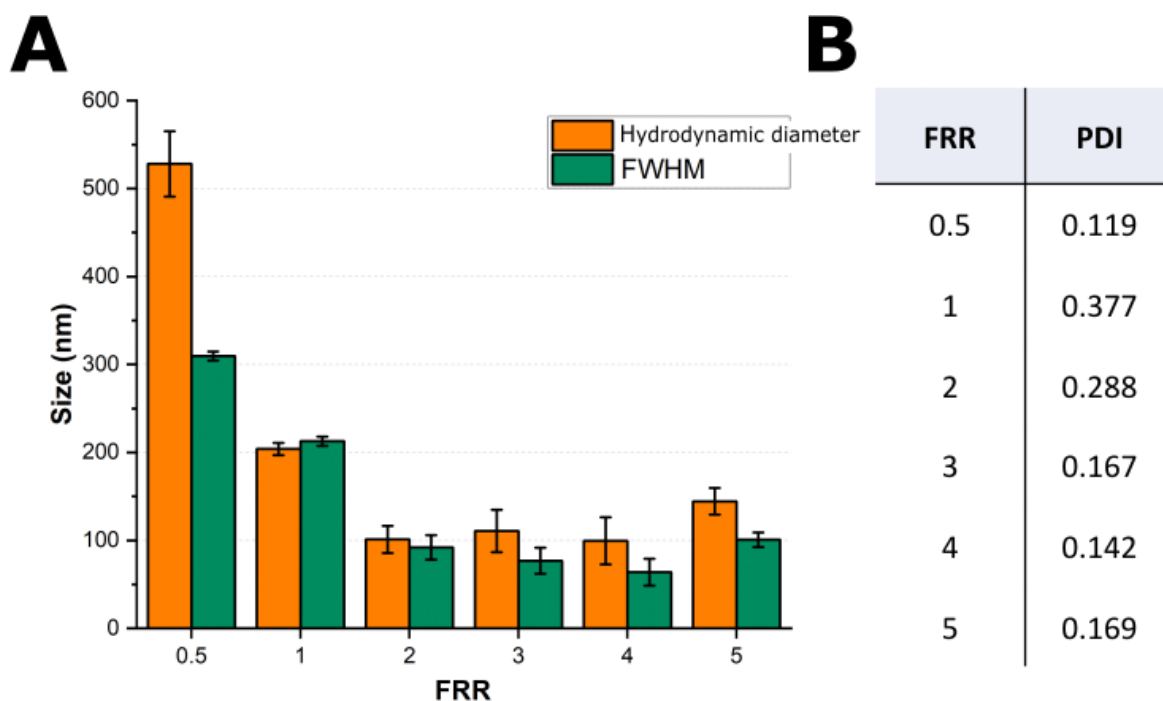


Figure 5.4) Impact of FRR on the resultant liposome distributions produced from an ethanol phase containing 0.1 mg mL⁻¹ DOPC. **A** Mean diameter and FWHM resulting from increasing FRR, distributions were produced from n = 3 samples. **B** Tabulated PDI values for FRR value.

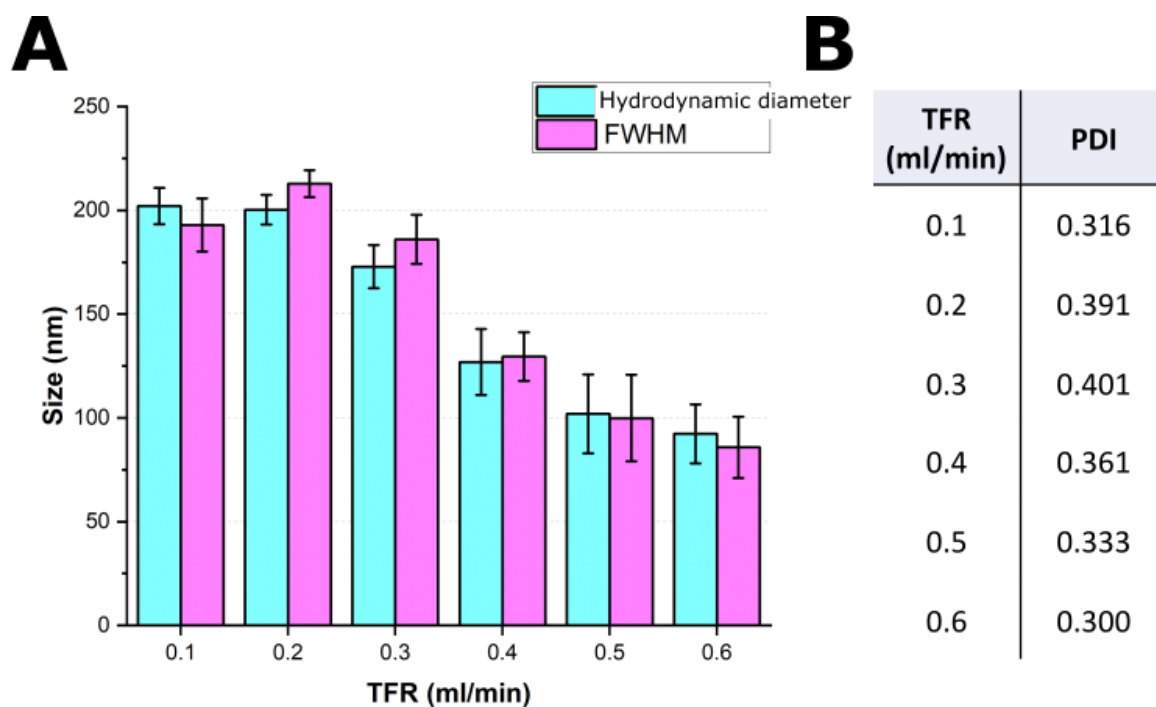


Figure 5.5) Impact of TFR on the resultant liposome distributions produced from an ethanol phase containing 0.1 mg mL^{-1} DOPC. **A** Mean diameter and FWHM resulting from increasing TFR, distributions were produced from $n = 3$ samples. **B** Tabulated PDI values for TFR value.

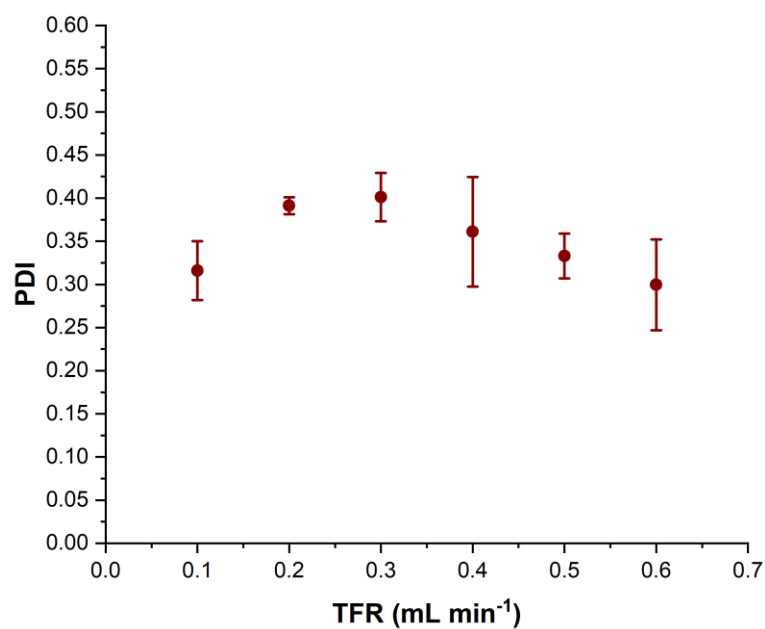


Figure 5.6) TFR against PDI plot for DOPC TFR sweep (error bars = 1 SD).

5.2.5 FRR and TFR comparison across AR

The FRR sweeps between the AR 50 and AR 161 devices gave similar profiles (Figure 5.7). Size control was observed from FRR 1 to FRR 2 with a decrease in mean diameter of 100 nm within each device. Beyond FRR 2, mean diameter remained within error with increasing FRR. TFR has remained a controversial subject within VFF literature, comparison of different AR devices perhaps highlights this, since device designs vary within greatly.^{59,62,64,134,135} The TFR plot for AR 50 demonstrated constant mean diameter with increasing TFR although within the AR 161 device, a decrease in mean diameter was observed. The decrease in size with increasing TFR could occur from a change in mixing-regime resulting in production of smaller resultant liposomes.⁶¹

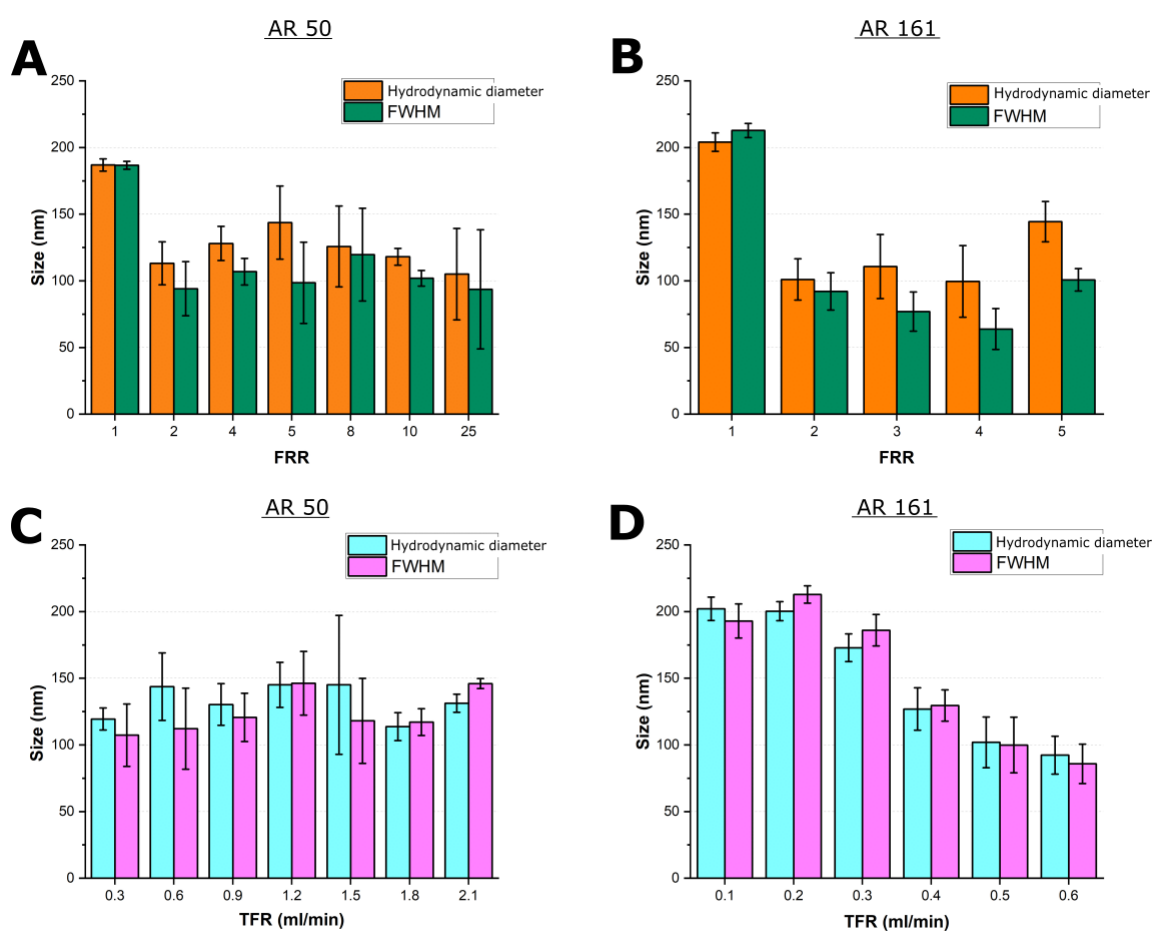


Figure 5.7) Comparison of FRR and TFR sweeps using 0.1 mg mL^{-1} DOPC within the AR 50 and AR 161 devices. **A** FRR sweep distribution data for the AR 50 device. **B** FRR sweep distribution data for the AR 161 device. **C** TFR sweep distribution data for the AR 50 device at FRR 5. **D** TFR sweep distribution data for the AR 161 device at FRR 1.

Through comparison of the TFR against PDI plots, the difference between each AR is best highlighted (Figure 5.8). Within each device, PDI was similar for all explored TFR values.

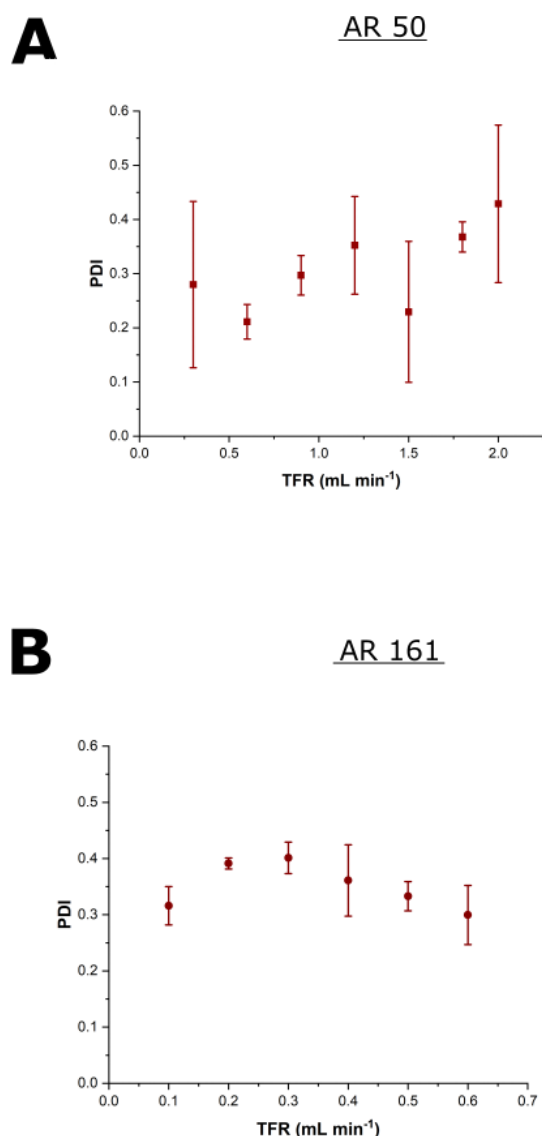


Figure 5.8) TFR against PDI plots for resultant distributions within the **A** AR 50 and **B** AR 161 devices (error bars = ± 1 SD).

5.3.1 Liposome concentration within VFF

Liposome concentration is essential for understanding dosage quantities in studies applying the liposomes. In this study liposomes were produced from three separate films; sizing was achieved by DLS, and concentration used MADLS. MADLS operates by measuring scattered light at multiple angles, analysis of the scattering patterns enables determination of particle concentration within monomodal samples.¹⁸⁵ Liposomes in this study were the control liposome formulation covered in Chapter 8 this is a formulation containing DOPC, CHOL and DSPEPEG₂₀₀₀ at a 47:7:46 molar ratio. Samples were formed at FRR 5 at TFR of 0.6 mL min⁻¹ using the AR 50 device and dialysed against PBS before DLS measurement.

An initial DLS scan was used to establish the average derived count rate (Chapter 2.1.2), plots indicated the sample mean diameters were centred around 50 nm (Figure 5.9A). Samples gave rise to differing FWHM and by relation different PDI values (Figure 5.9B). PDI values were below the < 0.2 range,

sample three exhibited the largest PDI value. Concentration was measured using MADLS and applying the determined count rate from the distribution plots (Figure 5.9C). The MADLS plot indicated all liposomes were below 100 nm in diameter, this agreed with the initial DLS scan. The mean particle concentration was 3.121×10^{11} , the standard deviation between samples was 1.841×10^{11} , this represents a 60% variation in sample concentration. The large uncertainty in the MADLS measurement likely results from the differing distributions between samples. For comparison, particle concentration estimates were performed to evaluate the success of the VFF production.

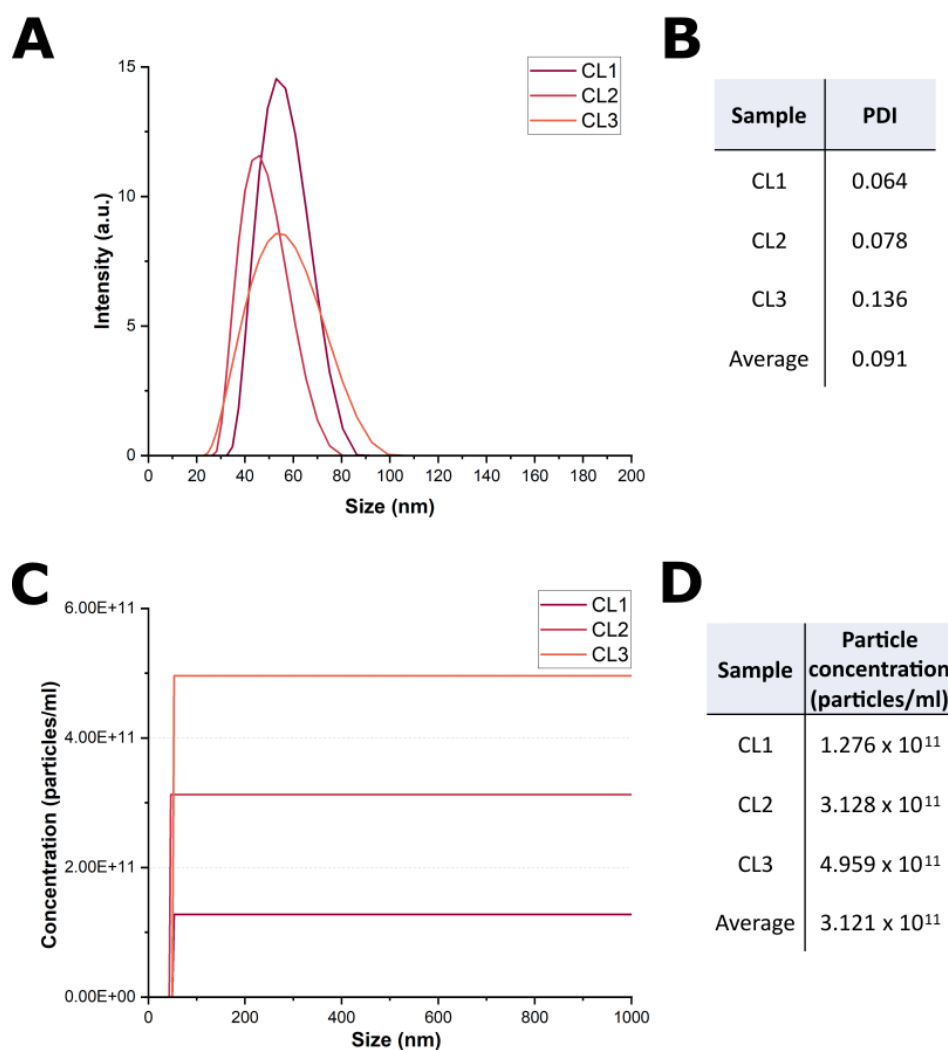


Figure 5.9) DLS and MADLS distribution data for the control liposome formulation. **A** DLS data for each lipid film replicate CL 1-3. **B** Corresponding PDI values for the DLS data for each lipid film replicate. **C** MADLS particle concentration data from the corresponding DLS samples. **D** Tabulated particle concentration determined by MADLS of each lipid film replicate.

For comparison, the number of vesicles was estimated using the lipid mass within the sample and the mean diameter of the CL liposomes determined in this work (Equation 11). The total number of DOPC molecules was estimated by calculating the total surface area of the liposome interior and exterior and dividing by the phosphatidyl choline head group surface area ($A_{PC} = 0.71 \text{ nm}^2$)^{186, 187}. Surface area of the liposome was calculated using an experimentally determined value for the mean radius ($26.41 \pm 2.49 \text{ nm}$) and DOPC bilayer thickness ($d = 3.59 \text{ nm}$) measured by Bryant *et al*¹⁸⁶. Values for A_{PC} and d were both taken from work by Bryant *et al*¹⁸⁶. The calculation assumes the sample is unilamellar and monodisperse, ensuring r_{ves} is representative.

$$N_{tot} = \frac{4\pi((r_{ves})^2 + (r_{ves}-d)^2)}{A_{PC}}$$

Equation 11. The total number of DOPC molecules (N_{tot}) was estimated from the choline head group surface area (A_{PC}), vesicle radius (r_{ves}) and bilayer thickness (d).

The number of vesicles per ml (N_{ves}) was then calculated using the number of moles per mL (M_{lipid}), Avogadro's number (N_A) and the total number of lipid molecules (N_{tot}) (Equation 12). M_{lipid} was calculated from the lipid concentrations applied within VFF, accounting for FRR dilution (1:6) and dilution to make the DLS sample (1:5). The calculation assumes lipid loss during VFF was minimal so the value for M_{lipid} is accurate, application of HPLC could be used to experimentally determine the lipid mass within the sample.¹⁸⁸ The final number of vesicles was calculated as $1.27 \times 10^{11} \pm 0.26 \times 10^{11}$, where the error is ± 1 SD from the upper and lower limits of the r_{ves} value (26.41 ± 2.49 nm). The calculated value was the same order of magnitude as the value determined by MADLS (Figure 5.9) which supported the validity of the measurement. Overall, all MADLS values were higher than the estimated value, which could suggest minimal lipid was lost in the VFF device.

$$N_{Ves} = \frac{M_{Lipid} \times N_A}{N_{tot} \times 1000}$$

Equation 12. The number of vesicles per ml (N_{ves}) calculated using the number of moles per mL (M_{lipid}), Avogadro's number (N_A) and the total number of lipid molecules (N_{tot}).

5.4 Ethanol impact within VFF

5.4.1 Ethanol impact within lipid bilayers

Ethanol lipid bilayer interactions are widely studied within the membrane biophysics field.^{178,189–192} Ethanol is reported to impact the acyl chain organization by binding to the lipid-water interface, which has been previously determined using NMR, XRD and DSC.^{189,193} Ethanol addition is reported to reduce membrane thickness in fluid lamellar samples, this predominantly occurs from three mechanisms; disordering, tilt angle change and interdigitation.^{189,191,193} As ethanol intersperses within the bilayer heterogeneity occurs in the acyl region, this leads to a change in alkyl chain orientation which increases the molecular area (Figure 5.10).^{178,192} The increase in molecular surface area gives rise to a lateral expansion and thinning within the bilayer structure.^{189,192} At above 10 mol% ethanol, the process of interdigitation of the opposing lipid leaflets can occur.¹⁸⁹ At very high ethanol fractions, ethanol solvation of lipid molecules results in micelle formation.¹⁹² Ethanol is reported to predominantly impact lipids with PC and PE headgroups, and its effect is also highly dependent on lipid phase leading to significant change for varied formulations.^{178,192,194} Within this work, application of DLS, TEM and fluorescent molecular rotors were used to explore ethanol impact within the AR 161 VFF device.

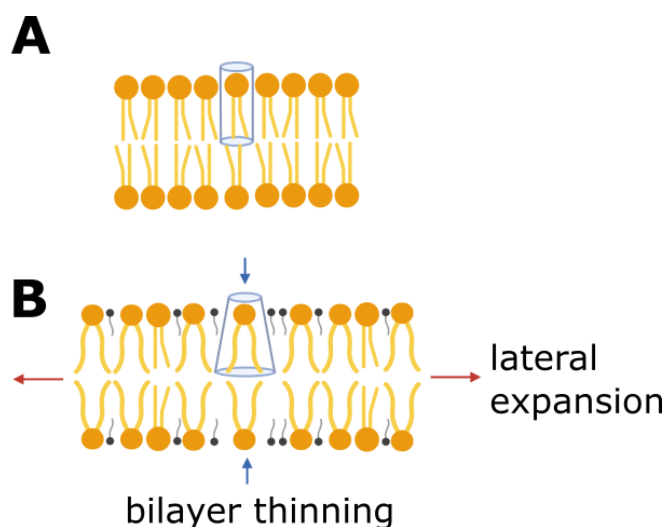


Figure 5.10) Cartoon representation of bilayer thinning and lateral expansion upon ethanol inclusion **A** Bilayer comprised of lipid molecules (yellow) **B** Bilayer comprised of lipid (yellow) and ethanol (black) molecules, direction of bilayer thinning, and lateral expansion is indicated.

5.4.2 Serial dilution DLS study

Serial dilution of VFF liposome samples, followed by DLS analysis was the first approach to study the impact of ethanol within VFF. Dilution in buffer reduces the volume fraction of ethanol within the sample, DLS characterises the impact on the mean diameter of liposomes. If ethanol is interspersed within the bilayer, liposomes should exhibit larger size from the effect of lateral expansion.^{190,195} Ethanol incorporation within the liposomes is likely stochastic, this would likely manifest as an increase in sample dispersity. A sample that exhibits ethanol removal from the bilayer would likely decrease in both mean diameter and FWHM.

Samples for the dilution study were produced by VFF using the AR 50 device with 0.1 mg mL^{-1} DOPC in the ethanol stream. Following co-flow, the VFF sample concentration was 0.02 mg mL^{-1} DOPC, samples were then diluted in the range 1:5 to 1:20 with PBS, a sample was made at 1:50 dilution although liposome concentration was below the DLS sensitivity. Following dilution, samples were mixed by aspiration and left at ambient conditions for 20 minutes. The ethanol content in the FRR 5 samples ranged from 682-171 mM, ethanol content of the FRR 25 samples ranged from 136-34.1 mM, with increasing dilution.

When the FRR 5 sample was diluted, minimal change in mean diameter and FWHM was observed (Figure 5.11). This could suggest dilution driven change in bilayer ethanol concentration, for the FRR 2 sample, could be insufficient to change the curvature of the liposomes. Alternatively, dilution may be insufficient to remove embedded ethanol molecules from the bilayer. This experiment could not distinguish the impact of ethanol decrease against the population biasing with dilution. The approach of using DLS is limited by the sensitivity, to explore samples containing higher ethanol fractions, the technique of TEM was applied.

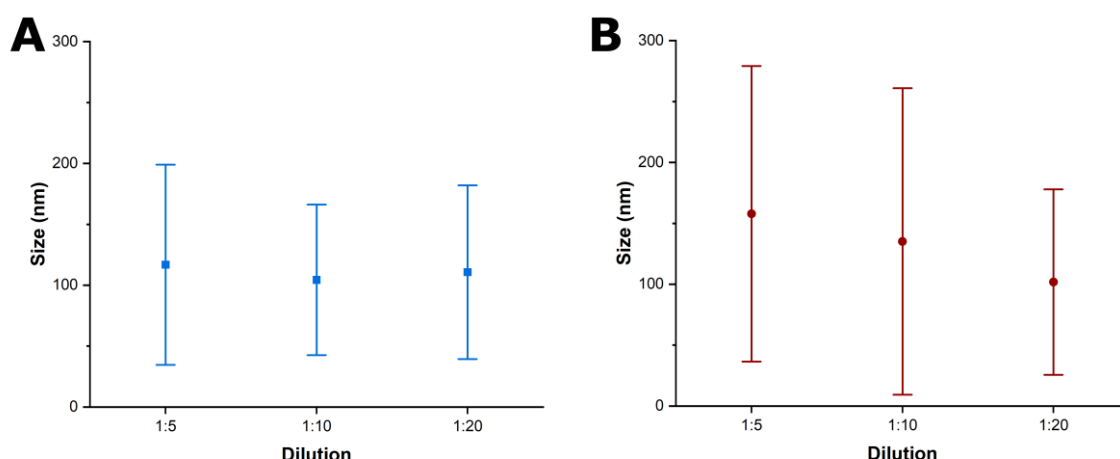


Figure 5.11) Mean diameter and FWHM (error bars) of samples at 1:5, 1:10 and 1:20 dilution in PBS from $n = 1$ samples. Samples were produced at **A** FRR 5 and **B** FRR 25.

5.4.3 Transmission electron microscopy of VFF samples

To explore the significant size shift between FRR 0.5 to FRR 5 and the 50 nm particle population, TEM was used to image the respective VFF samples (Chapter 2.1.4). TEM is the process by which small particles (< 200 nm) can be visualized, it operates by passing a beam of electrons through samples and imaging with a detector.^{196,197} TEM is an effective imaging technique for objects below the diffraction limit for conventional brightfield microscopy ($\approx 0.25\mu\text{m}$).¹⁹⁸ In practice, TEM is used to image structures between 5-500 nm in size, the images can be used to infer lamellarity and topography of lipid-based structures.^{196,199} TEM in this chapter was kindly undertaken by Dr Claudia Contini of the membrane biophysics group, Imperial College London.

The respective DOPC content for the samples was estimated using the applied flow rate and lipid concentration in the ethanol, DOPC content was determined as 0.05 mg mL^{-1} and 0.0167 mg mL^{-1} for FRR 0.5 and FRR 5, respectively. In Chapter 4.4.1, particles produced at FRR 0.5 and FRR 5 were found to result in significantly different mean diameters (Figure 5.12A). Within Chapter 5.2.4, the AR 161 device was found to produce bimodal distributions at low FRR, featuring a peak near 200 nm and a peak near 50 nm peak (Figure 5.12B).

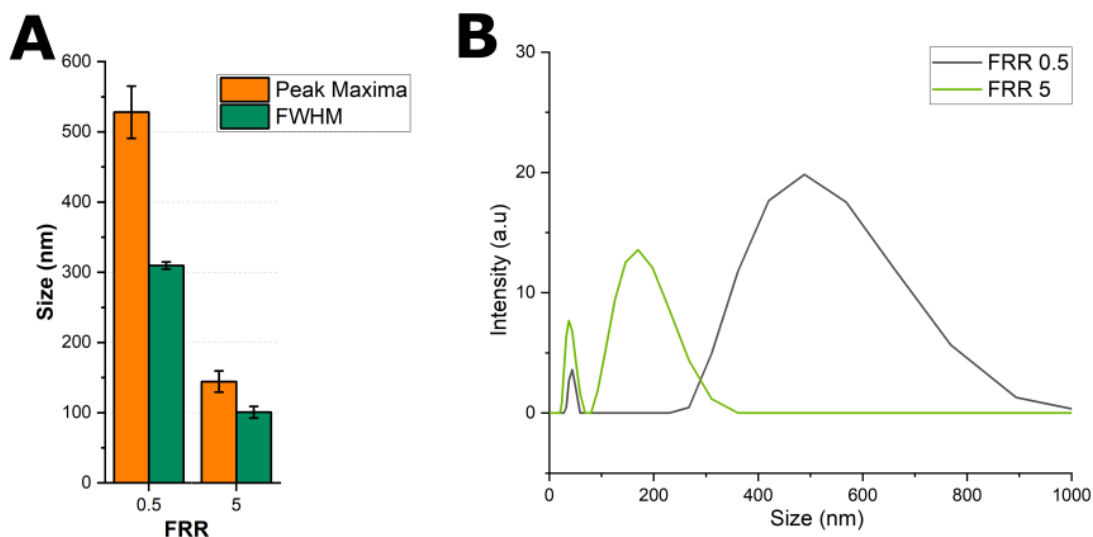


Figure 5.12) Samples used within the TEM study. **A** Mean diameter and FWHM plot for the FRR 0.5 and FRR 5, produced from $n = 3$ samples. **B** DLS plot at FRR 0.5 and FRR 5 from $n = 1$ samples.

The TEM images were first produced for the 0.02 mg mL^{-1} DOPC sample made at FRR 5 and TFR 0.6 mL min^{-1} , within the AR 161 device. This sample contained approximately 3.41 M of ethanol; ethanol content was calculated from the VFF dilution factor for the anhydrous absolute ethanol ($\geq 99.5\%$) central stream. Within the TEM images a large quantity of clustered $< 75 \text{ nm}$ particles were observed, clustering is likely a result of the TEM sample preparation method (Figure 5.13A). Presence of the 150 nm particles within DLS were typically embedded within smaller clusters (Figure 5.13B). The clustering reduces the clarity of the of the 150 nm particles, making assignment difficult. The 150 nm particles appear to be vesicles, as a darker circumference were observed in some images.

This sample confirms that the AR 161 DOPC samples are bimodal and could be a mixture of vesicles or coexisting vesicles and micelles. Micelles could be forming from the high 3.41 M ethanol content within the sample. In a study by Setiawan *et al*¹⁹⁵ ethanol addition is explored within a 50 mol% DOPC SLB. Setiawan *et al*¹⁹⁵ demonstrate an ethanol concentration of 1.0 M ethanol entirely disrupts the SLB, and suggest that above 0.8 M ethanol, the bilayer begins to undergo interdigitation. The SLB used by Setiawan *et al*¹⁹⁵ contained 40 mol% egg sphingomyelin and 10mol% CHOL. A CHOL concentration of above 5mol% is thought to protect bilayers from the effect of ethanol.¹⁹² Without the presence of cholesterol, it is reasonable to suggest that the ethanol concentration is far above the threshold for bilayer disruption within this study. At 3.41M ethanol content, ethanol could solvate DOPC stabilising the formation of curved micelles. Alternatively, the coexistence at low FRR within the AR 161 could be a result different mixing regime, from this data set it is currently unclear which is the dominant effect.

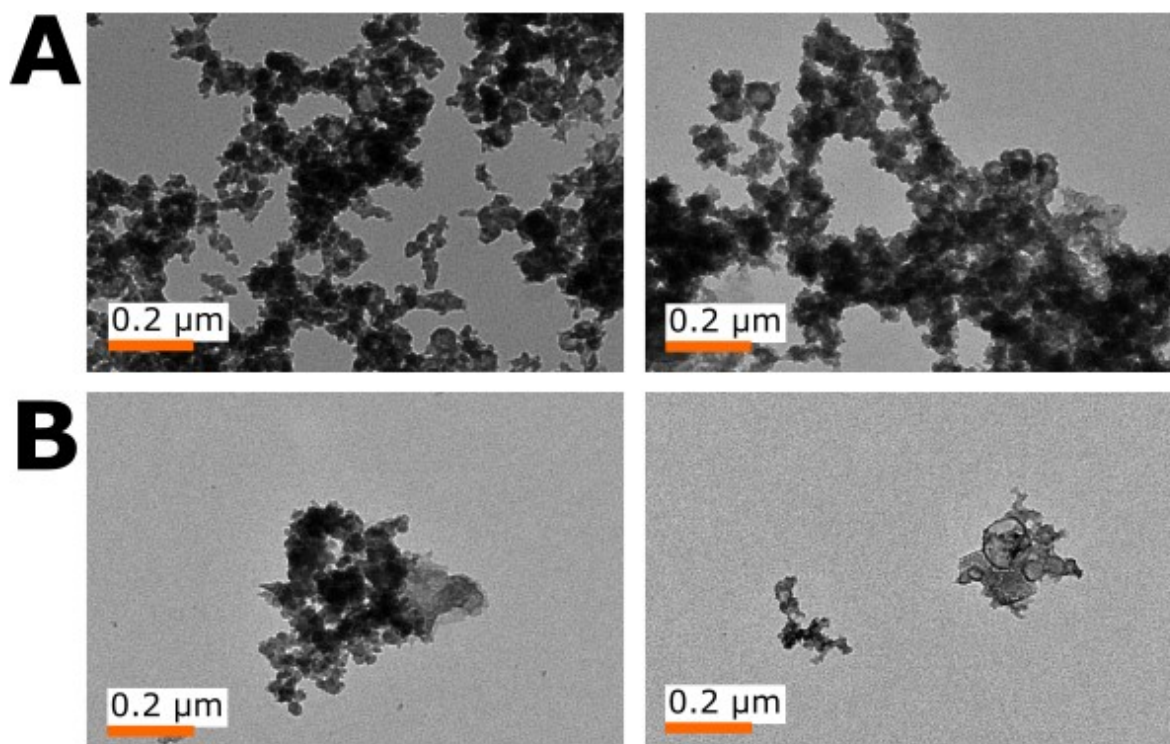


Figure 5.13) Samples containing 0.02 mg mL^{-1} DOPC produced from: FRR 5, TFR 0.6 mL min^{-1} within the AR 161 device. Samples were used without dialysis. **A** Images of larger clusters within the sample, containing predominantly $< 75 \text{ nm}$ particles (Scale bar = 0.2 μm). **B** Images of smaller clusters within the sample, showing $< 75 \text{ nm}$ particles aggregating around $>100 \text{ nm}$ particles (Scale bar = 0.2 μm). (magnification = SA 26000-52000x; defocus = -2.6 μm).

Imaging of a sample produced at FRR 0.5 enabled study of the large 528 nm mean diameter particles produced, the sample exhibited an apparent low PDI of 0.119. The 528 nm particles were observed to predominantly co-exist in clusters with the $< 75 \text{ nm}$ particles (Figure 5.14A). Inspection of non-clustered large particles indicated a faceted outline with a pore-like contrast gradient (Figure 5.14B). The assignment of the large, faceted particles is unclear from the images, and they could either be a cubosomal structure or clustered liposomes/micelles. Cubosomes often present a faceted structure with a well-ordered internal structure, high contrast imaging of cubosomes is shown in work by Demurtas *et al*^{197, 200–202}. If the particles are cubosomes, the ability to produce cubosomes using a continuous flow-based method has important connotations for the study and application of cubosomal drug delivery systems. The $< 75 \text{ nm}$ particles were observed within clusters, these were like the potentially liposomal or micellar particles within the FRR 5 sample (Figure 5.14C). Overall, TEM images suggested sample dispersity is greater than DLS typically presents and that low FRR samples require characterisation to ensure samples are liposomal.

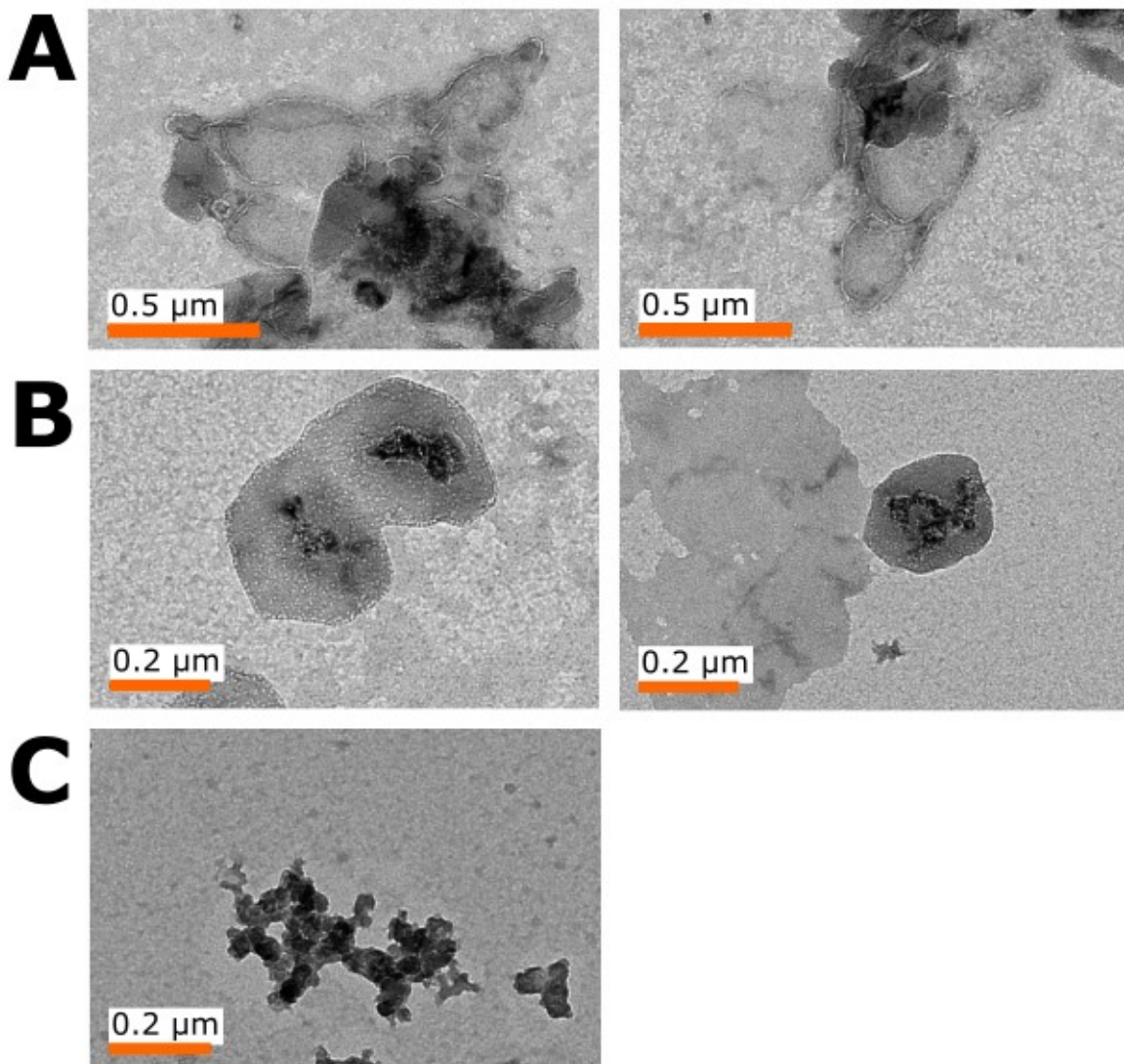


Figure 5.14) Samples containing 0.05 mg mL^{-1} DOPC produced from: FRR 1, TFR 0.2 mL min^{-1} within the AR 161 device. Samples were used without dialysis. **A** Images of larger clusters within the sample, containing 400 – 700 nm and < 75 nm particles (Scale bar = 0.5 μm). **B** Images of smaller clusters within the sample, showing 300 - 400 nm particles aggregating (Scale bar = 0.2 μm). **C** Images of clusters within the sample comprised of < 75 nm particles aggregating (Scale bar = 0.2 μm). (magnification = SA 26000-52000x; defocus = -2.6 μm).

5.4.4 Molecular rotor study of micro-viscosity

The impact of residual ethanol on the local membrane viscosity was explored with application of boron-dipyrin (BODIPY) molecular rotors. Miguel Paez-Perez within the Kuimova group, Imperial College London, kindly supplied BODIPY rotors and undertook the time resolved fluorescence spectroscopy for this chapter. BODIPY rotors were first developed as a means for imaging membrane viscosity within live cells, and have also been applied to model lipids systems, as in the work by Dent *et al*²⁰³ where DOPC, DPPC and CHOL containing bilayers were studied.^{204,205}

Mechanistically, specialised BODIPY molecular rotors are first intercalated into lipid bilayers; rotors are typically added during the formation of the lipid film. When membrane embedded rotors are excited, they undergo two forms of decay, either radiative fluorescent emission or a non-radiative pathway.²⁰³ The non-radiative pathway occurs by intra molecular relaxation and the rate of this is dependent on the local environment of the rotor. Determination of a local membrane viscosity is an important metric for liposomal suspensions, as bulk viscosity is unrepresentative of liposome properties. Quantifying the balance of the two decay rates allows determination of the local membrane viscosity.

The fluorescence of BODIPY fluorescent decay is measured by time resolved spectroscopy, this value is coupled to the local membrane viscosity.^{203,206} The lifetime of a molecular rotor τ_f can be related to the local viscosity η , using a variant of the Förster-Hoffmann equation, where k_r is the radiative decay constant, a and z are fixed dimensionless constants (Equation 13). If the local membrane viscosity is high, the lifetime of the molecular rotor is greater. In the work by Dent *et al*²⁰³ calibration is shown using a glycerol/methanol solution, higher glycerol fractions increase viscosity and were shown to increase τ_f . In this work, DOPC liposomes were produced by VFF and extrusion to enable comparison. VFF samples were dialysed against PBS buffer to explore membrane viscosity changes associated to ethanol.

$$\log \tau_f = \log \left(\frac{z}{k_r} \right) + a \log \eta$$

Equation 13. Where lifetime of a molecular rotor τ_f can be related to the local viscosity η , where k_r is the radiative decay constant, a and z are fixed dimensionless constants

In this work, samples were prepared with 0.5 mol% BODIPY rotor, at this concentration BODIPY is detectable and agglomeration within the bilayer is minimised.²⁰⁷ For VFF samples, BODIPY was added as part of the lipid film and dissolved into ethanol, prior to flow focusing. Lipid and BODIPY concentrations were varied for the different FRR applied, such that the end lipid and BODIPY concentration was fixed. Extrusion samples added BODIPY as part of the lipid film before hydration in buffer, extrusion was undertaken using a 200 nm pore membrane. Sample dialysis was achieved using Thermo Scientific™ Slide-A-Lyzer™ with a 3.5K molecular weight cut-off, samples followed the Thermo fisher protocol Slide-A-Lyzer™ and were dialysed for 12 hours against PBS at room temperature. Each condition was comprised of a minimum of 3 sample replicates, made from different lipid films. Time resolved spectroscopy measurements were all taken at 20°C (Figure S12.6).

Initially, micro-viscosity of a VFF sample produced at FRR 5 using the AR 50 device was compared with a sample produced by extrusion (Figure 5.15A). At FRR 5 the micro-viscosity mean value was found to be 96.7 ± 6.5 cP whilst the extruded mean value was 154.8 ± 25.3 cP. In the study by Dent *et al*²⁰³ the micro-viscosity for DOPC LUV was measured as 135 cP at 25°C, this is close to the extrusion sample value in this study, for DOPC SUV at 20°C. At FRR 5, the un-dialysed sample has an ethanol volume

fraction of 20%, this is 58.2 cP lower than the extruded value. As discussed in Chapter 5.4.1, ethanol can intersperse within the bilayer causing increased lipid chain splay and lateral expansion. With ethanol driven lateral expansion, an increase of fluidity within the alkyl region occurs, this decreases membrane viscosity.¹⁹² The rotor study provides evidence that ethanol led to a reduction in the micro viscosity in the membrane, the expected behaviour, since the bilayer becomes more fluid the BODIPY rotor will undergo less hindered non-radiative decay and report lower viscosity. The derived microviscosity values for VFF vesicles produced at FRR 5 and the extruded control sample were both comprised of over 4 replicates, the SD of the extruded sample was 4-fold higher than that of the VFF sample. Lower SD between samples could suggest that VFF produces more homogenous samples, this may be a result of the low length-scale of the mixing process.

The impact of sample dialysis was explored through comparison of dialysed and un-dialysed FRR 5 samples with the extruded samples (Figure 5.15B). Dialysis increased the mean membrane viscosity from 96.7 ± 6.5 cP to 113.8 ± 13.3 cP, this increase was lower than expected as the micro-viscosity of the extrusion sample was 150 cP, this implies there is residual ethanol retained in the bilayer after dialysis. The reliability of the dialysis method was shown to be low; one sample exhibited a micro-viscosity close to the un-dialysed value. The greatest viscosity of the dialysed sample was within the micro-viscosity range of the extruded sample. This set implies dialysis over the 12-hour period was insufficient for removing ethanol from low FRR VFF samples. To explore this further, different dialysis conditions and times could be applied.

By exploring different FRR values within VFF, different residual ethanol concentrations could be measured (Figure 5.15C). FRR was explored in the range 1-25, as FRR was increased the reported micro-viscosity increased. Within the FRR data, micro-viscosity increased with a potentially linear gradient, this implies that residual ethanol concentration predominantly impacts micro-viscosity, since lipid and rotor concentration were fixed between samples. The lifetime measurement is independent of rotor concentration and rotor concentration was measured to remain below a threshold where rotor agglomeration could occur. The FRR sweep implies high FRR is required to produce liposomes with a micro-viscosity that exhibits minimal impact of ethanol. The data suggests sample generation requires high FRR and a dialysis step, to minimise ethanol complications within future studies. As demonstrated in Chapter 4 high FRR is not always favourable in terms of distribution and the optimum FRR should be chosen on balance of these effects.

A further impact on micro viscosity could be the decrease in mean diameter associated with higher FRR, at smaller size and curvature lipid molecules exhibit a different chain splay.^{11,208} As discussed previously, in the case of ethanol addition, chain splay leads to a greater fluidity within the membrane. Analysis of this of the rotor study shows ethanol is most likely the cause for several reasons. In Chapter 4.4.1, mean diameter between FRR 5-25 within VFF AR 50 was similar, this means any difference in micro-viscosity between FRR 5-25 could be attributed to the ethanol fraction. At FRR 1 liposomes were closest in mean diameter to the extruded sample, demonstrating the large disparity in the micro-viscosity for these two samples. Additionally, dialysis is shown to have a measurable impact on micro-viscosity, this suggests the difference is attributed to the ethanol concentration within the system. For these reasons, it is likely that the impact of mean diameter on micro-viscosity is minimal across the 200-100 nm size range explored. This study makes light of an underappreciated issue within MHF/VFF, typically the approach of either high FRR or post-formation dialysis is taken when this work demonstrates the combination is required to produce liposomes fit for therapeutic application. Currently, the application of molecular rotors to study MHF/VFF sample quality is unreported and this work marks the first attempt to quantify ethanol impact and evaluate means to reduce residual ethanol.

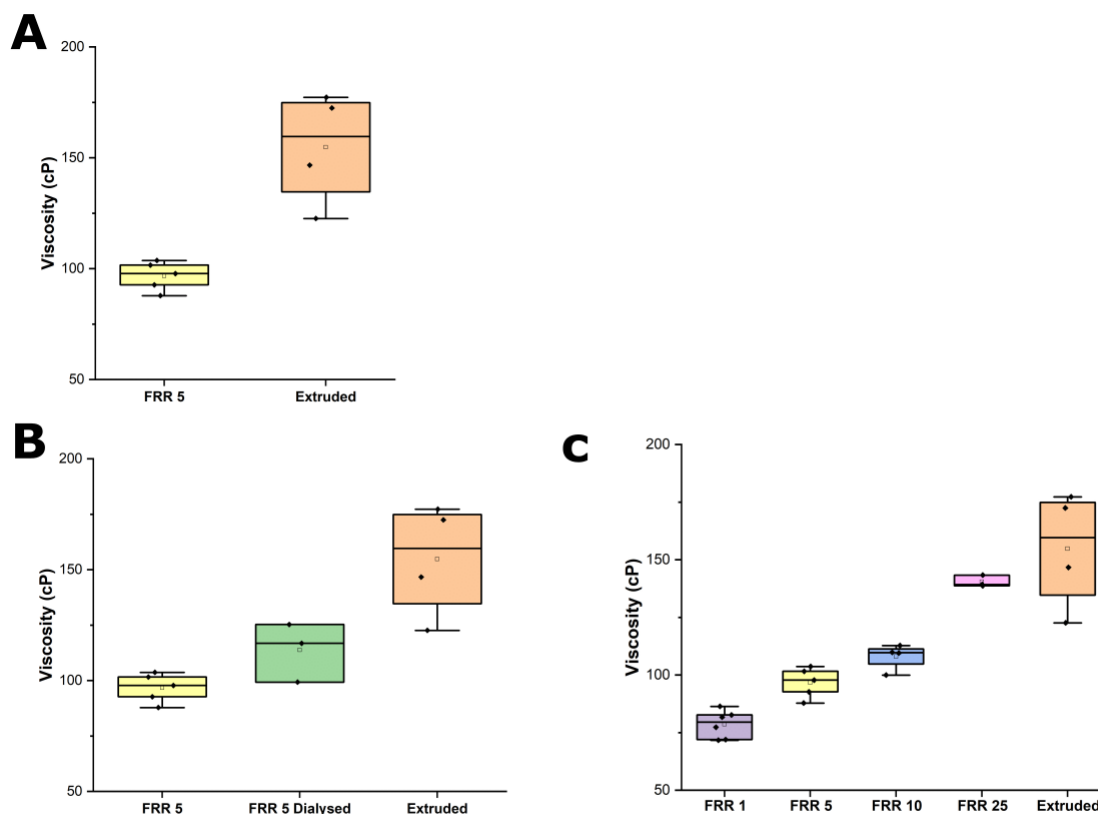


Figure 5.15) Sample micro-viscosity plots determined from BODIPY addition using time resolved fluorescence spectroscopy. **A** FRR 5 and extruded sample comparison. **B** Comparison between FRR 5 before and after dialysis with the extrusion reference. **C** Effect of varied FRR, compared to the extruded sample.

5.6 Conclusion and future work

5.6.1 Conclusion

This chapter initially presented a method for producing a higher AR PMMA based VFF device through application of adhesive backed PET films. Our device produced distinct liposomal distributions that were bimodal at low FRR, this highlighted a geometry dependence within VFF; a potential cause for the controversy around reported FRR and TFR impact. The effects within different devices are reported by Golden *et al*¹³⁴ and Jahn *et al*⁶⁰ for differing designs. The high AR device was able to produce liposomes at all explored FRR and TFR, these presented similar mean diameters to the AR 50 device. A decrease in mean diameter was observed in the TFR sweep which was unexpected, to explore this further a FRR sweep with fixed TFR could be performed to identify whether both FRR and TFR are impactful within the AR 161 device. Overall, the AR 161 device presents an interesting case study and offers a potentially viable fabrication for smaller channel sizes.

Through application of the MADLS technique, VFF liposome concentration was characterised ($3.121 \times 10^{11} \pm 1.841 \times 10^{11}$ particles mL^{-1} at FRR = 5, TFR = 0.6 mL min^{-1}) and compared to an estimated value ($1.27 \times 10^{11} \pm 0.26 \times 10^{11}$ particles mL^{-1}).

The combination of DLS, TEM and molecular rotor-based studies were employed to study the impact of ethanol on VFF produced liposomes. DLS studies were inconclusive as ethanol dilution occurred with liposome dilution and significant impact on the size wasn't observed. TEM study gave insight into particle structure at high ethanol fraction (66 vol%), where large agglomerates that were potentially cubic structures or liposomal aggregates were observed. The molecular rotor study quantified the impact of ethanol across FRR and compared with extrusion, the widely applied method of liposome production. The rotor study provided guidelines for producing samples that exhibited minimally perturbed micro-viscosity, both high FRR (> 20) and sample dialysis are required to obtain sample micro viscosity comparable to extrusion.

5.6.2 Future work

To further explore the impact of adhesive contamination, analysis of fluid run through the AR 161 device using IR or mass spectrometry could indicate if adhesive enters the AR 161 device. Particle concentration is important for cell-based study, to validate dosage, future work could be explored to alternatively measure using nanoparticle tracking analysis or resonant mass measurement (> 300 nm vesicles) and validate the MADLS measurement.^{209,210}

Imaging of the large structures observed in TEM using cryo-TEM could enable an accurate assignment, imaging was complicated by the dilute VFF samples, when compared with alternative methods. The use of XRD could also distinguish the nature of the large particles, however the sample concentration would need to be significantly increased to achieve this. Developing an integrated dialysis on chip would further improve resultant sample quality, in a continuous fashion and avoid batch-based sample purification.

6 Novec driven hydrodynamic focusing

6.1 Overview

A widely recognised limitation of VFF is the low liposome concentrations that results from dilution during the co-flow of miscible phases. Within this chapter, method development and preliminary data is presented toward the application of Novec flow focusing, a VFF adaptation that could result in higher lipid concentration post flow focusing. Novec flow focusing replaces the buffer outer-phase in conventional MHF/VFF with Novec, by exploiting Novec miscibility with ethanol and immiscibility with aqueous phases the approach seeks to improve liposome concentration following flow-focusing. Novec flow focusing produces two immiscible phases, the first is an aqueous liposomal suspension phase and the second is an ethanol enriched Novec phase, the phases are readily separated by manual aspiration. In this work, Novec MHF was confirmed to produce liposomes by cryo-TEM, distribution analysis by DLS demonstrated Novec MHF typically formed asymmetric distributions containing large particles (>1000 nm) this signified the application to therapeutic liposome production was unsuitable. Novec MHF inefficacy was attributed to Novec incompatibility with PDMS based devices and surface modification. When Novec was applied within VFF a significant improvement in resultant distribution was observed using DLS and a low PDI (< 0.1) was measured. MADLS comparison with conventional VFF samples suggested both the concentration and size of liposomes was larger within Novec VFF, large uncertainty in the measurement meant further samples are required to confirm the observation. Although promising, further work is required to establish an optimum procedure for Novec VFF this could be aided by fluid mechanic simulation and further clarity around the MHF/VFF liposomal assembly mechanism.

6.2 Introduction to Novec hydrodynamic focusing

6.2.1 Introduction to Novec hydrodynamic focusing

The main limitation of hydrodynamic flow focusing is widely considered to be the low concentration of the resultant liposomes.^{56,62,211} During hydrodynamic focusing, the co-flow of two miscible phases results in the dilution of lipid content within the central stream. An upper limit for hydrodynamic flow focusing is presented by the phospholipid solubility and liposome self-assembly before flow focusing.¹³⁵ To counter the dilution effect within MHF, this work adapted the MHF method by employing Novec™ 7100 engineered fluid, developed by 3M. Novec 7100 is comprised of an inseparable isomeric mix of methoxy-nonafluorobutane, other Novec fluids offer differing viscosities and solvent solubilities. Within engineering, low viscosity Novec engineered fluids are a greener CFC alternative which avoids ozone depletion.²¹² Within academia, Novec is applied as a microfluidic surface treatment, inert carrier fluid for single cell encapsulation and as a heat transfer fluid.^{213–217} In this work, Novec 7100 was utilised as it offers a low solubility in water (12 ppmw²¹⁸) whilst being miscible in ethanol.²¹⁸

Novec flow focusing involves a similar flow focusing to MHF/VFF, Novec MHF differs by replacing the outer buffer phase with Novec and adding a buffer fraction to the central ethanol (Figure 6.1). Co-flow enriches the Novec phase with ethanol increasing lipid concentration in the central stream. Following sample collection, a two-phase solution results subsequent manual extraction of the aqueous portion isolates the aqueous liposome suspension. Novec MHF results in a FRR independent lipid concentration as the concentration within the process is determined by the aqueous buffer fraction within the ethanol phase. A low concentration of buffer is required to ensure lipid molecules remain fully solvated and prevent self-assembly before flow focusing, lower buffer fraction should hypothetically result in a higher lipid concentration post flow focusing. The potential lipid concentrating behaviour within Novec hydrodynamic focusing was hypothesized to increase the resultant liposome concentration, this could advance the capabilities of hydrodynamic focusing within an academic setting. In addition to this potential, application of Novec may provide a greener alternative as mixing ethanol enriched Novec with buffer could regenerate the Novec and enable its re-use.

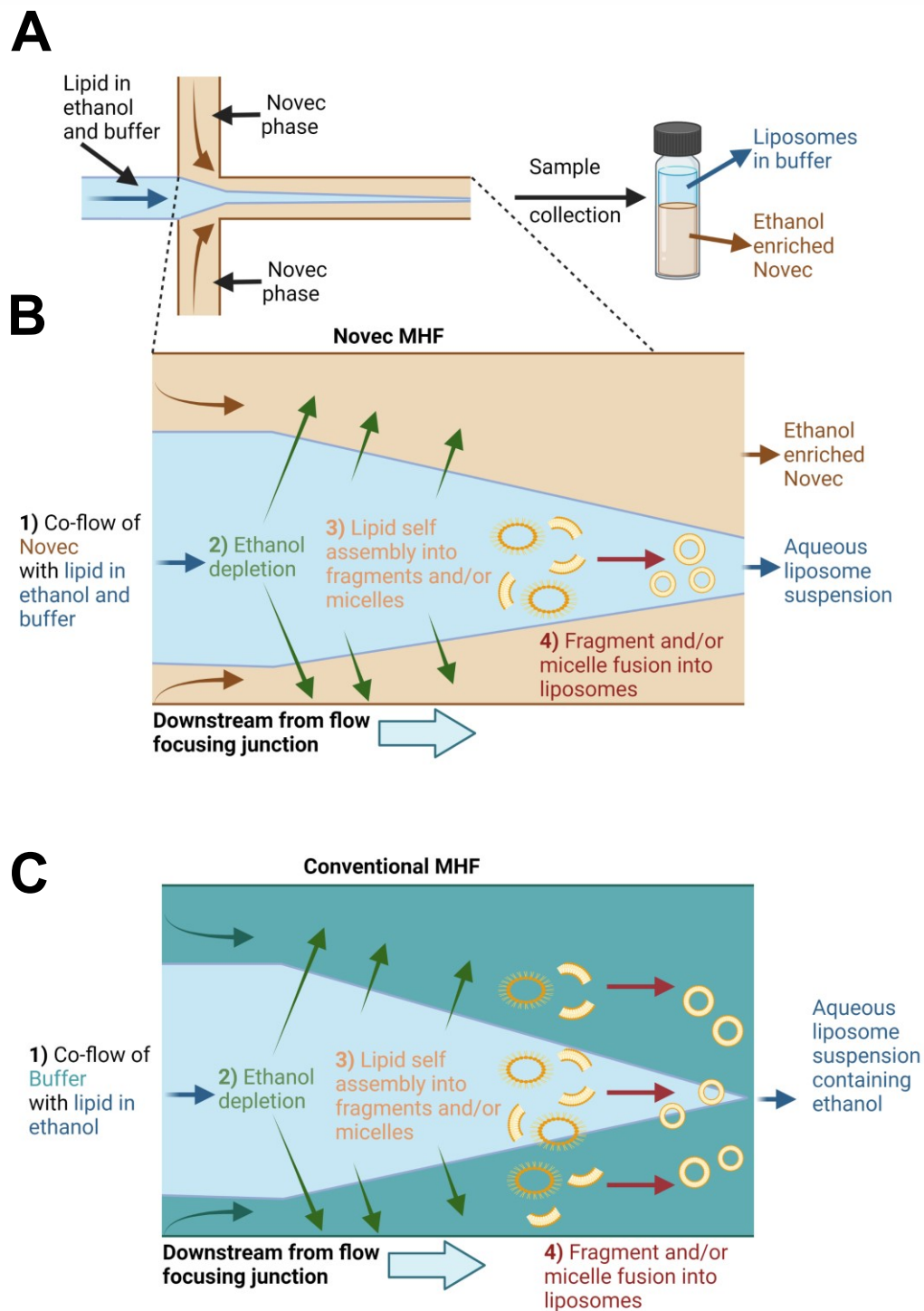


Figure 6.1) Depiction of the Novec MHF adaptation **A** Diagram of the flow focusing junction, flow direction is indicated by coloured arrows (left) and resultant two-phase sample produced is shown (right). **B** Depiction of the proposed mechanism of liposome formation after the flow-focusing junction, shown to the right are the outputs of Novec MHF (Figure not to scale). **C** Depiction of the proposed mechanism of liposome formation after the flow-focusing junction, shown to the right are the outputs of conventional MHF (Figure not to scale).

Mechanistically Novec flow focusing has similarities to conventional hydrodynamic focusing, both processes rely on the depletion of ethanol into the outer phase (Figure 6.1C). Since the purported mechanism for liposome formation within MHF/VFF is still unconfirmed this chapter speculates based on the conventional MHF liposome assembly mechanism hypothesized by Jahn *et al*^{59,61}. Within both systems it is likely once ethanol is sufficiently depleted the lipid molecules undergo self-assembly resulting in production of lipid intermediates which may be lipid fragments and/or micelles, the lipid intermediates then either close or fuse forming liposomes further downstream.

A major difference of Novec flow focusing is the diffusion rate of ethanol, when Novec 7100 is co-flowed with anhydrous ethanol a single-phase results, demonstrating ethanol diffusion within the MHF timescale and the need buffer in the ethanol phase to facilitate partitioning. Enthalpically it is reasonable to suggest the interaction of ethanol with Novec is weaker than that with water on a basis of hydrogen bonding, ethanol-water hydrogen bonds are particularly strong whereas ethanol-Novec hydrogen bonding is likely weaker.²¹⁹ Covalently bonded fluorine only participates in hydrogen bonding in an electron rich local intramolecular environment, the weak hydrogen bonds with Novec likely occur at the methoxy group.^{149,219,220} The low solubility of water within Novec (95 ppmw²¹⁸) supports the idea hydrogen bonding to Novec is weak. Weak dipolar interactions between Novec and ethanol are the likely enthalpic contributions within ethanol Novec mixtures.^{221,222} Ethanol solubility within Novec flow focusing is likely predominantly driven by entropy as the ethanol disperses into Novec. Based on these observations it is hypothesized the ethanol diffusion will be slower for Novec flow-focusing and by relation to the FRR trend established by Jahn *et al*⁶¹ Novec flow focusing may produce larger liposomes.

Hypothetically a further degree of control can be achieved within Novec flow-focusing by adjusting the buffer fraction within the ethanol phase. An increasing buffer fraction would reduce concentration of lipid within the produced sample as the buffer fraction determines volume of the aqueous fraction. When considering Fick's first law, increasing buffer content may also reduce the concentration gradient between the central and outer phase, this could act to reduce the diffusion rate of ethanol further increasing the size of resultant liposomes (Equation 14).^{182,221} Fick's first law relates the concentration gradient ($\frac{dc}{dx}$) at a given x coordinate to the diffusional flux (J), the amount of substance that flows through an area per unit time, by the diffusional constant (D) which is inherent to the solvent and diffusing substance. Fick's first law is an approximation given the potentially non-ideal nature of the solutions and dynamic flow conditions, a greater representation could be achieved through application of Navier-Stokes equations for incompressible laminar flow.^{182,223–225} Navier-Stokes equations represent the time-resolved nature, kinematic velocity and location dependent convection and diffusion rates.^{182,225} To effectively model Novec flow-focusing elements of two phase miscible flow and immiscible flow need to be encompassed. Dynamically, the interaction of Novec with the central phase likely differs along the channel length impacting the kinematic viscosity. As Novec depletes the ethanol, the central phase becomes predominantly buffer and the interaction with Novec should decrease. Fluid mechanic simulation could shed light on the variable kinematic viscosity along the channel length and the appropriate exit channel length for applied ethanol-buffer ratios within the central phase.

$$J = -D \frac{dc}{dx}$$

Equation 14. Where diffusional flux (J) is related to the diffusional constant (D) by the concentration gradient ($\frac{dc}{dx}$).

With regards to Novec flow focusing, liposome assembly is confined to the central stream whereas within conventional MHF liposome may also occur within the outer aqueous phase across a larger length scale. The confinement of liposome assembly to the narrow central stream within Novec flow focusing could result in a higher concentration of lipid intermediates. An increased concentration of lipid intermediates could result in the formation of larger vesicles, as a greater number of fusion events occur. Our hypothesis stems from work by Hood *et al*⁶⁴ who report increasing lipid concentration within conventional VFF results in larger liposome formation. When considering potential mechanisms and reasoning using previous DLS data, there could be numerous routes to the formation of lipid structures, though the process likely initiates with recruitment of lipids forming lipid intermediate structures (Figure 6.2). Once initiated, purely lipid recruitment onto the lipid intermediate could favour low dispersity micellar and liposomal samples. Purely direct formation could hypothetically be driven by a low concentration of lipid intermediates favouring micelle formation or rapid recruitment and closure of intermediates favouring liposomes. At a high concentration of lipid intermediates, fusion of the micelles and liposomes may result in larger liposomes with a greater sample dispersity. With the uncertainty around Novec flow-focusing a series of experiments were undertaken.

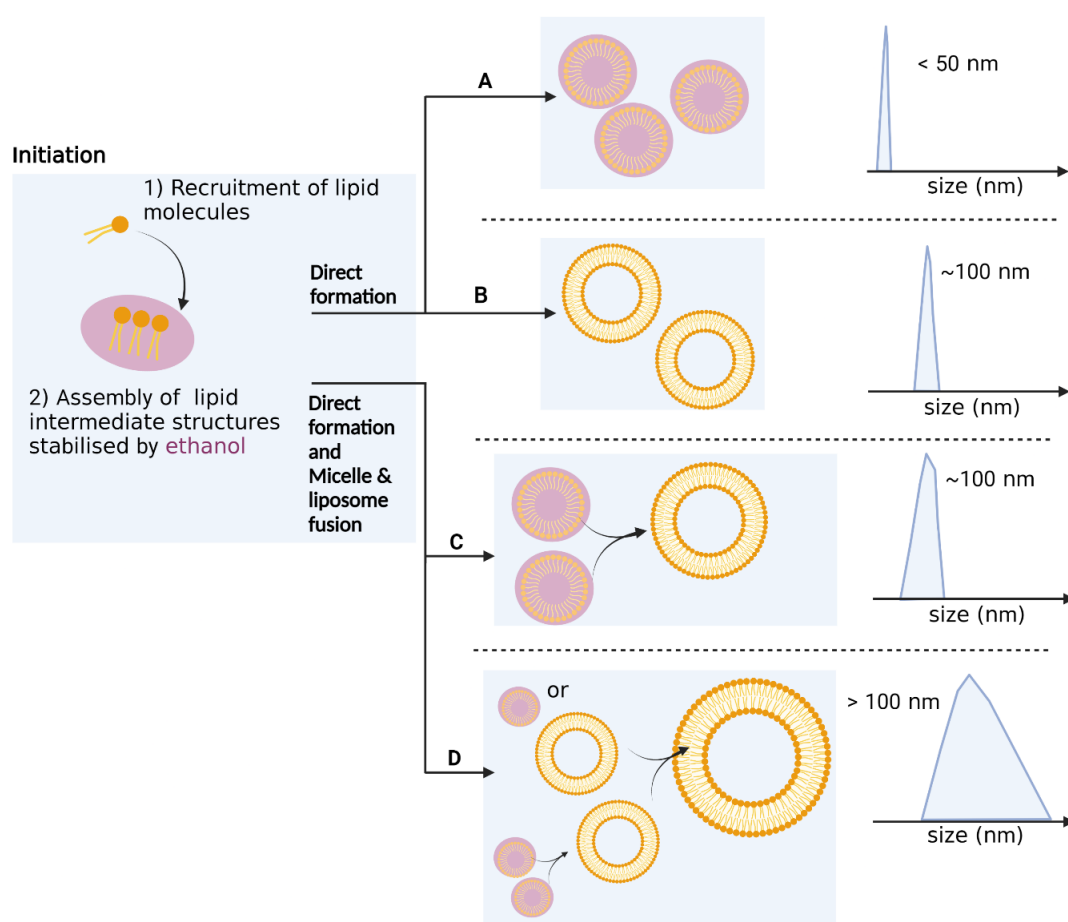


Figure 6.2) Hypothesized cartoon of micelle and liposome formation within VFF relative to the DLS characterised distributions. Assembly likely starts with lipid recruitment and formation of lipid intermediate structures. Ethanol stabilised structures are highlighted in purple and DLS distributions are arbitrary intensity **A** Low recruitment or lipid concentration results in formation of monodisperse micelles. **B** High recruitment or lipid concentration results in formation of monodisperse liposomes. **C** Low recruitment with high lipid concentration results in micellar fusion leading to liposomes with high dispersity. **D** Rapid recruitment and high lipid concentration result in inter-micellar and inter-liposomal fusion producing large polydisperse liposomes.

6.2.2 Data collection and analysis

Novec MHF/VFF was undertaken using designs produced in Chapter 3.2.2 and Chapter 4.2.3 respectively. Data sets within this Chapter used DOPC, a variable concentration was applied and highlighted in each section. Both Novec MHF and VFF were driven using syringe pumps loaded with PTFE plastic syringes and PTFE tubing connections. Novec MHF/VFF samples were collected off chip into glass vials, the aqueous phase was carefully aspirated using a pipette, whilst avoiding sample disturbance.

During Novec MHF, PBS was added to the lipid in ethanol solutions and vortexed prior to MHF application. PBS fractions were explored from 10 - 50 vol%, for the study a DOPC concentration of 0.01 mg mL⁻¹ (12.72 μM) was applied, DOPC concentration below the CMC in water (50 μM²²⁶) prevents lipid assembly prior to flow focusing. During MHF, a FRR of 10 and TFR of 0.11 mL min⁻¹ were applied and samples were collected after 3 minutes equilibration time. Samples were diluted 1:10 in PBS before measurement with DLS, at this dilution a count rate close to 200 kcps was achieved.

Within this chapter, the dilution used for Novec MHF/VFF DLS samples was 1:10 using PBS buffer. Novec samples were further diluted compared to conventional MHF/VFF samples this was to achieve a count rate near 200 kcps. Conventional MHF/VFF samples were diluted to 1:5 in PBS to achieve 200 kcps, at this count rate the sample was within the effective working range of the instrument. A DLS scan was first used to determine the kcps of the sample, this value was applied within the MADLS measurement to achieve particle concentration. When presenting particle concentration per mL, the value was scaled for sample dilution, ensuring it was representative of the particle concentration exiting the device.

6.3 Novec MHF optimisation

6.3.1 Novec MHF buffer content

PBS volume fraction was first explored within Novec MHF as the PBS fraction within the ethanol stream determines the resultant sample lipid concentration. Initial samples produced gave rise to wide asymmetric distributions (Figure 6.3). Broad unresolved populations and presence of multiple particle sizes establishing a trend difficult. At 50 vol% PBS, a population of particle around 50 nm was observed, this could be the presence of micelles. The broad asymmetric peaks could result from significant fusion of micelles and liposomes facilitated by the high concentration of lipid intermediates.

Particles larger than 2000 nm were observed in some samples, this was thought to be presence of Novec oil droplets. Careful sample aspiration was used for future samples, to minimize disruption of the Novec phase. Novec MHF samples were outside the diameter range for therapeutic application and the asymmetric distributions implied different particle populations were present. Sample quality was thought to be impacted by Novec absorption within the PDMS device, PTFE tubing and syringes.²¹⁸ A FRR sweep was undertaken to establish whether FRR could reduce sample dispersity.

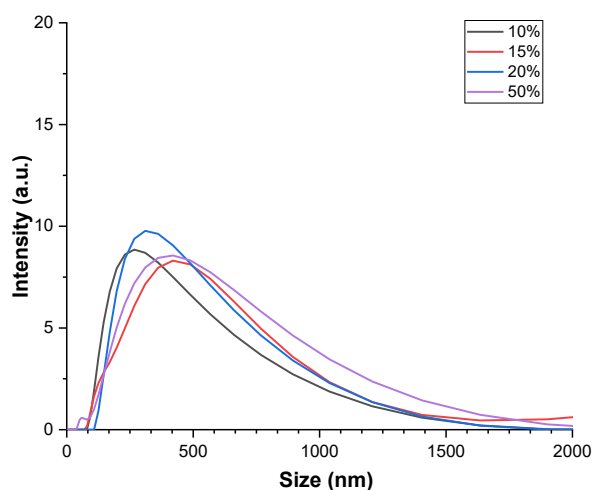


Figure 6.3) DLS of resultant populations formed from Novec MHF using 0.01 mg mL^{-1} DOPC with increasing PBS volume fraction from 10 – 50 vol% in the central stream, produced from $n = 1$ samples.

6.3.2 Novec MHF FRR sweep

FRR was explored from FRR 5 - 10, using 0.01 mg mL^{-1} DOPC within the central stream (Figure 6.4). As FRR was increased, samples became more asymmetric with presence of particles $> 1000 \text{ nm}$. It is likely the samples were bimodal, the FRR 8 samples exhibited a bimodal distribution using standard DLS analysis. Samples likely consisted of micellar $< 50 \text{ nm}$ and liposomal $75 - 1000 \text{ nm}$ populations. Samples exhibited large dispersity and large mean diameter at all explored FRR. Given the high sample dispersity, Novec was utilized within VFF with the intention of achieving lower sample PDI.^{64,123,135}

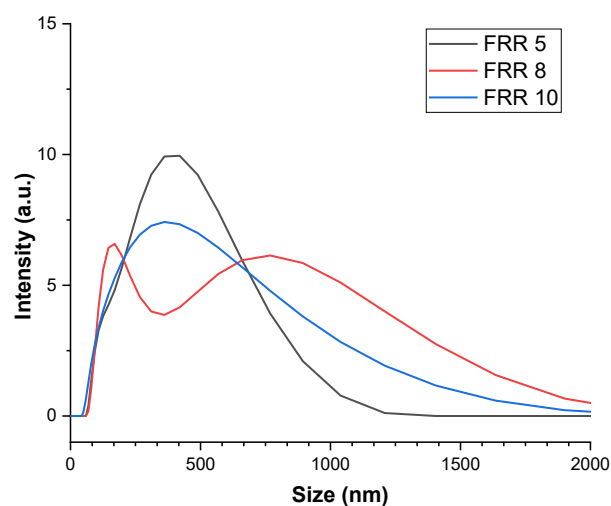


Figure 6.4) DLS of resultant populations formed from Novec MHF using 0.01 mg mL^{-1} DOPC with increasing FRR from 5 - 10 produced from $n = 1$ samples.

6.4 Novec VFF

6.4.1 Novec VFF application

Application of Novec VFF was first attempted using 0.1 mg mL^{-1} DOPC with FRR 1 and a TFR of 0.2 mL min^{-1} . To the ethanol and lipid stream, 20 vol% PBS was added this fraction was chosen to avoid preformation of lipid particles at the higher DOPC concentration (0.1 mg mL^{-1} , $127.2 \text{ }\mu\text{M}$). At $127.2 \text{ }\mu\text{M}$ DOPC the sample was above the DOPC cmc ($50 \text{ }\mu\text{M}^{226}$) in water although DLS analysis indicated the high ethanol content (80 vol%) was sufficient to prevent preformation of lipid particles. Lower FRR was applied based on the Novec MHF FRR sweep, which indicated lower FRR results in a reduces sample dispersity. The sample was manually aspirated from the two-phase device output and then diluted 1:10 with PBS before DLS analysis. The hypothetical lipid concentration within the sample was estimated to be approximately 20-fold higher than conventional VFF. The lipid concentration assumes lipid is insoluble in the Novec phase, however in practice lipid may be soluble in ethanol enriched Novec. Application of buffer containing lipid and Novec interfacial tension measurements could potentially shed light on lipid surface activity within the system. Once aspirated the final sample volume was estimated as $275 \text{ }\mu\text{L}$ using a Gilson pipette, $200 \text{ }\mu\text{L}$ was the expected sample volume as this was the quantity added to the central stream the measured sample volume indicated the sample contained $\approx 75 \text{ }\mu\text{L}$ ethanol.

When analysed by DLS, the sample was found to have a peak mean of 151.8 nm and FWHM of 47.9 nm (Figure 6.5). A PDI value of 0.034 was calculated for the sample, this was lower than any of the conditions explored within conventional VFF, for a DOPC sample. As the sample presented lower PDI with a peak mean below 200 nm , the Novec VFF could be applicable for therapeutic application. Conventional VFF at identical flow conditions produces 12 mL per hour of liposome suspension, with a 0.05 mg mL^{-1} DOPC concentration. With Novec VFF, when using the measured sample volume, the sample production rate is estimated to be 1.65 mL per hour of liposome suspension, with an approximate DOPC concentration of $\approx 0.36 \text{ mg mL}^{-1}$. The ability of Novec flow focusing to increase lipid concentration post flow-focusing could circumvent the need for batch-based concentrating steps such as centrifugal separation.²²⁷ To study the form of the produced particles and confirm their lamellarity, cryo transmission electron microscopy imaging was undertaken.

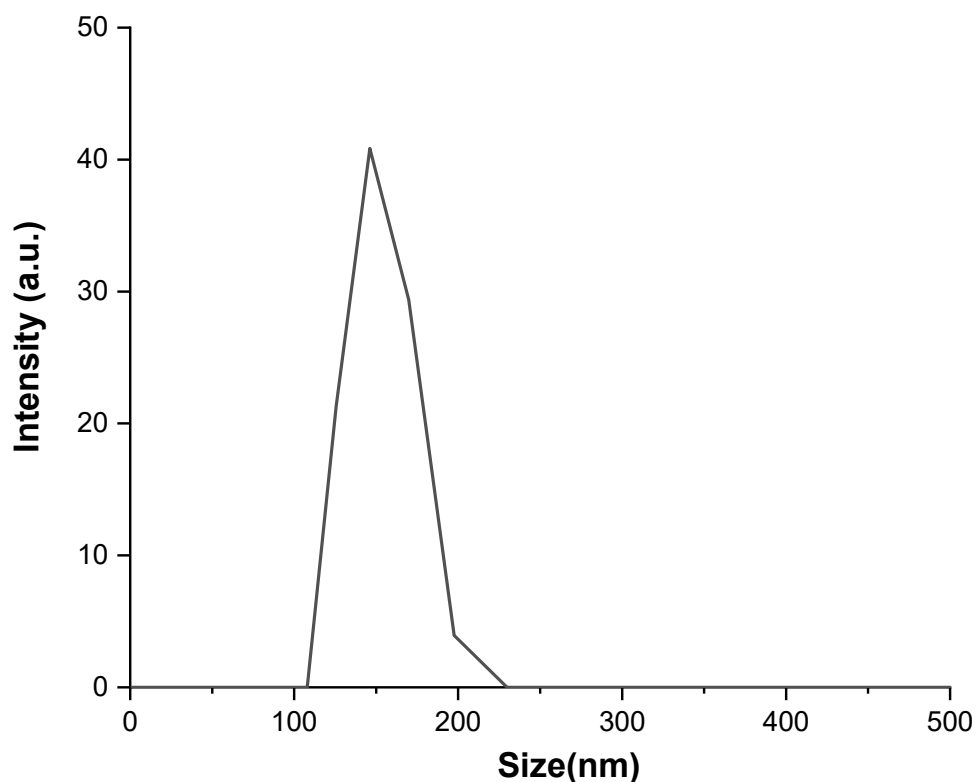


Figure 6.5) DLS of the sample produced by Novec VFF using 0.1 mg mL^{-1} DOPC in 80:20 Ethanol:PBS and a FRR 1 with TFR 0.2 mL min^{-1} $n = 1$ replicate.

The cryo-TEM imaging of the DLS characterised sample was undertaken less than 12 hours after sample generation and the sample was not dialyzed. Reduction of ethanol content by dialysis could result in typically smaller liposomes by lowering the swelling effect of ethanol discussed in Chapter 5.4.1. Cryo-TEM images suggested the structures formed were unilamellar liposomes, this is implied from contrast between the object centre and edge (Figure 6.6).¹⁹⁶ Unilamellarity is implied from the absence of concentric ring structures within the structures. The average estimated size of the liposomes was $148.13 \pm 48.48 \text{ nm}$, this value was within the DLS measured range. The cryo-TEM images confirms that Novec VFF likely forms liposomes that are suitable for drug delivery application. After studying the structure by EM, the impact of buffer content was evaluated for Novec VFF.

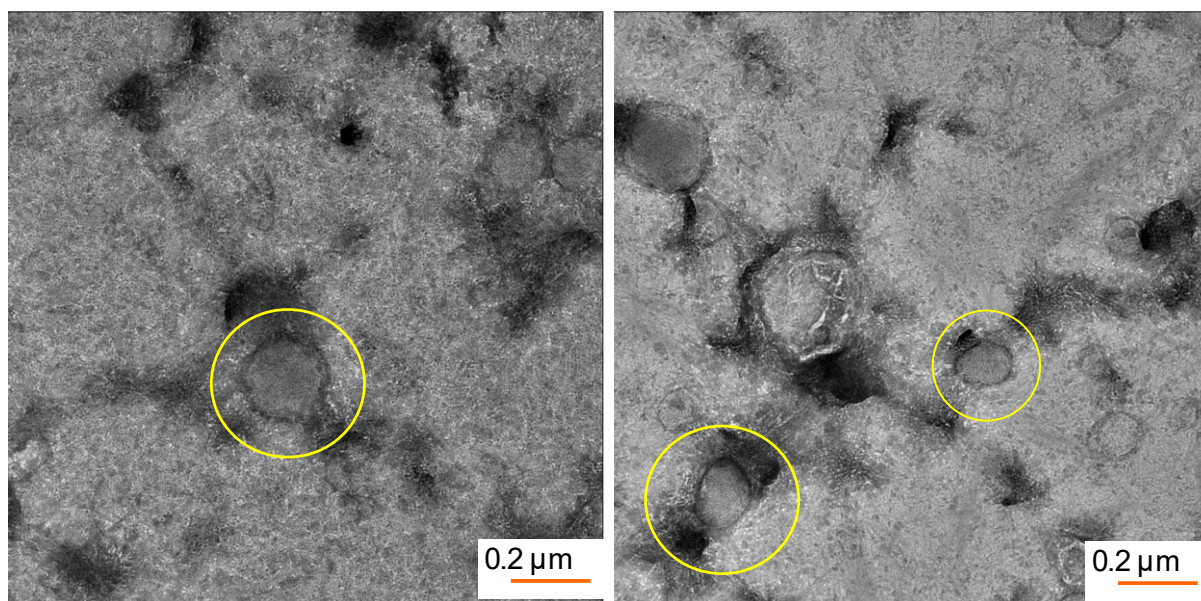


Figure 6.6) Cryo-TEM images of the sample produced by Novec VFF using 0.1 mg mL^{-1} DOPC in 80:20 Ethanol:PBS under a FRR 1 and TFR of 0.2 mL min^{-1} (Scale bars = $0.2 \text{ }\mu\text{m}$).

6.4.2 Buffer fraction in Novec VFF

To the central ethanol solution, 10 - 40 vol% PBS was added to establish the optimum buffer content for VFF (Figure 6.7). The PBS fraction was chosen to ensure preformation of vesicles would not occur prior to flow focusing and such that the concentrating effect of Novec flow-focusing was above 2-fold. Lower PBS fractions (< 10 vol%) were avoided as Novec volumes beyond the setup capacity (>12 mL) were required to achieve sufficient liposome sample volumes (> 200 μL). Samples were collected and diluted for DLS using the same method as in previous sections. Our data indicates as the buffer content was increased, mean diameter remained close to 280 nm from 10 – 30 vol% PBS, at 40 vol% PBS mean diameter decreased to 139 nm. Increasing PBS volume fraction was shown to decrease PDI from 10 - 20 vol% PBS although at 30 vol% PBS, PDI increased (Table 3).

At 40 vol% PBS, the higher PBS fraction resulted in a decrease in mean diameter this may suggest the reduction in the ethanol concentration gradient does not increase mean diameter and the concentration of lipid intermediates within the central stream determines resultant liposome size. At 40 vol% buffer, the central stream is likely broader reducing the concentration of lipid intermediates within the stream and by relation liposome size. In Chapter 5.4, ethanol impact was studied and within the bilayer could swell liposome size and increase PDI. Here, the samples produced with a higher PBS volume theoretically contain less ethanol, so could reasonably be expected to be smaller in size.

PDI values were lowest below 20 vol% PBS although the mean diameter exceeded 200 nm, which is unsuitable for therapeutic application. Within Novec 7100 VFF, formulation is likely key to the size control of resultant liposomes as Novec 7100 VFF results in larger liposomes than equivalent flow conditions within conventional VFF. Larger liposomes could result from a slow molecular diffusion of ethanol into the Novec phase. With the aim to reduce the size of resultant liposomes, varied FRR was explored within Novec VFF.

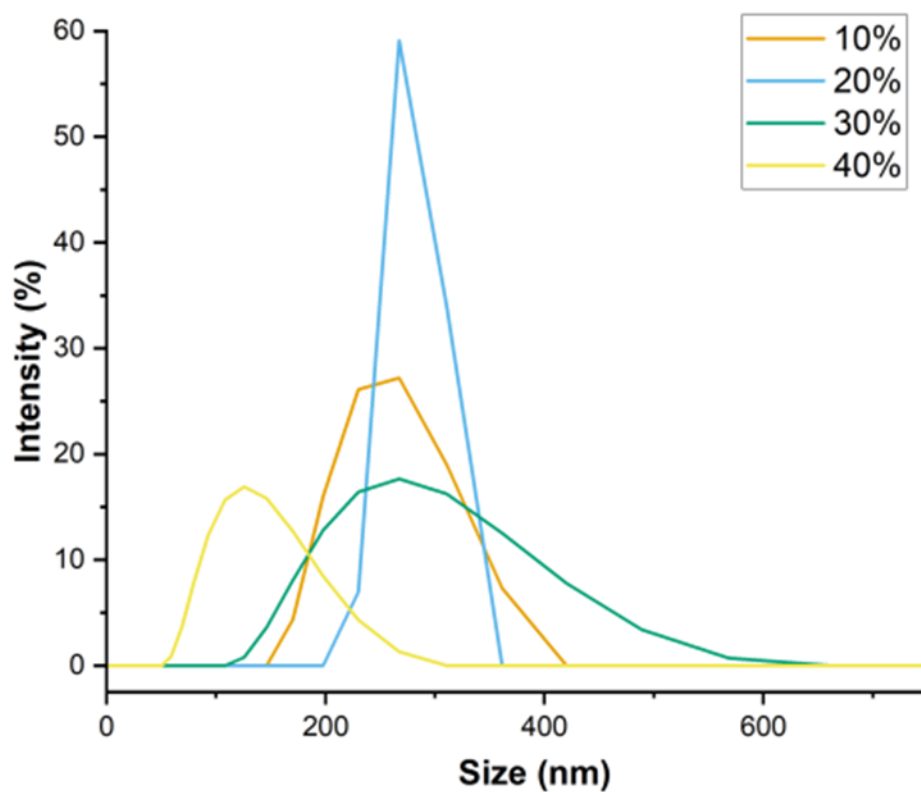


Figure 6.7) DLS distribution of samples containing 10 – 40 vol% PBS within the central ethanol stream. Samples were produced by Novec VFF using 0.1 mg mL^{-1} DOPC, using a FRR 1 and a TFR of 0.2 mL min^{-1} .

PBS content (vol%)	Mean diameter (nm)	FWHM (nm)	PDI
10	262	125	0.079
20	281	56.6	0.014
30	289	202	0.170
40	139	100	0.181

Table 3) Tabulated mean diameter, FWHM and corresponding PDI values for 10 – 40 vol% PBS within the central stream.

6.4.3 FRR sweep in Novec VFF

FRR was explored from FRR 0.5 - 5 for using 0.01 mg mL^{-1} and 0.1 mg mL^{-1} DOPC. A lower range was explored than used in Novec MHF, as preliminary data suggested lower FRR results in a lower PDI of resultant liposomes. A PBS fraction of 20 vol% was applied within the ethanol phase, this was shown to produce the lowest PDI distributions. Data points were comprised of $n = 3$ lipid in ethanol replicates, to provide appreciation for sample reproducibility within Novec VFF.

Initially a 0.01 mg mL^{-1} DOPC solution was used within the Novec VFF FRR sweep (Figure 6.8A). Liposomes at FRR 0.5 were 375 nm in diameter, at FRR 5 an increase in mean diameter to 440 nm was observed. The increase in mean diameter at FRR 5 was unexpected as conventional VFF mean diameter decreases at higher FRR, as demonstrated in Chapter 4.4. At FRR 0.5 the PDI was 0.114, PDI increased at higher FRR values, which was also observed in the Novec MHF FRR sweep. PDI likely increases with the increasing TFR applied within the set, at FRR 1 TFR was 0.2 mL min^{-1} and at FRR 5 TFR was 0.6 mL min^{-1} . This data set exhibits minimal liposome size control over the FRR explored.

For the higher 0.1 mg mL^{-1} DOPC concentration, FRR 0.5 produced liposomes with a 272 nm mean diameter (Figure 6.8B). At FRR 1 the mean diameter decreased to 226 nm and at FRR 5 mean diameter increased to 321 nm. Overall, the data suggested that FRR is independent of mean diameter within the range applied, a larger FRR range would help confirm any FRR trends. The average mean diameter decreased with the higher DOPC concentration, conversely to conventional VFF where higher lipid concentration typically leads to larger liposomes.¹³⁵ The opposite trend with lipid concentration suggests that the liposome formation mechanism within Novec hydrodynamic may differ from conventional hydrodynamic focusing. Adding greater quantities of lipid ($> 0.1 \text{ mg mL}^{-1}$) to the ethanol and PBS central phase has the potential to form smaller liposomes although higher lipid concentrations will require testing for preformation of vesicles before flow focusing.²²⁶

For 0.1 mg mL^{-1} PDI values increased with increasing FRR and TFR, similarly to samples produced from 0.01 mg mL^{-1} DOPC (Table 4). PDI values for 0.1 mg mL^{-1} DOPC were lower across the study, this suggests higher lipid concentration may be more favourable for reducing sample dispersity. When considering the hypothesized mechanism, lower PDI suggests fewer lipid intermediate fusion events occur as each could act to increase sample dispersity the dominant mechanism may be direct formation. An increased lipid concentration would increase the concentration of lipid in the central stream, this could increase the rate of lipid recruitment and favour direct formation, resulting in the low PDI observed. At 0.1 mg mL^{-1} lipid concentration, liposome assembly may occur without fusion of lipid intermediates an alternative could be the nucleation and closure of lipid structures. After exploring the FRR resultant distributions, a MADLS study was undertaken to determine the concentration of liposomes produced.

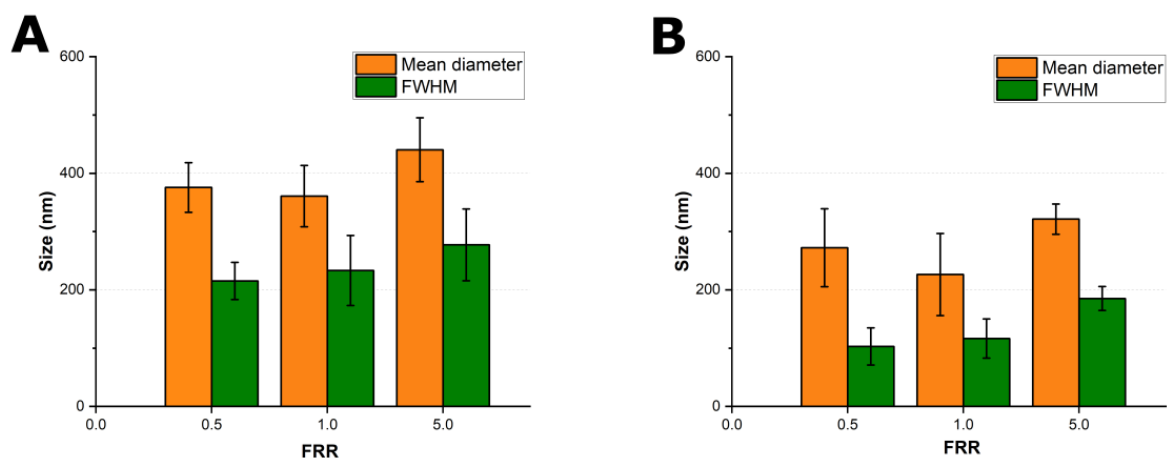


Figure 6.8) FRR sweep data produced using a **A** 0.01 mg mL⁻¹ DOPC and **B** 0.1 mg mL⁻¹ DOPC solution in 80:20 Ethanol:PBS as the central phase. A fixed ethanol stream flow rate of 0.1 mL min⁻¹ was applied with an increasing Novec flow rate to achieve higher FRR.

DOPC (mg/ml)	FRR	PDI
0.01	0.5	0.114
	1	0.145
	5	0.137
0.1	0.5	0.050
	1	0.092
	5	0.115

Table 4) Tabulated PDI values for increasing FRR at 0.01 mg mL⁻¹ DOPC and 0.1 mg mL⁻¹ DOPC within the ethanol phase.

6.4.4 Particle concentration in Novec VFF

Particle concentration was explored within conventional and Novec VFF using a 0.1 mg mL^{-1} DOPC solution. The conventional VFF sample applied a FRR of 5 and TFR 0.6 mL min^{-1} , these conditions were found to produce low dispersity and a therapeutically relevant diameter below 200 nm. Novec VFF was performed using 0.1 mg mL^{-1} DOPC containing 20 vol% PBS in the ethanol stream and a FRR of 1, with a TFR of 0.2 mL min^{-1} . The conditions applied within Novec VFF produced the lowest dispersity and mean diameter within Chapter 6.4.3, of the conditions explored. Samples were prepared for MADLS as described in Chapter 6.2.2. Samples A, B and C represent the 3 sample replicates derived from independent lipid films used to make the central phase.

The determined conventional VFF samples average mean diameter was $57 \pm 13 \text{ nm}$ with an average PDI of 0.023 ± 0.010 (Figure 6.9A). Sample mean diameter and PDI were both lower than in Chapter 4.4.1, the average mean diameter was 41 nm less than the previously determined value. Particle concentration was similar between samples A and C, the concentration of sample B was an order of magnitude larger (Figure 6.9C). Sample B presented a similar particle concentration to the CL samples within Chapter 5.3.1, the CL samples exhibited a similar mean diameter. The large disparity between samples may be a result of lipid loss to Novec, uncontrolled mixing conditions or lipid loss within the device.

Novec VFF resulted in an average mean diameter of $61 \pm 41 \text{ nm}$ with an average PDI of 0.042 ± 0.020 (Sample 6.9B). Sample PDI was the lowest recorded for Novec VFF, sample C exhibited a bimodal distribution and the reported PDI is for 200 nm population. Particle concentration was similar for Novec samples B and C although sample A was an order of magnitude lower (Figure 6.9D). Sample A was expected to exhibit a higher concentration than sample C, as it exhibits a bimodal distribution containing larger particles, this result could suggest lipid loss to the Novec phase. The particle concentration data suggests Novec VFF may produce higher liposome concentrations ($1.31 \pm 1.83 \times 10^{11} \text{ particles mL}^{-1}$) than conventional VFF ($3.75 \pm 2.99 \times 10^{10} \text{ particles mL}^{-1}$), large error (1 SD) in the study meant firm conclusions were not made (Table 5). When compared with the calculated particle concentration derived from the estimated lipid concentration, samples were within an order of magnitude of the calculations. Notably the bimodal Novec sample exhibited the farthest deviation from the calculation, as previously mentioned the calculation is a poor estimate for multimodal samples. The closeness of the calculations to the MADLS values could suggest minimal lipid loss occurs within Novec flow focusing.

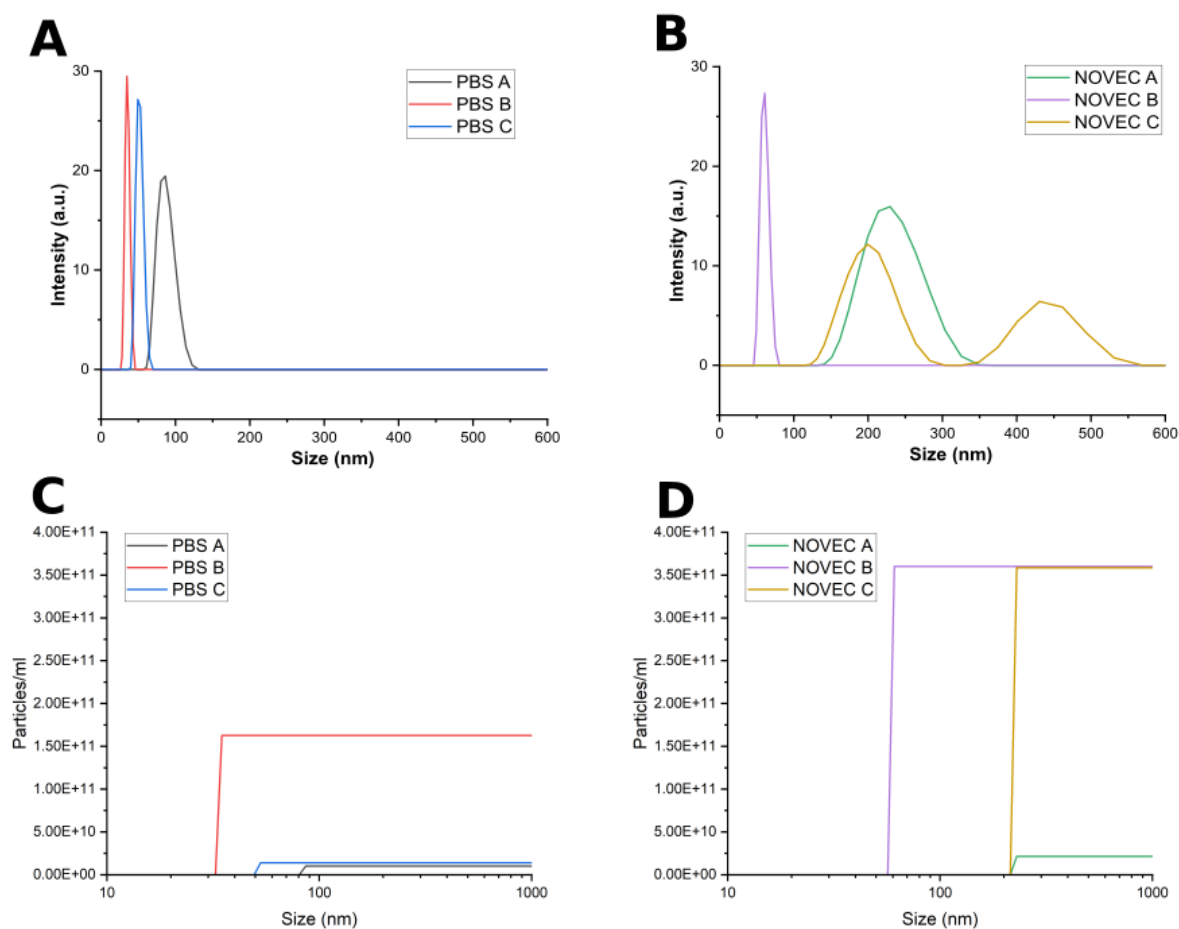


Figure 6.9) DLS particle distribution for **A** Conventional VFF and **B** Novec VFF with corresponding sample MADLS particle concentrations for **C** Conventional VFF and **D** Novec VFF.

VFF	Mean diameter (nm)	FWHM (nm)	PDI	MADLS Particles ($\times 10^{11}/\text{ml}$)	Calculated Particles ($\times 10^{11}/\text{ml}$)
PBS A	86	28	0.035	0.102	0.109
PBS B	35	8	0.017	1.63	0.699
PBS C	51	12	0.018	0.140	0.319
Novec A	231	91	0.054	0.215	0.221
Novec B	60	14	0.019	3.60	3.42
Novec C	201	78	0.053	3.59	0.292

Table 5) Tabulated mean diameter, FWHM, PDI and particle concentration for each conventional VFF and Novec VFF sample. PDI for unrepresentative multimodal distributions are highlighted in red. Particle concentrations were calculated using the approach in Chapter 5.3.1 for comparison with the MADLS measured values.

6.5 Conclusion and future work

6.5.1 Conclusion

Within this chapter an adaptation of MHF using Novec engineered fluid was established. Preliminary data suggested Novec MHF was producing detectable amounts of particles. Novec MHF distribution optimisation was attempted by varying buffer content in the solvent stream as well as FRR. The resultant distributions remained large and bimodal. In its current state Novec 7100 MHF would be ineffective at producing therapeutically relevant liposomes, this may stem from incompatible surface chemistry and PDMS incompatibility with Novec.

After transferring to Novec VFF, lower mean diameter and PDI distributions were observed, when compared with Novec MHF. Cryo-TEM was used to image the < 200 nm particles, they were thought to be liposomal based on the observation of thick outer edges. Novec VFF was optimised through buffer content, lipid concentration and FRR. A buffer content of 20 vol% was shown to result in the lowest PDI, the low buffer content results in a favourable higher resultant lipid concentration. FRR exhibited minimal impact in the range explored, suggesting Novec VFF may differ mechanistically from conventional VFF. When lipid concentration was increased, mean diameter of liposomes reduced, the opposite of the trend observed in conventional VFF.

Finally, the MADLS technique was used to characterise and compare liposome concentrations at optimal flow condition for conventional VFF and Novec VFF. The data suggested Novec VFF may produce higher liposome concentrations when compared to conventional VFF using an identical lipid concentration within the ethanol phase. A drawback of Novec VFF is the lower liposome production, although the higher liposome concentration could subvert the need for batch-based concentrating methods and retain continuous liposome production.

6.5.2 Future work

Disparity between Novec samples implies further work is required to improve the efficacy and reliability of Novec VFF, for it to see wider application. Application of glass syringes and Novec compatible tubing could improve the practical setup of the device. Characterisation of Novec interfacial tension with aqueous DOPC solution could determine whether DOPC is lost to the Novec phase. Exploration into residual ethanol content could elude the mixing efficacy, as the residual ethanol may be high resulting from the slower diffusion of ethanol into Novec. Further lipid concentration exploration, Novec variant implementation and FRR study could further improve resultant distributions for therapeutic application. Exploration into lower viscosity Novec could be investigated with the aim to reduce liposome diameter.⁵⁹

7 Design and evolution of tissue-on-chip devices

7.1 Overview

TOC and OOC devices are typically highly specialized and can be designed to meet a suite of applications. In this chapter, the development of a reductionist TOC device for the study of drug delivery liposomes, in flow. The optimisation and design choices for the development of the PDMS TOC device are then presented, with a focus on the seeding methodology and surface chemistry. A key advantage over conventional TC is the ability to incorporate *in vivo* topography, attempting to achieve this using a TOC device and present further optimisation. TOC devices were successfully fabricated and seeded with H9C2 cells, and present preliminary surface optimisation for H9C2 adhesion under flow. When scaling to 3D tissue constructs, more accurate representation of *in vivo* tissues can be achieved through the implementation of a model ECM. Finally, the generation of 3D bio-printed tissue constructs for the testing of liposome efficacy and with promise for experimental scalability is presented. Bio-printed culture incorporates an ECM and the study of liposomal transport through ECM is potentially critical to understanding *in vivo* therapeutic efficacy.

7.2 Tissue-on-chip design

7.2.1 Designing a tissue-on-chip for drug efficacy testing

Within this chapter TOC were designed as models for testing the success and efficacy of drug delivery liposomes. TOC designs can allow for advances on conventional tissue culture with improved biomimicry and control over the biophysical environment. To facilitate study, a TOC device must include optical clarity, biocompatibility, and gas permeability.^{96,98,114,228} An effective TOC model for drug delivery application should be scalable, offer sufficient cell capacity and longevity. Cell capacity and scalability enable meaningful data collection by providing larger sample sizes for data acquisition.^{4,44} Cell longevity is important for achieving cell maturation and studying physiologically relevant timescales of drug dosage.^{4,44,110,229–232} Finally, the device should minimally absorb the therapeutic liposomes, this ensures dosage concentrations and delivery kinetics remain accurate. TOC devices within this chapter are discussed and analysed based on their ability to meet these criteria.

Models within this chapter were designed for the housing of H9C2 cells, a clonal line of rat cardiomyocyte cells.²³³ H9C2 cells are advantageous to TOC development as they are a proliferative cardiomyocyte line, this allows large-scale culturing practices and seeding below confluency within TOC models. A series of toxicity studies have been performed using H9C2 cells and they have been shown to exhibit similar physiological responses to primary human cardiomyocytes, this retains their relevance for testing liposomal drug delivery systems.^{233–236} Application of H9C2 cell to heart-on-chip devices has been reported, indicating their suitability for TOC devices.²³⁷ The use of primary or human iPSC cells to succeed H9C2 usage as cells with greater clinical significance. On a basis of time and cost limitations, purely H9C2 cells were implemented, although TOC designs were intended to remain functionable for primary and iPSC derived cultures.

7.2.2 Fabrication of Tissue-on-chip device

The first TOC design was made entirely in PDMS using soft lithography methods described in Chapter 2.2.1. The design was comprised of a sealed channel, with 1 mm biopsy punched inlet and outlet holes (Figure 7.2A). TOC models were made from a channel segment and base, the channel segment was cast directly on the wafer face and the base segment was 0.2 mm thick level cured PDMS. For the grooved TOC device, the base was produced from a wafer cast and applied PDMS was weighed to achieve 0.2 mm thickness. Prior to plasma treatment, scotch tape was applied and removed to clean the bonding surface, plasma treatment was applied (Harrick, 18 W, air O₂, 2 minutes exposure). The plasma treated PDMS pieces were gently pressed together in a hinge motion to minimise trapping of air bubbles (Figure 7.1). The sealed pieces were then gently depressed to ensure contact and displace further air bubbles. Surface treatment was applied immediately after sealing as the surface was activated, this was achieved using a syringe and needle to flow directly into the inlet.

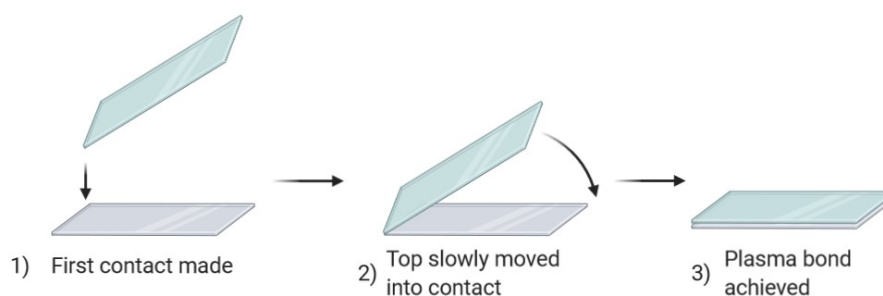


Figure 7.1) Procedure to bond PDMS while minimising air trapping.

The base thickness was chosen to enable clear imaging of the device when using inverted microscopes and ensure gas permeability of the device, a critical property for maintaining cell culture. The channel shape was chosen to achieve a 0.2 cm^2 seeding area within the rectangular segment, at confluence this has an approximate capacity of 5000 H9C2 cells (Figure 7.2C). The seeding area (0.2 cm^2) is a close match to the area used for well plate assays (0.25 cm^2 , Falcon 96 well half-area) to ensure sufficient cells were contained to produce statistically significant TOC replicates. The larger surface area was chosen to account for the likely lower confluency achieved with TOC, arising from the increased cell handling stages, larger surface areas would reduce the benefit of miniaturisation and scalability for the device. The wide channel dimension was chosen to achieve low velocity at the channel edges, reducing shear rate exposure to seeded cells this is an inherent property of cell cultures maintained under flowing media and can be utilised as a biophysical control aiding replication of certain disease states.^{122,182,236} With a channel depth of 0.27 mm , the total channel volume was $8 \mu\text{L}$ (Figure 7.2B). The channel volume was chosen to miniaturise the overall system such that a 1 mL syringe of media could sustain the device for several days. An experimental setup was then designed to perfuse the device with media, low volume devices require a flow of media to replenish nutrients and facilitate cell growth.

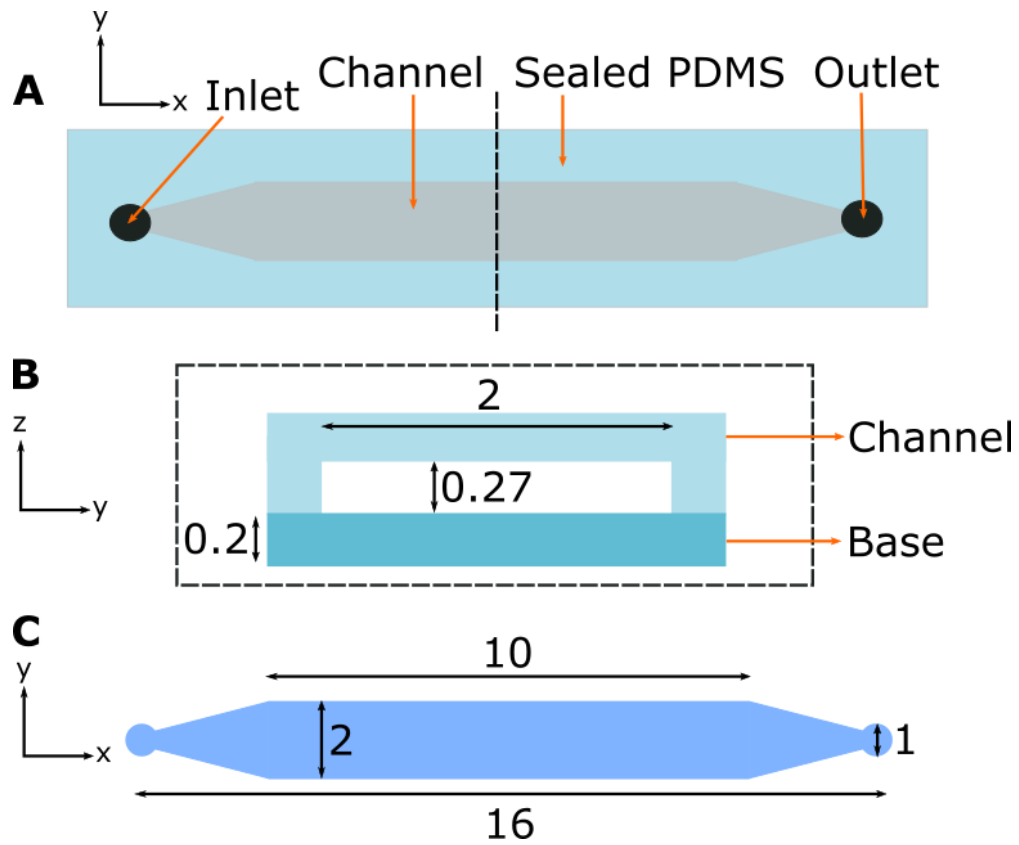


Figure 7.2) Schematics of tissue-on-chip design with reference axes shown left, dimensions are in mm. **A** Bird's eye view of the PDMS design **B** Central cross-section of the channel **C** Dimensions of channel area.

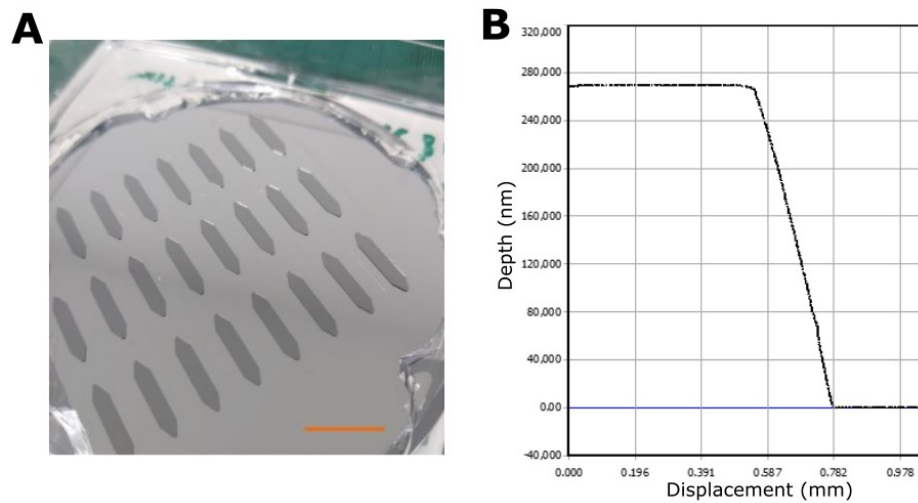


Figure 7.3) **A** Image of wafer used to cast the channel structure segments (Scale bar = 10 mm) **B** Profilometry scan of channel structure.

7.3 Tissue-on-chip optimisation

7.3.1 Development of biocompatible PDMS coating

Compatibility of surfaces was explored using 10 mm diameter PDMS inserts adhered into falcon 12 well plates. Inserts were adhered using pipetted PDMS (10:1 silicone : curing agent), this prevented detachment of inserts during seeding and imaging. Native PDMS, collagen-1 and fibronectin coated PDMS were explored within this section. Fibronectin is abundant within the myocardium and col-1 is a highly abundant structural protein within the body, both see application as biocompatible surface coatings.^{238–242} The collagen surface treatment contained 50 $\mu\text{g mL}^{-1}$ type 1 collagen (col-1), which was prepared by dilution with sterile de-ionized water (Invitrogen) and correction to pH 7.2 with glacial acetic acid (Sigma Aldrich).²⁴³ The fibronectin surface treatment was prepared analogously, using a half the concentration of the col-1 solution (25 $\mu\text{g mL}^{-1}$) as fibronectin units are approximately double the molecular weight of col-1, this retains a similar protein coverage.^{125,147,243} Collagen and fibronectin treatments were applied directly to the surface and incubated for 12 hours at 4°C. After incubation, the surfaces were washed with sterile DIW and left in a cell hood to dry. When dry, PDMS discs were subjected to UV-C sterilisation for 45 minutes, prior to cell seeding. Inserts were seeded with 2 mL of a 1×10^5 cells mL^{-1} solution of H9C2 cells in DMEM, this was left for 12 hours before imaging. The water CA was measured to be $102.6 \pm 4.4^\circ$ and $68.9 \pm 7.3^\circ$ for the fibronectin and col-1 surfaces respectively ($n > 24$ samples, Supplementary S12.4).

In its native state PDMS was expected to show low cell adhesion, as it is the least biocompatible surface. After seeding the un-treated PDMS insert, imaging was undertaken using a DIC microscopy to visualize cells. The native PDMS insert showed low rates of cell adhesion, cells are the elongated structures presented (Figure 7.4A). As H9C2 cells adhere they became elongated, this differentiates cells from the air bubbles present within the PDMS used to adhere the insert. PDMS coated with fibrinogen exhibited higher confluency than native PDMS, cells were distributed evenly across the surface which implies the treatment was effective (Figure 7.4B). When coated with col-1, high confluency areas were observed, suggesting the surface treatment was successful (Figure 7.4C). Observation of high confluency regions implies the cells were proliferating, within the 12-hour experiment. Surfaces that encourage proliferation are favourable for TOC devices, proliferation improves upon seeding density by increasing cell coverage. In this study, col-1 was considered the most suitable surface for the TOC device. The myocardium ECM is predominantly comprised of type 4 collagen (col-4) and laminin, col-1 structural similarities to col-4 may aid adhesion and proliferation of H9C2 cells.¹⁰⁴ Col-1 was then employed within the TOC to explore seeding efficacy.

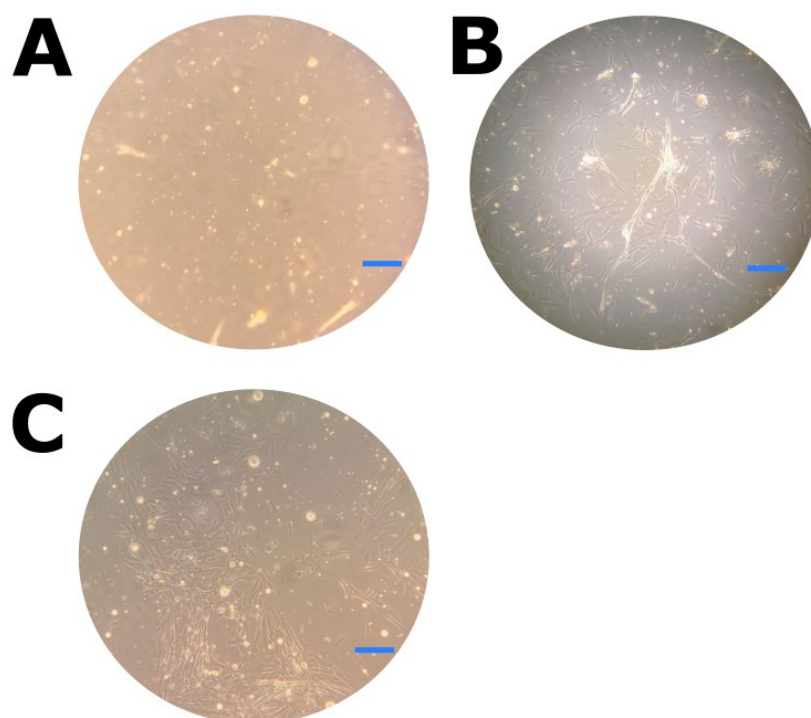


Figure 7.4) DIC images of resultant H9C2 cell densities imaged at the centre of PDMS inserts that were **A** Untreated (Native) **B** Fibrinogen coated and **C** Col-1 coated. (Scale bar = 0.2 mm)

7.3.2 Seeding of Tissue-on-chip

TOC devices were prepared as outlined in Chapter 2.2.8. After plasma bonding, coating solutions were introduced into the TOC devices and left for 12 hours to adhere at 4°C. Two coatings were employed within this Chapter, the col-1 direct coating and PVA coating. The PVA coating solution was 0.5 wt% PVA (Sigma 87 - 90% hydrolysed 30 - 70 kDa) in 50:50 IPA:DIW. The PVA coating was developed from work by Trantidou *et al*¹³⁷ and characterization of this surface is shown in Chapter 3.2.5. PVA is a hydrophilic modification, its application for cell adhesion in this work was hypothesized to offer several advantages over col-1. PVA coating subverts the need for proteins and the high temperature stability of PVA ($\approx 200^{\circ}\text{C}$) could allow treated PDMS to be sterilised by autoclaving (120 - 140°C). Use of synthetically derived coating could also improve coating reliability and reproducibility, by avoiding animal extracted products.

Once coated, the models were washed in sterile DIW and sterilised by UV-C exposure for 45 minutes. Sterilised models were purged with DMEM using a Gilson p100, a segment of silicone tubing was used to mate the p100 tip with the metal hub of the device. Models were seeded using a H9C2 suspension containing 1×10^6 cells mL^{-1} , this was added using a Gilson p100 and cells were left for 20 minutes to adhere. Adherence time was chosen to minimize the time out of media perfusion whilst facilitating adherence, which was confirmed by microscopy. After adhesion, excess cell suspension was displaced using DMEM, to avoid bubble accumulation in the device and remove unadhered cells. The device was attached to the syringe pump as described in Chapter 2.4.7 and perfused overnight at a flow rate of $1 \mu\text{L min}^{-1}$. At $1 \mu\text{L min}^{-1}$ the channel media volume was replenished every 8 minutes and a total volume of 720 μL of media was required (for a 12-hour overnight incubation period).

The centre of the device was imaged to visualize cell density within each coated device (Figure 7.5). The col-1 coating demonstrated a lower resultant seeding density, despite the higher seeding density (4.0×10^4 cells cm^{-2}) than that applied used for cell passaging Chapter 2.5.1 (2.0×10^4 cells cm^{-2}). A higher seeding density was applied for the TOC device as the seeding process was expected to result in lower cell viability where cells typically spent 10-15 minutes in suspension during the seeding process. The low confluence within the col-1 device was thought to be a product of the seeding process, and the shear driven detachment of cells. Despite the low perfusion flow rate, cells on the col-1 surface were prone to detachment, this was confirmed by observing the device under flow. Cell detachment implies cells were weakly adhered and that the col-1 surface is potentially labile under flow.

The PVA surface yielded visibly high cell density in comparison to the col-1 surface. PVA is widely applied as a 3D biopolymer to create porous scaffolds or bases to facilitate cell incorporation.^{244–246} PVA use in 3D scaffolds has been reported in conjunction with H9C2 cells, implying its suitability for TOC models utilising these cells.²⁴⁵ Application as a biocompatible PDMS coating for seeding H9C2 cells has not yet been reported. This study demonstrated preliminary data for the efficacy of PVA coatings within TOC devices where PVA was shown to facilitate adhesion for H9C2 cells overnight, under the perfusion of media (Figure 7.5). The PVA coated model exhibited areas of high cell density after the 12-hour period in which clustering of cells occurred, this implied cells were proliferating. With established surface treatments, the TOC model was adapted to further increase the biomimicry.

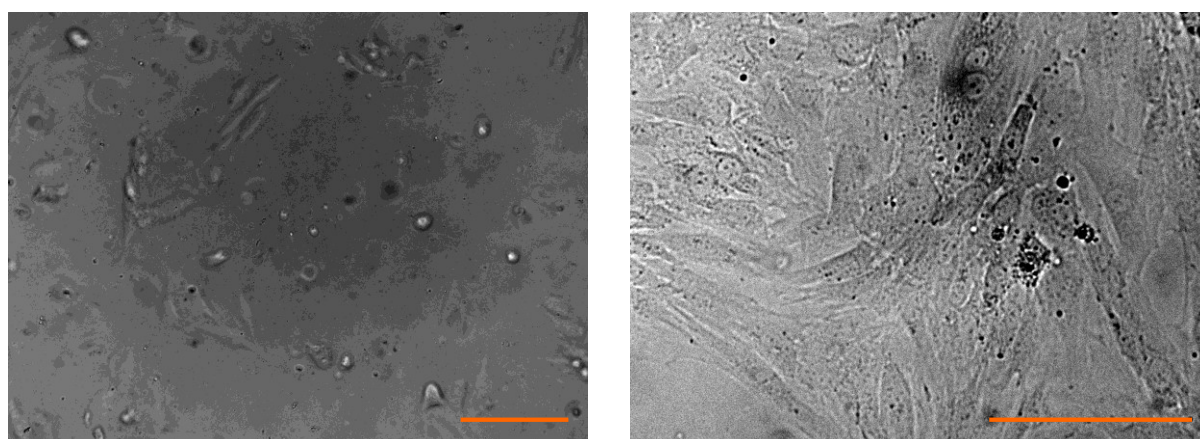


Figure 7.5) DIC images of the TOC channel centre after 12 hours incubation, showing the adherence of H9C2 cells following seeding at 4.0×10^4 cells cm^{-2} within a (left) Col-1 coated channel and (right) PVA coated channel (Scale bars = 0.1 mm).

7.4 Grooved tissue-on-chip fabrication and optimisation

7.4.1 Development of grooved Tissue-on-chip

Cardiomyocytes *in vivo* exist within long filament structures, where within the filament cardiomyocytes are elongated and axially aligned.^{97,247,248} To recreate myofilaments in a reductionist 2D model, microgrooves from work by Morez *et al*²⁴⁹ were integrated within our TOC design. Morez *et al*²⁴⁹ implemented PDMS based micro-grooves *in-vitro*, to enhance differentiation of cardiac progenitors into cardiomyocyte-like cells. The ability to achieve enhanced differentiation

demonstrates cardiomyocyte sensitivity to topographical cues. By recapitulating this within our TOC device, the TOC could better represent *in vivo* behaviour.^{249,250}

The channel structure from the TOC device was left unchanged, however within the base parallel micro-grooves were incorporated in the rectangular channel section (Figure 7.6A). The grooved TOC was comprised of a channel structure segment and the grooved base, both segments were cast from wafer designs (Figure 7.6B). The device was sealed using plasma bonding, alignment of the grooved structure was achieved manually. The grooves ($20\ \mu\text{m} \times 50\ \mu\text{m} \times 10\ \text{mm}$, width $\times$ depth $\times$ length) ran parallel to the intended flow direction and were designed to house H9C2 cells which were measured to be $18.3 \pm 0.608\ \mu\text{m}$ ($n = 6$ samples, containing > 1000 cells) by flow cytometry (Supplementary S12.3). The $20\ \mu\text{m}$ grooves were chosen to house the H9C2 cells and encourage longitudinal growth, parallel to the groove (Figure 7.6C).

The wafers used to generate the grooved structure were made by an adapted SU8 baking and development procedure (Figure 7.7A). The grooved wafer fabrication was optimised to prevent thermal shock, which led to warping and detachment of the $50\ \mu\text{m}$ deep SU8 structure (Chapter 2.2.1). Patterned wafers remained intact for around 30 uses, after which SU8 structure began to detach. The wafer applied within this section was measured to have an SU8 depth of $51\ \mu\text{m}$, by profilometry (Figure 7.7C).

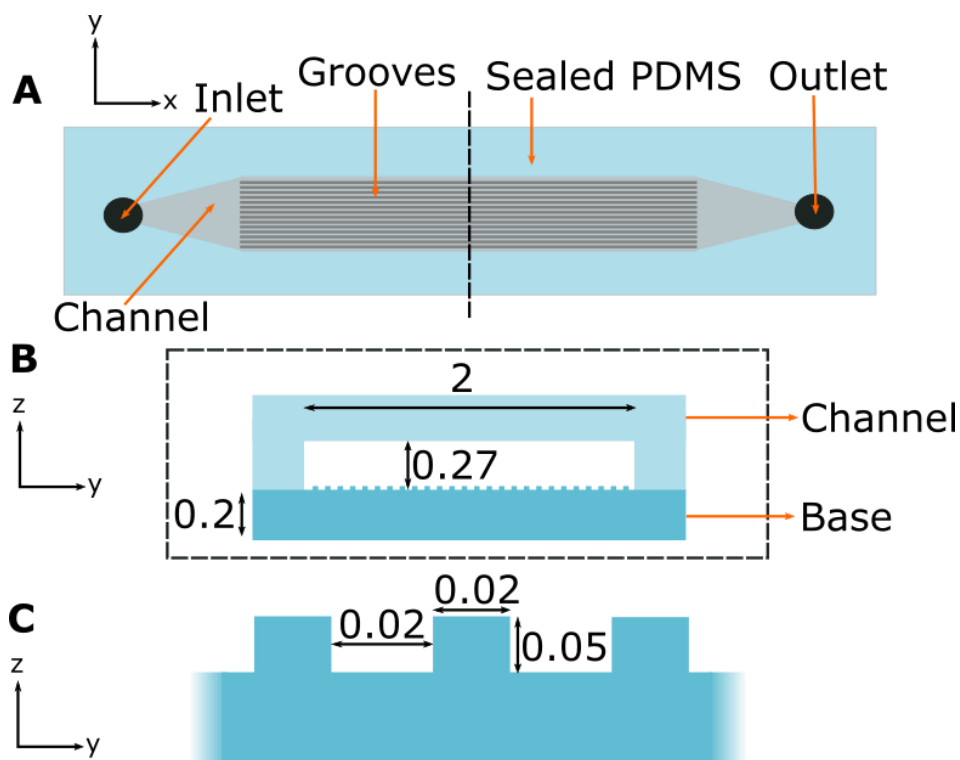


Figure 7.6) Schematics of the grooved tissue-on-chip design with reference axes shown left, dimensions are in mm. **A** Bird's eye view of the grooved PDMS design **B** Central cross-sectional of the channel, with grooves visible in the base of the channel **C** Magnified view of the grooved structure in the channel base.

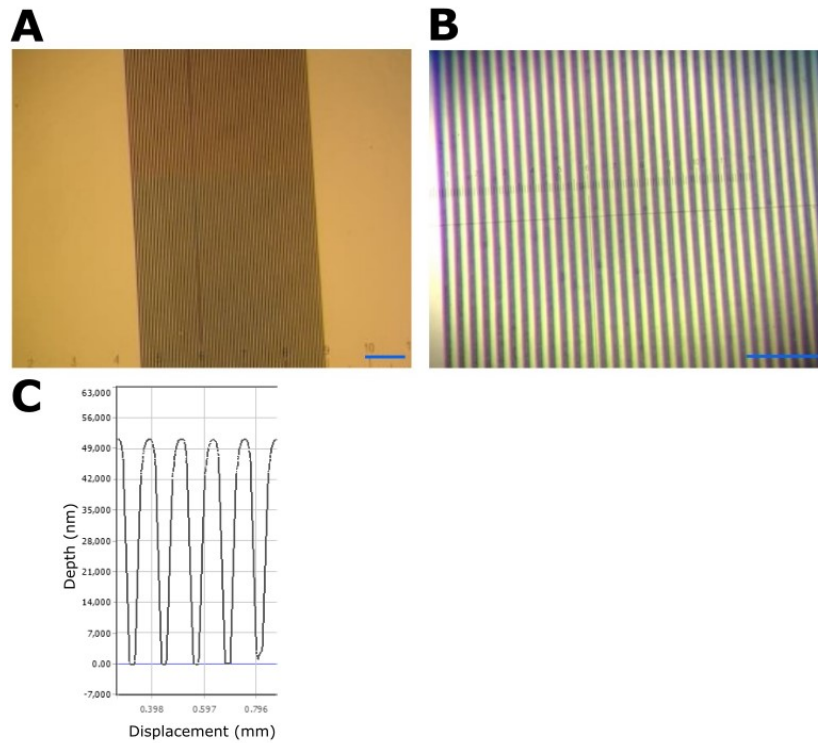


Figure 7.7) Images of grooved structure, scale bar = 0.5 mm **A** Brightfield image at 5x magnification **B** Brightfield image at 10x magnification **C** Profilometry of grooved structure.

7.4.2 Optimization of seeding

When the device was seeded by the method outlined in Chapter 7.3.4, accumulation of cells at the channel taper and edge was observed (Figure 7.8). Accumulation of cells was thought to be caused by using manual introduction of the cell suspension into the TOC device. An alternative seeding method using applied flow from a microfluidic pump was explored. Use of a microfluidic pump allows defined seeding flow rates at the cost of a slower seeding process.

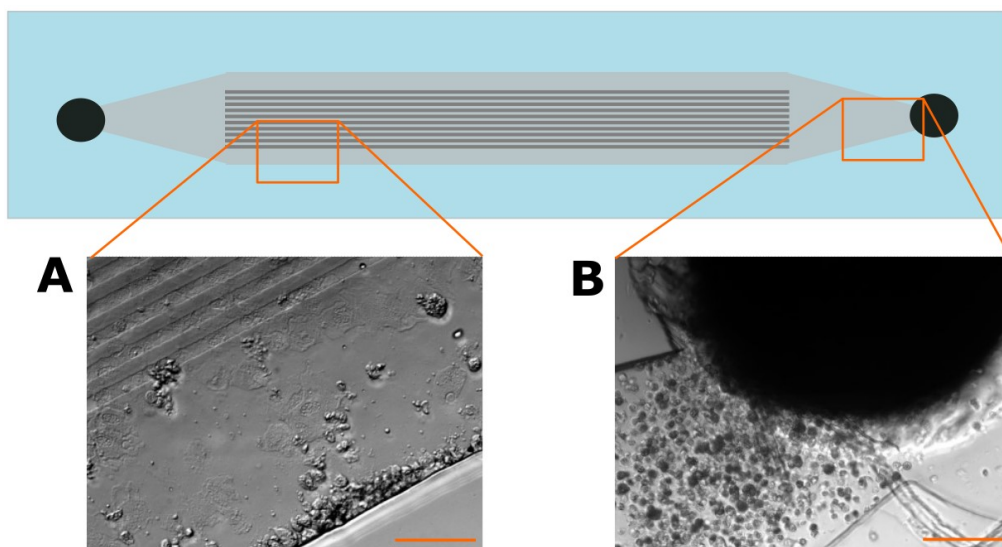


Figure 7.8) Imaged seeding imperfections from the manual seeding process **A**) Accumulation of cells at the channel edge **B**) Accumulation of cells near the channel inlet (Scale bar = 0.1 mm).

To achieve controlled seeding, H9C2 cell suspensions (1×10^5 cells mL^{-1}) were added to a 1 mL syringe and then connected to the TOC using silicone tubing. Devices were imaged when under flow to determine an effective flow rate for seeding the TOC devices. Initially a flow rate of $1 \mu\text{L min}^{-1}$ was applied, at this flow rate cells were distributed across the channel width during flow. At $10 \mu\text{L min}^{-1}$, cells underwent flow focusing and were predominantly near the channel centre (Figure 7.9). This data set implied the use of lower flow rate is favourable to achieve even seeding distributions within TOC devices. Lower flow rates have the additional benefit of reducing the shear induced damage on cells under flow.²⁵¹ At both applied flow rates accumulation at the device edge and taper was prevented suggesting the accumulation was driven by high flow rate, manual seeding ($> 10 \mu\text{L min}^{-1}$). At $1 \mu\text{L min}^{-1}$ the TOC device could be filled in 8 minutes; lower flow rates increase the time cells spent in suspension, which could prove detrimental to cell viability. The cell concentration within the seeding solution was then increased to 1×10^6 cells mL^{-1} , at this concentration the seeding density was sufficiently high. The ability to produce TOC devices with comparable cell densities is essential for the reproducibility of subsequent experiments (Figure 7.10). With a seeding procedure developed, two surface coatings were explored within the grooved TOC device.

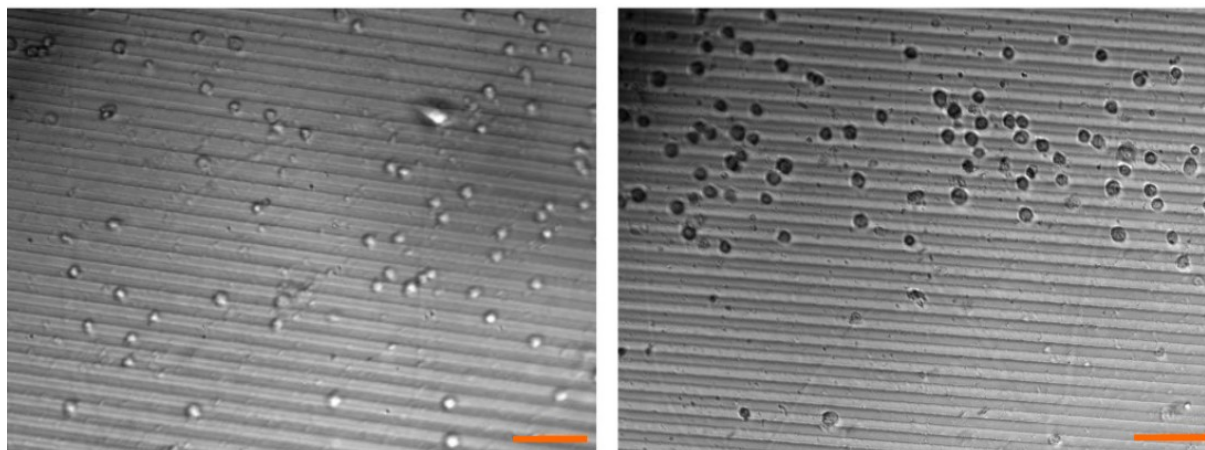


Figure 7.9) Images of seeding under flow using a 1×10^5 cells mL^{-1} seeding solution at **(Left)** $1 \mu\text{L min}^{-1}$ and **(Right)** $10 \mu\text{L min}^{-1}$ (Scale bar = 0.1 mm).

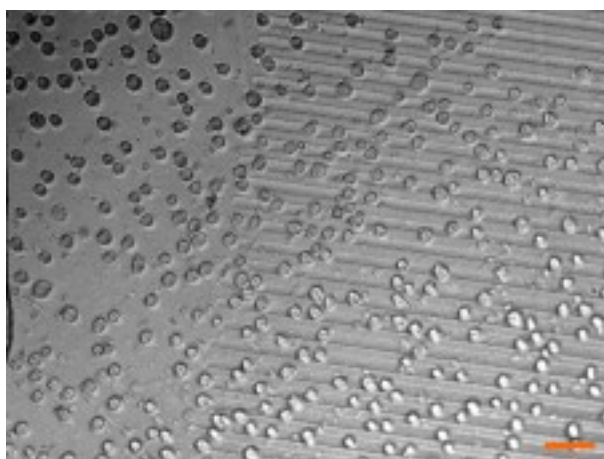


Figure 7.10) Image of seeding under flow at $1 \mu\text{L min}^{-1}$ using a 1×10^6 cells mL^{-1} seeding solution (Scale bar = 0.1 mm).

7.4.3 Seeded grooved device

The col-1 surface treatment was first employed within the grooved TOC device; seeding was undertaken using the seeding in flow method developed in Chapter 7.4.2. In the preliminary data set, cells were observed to both align with the grooves and adhere while remaining circular (Figure 7.11). Seeding density was even across the highest and lowest sections of the grooves, this suggests the effective seeding area is unaffected by addition of grooves. Retaining the seeding area of the non-grooved TOC device is essential to retain the intended cell capacity. Higher magnification images suggested cells could adhere across grooves, when in a non-elongated form.

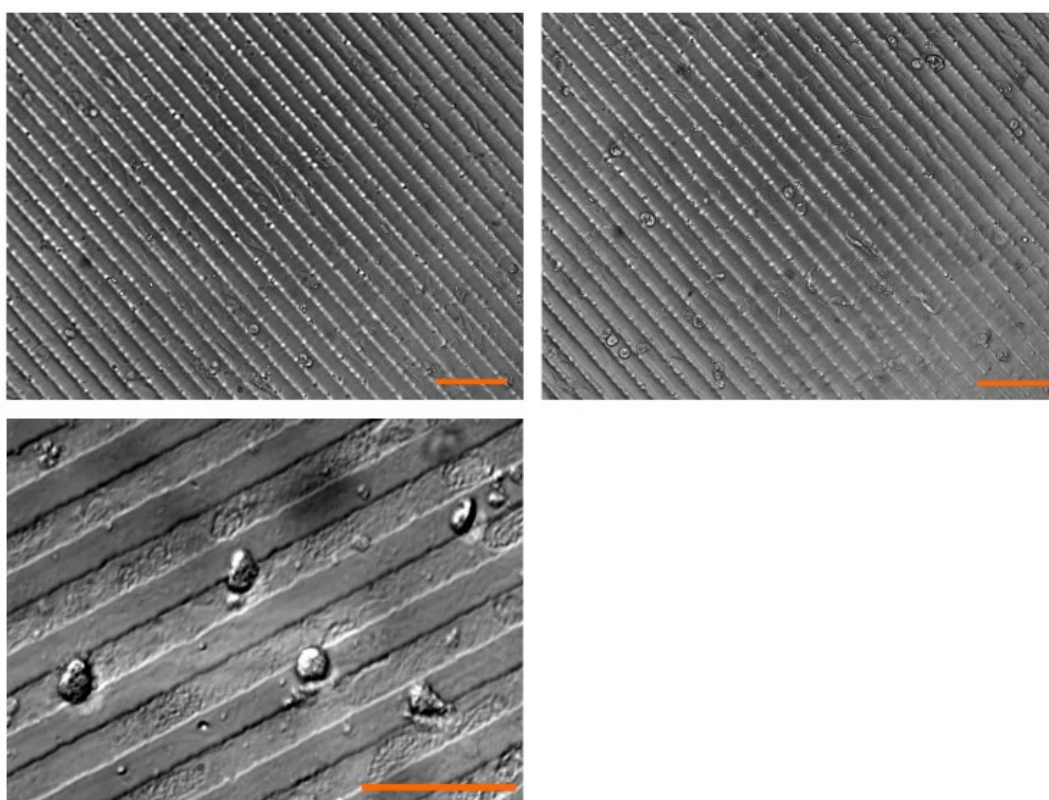


Figure 7.11) DIC microscopy images of H9C2 seeded grooved, TOC device coated with col-1. Cells are shown both confined and atop the grooved structures and under higher magnification were shown to adhere across the groove structures (Scale bars = 0.1 mm).

When compared to the col-1 surface, PVA coated microgrooves resulted in a higher confluence and alignment (Figure 7.12). Within the grooves cells aligned parallel to flow, spatial confinement suggests the cell diameter was close to the groove size. Cells were observed in a non-elongated state, similarly to the col-1 device, this could suggest local surface imperfections or shear driven cell damage. The preliminary microscopy data suggests PVA is effective at facilitating cell adhesion within TOC devices (Figure 7.12). The resultant cell adhesion appeared to be below the initial seeding concentration, it is likely cells were detaching during media perfusion. Detachment was thought to arise from the direct coating application, as both col-1 and PVA are soluble in media. A covalently linked surface treatment was next explored to reduce cell detachment during perfusion.

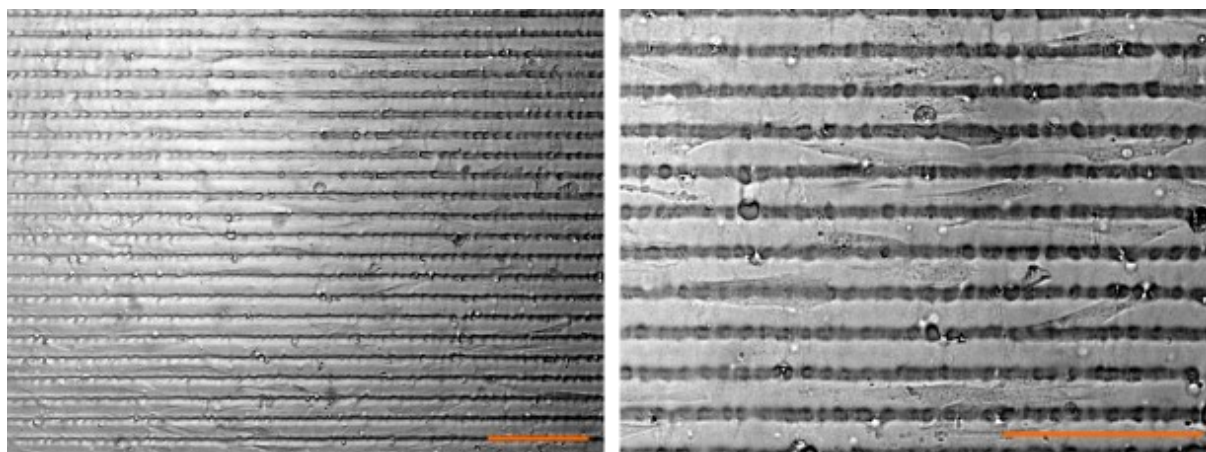


Figure 7.12) DIC microscopy images of H9C2 seeded, grooved TOC device coated with PVA. Where H9C2 cells are shown to align within the grooved structure (dark grey), running parallel (Scale bars = 0.1 mm).

7.4.4 Development of covalent biocompatible treatment

Work by Gunda *et al*¹²⁵ was adapted to produce two surfaces with covalently linked col-1. The first was linked to PDMS by an APTES linker, APTES treatment was applied post-plasma exposure using the procedure from Chapter 2.4.3. APTES application was optimized to reduce formation of polymerized APTES, which resulted in clouded PDMS.²⁵² After APTES functionalisation, the surface was left in contact with a 50 $\mu\text{g mL}^{-1}$ col-1 solution used in Chapter 7.3.3 and left for 2 hours at 4°C to adhere.

The next surface applied had an additional linker of glutaraldehyde in between APTES and col-1. Work by Gunda *et al*¹²⁵ reported application of GA linker increases the surface hydrophilicity which led to a more homogenous surface immobilization of an antibody. Gunda *et al*¹²⁵ measure the water CA on the APTES silanized PDMS surface to be $63 \pm 4^\circ$ and report addition of GA led to a lower CA of $51 \pm 3^\circ$. Our work established that col-1 leads to an increase in hydrophobicity of both the APTES and APTES-GA functionalized PDMS. The water CA of the APTES linked col-1 surface was determined to be $69 \pm 6^\circ$, this was lower than the APTES-GA linked col-1 surface value of $75 \pm 5^\circ$ (Figure 7.13). In this work, Col-1 immobilization led to an increase in hydrophobicity on both modified surfaces. Col-1 exists in a polypeptide triple helix structure with a hydrophobic side, the other side is scattered with hydrophilic lysine residues which are reported to facilitate binding to hydrophilic surfaces.^{103,238,240,253} It follows that immobilization of the col-1 hydrophilic side onto a hydrophilic surface will increase hydrophobicity.²⁵³ Addition of the GA linker appears to result in a more hydrophobic surface with col-1 immobilization, this suggests GA hydrophilization of the surface can facilitate more effective col-1 immobilization.

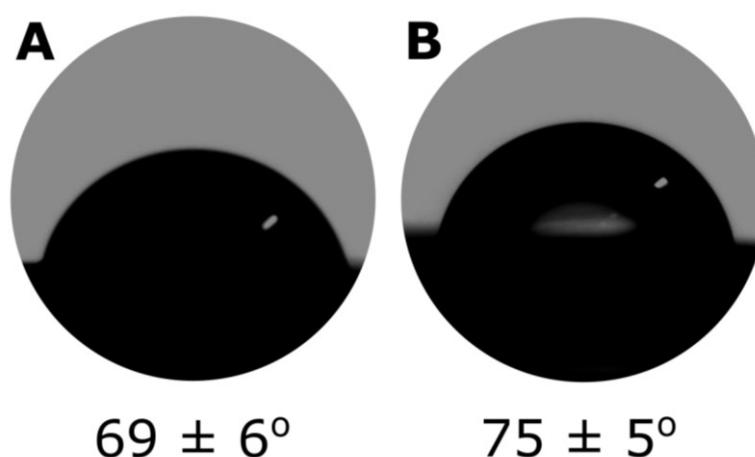


Figure 7.13) Image of DIW water droplet on PDMS functionalised with **A** APTES and col-1 and **B** APTES-GA and col-1. CA measurements were determined from $n = 39$ measurements from over 6 independently functionalised surfaces.

To explore the compatibility of the col-1 surface modifications, cell attachment was quantified on conventional TC ware and col-1 modified PDMS. PDMS inserts immobilized col-1 using both APTES and APTES-GA linkers, the PDMS inserts were placed into well plates and each well was seeded with H9C2 cells using 2 ml of a 3×10^4 cells mL^{-1} solution. A lower seeding concentration was chosen to minimise formation of cell clusters, which reduce image clarity. Seeding was undertaken using a pipette controller on the gravity driven setting ('g') to produce even cell distributions, across the seeded surfaces. After seeding, cells were left for two hours to adhere before media was aspirated. A total of 12 wells were used for each surface and seeding applied the same cell suspension. TC ware samples were seeded in a Falcon 12 well plate functionalised with the Corning® Primaria™ surface.²⁵⁴ Corning® Primaria™ surfaces utilise a proprietary technique to modify polystyrene with negative oxygen and positive nitrogen functional groups through application of vacuum-plasma.²⁵⁴ Within the study, following media removal, surfaces were washed with three 2 ml PBS portions and fixed using paraformaline. Cells were then permeabilized using triton and stained with Coomassie blue. Coomassie blue binds non-specifically to proteins staining cells blue, this made cell images clearer under DIC microscopy.

Seeded surfaces were imaged using brightfield microscopy, 6 images were taken within each well and tiled without overlap. When imaged, cells on the TC ware surface appeared larger than the PDMS inserts, this was attributed to the 1 mm thick PDMS differing the refractive index from that of air and glass (Figure 7.14). Substrate dyeing was observed with PDMS inserts; this was thought to be Coomassie blue binding to surface immobilized col-1. To pre-process and analyse the data, an image analysis pipeline was developed in Cell profiler. Images were first inverted and then object identification was performed; colour inversion enables more effective object identification within Cell profiler (Figure 7.15). After object identification, a threshold was determined for the filtering of Coomassie blue surface deposits and removal of inseparable cell clusters. Filtering of surface deposits led to an image of stained cells within the image, this image was used for object quantification and analysis.

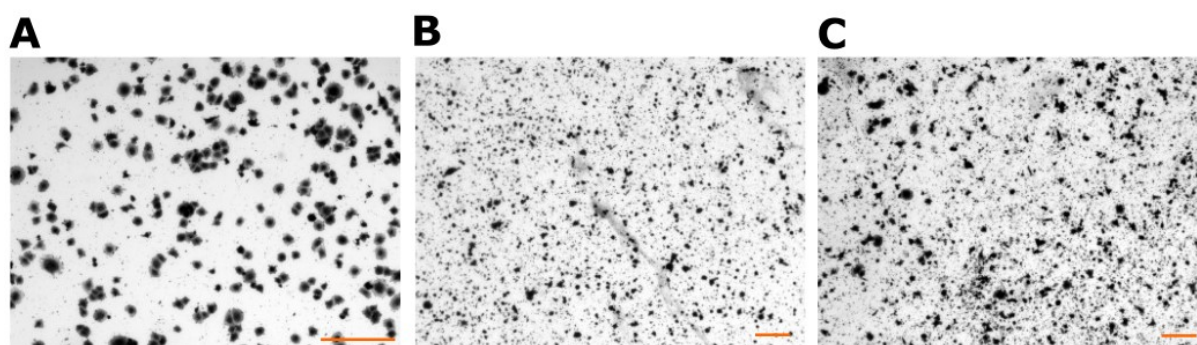


Figure 7.14) Images of fixed cells stained with Coomassie blue on **A** Falcon TC well **B** Co1-1 coated PDMS insert with an APTES linker **C** Co1-1 coated PDMS insert with an APTES-GA linker (Scale bar ≈ 0.1 mm).

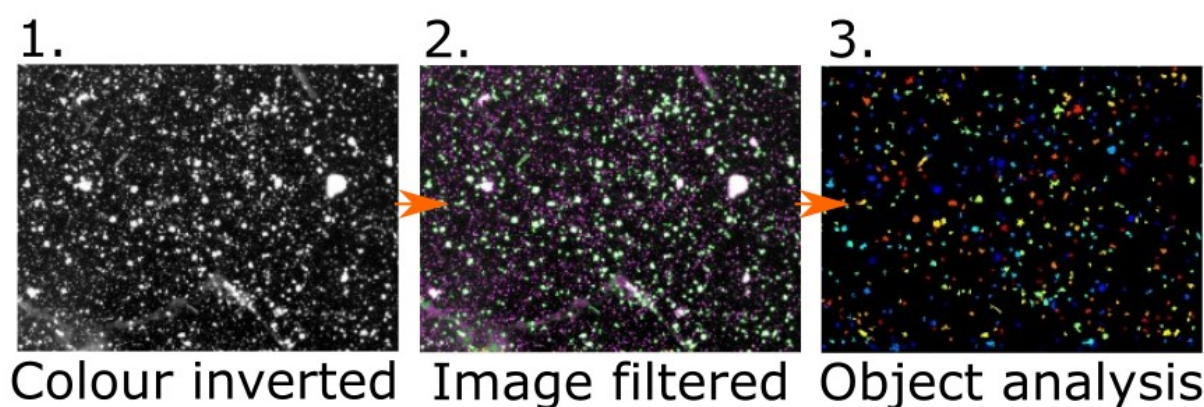


Figure 7.15) Example outputs of the image analysis pipeline developed in Cell profiler **1** Colour inverted raw image **2** Object identification image with the accepted cells (green) and rejected surface bound Coomassie blue and cell clusters (pink) **3** Processed image containing fits to identified cells, highlighted in multicolour for clarity of separate cells.

The processed images were used to determine a mean cell count per image (Cells pix^{-2}), then a scalar (pix cm^{-2}) was calculated to convert this into cells cm^{-2} . A scaling factor was required to compare seeding density within TC ware and PDMS inserts. The scaling factor was calculated by comparison of the mean cell diameter measured within cell profiler in pixels, to the mean diameter determined by flow cytometry ($18.98 \mu\text{m}$) (Supplementary S12.3). The scaling factor was an approximation, as cells measured by flow cytometry were detached, cells are typically larger when adhered and will vary in size dependent on the surface. Conventional scaling using a calibration slide was unrepresentative as the cell bound substrate varied. The use of a 2-hour seeding time ensured cells remained approximately circular, elongation would make the scaling factor less representative and the cells harder to differentiate. The hypothetical maximum for cell density was 1.58×10^4 cells cm^{-2} , based on the cell concentration and viability of the seeding solution. Initial cell viability, within the seeded solution, was determined to be 94.6% by flow cytometry. Realistically, cell density will be below the maximum, this results from cell detachment and viability decrease during the seeding process.

When converted to cells cm^{-2} the surfaces could be compared, the highest seeding density was observed in TC ware (Figure 7.16). Within the TC ware a value of $7.98 \times 10^3 \pm 4.97 \times 10^2$ cells cm^{-2} was calculated, this is approximately 50% of the hypothetical maximum, indicating a significant reduction in viability during the adhesion, washing and staining processes. For the APTES col-1 and APTES-GA col-1 functionalised PDMS inserts, cell densities of $3.28 \times 10^3 \pm 3.71 \times 10^2$ cells cm^{-2} and $3.10 \times 10^3 \pm 2.70 \times 10^2$ cells cm^{-2} were determined, respectively. The functionalised PDMS inserts were approximately 20% of the hypothetical maximum, this suggests the coating is less effective than

conventional TC. Lower seeding density could arise from uneven or incompatible surface coating and shear detachment of the seeded cells during the washing process. All these factors are detrimental for TOC model development. Addition of the GA linker remained within the observed cell adhesion of the APTES col-1 surface. Based on the CA data, a greater rate of col-1 linkage was implied in the GA linked surface, this was expected to encourage cell adhesion. Although, application of the GA linker requires an additional step within the functionalisation method, and it didn't impact cell adhesion. Based on the additional fabrication stage and similar seeding density, GA linker use was discontinued. This data set gives insight into the required cell concentrations for seeding TOC devices and highlights the non-trivial process of PDMS biocompatible modification.

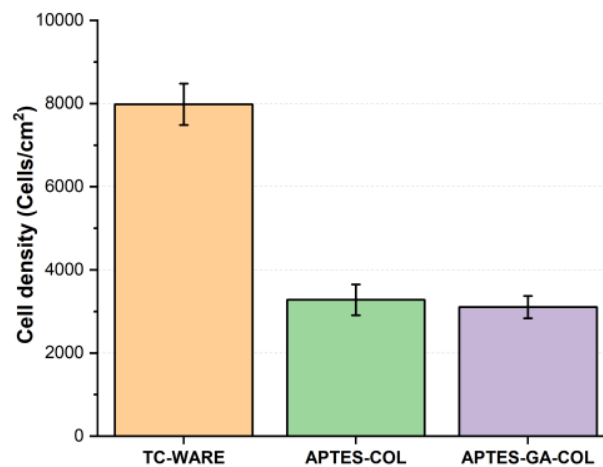


Figure 7.16) Resultant cell densities for conventional TC ware and col-1 functionalised PDMS inserts ($n > 77$ images, error bars = ± 1 SD).

To further explore the biocompatibility of the surfaces a 5-ethynyl-2'-deoxyuridine (EdU) assay was applied, to explore H9C2 cell proliferation (Figure 7.17). Cell proliferation can be used as a metric to describe the biocompatibility of a substrate. Whilst preliminary data for the proliferation was collected, disruptions from onset of COVID-19 based restrictions prevented completion of these experiments. TOC development was transferred to a 3D bioprinted tissue model, this was to address the long cleanroom-based fabrication times required for meaningful study within TOC. Although promising, TOC devices are an exceptionally time-consuming process from PDMS manufacture to surface treatment and typically 20 - 30% of devices fail from either poor bonding quality, alignment in the case of the grooved device or leakage under perfusion. The device cell capacity (0.2 cm^2) is comparable to that of a single well (0.25 cm^2) used in the cell validation studies within Chapter 9, as such to achieve meaningful study, at least 30 devices are required ($n = 6$ measurement replicates and $n_{\text{bio}} = 5$) for a single treatment. Bioprinting was sought to achieve scalability by printing directly into well plates and further improve upon the biomimicry through absence of shear flow and presence of ECM.

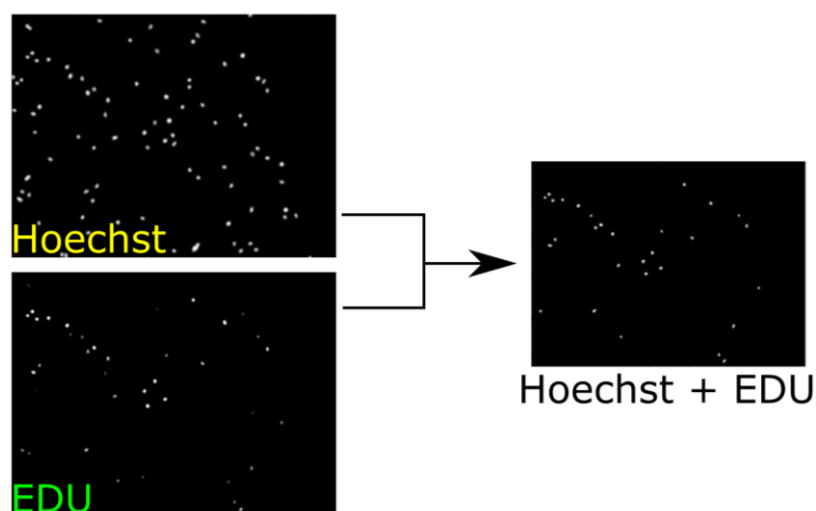


Figure 7.17) Images of EdU migration assay, showing the Hoechst labelled total cell population and the EdU stained, migrated cell population. Hoechst and EdU positive nuclei were proliferated cells, post adhesion.

7.5 Development of 3D bioprinted tissue model

7.5.1 3D bioprinting application

The process of 3D bioprinting is rapidly becoming a broad field of research; bioprinting is applied in tissue engineering, OOC and drug efficacy screening.^{97,248,255–258} Bioprinting employs various methodologies, the two most common are FDM and SLA.²⁵⁹ FDM is the focus of this work, it involves the direct deposition of a cell-laden hydrogel (Figure 7.18).^{248,259} The hydrogel is commonly referred to as a bioink, it usually contains components of the *in vivo* ECM and cells. FDM involves a bioink loaded syringe, the syringe is housed in printhead, providing horizontal and vertical control by displacement. Printing occurs with pressurization of the syringe and subsequent material extrusion; the applied printing pressure determines the filament deposition rate. During printing, bioinks are extruded through an extrusion tip, this determines the diameter of the resultant gel filament. Bioprinting results in a 3D structure composed of a patterned continuous filament, this structure can be made from multiple vertical layers to increase height.²⁵⁸

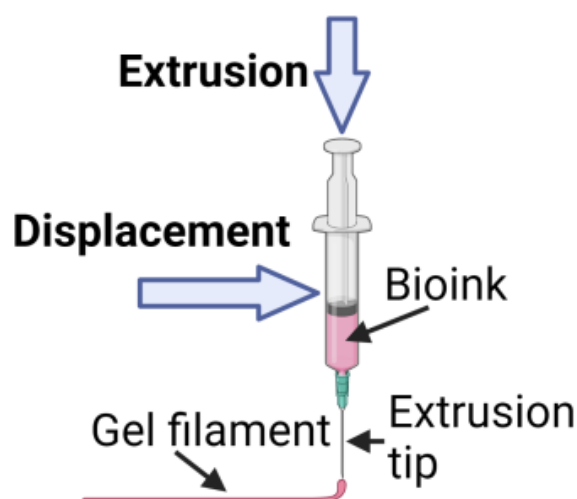


Figure 7.18) Schematic representation of FDM 3D bioprinting where extrusion drives the formation of a gel filament through an extrusion tip. The resulting gel filament is comprised of bioink and is patterned by the displacement of the print head forming a model tissue.

Within this work, bioprinting was applied to produce models with elements of biomimicry that cannot be achieved using conventional tissue culturing methods. Bio printed models were designed with the goal of screening the efficacy of liposomal drug delivery systems to models of cardiac tissue. Bioprinting offers the ability to reproduce structures that emulate cardiomyocyte topography *in vivo* (Figure 7.19). Heart muscle is composed of myofilament strands comprised of cardiomyocyte cells, these structures are bundled in a primarily parallel alignment with cells aligning axially.^{260,261} Within a healthy human heart these filament structures are around $22.6 \pm 2.4 \mu\text{m}$ and within a rat they are typically $17.7 \pm 1.5 \mu\text{m}$.^{261–263} Myofilaments are amenable to FDM bioprinting as the tubular structure can be replicated through extrusion of a gel-based ECM. Application of a hydrogel ECM enables structural retention and can mimic the *in vivo* ECM by incorporating structural proteins found within heart tissue whilst enabling media perfusion.

A further advantage of bioprinting is the ability to study different ECM composition by altering the hydrogel constituents. This would enable the study of the impact of ECM on drug delivery within the model, this could be especially advantageous for study of liposomal delivery.^{24,176,264} There is a significant increase in length scale of a liposomal drug delivery system over a free molecule. Particles are known to undergo size filtration within the ECM, ECM can also absorb particles based on composition.^{176,265} Understanding liposomal mobility within model ECM is critical for determining the efficacy of liposomal delivery *in vivo*.^{177,264} To develop a 3D printed model, bioink development was first explored with a focus on biomimicry and printability.

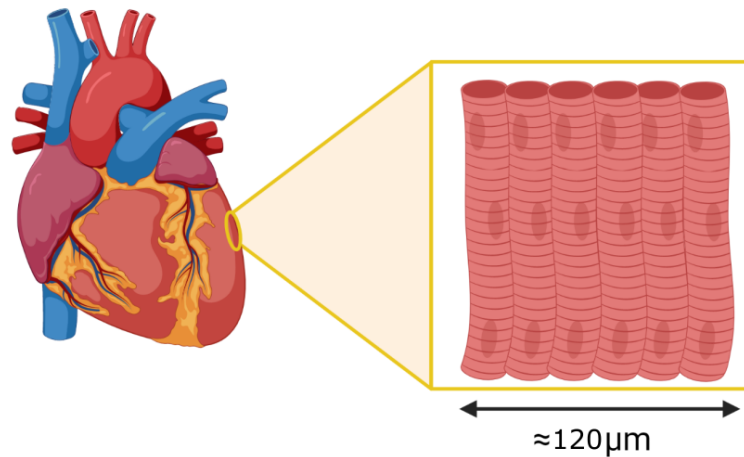


Figure 7.19) Representation of myofilament arrangement within heart muscle tissue.

7.5.2 Development of bioink

The first bioink was adapted from work by Wang *et al*²⁴⁸, in which a model of functional cardiac tissue was developed with a focus on tissue engineering and pharmaceutical studies. The active element of the bioink is fibrinogen, this allows a 2-stage printing and curing process. Post-print curing is used to avoid printing of high viscosity bioinks, this is a pressure intensive process with the potential of causing cellular damage and high potential of print nozzle blockage.^{248,256} After printing, models are submerged in a thrombin curing solution, this causes fibrinogen clotting; fibrinogen clotting is well documented within literature.^{266,267} Curing results in a set, cell-laden hydrogel that is insoluble in cell media. The clotting inhibitor aprotinin was added to prevent clotting before extrusion. The other components were the viscosifying agents' gelatine, hyaluronic acid, and glycerol.

Preparation was first optimised to achieve a homogenous, sterile gel. Components were mixed in batches, in 90:10 DMEM:Glycerol under stirring within a 60°C water bath. Stirring was applied for two hours at a low spin rate, this produced a homogenous solution without introducing air. The mixed solution was then passed through a 0.2 μm filter at 60°C to sterilise the gel. Keeping the gel at 60°C enabled filtration by reducing viscosity. When filtered, the solution was loaded into a syringe and purged of residual gas, syringes were sealed and stored within at 4°C. Bioink preparation resulted in syringes that contained no observable air bubbles, typically 10% of material was lost during the work up (Figure 7.20A). The final concentrations of each substituent were altered from work by Wang *et al*²⁴⁸ to work more effectively in a Cellink Inkredible+ bioprinter, this was empirically achieved through assessment of print quality and frequency of nozzle blockages.

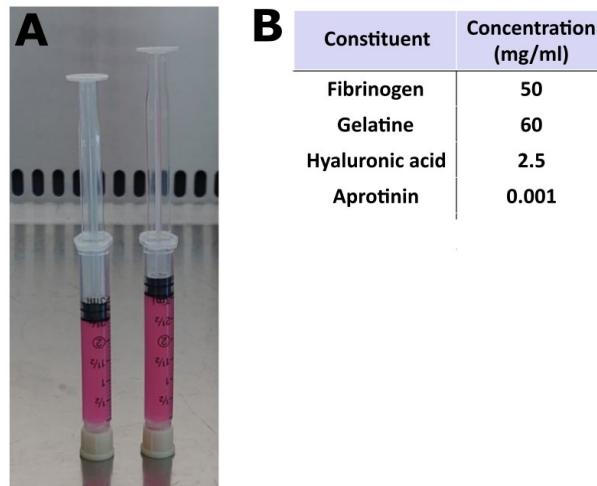


Figure 7.20) **A** Image of Cellink containing syringes produced at the end of the workup, prior to H9C2 cell loading **B** Bioink constituents.

The bioink was first used to print a 20 by 20 mm spiral square within a petri dish, to assess print quality and reliability. Extrusion used a 200 μm ID syringe tip, printing was set to a single layer height resulting in 200 μm print depth. The print head was heated to 37°C prior to printing, a print speed and pressure of 10 mm s⁻¹ and $\approx$ 22 KPa were used, respectively. During printing, a HEPA filter fan was used to retain sterility within the printing area. At first, prints generated an even spiral square structure with the fine print height (Figure 7.21A). Later prints (> 20 minutes operating time) using the same ink exhibited defects resulting in resolution loss. Within some prints, the spiral patterning was lost as printed filaments merged, this was thought to occur from low viscosity portions of the bioink indicating poor bioink homogeneity (Figure 7.21B). Extreme blockages occurred when material agglomerated in the print nozzle, this resulted in material surges which created amorphous structure (Figure 7.21C). Blockages occurred from fibrinogen prematurely clotting and gelatine becoming viscous, at the cooler nozzle tip. Use of the HEPA filtered air flow within the hutch was found to greatly impact clotting of the gelatine at the nozzle, likely due to the increased air flow the filtration produces. Since application of HEPA filtration was found to severely impact utilization of gelatine based custom bioinks, further reduction of gelatine content can impact the print quality because of lower viscosity. HEPA filtration is required to retain a sterile environment, to avoid the gelatine clotting issue a commercial bioink was investigated.

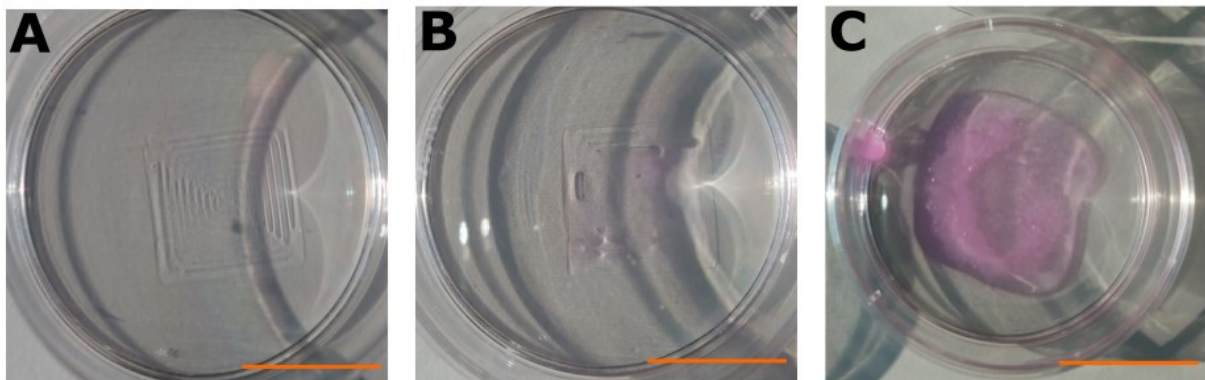


Figure 7.21) Resultant 20 x 20 mm spiral square prints with **A** low defects, **B** resolution loss from filament merging post-extrusion and **C** significant resolution loss from print head blockage (Scale bars = 20 mm).

The Cellink bioink used is composed of alginate, cellulose nanofibrils and polysaccharides.²⁶⁸ The alginate-based bioink is cured after printing using a sterile curing solution provided by Cellink. Alginate is the active curing agent, when a CaCl_2 curing solution is applied it gels.²⁶⁹ Cellulose nanofibrils are employed to achieve temperature independent viscosity within the Cellink. The ink is semi-translucent when printed, therefore amenable to imaging techniques. Printing with the alginate-based ink gave reliable print quality, through the reduction of blockages (Figure 7.22). Prints were successfully undertaken with multiple layers, indicating stable print structure. When cured, the structure retained a cloudy white colour and became stable in media. Alginate-based bioink offer little biomimicry, in terms of ECM protein contents, when compared to the bioink developed by Wang *et al*²⁴⁸. With a suitable ink chosen, based on practical application, the print methodology was developed.

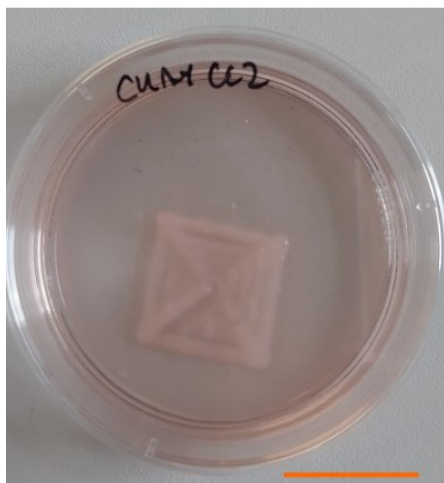


Figure 7.22) Cellink alginate based 20 x 20 mm square spiral structure submerged in DMEM media (Scale bar = 20 mm).

7.5.3 Development of 3D bioprinting methodology

Print models were designed in Autodesk fusion 360, models were then transferred to Cellink Heartware to set printing parameters and finally the designs were sliced using SLICR to generate a printer compatible script. The SLICR outputs were corrected by manual editing of the G-code, this was required to meet the demands of printing into a well plate. By printing into a well plate, scalability and reproducibility could be achieved in subsequent studies. The bioprinted model scalability comes with the drawback of losing precise control of media flow and drug delivery under flow, that are possible in a microfluidic TOC. However, within the body, cardiomyocytes are not exposed to shear which makes the bioprinted model closer to *in vivo* conditions.

Initially designs were developed for printing 0.13 cm² area circles (0.4 cm diameter) of tissue into a falcon 96 well plate with a 1 cm² well seeding area (Figure 7.23A). The discs were comprised of a circular outline which contained the gel, infill was achieved using 3-4 parallel lines per disc to replicate myofilament structure. Discs were designed in a 6 x 10 array and the design aligned the discs to the well centre. A print retraction was implemented in the print path (light blue line), this was to prevent the extrusion tip from catching on the well during relocation (Figure 7.23B, Figure 7.24). Extrusion in the plane above the discs (dark blue filament) was used to load the material into the extrusion tip and intermittently displace blockages.

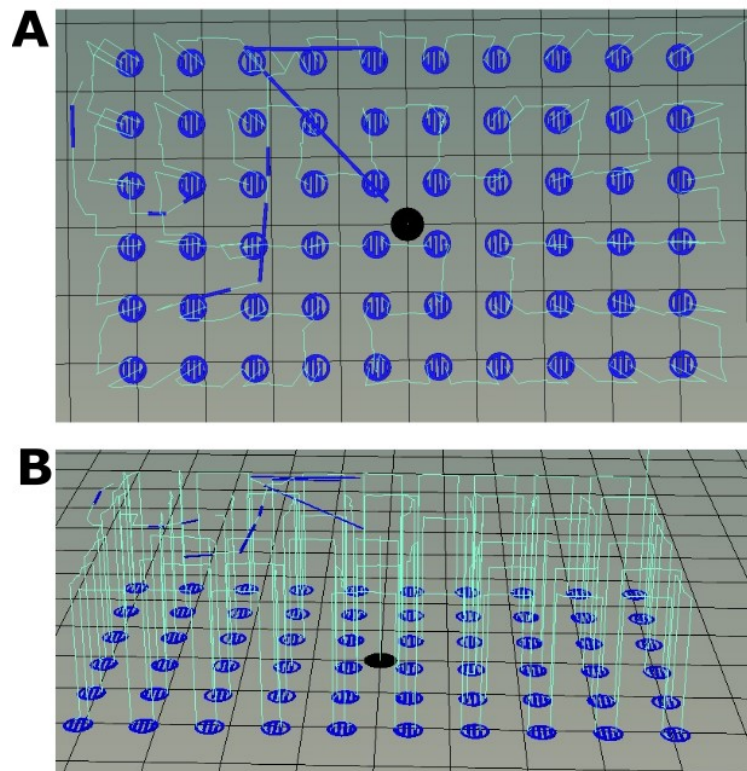


Figure 7.23) Edited SLICR output for the 6 x 10, 0.4 mm diameter disc array detailing the print structure (dark blue) and print head travel coordinates (light blue) for **A** bird's eye view and **B** side on view (Grid squares = 5 x 5 mm). The intended print structure is an array of discs (dark blue) designed to be set into a Falcon 96 well plate, the discs feature a round outline, which is printed first, and parallel line infill is then printed to recreate a parallel filament like structure. The printer path (light blue) details the motions taken by the print head in between printing the disc structures, the print head was raised in between the wells within the plate to avoid the print head catching.

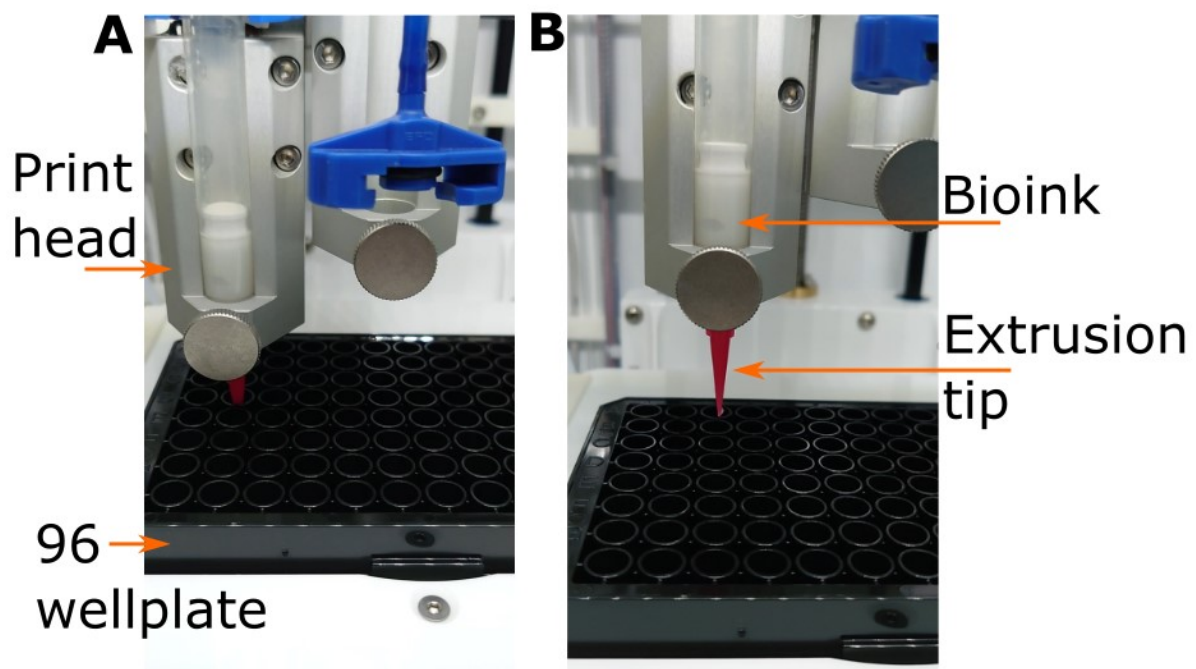


Figure 7.24) Images of print head during the printing of the 0.4 mm diameter disc structures shown in the SLICR output showing the print head in the **A** lowered and **B** retracted state.

When produced, the 4 mm discs were of variable print quality, whilst the opaque well plates meant quality could not be assessed during printing. Upon media addition, discs often detached and began to float in the media. To prevent detachment, the print was scaled up to work in a falcon 6 well plate with a 9.6 cm² well seeding area (Figure 7.25A). Tissue constructs were designed as 10 x 10 mm squares with a 0.2 mm depth, they were comprised of a square outline and parallel strand infill (Figure 7.25B). The total print volume was approximately 32.7 μ L of gel which contained 3.2×10^5 cells, an order of magnitude higher than the TOC device developed in Chapter 7.3.1. Scale up of the print enabled assessment of print quality during printing and resulted in reduced detachment of the printed construct, when media was added to the well (Figure 7.25C). Scale up reduces the ability to explore different experimental conditions in a single well plate however, it increases the sample size and cell count per well.

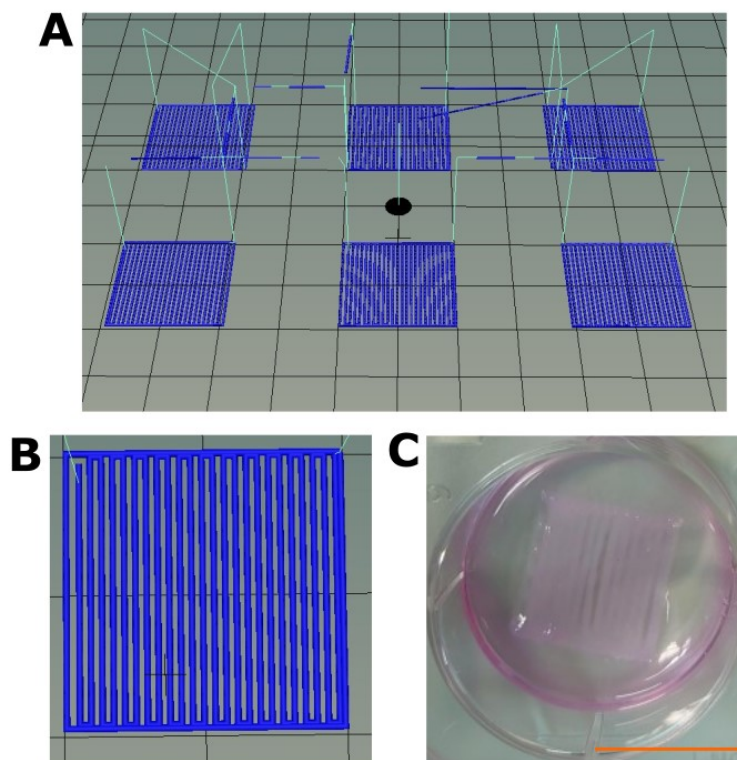


Figure 7.25) Images of the edited SLICR output for the 6 x 2, 10 x 10 mm square array detailing the print structure (dark blue) and print head travel coordinates (light blue) for **A** side on view and **B** bird's eye view of the square construct (grid squares = 5 x 5 mm). The printed structure (dark blue) detail the square outline which is first printed, followed by the parallel line infill to recreate the filament like structure within the myocardium. The print head travel (light blue) details the print head movement in between prints showing the lift out of individual wells to avoid catching on the well edge, in between prints **C** Resultant printed construct formed from cell-laden hydrogel in DMEM after the curing stage (Scale bar = 10 mm).

Printed 10 x 10 mm constructs had a lower rate of failure from delamination, the size increase on previous designs meant defects were less impactful on the total structure (Figure 7.26). Upon media addition smaller printed structures could partially detach, here the increased size prevented floating (Figure 7.26B). Application of a rigid printed support structure could prevent detachment, at the cost of longer print times. Alternatively, chemical modification of the well surface could be explored to facilitate better gel adhesion, plates used were optimized for cell culture. Nozzle blockages still occurred with the alginate bioink, however by using manual pressure adjustment during printing this could be partly mitigated to reduce the impact on the print quality (Figure 7.26C). Overall, the scale up presented a marked improvement on previous designs, typically 1 in 6 prints exhibited poor quality.

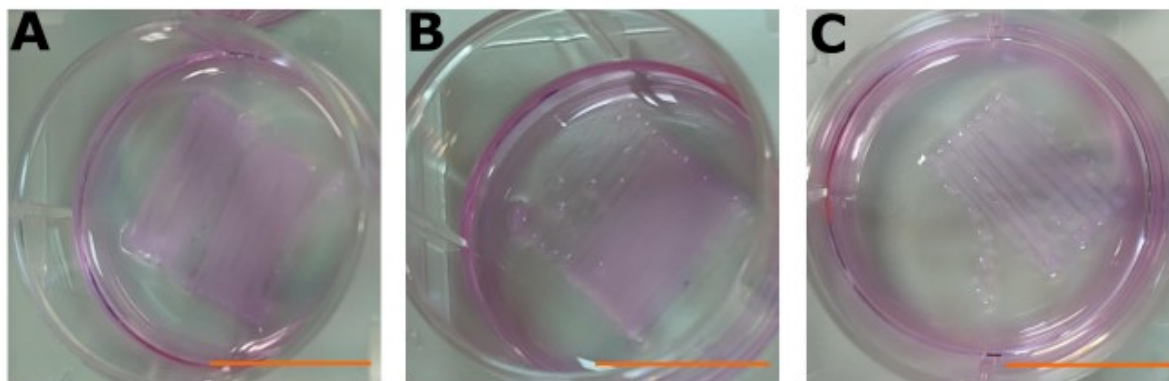


Figure 7.26) Prints imaged after curing and immersion in media. The series of prints show **A** reasonable quality print with a central defect indicated by increased spacing between the parallel infill **B** print exhibiting a partial delamination indicated by the beaded print structure shown to the top and **C** a print exhibiting significant delamination and poor print quality as little of the print structure remains (Scale bars = 10 mm).

7.6 Conclusion and future work

7.6.1 Conclusion

In this chapter, a straightforward PDMS based cardiac tissue model for the efficacy testing of drug delivery liposomes is developed. A second iteration of the model incorporated micro-patterning of cardiomyocytes to better mimic cardiomyocyte alignment *in vivo*. Initial surface coating study demonstrated efficacy of col-1 and synthetic PVA coatings. The PVA surface has the potential to result in a reproducible, biocompatible surface coating for static cell culture. By exploring surface coatings in flow, it was determined PVA and non-covalently bound col-1 surfaces are ineffective at maintaining cell adhesion under flow, using microscopy. Covalently linked col-1 could be the most feasible surface for the TOC device, as seeding was successfully achieved on static samples. Determination of seeding density by image analysis demonstrated the low cell adhesion of col-1 immobilized surfaces relative to TC ware this outlines the need for effective PDMS surface modifications for TOC.

A bioprinted tissue model was developed to counter the intensive production and low reproducibility of our TOC device. The bioprinted model captured *in vivo* structure by replicating myofilament topography and cardiomyocyte micro-environment by avoiding media shear. Printing into tissue well plates facilitated scalability and compatibility with plate-reading analysis machines. Through optimisation of bioink and printing procedure a cell-laden structure suitable for testing drug delivery liposomes *in-vitro* was achieved.

7.6.2 Future work

A potential drawback of the PVA coating is the absence of literature around H9C2 cell adhesion. Future work into cell gene expression on the PVA surface would provide critical information about the impact of using a PVA surface, as topography and surface chemistry are reported to impact H9C2 cell gene expression.²⁵⁰ Within the grooved device, increased groove depth could be investigated to result in more effective confinement of cells, although this may increase the trapping air bubbles, which are

detrimental to cell viability. Exploration into gelatine-methacrylate (GelMA) printing could offer a viable alternative to alginate-based inks.^{270,271} GelMA offers closer representation of the heart ECM, when compared to alginate. Through the use of light based curing, GelMA can utilise lower viscosity printing to avoid nozzle blockage and offer rapid curing without need for successive treatment.

8 – Design and stability of therapeutic liposomes

8.1 Overview

Liposome phase, stability and encapsulation are important characteristics for drug delivery *in-vivo*. This chapter applied the method of XRD to characterise the bilayer phase, phase assignment can aid understanding the efficacy of encapsulation and infer liposome stability. The encapsulation of fluorescent dyes was then used to determine encapsulation success during VFF and elucidate the stability of encapsulated systems. Stability was explored over two-week period as this is a practical representative period for *in-vitro* drug efficacy screening. Encapsulation within VFF is still an area of uncertainty and currently a method to determine an accurate encapsulation efficiency is unestablished. Understanding long term stability of encapsulating liposomes is critical for application within cell studies and practical handling. Finally, the concentration and media stability of the liposomes is characterised. Liposome concentration is an important benchmark for comparison with the commonly applied method of extrusion, this can aid comparison with literature based liposomal studies. Media stability is an essential characteristic for the application of liposomes *in-vitro*, osmotic pressure can rupture liposomes resulting in a premature release of cargo.

8.2 X-ray diffraction

8.2.1 X-ray diffraction overview

X-ray diffraction is a method commonly used for studying the phase and structure of lipid membranes.^{7,272–275} When exposed to highly collimated x-rays, samples produce both a small angle scattering (SAXS) and wide angle scattering (WAXS) spectra (Figure 8.1). Long-range structure is determined from the SAXS spectra, these spectra are comprised of a series of Bragg peaks.^{272,276,277} When Bragg peaks are assigned an index, the index relates to the peaks relative position, each peak corresponds to a diffraction from a Miller plane, inherent symmetry properties of crystal structures. By relation of characteristic structures to the SAXS spectra, a phase assignment can be made. X-ray diffraction can also be used to probe the short-range structure through analysis of WAXS. WAXS provides information about the packing of lipid acyl chains within membranes, this enables further differentiation between samples, especially lamellar phase subsets.^{7,272} An in-depth introduction into the fundamentals of X-ray diffraction is provided by Woolfson²⁷⁷ and Bragg²⁷⁶, whilst an accessible overview is written by Tyler²⁷² for further information. Phase assignment provides important context for liposomal drug delivery as phases exhibit widely different parameters such as permeability and membrane viscosity, an extensive review for phase assignment is published by Seddon^{7, 274,275,278}

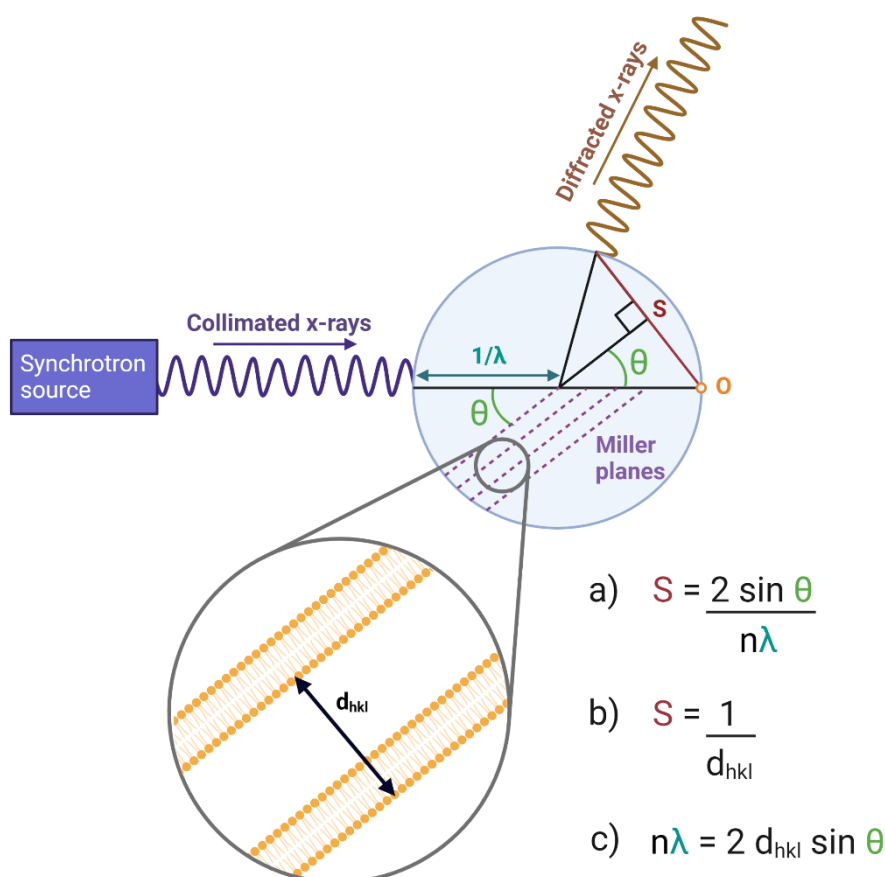


Figure 8.1) Schematic of SAXS XRD within an Ewald sphere of radius $1/\lambda$ where λ is the wavelength of the x-ray source. Incident collimated x-rays are diffracted by the angle θ (Green) from the Miller plane. Within the Ewald sphere, x-rays are displaced by vector S (brown) from the reciprocal lattice origin O (Yellow). Miller planes are a symmetry property of lamellar bilayers and are related to the repeat spacing d_{hkl} (Black). Within the Ewald sphere Bragg's law can be applied which derives equation a) and S is related to d_{hkl} by equation b) and so it follows, by removal of S , that equation c) results.

8.2.2 Control liposome formulation SAXS and WAXS spectra

First the SAXS spectra of the CL formulation were recorded at 20°C and 37°C and then labelled (Figure 8.2). These temperatures were chosen as they closely represent the ambient storage conditions and physiological body temperature which is also used during cell culturing. Once labelled the peaks were indexed to 2,3,4,5 and 2,3,4 for the 20 °C and 37 °C samples, respectively. This pattern of peaks suggests the samples were both lamellar phases, the corresponding Miller indices for each peak are presented (Table 6). The first peak at index one, was not recorded using the detector settings although the clarity of successive peaks enabled assignment. A repeat spacing of 179.5 Å and 172.1 Å was calculated for 20°C and 37°C using Equation 15. The repeat spacing is the distance between repeat points within the lamellar lattice, for the lamellar phase this distance encompasses both bilayer thickness and water layer thickness between lamellar sheets.⁷ Despite the samples predominant DOPC molar constituent, the calculated repeat spacings were larger than a literature value determined by Bryant *et al*¹⁸⁶ for a pure DOPC system (63.78 Å). The larger spacing within the control formulation likely results from the pegylated lipid inclusion, sterically bulky PEG could act to increase the repeat spacing within the water layer between lamellar sheets. Hristova *et al*²⁷⁹ estimate PEG₂₀₀₀ contributes approximately 65 Å from each bilayer surface (DSPE-PEG:DSPC mixture), so it is reasonable that the control formulation is within 130 Å of the purely DOPC sample. The SAXS data supports that the DSPE-PEG₂₀₀₀ fraction was chosen to remain in the brush like conformation, where PEG is fully extended. The brush formation is favoured for therapeutic application and the fully extended chains likely contributing to the lamellar spacing.^{29,279}

$$\text{Repeat spacing} = \frac{2\pi}{\text{Inter peak spacing}}$$

Equation 15. Where the repeat spacing, a structural symmetry property, is related to the inter-peak spacing, for a lamellar phase.

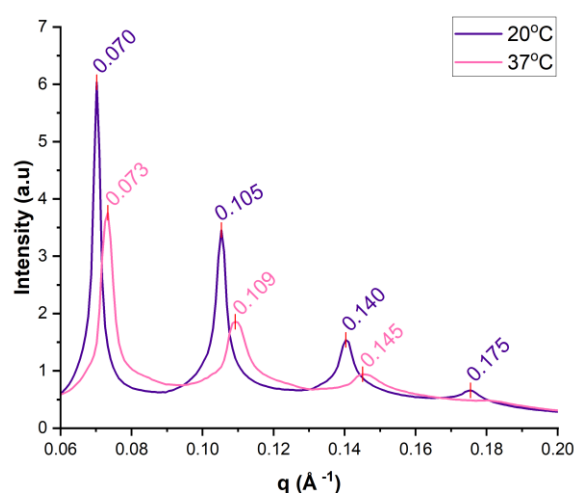


Figure 8.2) SAXS spectra of the control liposome formulation at 20°C (Purple) and 37°C (Pink). Peak maxima locations are highlighted.

	20°C	37°C
Peaks ($\text{\AA}^{-1}$)	0.07, 0.105, 0.14, 0.175	0.073, 0.109, 0.145
Index	2,3,4,5	2,3,4
Miller indices	(200),(300),(400),(500)	(200),(300),(400)
Inter-layer spacing ($\text{\AA}$)	179.5	172.1

Table 6) Tabulated peak locations, assigned indexes and the corresponding Miller indices and repeat spacing for the control liposomes formulation at 20°C and 37°C.

To study the stability of the control formulation, a temperature sweep was performed between 20-60°C at 5°C intervals, then the SAXS and WAXS was collected for each temperature point (Figure 8.3). The SAXS shows the 2, 3, and 4 index peaks for all temperature points, when increasing the temperature peaks become broader, this arises from temperature fluctuations and is commonly seen in soft matter ordered structures.^{7,272} Across the temperature sweep the formulation remained lamellar, this demonstrates temperature stability of the sample within the range tested. Phase changes within the temperature range could suggest potential instabilities for the liposomal system, phase changes can impact cargo release of liposomal systems.^{280–282}

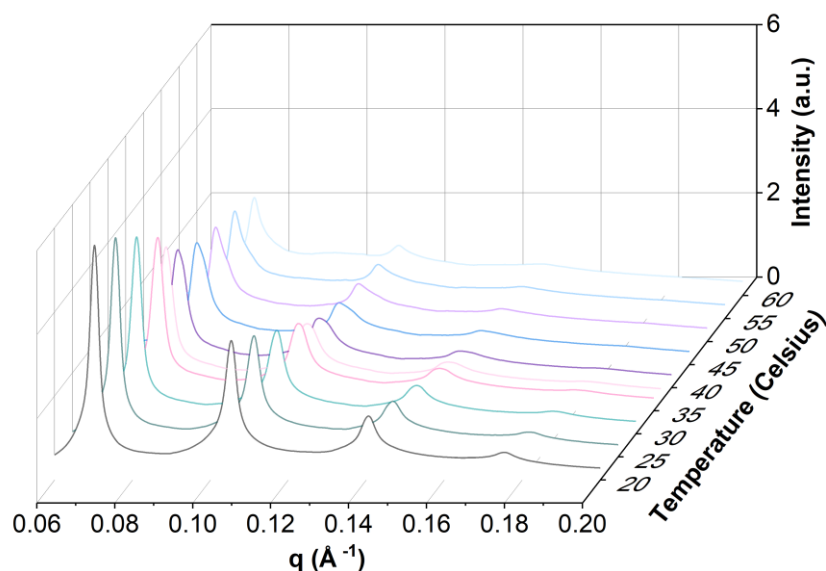


Figure 8.3) SAXS spectra of the control liposome formulation at 20 - 60°C. With heating at the 20 – 60 °C temperature range explored the peaks broadened and the phase remained.

The WAXS was explored to study the temperature impact on the short-range ordering within the acyl chains (Figure 8.4). Within the WAXS broad peaks were observed at 1.4 and $2 \text{ \AA}^{-1}$. The peak at $1.4 \text{ \AA}^{-1}$ indicates the sample is the fluid lamellar (L_α) phase (Chapter 1.2.2), the broad nature of the peak implies a fluid nature of the lipid chains.^{7,208} A gel phase (L_β) would exhibit a narrow peak resulting from the ordered all trans lipid chains. The peak at $2 \text{ \AA}^{-1}$ indicates the presence of water, this is expected as the sample is 70 wt% water. When temperature was increased, the sample remained unchanged which demonstrated temperature stability within the lipid chain region. Overall, SAXS and WAXS data allows assignment of the formulation to the L_α phase and demonstrates the formulation is thermally stable within the experimental range. These data sets demonstrate support findings about the suitability of PEGylated formulations within the clinical setting, from the favourable stability between room temperature and body temperature.

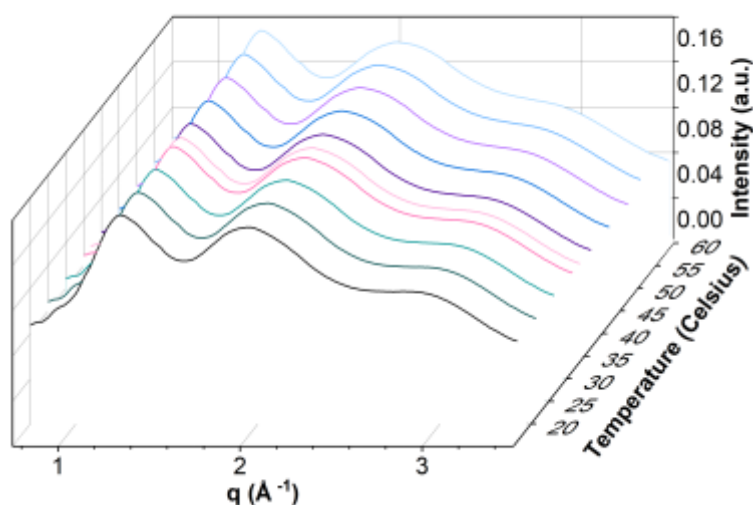


Figure 8.4) WAXS spectra of the control liposome formulation at 20-60°C where peaks are shown at $1.4 \text{ \AA}^{-1}$ (L_α) $2 \text{ \AA}^{-1}$ (water). The peaks subsisted within the 20 – 60 °C temperature range explored.

8.3 Liposomal carboxyfluorescein encapsulation

8.3.1 Carboxyfluorescein introduction

Carboxyfluorescein (FAM) is a fluorescent dye that is commercially available as a mixture of the 5 and 6 isomers (Figure 8.5).²⁸³ The absorption and emission peaks of FAM are at 495 nm and 517 nm respectively.^{283,284} FAM is readily soluble in an aqueous solution which enables its application to liposomes, FAM is typically encapsulated within the aqueous core.^{284,285} FAM is self-quenching at high concentrations ($\geq 100 \text{ mM}$ ²⁸⁴) which makes it applicable to release assays. As a liposome leaks FAM or is lysed, an increase in fluorescent signal can be detected and quantified by fluorescent microscopy (Figure 8.6).²⁸⁴ In this work, FAM was used in place of calcein another commonly applied self-quenching dye as FAM offers improved solubility.²⁸⁶ Solubility across a broad pH range is critical for direct encapsulation during VFF, where a sharp pH change occurs with ethanol and aqueous mixing.²⁸⁵

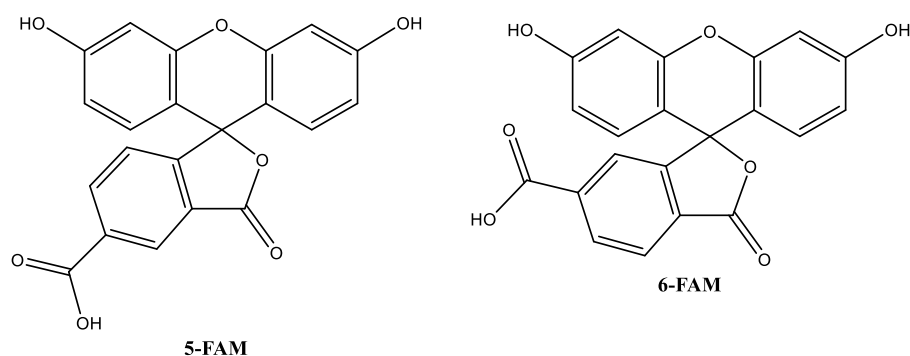


Figure 8.5) 5-FAM and 6-FAM structures

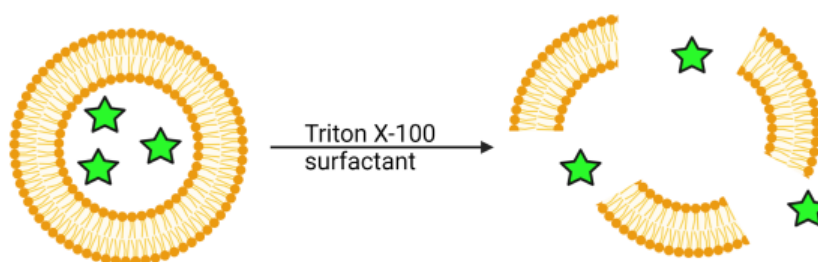


Figure 8.6) Schematic representation of FAM (green) encapsulation in a liposome (yellow) and subsequent release after lysis.

8.3.2 Carboxyfluorescein release assay

To encapsulate 5(6)-FAM (Novabiochem) during VFF, a FAM solution was used for the aqueous outer phase, this was co-flowed with an ethanol solution containing the control liposome formulation (Molar ratio 47:7:46 DOPC:CHOL:DSPEPEG₂₀₀₀). The outer phase was a 100 mM FAM solution in PBS and corrected to pH 10 using 1M NaOH. The ethanol stream used contained 0.1 mg mL⁻¹ of lipid for liposome formation at FRR 5, the lipid concentration was increased to 0.2 mg mL⁻¹ and 0.5 mg mL⁻¹ for the FRR 10 and FRR 25 samples, respectively. Application of higher lipid concentrations mitigates the dilution effect at higher FRR this ensures the final lipid concentration in all samples was 0.02 mg mL⁻¹. Liposomes were formed with a fixed ethanol flow rate of 0.1 mL min⁻¹ and the outer aqueous flow rate was increased to achieve the FRR range. The extrusion sample was prepared by hydration of the control liposome film at 0.02 mg mL⁻¹ using the FAM solution, extrusion was undertaken with a 200 nm filter for 30 cycles. After co-flow and extrusion, samples were purified on a SephadexTM column to remove unencapsulated FAM.

The extrusion sample was shown to have a 14-fold increase in fluorescence intensity after lysing. This was the largest change observed within the data set (Figure 8.7). Increasing FRR from 5 - 25 appeared to show minimal impact on the intensity after lysis, although a decrease in the intensity prior to lysis was observed. The decreasing intensity prior to lysis may be a result of reduced passive leakage from smaller SUV that are produced at higher FRR. The greater change in intensity observed for the extrusion sample was expected as the extrusion process is batch based and samples are equilibrated during the extrusion cycling. The encapsulation within VFF is also expected to be lower from the dilution with the central stream, thus the comparison between VFF and extrusion is inaccurate. VFF samples appear to show a greater intensity before lysis, this could be the result of FAM leakage.

Overall, this set shows aqueous encapsulation can be achieved directly during VFF at a likely lower encapsulation efficiency than extrusion. Since minimal difference was observed with increasing FRR, size control could still be achieved through FRR shift without impacting the encapsulation efficiency of the method.

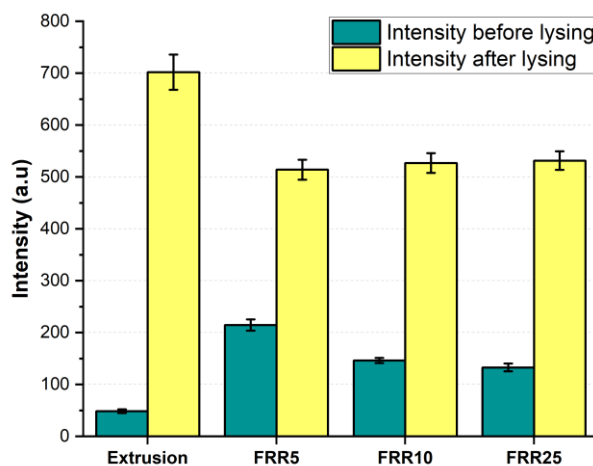


Figure 8.7) Fluorescent intensity of 100 mM FAM containing liposomes before and after lysis ($n = 3$ samples error bars = 1 SD).

8.3.3 Control liposome encapsulation over extended time

To determine the leakage over an extended period, fluorescence intensity was measured before and after lysis at several intervals. Long-term leakage is an important practical consideration, as it denotes the time-frame liposome formulations remained stable at the desired initial encapsulation. Understanding the leakage profile can also support future studies involving long term liposome incubation with cells. A total of 3 film replicates were used for each data point and the change in fluorescent intensity after lysis, ΔE was plotted with error bars of 1 standard deviation. Both samples were purified by size exclusion prior to study, the first time point was around 12 hours post formation for all samples. For the scatter plot, ΔE was normalized to aid comparison and an exponential fit was performed on the data. Identical sampling and lysis methods were used from Chapter 2.3.2, samples were stored at room temperature in a dark environment between readings.

The initial change in intensity (ΔE) was 350 for the VFF samples, this value remains similar for around 10 days (Figure 8.8A). Above 10 days, a decrease in ΔE was observed, this likely indicates leakage from the liposomes. With the extrusion sample an initial ΔE value of 650 was observed, this value was greater than the VFF sample and expected based on previous data (Figure 8.8B). The ΔE exhibited an apparent increase between day 1 and 4, this was attributed to a potential photobleaching of the sample occurring which would cause a lower initial fluorescence reading. Above day 4, a sharp decrease in ΔE was observed and a further decrease occurred at day 10 with a lower magnitude.

By plotting the normalized ΔE , the leakage profiles of VFF and extrusion produced samples could be compared (Figure 8.8C). Both samples exhibited the apparent increase in encapsulation between day 1 & 4, this was attributed to photobleaching of the sample before lysis. When exponential fits are compared, the leakage profile for the extrusion sample was steeper which suggests VFF samples leak at a slower rate. The reducing leaking of VFF sample could arise from the lower concentration gradient which results from the likely lower initial encapsulation. VFF liposomes also contain residual ethanol, this could act to stabilise the membrane preventing encapsulant leakage. Ethanol incorporates within the bilayer, fluidizing the bilayer which could impede molecule permeation. Alternatively, the lateral expansion which reduces bilayer thickness could reduce the size of the barrier to molecular diffusion out of the liposome. These data sets imply a practical window for the control liposome formulation of 4 days, above which leakage would cause issues with drug delivery studies. The next encapsulant explored was menadione as it is the antagonist studied within Chapter 9.

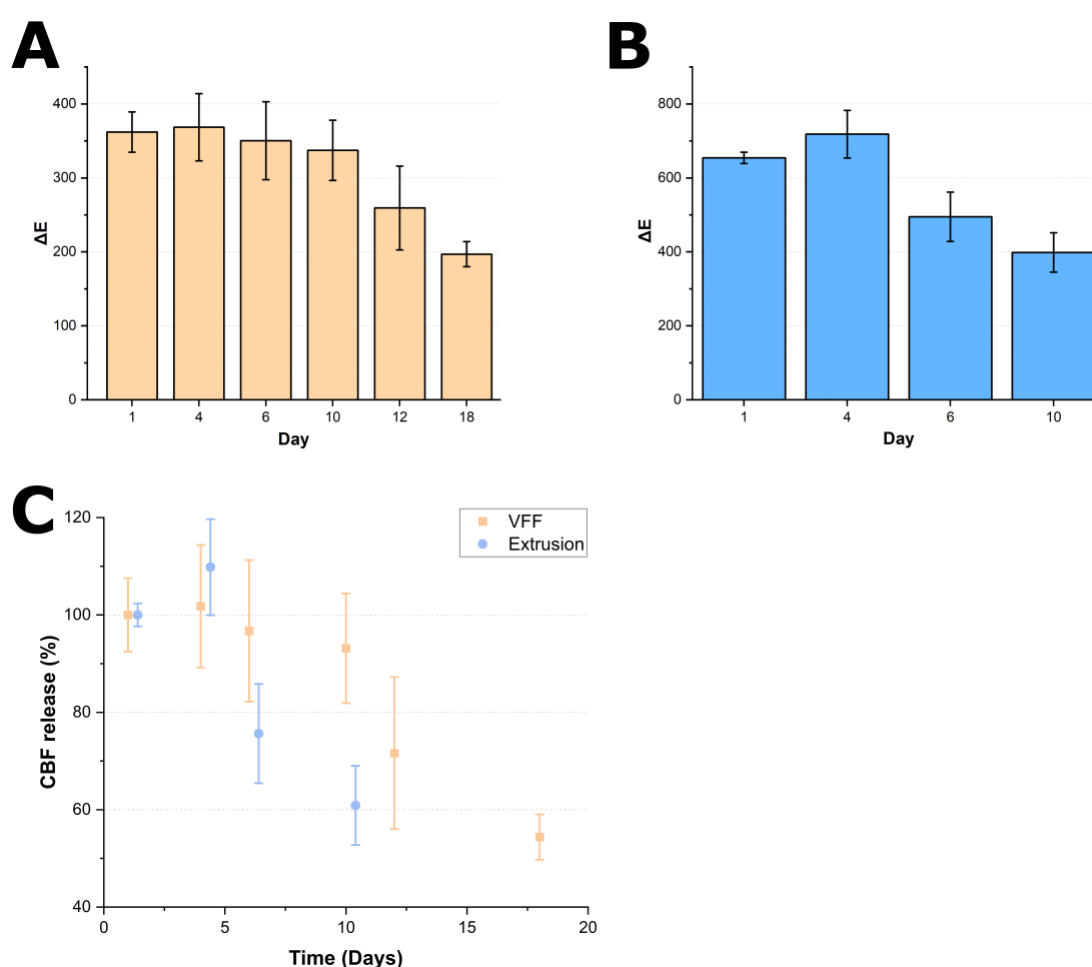


Figure 8.8) Liposome samples containing 100 mM CBF, measured before and after lysis to achieve the change in intensity (ΔE) over an extended period. Recorded changes in fluorescence after lysis for **A** VFF produced sample and **B** extrusion sample both $n = 3$ samples error bars = ± 1 SD. **C** Normalized change in intensity after lysis for VFF (Cream) and extrusion (Blue) (Extrusion sample is offset for clarity $n = 3$ samples, error bars = ± 1 SD).

8.4 Menadione liposomal encapsulation

8.4.1 Menadione introduction

Menadione which is also commonly referred to as vitamin K₃ is a known oxidative antagonist for cardiomyocytes (Figure 8.9A).^{287,288} The action of menadione will be discussed further within Chapter 9 where it is applied to cell culture in a free and encapsulated form. Menadione is comprised of a bicyclic structure, this structure presents a low water solubility ($< 1 \text{ mg mL}^{-1}$). Menadione is a fluorophore with a 331 nm absorption wavelength, this makes it amenable to an absorbent release assay (Figure 8.9B). As menadione is lipophilic, it's expected it to be incorporated into the hydrophobic core of liposome membranes (Figure 8.10).²⁷⁸ Analysis of menadione release can confirm encapsulation success within samples although encapsulation within the lipid leaflet means quantification of EE and release is less meaningful when compared with FAM release. When lysed, menadione likely exists within the lipid fragments and micelles that result instead of being fully released into the bulk aqueous phase where it is detectable. Application of a long-term dialysis release assay could provide accurate EE data.

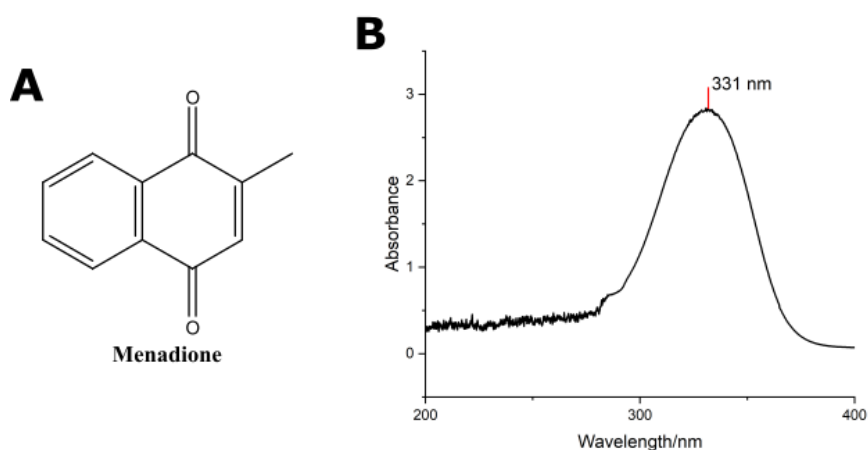


Figure 8.9) **A** Structure of menadione (vitamin K₃) **B** The absorbance spectrum of 0.25 mg mL^{-1} menadione in ethanol, ethanol was used for the effective solubility of menadione.

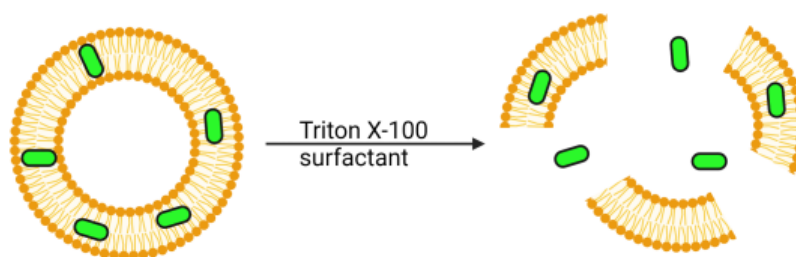


Figure 8.10) Schematic representation of menadione (green) encapsulation in a liposome (yellow) and subsequent release after lysis.

8.4.2 Menadione release assay

Liposomes were produced by VFF using FRR 5 and TFR 0.6 mL min⁻¹ and the control liposome formulation (Molar ratio 47:7:46 DOPC:CHOL:DSPEPEG₂₀₀₀) and menadione was encapsulated directly using the method from Chapter 2.3.5. Samples were analysed immediately after collection off chip and 12 hours after collection (Figure 8.11). When the immediate, 0-hour sample was lysed an increase in absorption was shown indicating a change in the local environment of menadione. The change post-lysis may indicate menadione was successfully encapsulated within the lipid core as free menadione may exhibit higher absorption if the liposome structure scatters light.¹⁸⁵ Samples were collected and run after 12 hours, one set was stored at room temperature and the other was dialysed. When lysed, the 12-hour samples exhibited a greater increase in absorption this implied a larger quantity of menadione was released. Samples initially contained approximately 17 vol% ethanol, the 12-hour samples likely contained less ethanol from the evaporation and dialysis of the samples, this was thought to be the cause of the greater increase in absorption. The pre-triton reading was unchanged for all data points which suggested that a difference in release of menadione was attributed to the increased absorption. Ethanol may result in reformation or stabilization of lipid particles or structures that retain menadione preventing its release into the bulk aqueous phase and subsequent detection by UV-Vis.

This data set confirmed the success of encapsulation of menadione and demonstrated the impact of residual ethanol on the release of encapsulant. Quantification of the encapsulation of menadione was unachievable using this method, future studies encapsulated lower concentrations of menadione (< 90 µM), ensuring the solubility limit was not approached. The ability of VFF to facilitate encapsulation of molecules with low water solubility is also demonstrated, this is especially critical for drug delivery of hydrophobic therapeutic molecules.^{19,24} The concentration of menadione encapsulated by VFF could potentially be greater through exploitation of the higher ethanol solubility of menadione, versus aqueous solubility that is required for extrusion-based encapsulation. When produced by extrusion, menadione was added to the lipid film by a chloroform stock and when hydrated, the solution was full of precipitate. The impact of menadione encapsulation was next explored with a focus on particle size and concentration. This set demonstrates the difficulty in quantifying encapsulation efficiency for hydrophobic moieties.

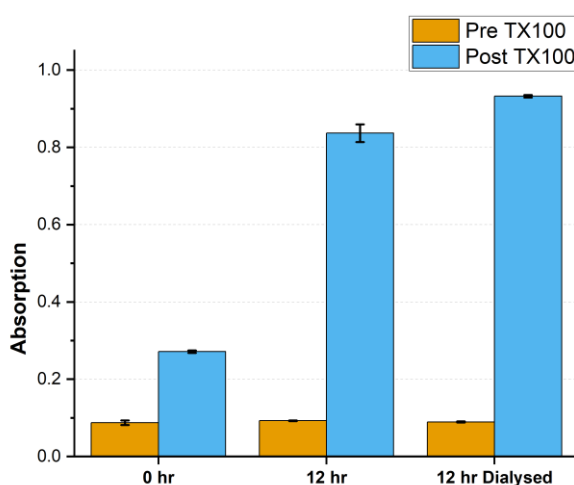


Figure 8.11) Menadione UV-Vis maxima absorption before and after lysis for VFF samples ($n = 3$).

8.4.3 Impact of menadione on particle size and concentration

As menadione is thought to be encapsulated within the liposomal bilayer, particle sizing and concentration analysis was used to explore the impact of menadione concentration. The only example of menadione membrane study was undertaken by Monteiro *et al*²⁸⁹, where menadione was found to distribute across the bilayer using ³¹P nuclear magnetic resonance. Liposomes in this study were produced by VFF using FRR 5 and TFR 0.6 mL min⁻¹ and the control liposome formulation (47:47:6 DOPC:CHOL:DSPEPEG₂₀₀₀). Zeta potential analysis of the three CL replicates determined a + 6.81 ± 4.00 mV surface charge, in the neutral range (± 10 mV), this was expected from the predominantly DOPC and DSPE-PEG₂₀₀₀ formulation (Supplementary S12.1). Menadione was added to the ethanol stream prior to VFF at a concentration of 0.4 mg mL⁻¹, encapsulation was achieved during flow. The final menadione concentration used in the sample was 46 µM, this concentration was applied in latter cell-based studies. Samples were comprised of 3 film replicates, sizing and concentration analysis were performed on the same corresponding samples (Figure 8.12).

Particles produced by VFF both exhibited mean diameters < 100 nm, ensuring they are therapeutically relevant (Figure 8.12). A mean diameter of 52.8 ± 2.5 nm and 73.8 ± 5.1 nm was measured for the control liposome formulation and menadione loaded control liposomes, respectively (Table 7). Encapsulation of menadione was shown to swell the liposomes diameter by approximately 21 nm, indicating encapsulation is likely occurring within the lipid bilayer. Whilst an increase in PDI of 0.04 was also demonstrated with menadione encapsulation, the PDI of the CLM liposomes was still below the 0.2 guideline. The increased PDI observed could be a result of menadione influence in the VFF liposome formation process or the stochastic process of incorporation into the membrane. Particle concentration was largely similar between samples, one CLM sample exhibited a significantly higher concentration which led to a greater average particle concentration. Overall, menadione encapsulation was thought to demonstrate minimal impact on particle concentration. This data set demonstrates the importance of size determination as encapsulation within the lipid membrane can greatly impact mean diameter and PDI impacting the application as therapeutic liposomes.

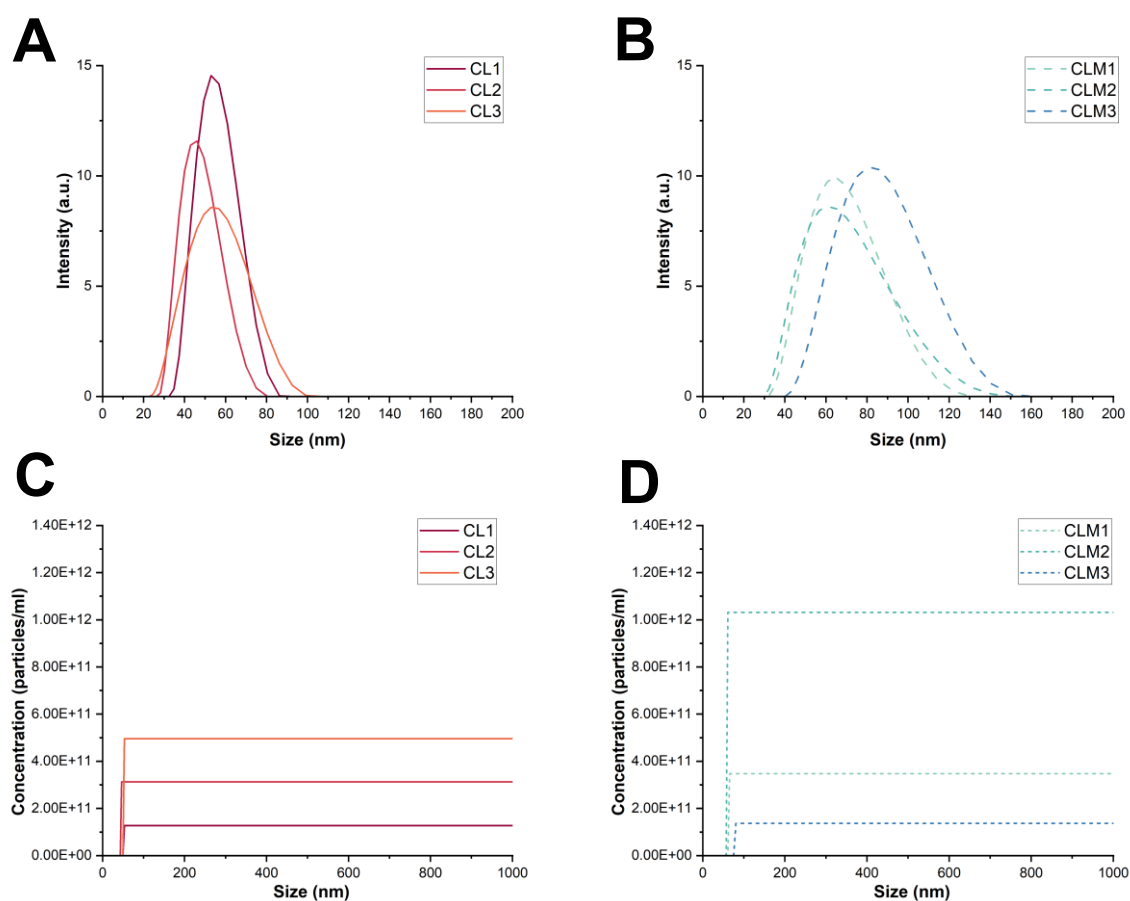


Figure 8.12) Distribution data for 3 film replicates of the **A** control liposomes formulation (CL) and **B** control liposome formulation loaded with menadione (CLM). Particle concentration data for the **C** control liposomes formulation and **D** control liposome formulation loaded with menadione.

	Mean diameter (μm)	PDI	Particle concentration (1×10^{11} particles/mL)
Control liposomes (CL)	52.8 ± 2.5	0.091	3.12 ± 0.92
Menadione loaded control liposomes (CLM)	73.8 ± 5.1	0.130	5.05 ± 2.34

Table 7) Tabulated mean diameter, PDI and particle concentration for the control liposome (CL) and control liposome encapsulating menadione (CLM).

8.5 Formulation stability

8.5.1 Liposome media stability

Liposome stability within cell culturing media is critical for future studies. Cell culturing medium contains an array of lipoproteins, salts, proteins, amino acids, and sugars these can interact with liposomes causing potential rupturing and surface modification.^{290,291} In this work the culturing medium was ATCC DMEM (high glucose), with additional L-glutamine, antibiotic-antimycotic and FBS. The osmolarity of the media was measured and then size analysis was undertaken for samples in media.

8.5.2 Impact of media on particle size

The osmolarity of PBS and the culturing media (DMEM) were measured using an osmometer (Löser). Osmometry indirectly measures the salt content within an aqueous sample by measuring the change in the freezing point of the solution.^{292,293} PBS is a close representation of the salt content within a VFF sample, the dilution with ethanol in VFF would lead to a lower value. A total of eight samples were taken for each medium and the average $\pm$ SD is presented. PBS was determined to have 304.3 ± 0.6 mOsm and the DMEM medium was 284.7 ± 0.6 mOsm. The osmolarities represent the determined salt concentration based on freezing point drift, these values indicate PBS has a higher salt content. A large difference in osmolarity could cause an osmotic gradient which can lead to rupture. The PBS and DMEM values are close in magnitude, this means vesicles are unlikely to rupture due to osmotic pressure when diluted in DMEM media. The pH of liposomes in PBS was measured as $\text{pH } 7.5 \pm 0.1$, which is close to the media pH value of $\text{pH } 7.4$. Close pH values suggest rapid ion exchange would not occur when PBS liposomes are mixed into DMEM media, which suggests stability with regards to pH.

A DLS size comparison was performed on a sample diluted 1:5 in PBS and the same sample diluted 1:5 in DMEM culturing medium (Figure 8.13). The dilution samples were comprised of 3 film replicates, mixing was achieved using pipette aspiration within the cuvette and samples were left for 5 minutes before measurement. In PBS the mean diameter was close to 90 nm, when the sample was diluted in DMEM a mean diameter of 80 nm was observed. The decrease in mean diameter between PBS and DMEM remained within the error, implying a minor perturbation. Overall, DMEM dilution appeared to have minimal impact on liposome size and particles were still observed, suggesting rupturing did not occur within the timeframe studied.

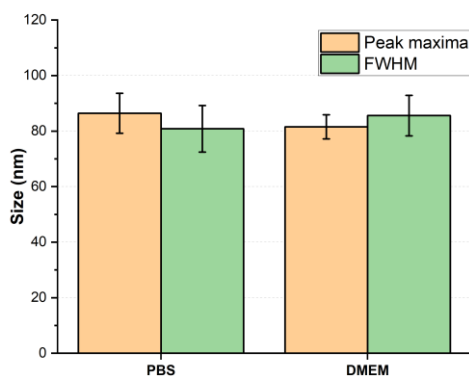


Figure 8.13) Liposome distribution of a sample diluted in PBS and cell culturing medium DMEM ($n = 3$, error = 1 SD).

8.6 Conclusion and future work

8.6.1 Conclusion

Use of SAXS and WAXS enabled the characterisation of the CL liposomal formulation as fluid lamellar. Fluid lamellar assignment has important connotations for ethanol inclusion within the bilayer, as ethanol will induce lateral expansion and swelling for the L_α phase.¹⁹⁴ Characterisation of FAM encapsulation demonstrated VFF encapsulation is comparable at different applied FRR. VFF was shown to exhibit a lower encapsulation than extrusion, which is likely a result of the dynamic mechanism of liposome formation. Long term stability studies suggested VFF produced liposomes exhibited greater stability than an extruded analogue although this may be a result of the lower encapsulation. Minimal encapsulant loss over a 10-day period ($\Delta E = 362 \pm 27$ to $\Delta E = 337 \pm 40$) meant application of liposomes to weeklong studies may be viable, within cell culture. By raising the storage temperature to 37°C and incorporating liposome samples diluted in FBS-media the stability of liposomes could be more effectively established. Raised temperature would likely impact liposome cargo retention and size.

Menadione encapsulation demonstrated the ability of VFF to directly encapsulate lipophilic molecules in flow. Encapsulation of hydrophobic moieties using VFF are currently unreported and the application of a PMMA device could aid ensuring minimal encapsulant loss to the device, especially when compared to PDMS devices. Although, the menadione encapsulation efficiency for direct encapsulation is potentially lower than extrusion and difficult to quantify.^{62,123} Menadione concentration was shown to be impacted by sample age, suggesting further encapsulation occurred with equilibration which further implies low encapsulation efficiency during VFF. A particle concentration study with CL liposomes and menadione encapsulating CL liposomes, suggested encapsulating menadione results in a liposomal swelling. Particle concentration was found to vary between CL samples, the concentrations remained within error of each other.

Characterisation of media osmolality gave a similar value to PBS buffer, suggesting the difference in salt concentration is unlikely to rupture liposomes. When diluted, mean diameter and FWHM remain unaffected, within error. Media dilution is critical for application to TC and these studies suggested liposome would remain intact when applied to TC, preventing premature encapsulant leakage.

Overall, this chapter provided important context for subsequent liposomal delivery studies in Chapter 9.

8.6.2 Future work

For future study, menadione encapsulation could be evaluated by measuring micro-viscosity using BODIPY molecular rotors and employing an extrusion control. Menadione inclusion would likely increase the micro-viscosity of the membrane, as closer packing is induced in the alkyl tail region. A longer term study may be required to observe the effects of media dilution, if at all on the membrane stability. Investigating this media stability at 37°C could also further replicate the application conditions. Finally, exploring the encapsulant leakage within a media environment could also further understanding into the stability of liposomal structures.

9 – Drug delivery to tissue culture and micro-engineered heart tissue

9.1 Overview

Evaluating success of liposomal delivery *in-vitro* is an ever-increasing field, driven by the development of increasingly specific drug delivery liposomes. Within this chapter toxicity of menadione solution on H9C2 cells is measured using a Hoechst/DRAQ7 alive dead assay. Through encapsulation of menadione within liposomal formulations, the alive dead assay was extended to study liposomes and assess liposomal facilitated menadione delivery. Liposomal delivery is then explored within our bioprinted tissue constructs, enabling exploration of liposomes within a 3D ECM. Extending study of liposomes to model ECM is critical to understanding drug delivery *in-vivo* as the ECM represent a critical diffusional barrier to drug delivery. By implementing VFF liposomal production, this chapter demonstrates the full lifecycle of liposomal formulation and efficacy testing.

9.2 Menadione driven cell toxicity

9.2.1 Menadione mechanism

Menadione is from the quinone family of molecules (Figure 9.1). Within the field of cell biology, menadione has seen significant use as a source of cellular ROS.^{294,295} The high lipophilicity of menadione means it readily enters cells, where menadione can undergo one electron reduction to form semi-quinone radicals and subsequent reactions with oxygen, producing oxygen radicals intracellularly.²⁸⁸ Production of high levels of intracellular ROS results in an oxidative cell stress, with cardiomyocytes this can mimic the cardiomyocyte damage observed following myocardial infarction.^{288,294,296} Menadione at high concentrations can also inflict mitochondrial DNA damage, lipid peroxidation and cell death.^{287,288,294,295} Menadione is reported to facilitate cell-death by activating programmed apoptotic pathways.²⁹⁵ Cell death is a commonly quantified attribute within the study of therapeutic molecules.²⁹⁷ By determination of changes in cell viability, the fraction of alive cells, the impact of therapeutic intervention or antagonism can be evaluated. This chapter first explores cell viability with free menadione and liposomal encapsulated menadione treatment, then the efficacy of liposomal hydrophobic drug delivery, *in-vitro*.

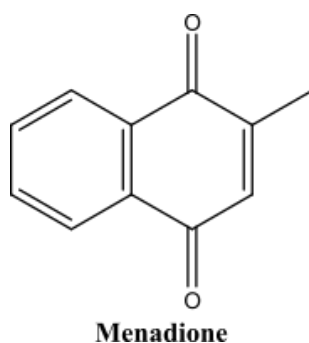


Figure 9.1) Chemical structure of Menadione (Vitamin K₃).

9.2.2 Liposomal targeting

Targeting liposomes explored within this Chapter applied several liposome-linked targeting peptides. The first targeting peptide was developed by Dasa *et al*³⁹ for application in post-MI therapeutic intervention. Dasa *et al* linked the peptide to DSPE-PEG₃₀₀₀ and determined the peptide linked liposomes accumulated within the infarct border zone of rats subjected to MI. Within our work, the peptide linked entity was kindly supplied by Siva Sai Krishna Dasa of the University of Virginia. The next peptide explored was the CHP, first reported by Vandergriff *et al*⁴⁰. In the study by Vandergriff *et al*, CHP was linked to exosomes and studied *in-vitro* and within mouse models. CHP exosomes were shown to accumulate within animal heart sections and reduce apoptosis within *in-vitro* infarct models. The CHP peptide has the sequence CSTSMLKAC and a scrambled analogue CSKTALSMC was also applied within the study. The scrambled analogue was applied from work by Vandergriff *et al*⁴⁰ and features a scrambled internal sequence, retaining the cysteine termini is essential to facilitate coupling with lipid-maleimide, resultant lipid-peptide entities can then be incorporated into liposomes readily. Within our work, the CHP peptide was synthesized using Genscript peptide synthesis service and linked to DSPE-PEG₂₀₀₀ using the procedure from Chapter 2.3.3.

9.2.3 Menadione titration in tissue culture

To explore the liposomal delivery of menadione, the effect of menadione in solution was first explored using the method from Chapter 2.4.2. Menadione (0-110 μM) was added to H9C2 cells seeded within a 96 well (0.5 cm^2 well area) plate, at time intervals of 2, 4 and 8 hours before measurement (Figure 9.2). All cell work within this Chapter is expressed in terms of the measurement replicates (n) and biological replicates (n_{bio}). The total number of measurements taken is equal to $n \times n_{\text{bio}}$ wells.

A 2-hour incubation with menadione was shown to have minimal impact on cell viability. At 4-hour incubation time, significant cell death was observed above 50 μM menadione and increasing further to 110 μM menadione, cell viability was 50 %. At 8-hour incubation time, significant cell death was observed at 30 μM menadione and < 20 % viability was observed above 80 μM menadione.

At 2-hours, the incubation time seemed insufficient for menadione to enter the cell, generate significant ROS and facilitate apoptosis. Within our study, lower incubation time resulted in lower SD between readings suggesting a more reliable measurement this is likely due to reduction in measurable cells, significant cell delamination was observed at high cell death fractions. From the application of the viability assay, a final menadione encapsulation concentration of 70 μM per well and incubation time of 3-hours were decided, to achieve 70 - 80% viability within the latter menadione liposome delivery studies.

The applied menadione dosage assumes 100 % encapsulation efficiency, this is an approximation as menadione exhibits low water solubility therefore will exist within the suspension buffer and may also have leaked into the PMMA VFF device.¹⁴¹ The viability assay measurement is dependent on retaining a sufficient alive cell count to determine viability, at high cell death measurement is complicated by increased rates of cell detachment. At 70 μM with a 3-hour incubation, the viability is expected to be 70 – 80 %, this represents an observable decrease without impacting the measurement validity.

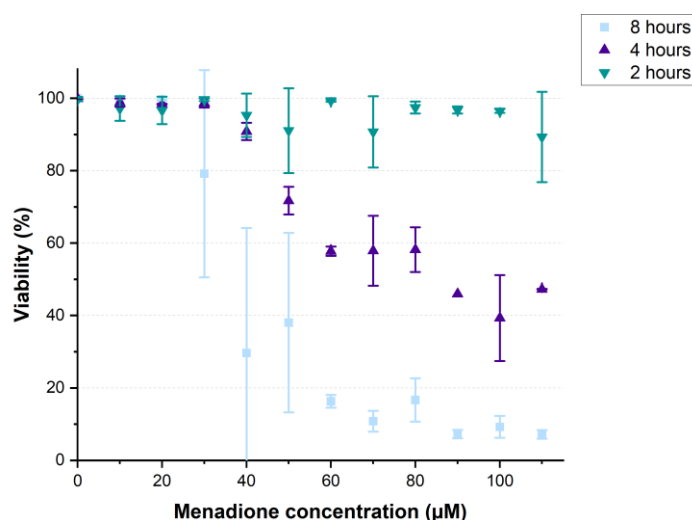


Figure 9.2) Menadione concentration impact on H9C2 cell viability at 2–8-hour incubation periods $n = 2$ measurement replicates, taken from $n_{\text{bio}} = 3$ separate passages.

9.3 Menadione delivery in tissue culture

9.3.1 Investigating addition to tissue culture

To explore toxicity of liposomes without menadione encapsulant, the control liposome (CL) formulation was first applied. The CL formulation was applied within Chapter 8.3.2, and is comprised of a 47:46:7 DOPC:CHOL:DSPE-PEG₂₀₀₀ molar ratio. Within this chapter, VFF was used to produce 52.8 ± 2.5 nm diameter liposomes at FRR 10, TFR 1.1 mL min^{-1} . Dialysed liposomes were added at 10 - 20 vol% per well (10 - 20 μL) and incubated for 3 hours; the 80 – 90 vol% was DMEM. The final liposome and lipid concentrations ranged from $3.12 \pm 0.92 \times 10^{10}$ liposomes mL^{-1} and 0.5 mg mL^{-1} lipid to $6.24 \pm 1.84 \times 10^{10}$ liposomes mL^{-1} and 1 mg mL^{-1} lipid. Liposome concentrations were estimated from the MADLS study within Chapter 8.4.3. The cell death assay was performed using $n = 10$ wells per condition and $n_{\text{bio}} = 1$, for 10 measurements per point. CL addition was explored in premixed CL solutions in DMEM and by direct addition of CL followed by DMEM dilution, within the well. Conditions were tested to ensure application of higher concentration liposomes (direct addition) followed by dilution did not impact viability from the short-term higher liposome concentration. A media control was used throughout the study to compare the viability decrease associated with media change and the staining process. Our data suggests adding 10 - 20 vol% CL has minimal impact on cell viability (Table 8). This is important as a liposome associated toxicity could complicate studies using menadione loaded liposomes.

Dosage method	Cell viability (%)	$\pm\text{SD}$ (%)
Media	94.5	6.7
Pre-mixed		
10% CL	94.2	5.4
20% CL	94.9	4.3
Direct addition		
10% CL	99.0	2.1
20% CL	97.4	2.1

Table 8) Viability after 3-hour incubation with 10-20 vol% CL in DMEM by two addition methods ($n = 10$, $n_{\text{bio}} = 1$).

Addition of dialysed and undialysed CL was explored at higher volume fractions, over the course of 3-hours (Table 9). Dialysis was used to explore whether the residual ethanol content within the lipid bilayer could result in a significant viability decrease and further complicate menadione assays. A total

of $n = 10$ replicates were used for the $n_{\text{bio}} = 3$ repeats, values are the mean of the 30 recorded values. Media and 70 vol% PBS controls were applied, and the impact of the media and PBS diluted media were shown to be minimal. PBS diluted media is a more representative control as liposome suspensions were in PBS and acted to dilute media. Overall, sample remain within experimental error indicating dialysing samples was non-essential, this suggests residual bilayer ethanol has a low impact on cell viability within the explored time frame. Minimal ethanol toxicity was unexpected, this is likely a result of the 3-hour time frame studied, and ethanol toxicity may be more problematic for longer incubation periods. Removing the need for dialysis is important, given menadione loaded liposomes could further lose encapsulant during the dialysis process. As such, a 3-hour time frame for study was considered favourable.

Treatment	Cell viability (%)	$\pm$ SD (%)
Media	97.4	1.1
70% PBS	97.6	1.1
Dialysed		
30% CL	97.2	2.8
70% CL	93.8	3.2
Un-dialysed		
30% CL	96.5	2.8
70% CL	91.1	6.1

Table 9) Viability after 3-hour incubation with 30vol% and 70vol% CL in DMEM pre and post dialysis ($n = 10$, $n_{\text{bio}} = 1$).

9.3.2 Preliminary targeted menadione delivery to H9C2 culture

After determination of liposome and ethanol associated toxicity, a preliminary study was performed with liposomes containing menadione. Liposomes contain 70 μM menadione that was encapsulated directly using VFF, where menadione was added to the lipid film in a chloroform stock. Within this chapter, VFF at FRR 10, TFR 1.1 mL min^{-1} produced 73.8 ± 5.1 nm liposomes containing menadione. Dasa liposomes were produced from the CL formulation, with addition of a DSPE-PEG₃₀₀₀-I1 linked peptide at 47:46:6:1 DOPC:CHOL:DSPE-PEG₂₀₀₀:DSPE-PEG₃₀₀₀-I1 molar ratio. Volume fraction of liposomes was increased from 10 - 50 vol% representing 7 - 35 μM menadione concentration, assuming menadione loss during VFF was minimal and 100% encapsulation efficiency. The menadione stock in DMEM was increased from 0 - 100 vol% representing 0 - 70 μM menadione concentration. Cells were incubated with liposomes for 3 hours, $n = 8$ measurement replicates at $n_{\text{bio}} = 1$ were performed.

Increasing volume fraction of CL had minimal impact on viability, conversely increasing Dasa liposomes vol fraction decreased cell viability (Figure 9.3). The menadione solution control showed a decrease in viability with increasing fraction, as expected. At 50 vol% Dasa liposomes, a mean viability of 40 % was observed, demonstrating potential liposome-facilitated delivery of menadione. At 50 vol% liposome, the menadione concentration was 35 μM , cell viability at 50 vol% Dasa liposome was comparable to the value at 70 μM free menadione, within this study. The efficacy of liposomal delivery was expected to be lower than free menadione, as the kinetics for liposomal delivery are likely slower. The observation of more effective liposomal delivery could suggest an endocytosis facilitated delivery may occur resulting in high localised menadione concentration within the cells which may induce higher cell mortality. This data set provided promising evidence that an alive/dead assay can be used to study liposomal delivery of menadione. To further understand liposomes localization within the study, Texas red lipid was incorporated into the formulation.

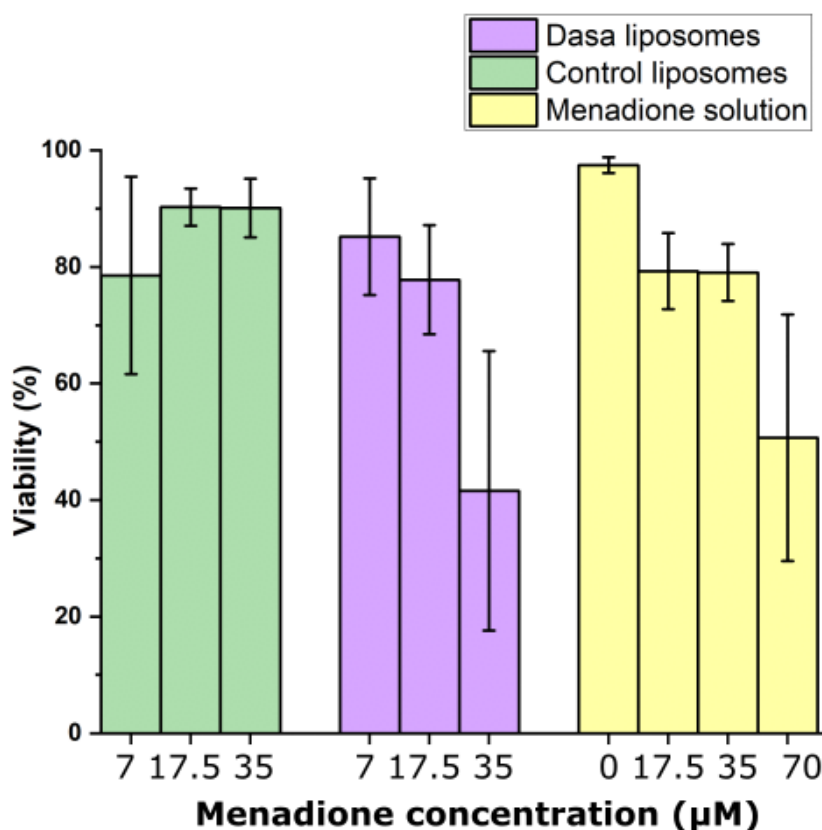


Figure 9.3) Impact on cell viability of increasing volume fraction of CL, Dasa liposomes relative to a free menadione solution ($n = 8$ measurement replicates at $n_{bio} = 1$).

9.4 Liposome accumulation *in-vitro*

9.4.1 Imaging liposome accumulation

After producing preliminary liposome toxicity studies, incorporation of Texas red into the liposomes was used to study liposome-cell localization (Figure 9.4). In this study, 3 mol% DHPE-Texas red was added to the lipid film prior to VFF, 3 mol% was chosen to provide sufficient signal within the study, while minimising structural impact. A purely DOPC formulation was tested in addition to the CL and Dasa formulations. Cells were incubated with 50 vol% liposomes for 12 hours, lower periods were found to result in a low Texas red signal. A total of $n = 10$ replicates were performed with $n_{\text{bio}} = 3$ replicates for 30 measurements per data point. After incubation, cells were washed with 2 media portions and stained with Hoechst. Image analysis was used to identify cell nuclei, the mask produced was then doubled in size and the overlap with the Texas red channel was used to quantify the fraction of Texas red positive cells. The Hoechst mask was doubled to capture the cytoplasm area within cells, where Texas red was observed. Generally, Texas red accumulation was observed in the cytoplasm rather than the cell nucleus. The method of determining Texas red positive cells was an approximation, fits to the Texas red channel were achieved despite the image grain complicating object acquisition. The grainy Texas red image could suggest liposomes were predominantly adhering to the cell membrane.

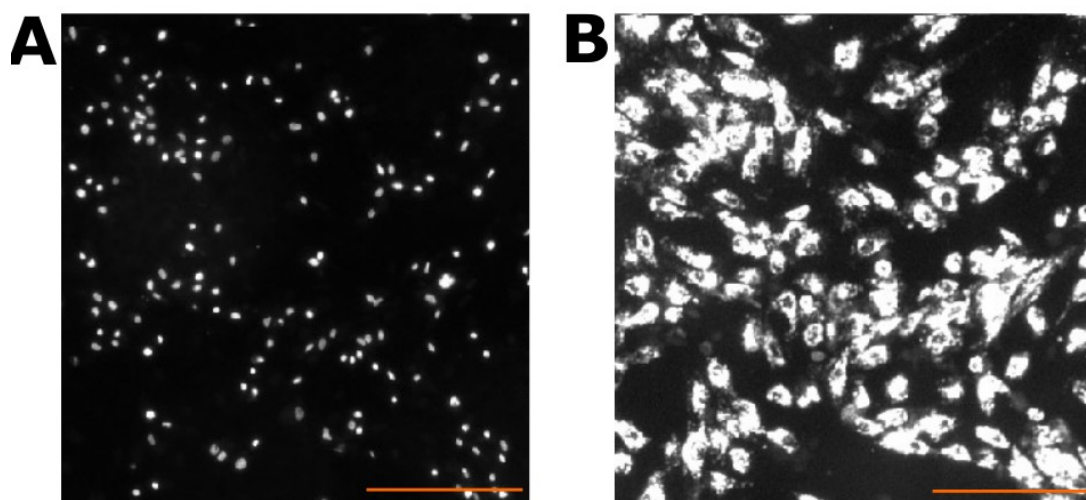


Figure 9.4) Images of cells incubated with DOPC Texas red liposomes and stained with Hoechst **A** Hoechst channel image displaying the live cell stained nuclei **B** Texas red image displaying the areas where liposome accumulation occurred, within the same area (Scale bar = 400 μm).

9.4.2 Comparing liposome accumulation

Qualitative comparison of cells treated with DOPC, CL and Dasa liposomes showed varying Texas red channel intensity (Figure 9.5). DOPC liposomes resulted in the highest intensity, this suggests the DOPC liposomes were the most fusogenic or cell adherent. The presence of pegylated lipids in the CL and Dasa formulations was thought to contributed to the lower Texas red intensity observed over the

12-hour period by reducing the interaction of liposome with H9C2 cells. When quantified, an increased frequency of Texas red positive cells was observed with DOPC liposomes (Figure 9.6). Reduced Texas red signal was observed in CL and Dasa samples, the mean value for Dasa liposomes was higher than CL. The data could suggest the Dasa formulation is more fusogenic or cell adherent than the CL formulation. This data effectively shows both CL and Dasa liposome can adhere or fuse with H9C2 cells. When performing liposome toxicity assays, the viability decrease observed is likely a result of adherent or fused liposomes facilitating in cell death.

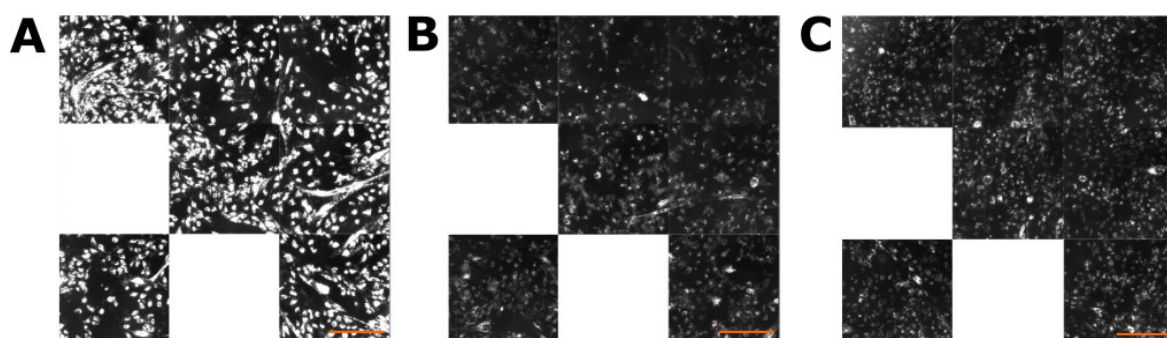


Figure 9.5) Images of cells incubated with Texas red liposomes of the formulation **A** DOPC **B** CL and **C** Dasa. Images taken from the well centre excluding the bottom and left tiles where pipetting was undertaken and could reduce cell confluency.

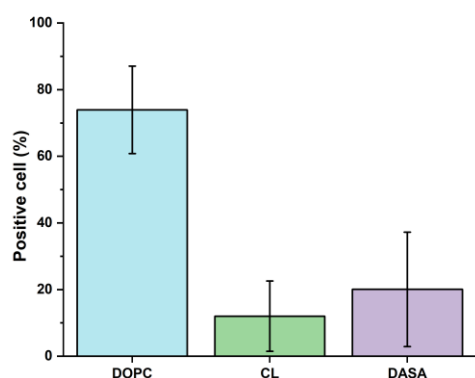


Figure 9.6) Texas red positive cell fractions for DOPC, CL and Dasa formulations $n = 10$, $n_{bio} = 3$.

9.5 Menadione liposomal delivery

9.5.1 Control and Dasa liposome toxicity

With the localization of liposomes to H9C2 cells established, toxicity of menadione CL liposomes was studied (Figure 9.7). For the control treatment with media and 70 μ M menadione solution were used for low and high cell death respectively. The CL liposomes were produced by VFF with a 70 μ M menadione concentration and added at 90 vol% fraction, for a 63 μ M menadione concentration. Viability with media change was determined to be 93.0 ± 6.5 %, this was within error of the menadione control liposomes (84.4 ± 11.7 %) (Figure 9.7). A low viability of 52.5 ± 20.1 % was determined for the

70 μM menadione solution. From this data set, control liposomes encapsulating menadione may exhibit an inherent toxicity, from the leakage of menadione although this value is low when considering experimental error. The potential viability decrease associated with the menadione CL was still far below the effect of free menadione, suggesting significant leakage of menadione did not occur during the study. The viability of targeting Dasa liposomes was then explored, to establish whether targeting provided a distinguishable effect.

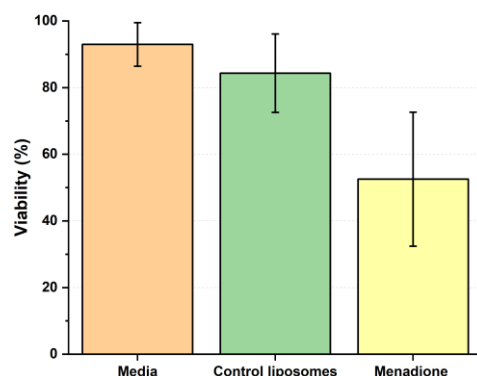


Figure 9.7) Viability of cells treated with media, CL containing menadione (63 μM) and menadione (70 μM) in solution $n = 6$, $n_{\text{bio}} = 5$.

To establish the effecting of peptide-based targeting, Dasa liposomes were produced with a 70 μM menadione concentration, a control of media and menadione containing control liposomes was applied (Figure 9.8). Liposomes were added at 90 vol% with a total of $n = 6$ replicates and $n_{\text{bio}} = 5$. The results were plotted with the menadione control used in the control liposome toxicity assay. Media change exhibited a 93.9 ± 6.4 % viability and control liposomes containing menadione exhibited a viability of 90.1 ± 3.3 % (Figure 9.8). Dasa liposomes exhibited a viability of 36.3 ± 12.4 % which was below the value for the 70 μM menadione solution. The menadione concentration of the diluted Dasa liposome sample was 63 μM and the lower concentration and liposomal encapsulation of menadione was expected to lower the impact on viability, when compared to the free menadione solution. This data set demonstrated a significant change in viability between CL and targeting Dasa liposomes, this implies the Dasa peptide is facilitating menadione delivery to H9C2 cells. The mechanism for Dasa liposomal delivery or accumulation is unreported, our data suggests inclusion of the peptide entity does improve menadione delivery. Next a comparative study was developed to explore different targeting entities.

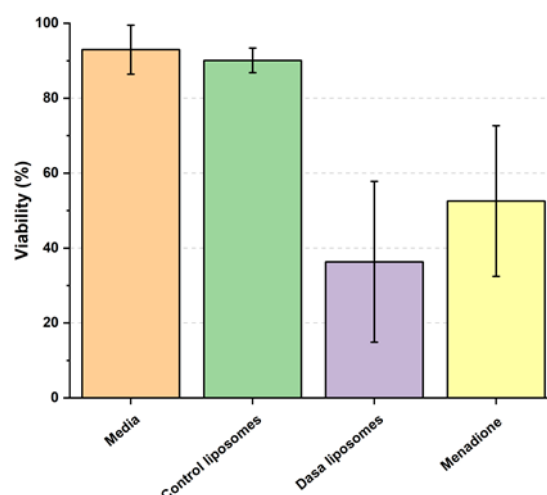


Figure 9.8) Viability of cells treated with media, control liposomes (63 μ M menadione), Dasa liposomes (63 μ M menadione) and menadione in solution (70 μ M) $n=6$, $n_{bio}=5$ (Error = ± 1 SD).

9.5.2 Comparison of targeted delivery

In this study, viability was explored with menadione containing Dasa liposomes and CHP liposomes. For controls, media, CL liposomes with menadione, scrambled CHP liposomes and menadione solution were applied. Scrambled CHP is a randomized AA sequence using the same constituent AA as CHP, this control infers specificity to peptide sequence. A total of $n=10$ measurement replicates and $n_{bio}=6$ were collected. The end menadione concentration was 63 μ M for all samples, except the media control. CHP liposomes and the scrambled analogue were synthesized analogously to Dasa liposomes with a 47:46:6:1 DOPC:CHOL:DSPE-PEG₂₀₀₀:DSPE-PEG₃₀₀₀-I1 molar ratio.

Treatment with media and CL liposomes gave viabilities of $81.9 \pm 6.8\%$ and $72.8 \pm 6.9\%$ respectively (Figure 9.9). The viability for the media and CL liposome controls were lower than in previous studies, this could be the result of the latter cell passages employed within the study, the error within this series was also larger. The 9.1 % decrease in viability between media and CL liposomes was comparable to previous data (Figure 9.9). The menadione control gave a viability of $63.9 \pm 11.5\%$, this was in the expected range based on data for 70 μ M menadione. The viability with CHP scrambled, CHP and Dasa liposomes was $61.1 \pm 22.2\%$, $68.9 \pm 20.5\%$ and $67.9 \pm 23.1\%$ respectively.

The scrambled CHP liposomes presented the lowest observed mean viability within the study however large error in the result the reduction was within error of all other treatments. The similarity between the different peptide residues may suggest the interaction is non-specific and facilitated by the presence of randomized peptide residues. CHP and Dasa liposomes both induced similar viability, the range of the standard deviation was within the media, CL and menadione viability suggesting the alive/dead assay was ineffective at distinguishing delivery efficacy for this study. The impact of Dasa liposome was less conclusive than the previous study (Figure 9.8). To conclude, this data set shows addition of peptides, to pegylated liposomes containing menadione resulted in indistinguishable impact, from treatment with media. From this work, the action of the peptide is unclear and could be

from endocytosis facilitated delivery or improved interaction with the H9C2 cell surface. Variable liposome concentrations were explored to establish the dose dependence of liposomal menadione.

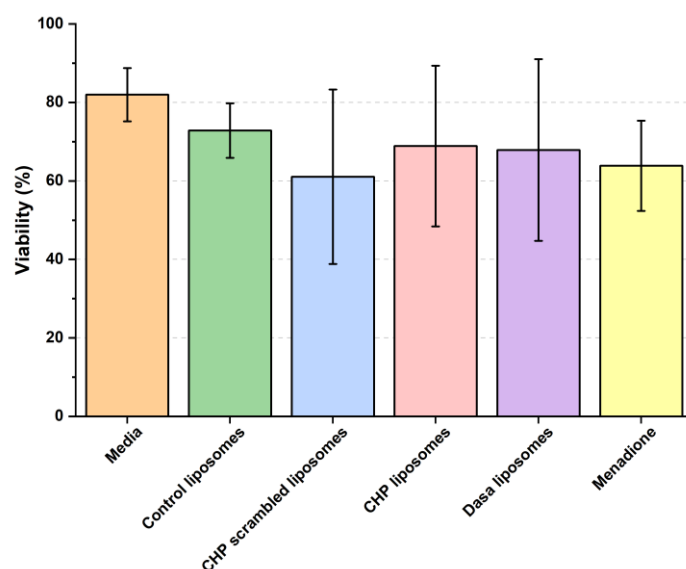


Figure 9.9) Viability of cells treated with media, control liposomes (70 μ M menadione), CHP scrambled liposomes (70 μ M menadione), CHP liposomes (70 μ M menadione), Dasa liposomes (70 μ M menadione) and menadione in solution (70 μ M) $n = 10$, $n_{bio} = 6$ (Error = ± 1 SD).

9.5.3 Menadione liposome titration

By exploring applied liposome concentrations, a concentration dependent toxicity was established for the 70 μ M menadione liposomes. Within the study the control liposomes and Dasa targeting liposomes were studied to follow from work by Dasa *et al*³⁹. Liposomes were diluted at 10 – 90 vol% in cell media, representing a menadione concentration of 7 – 56 μ M within the samples.

Within the explored range, application of control liposomes containing 70 μ M menadione exhibited minimal impact on viability (Figure 9.10). To achieve a dose response using the control liposome formulation, a higher menadione encapsulation concentration or longer incubation is likely required. At high menadione concentration, the solubility limit of menadione may be reached reducing the validity of the study. Dasa liposomes resulted in a viability decrease from 84.09 ± 8.70 % to 36.34 ± 21.44 % from 20 vol% to 90 vol%, respectively. An approximate dose response fit was performed on the titration data set, from this an EC_{50} of 51.68 μ M was determined where the EC_{50} is the menadione concentration value at 50 % viability, for free menadione the EC_{50} value is between 80 – 90 μ M, at a 4-hour incubation time (Figure 9.11). The EC_{50} for Dasa liposomal menadione was expected to be lower than the free menadione value, this could suggest liposomal delivery can improve the delivery of menadione. The control liposome EC_{50} suggests the effects of inherent liposome toxicity are minimally impactful.

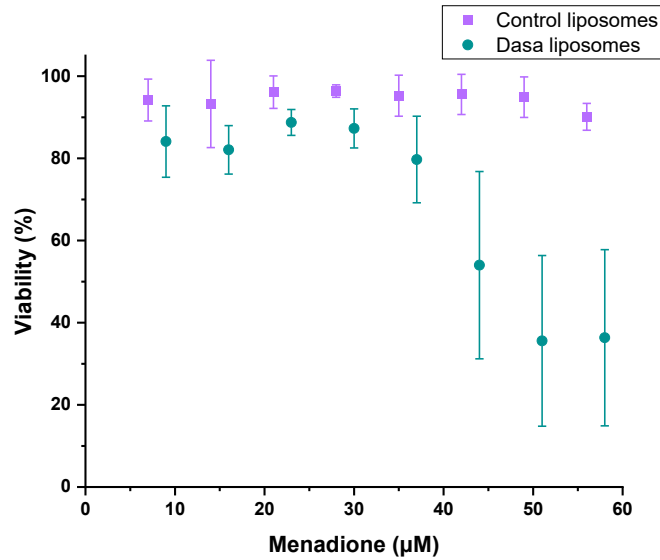


Figure 9.10) Viability of cells treated with 10 – 90 vol% menadione containing control liposomes and Dasa liposomes.

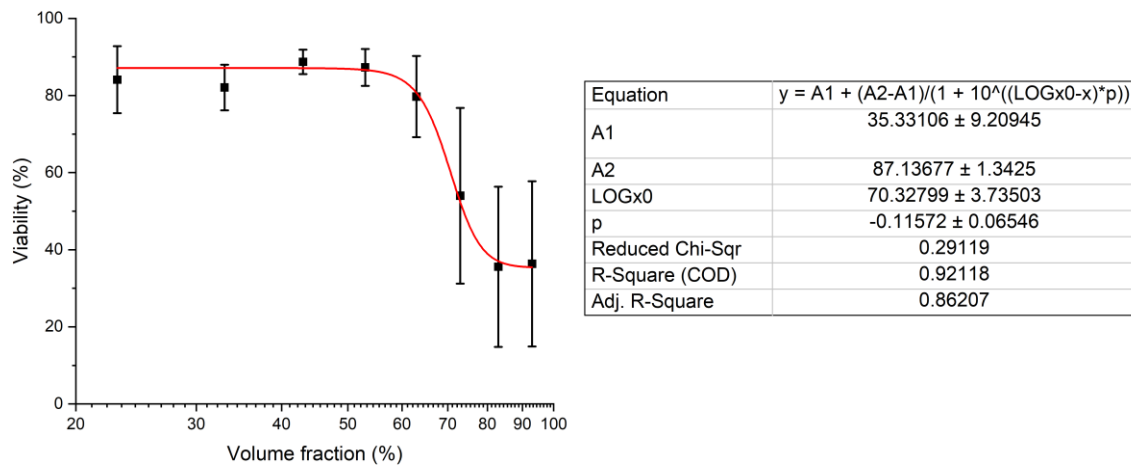


Figure 9.11) Dose response fitting to the Dasa liposome toxicity at 20 – 90 vol%.

9.6 Liposomal delivery to micro-engineered heart tissue

9.6.1 Assay design

In the final part of this work, the toxicity of menadione liposome was explored within the H9C2 bioprinted tissue model. The model was printed into a 6 well plate using 1×10^5 cells mL^{-1} applying the procedure developed within Chapter 7.5.3. Constructs were incubated in media overnight before treatment with either fresh media or liposomes at 90 vol% (4.5 mg mL^{-1} lipid content, $2.81 \pm 0.84 \times 10^{11}$ liposomes mL^{-1} , 70 μM menadione) and subsequent incubation for 3 hours (Figure 9.12A). the total cell count of each printed construct is estimated to be 3.2×10^5 cells, this value will vary based on print defects such as nozzle blockages. High liposome concentration was chosen to mitigate the barrier to diffusion the alginate gel poses, and 3-hour incubation was chosen to minimize the cell

death attributed to menadione activity. Initially Hoechst and DRAQ7 stains were applied, application of Hoechst resulted in non-specific staining of the ECM, which made determining viability unfeasible. A potential substitute for Hoechst could be Calcein-AM which is converted into fluorescent Calcein by intracellular esterase's, however a suitable filter set was unavailable to utilise this stain within the study.²⁹⁸

To avoid Hoechst, this study employed DRAQ7 as a dead nuclei stain and Texas red within the liposomes, to confirm localization (Figure 9.12B). The DRAQ7 stain highlighted a significant portion of dead cells, as bioprinting retains cells within hydrogel dead cells cannot be aspirated and are present within samples at higher fractions than conventional tissue culture. Additionally, because of the long time (>20 min) spent in suspension and the applied shear during bioprinting, viability was expected to be reduced. The Texas red fluorescent channel showed accumulation within cells and the ECM, cell accumulation was evidenced by localized high intensity within cells and ECM accumulation was shown by the diffuse bright background in the image. This study employed lower incubation time than the previous Texas red accumulation study in Chapter 9.4.1 (12 hours), as the higher lipid concentration aided Texas red accumulation.

The Texas red stain showed preliminary evidence that liposomes could diffuse through the ECM and were able to reach cells within the ECM. The intention for the Texas red channel was to produce an approximate image of cell localization and then overlap with the DRAQ7 image to determine the fraction of dead cells that exhibit liposome accumulation. Although, the grainy Texas red images made development of an image mask infeasible and by relation, the overlap determination. This study instead employed a total dead cell count from the DRAQ7 channel, this can evaluate liposome efficacy, with the assumption that cell density and by relation print quality, was uniform within the studied image tiles. Print defects were observed within the study; thus, the results of the study were considered are inconclusive, although act to show novel, evidenced application of therapeutic liposomes within a bioprinted tissue model. Images were produced from $n = 64$ tiles, and a total of $n_{\text{bio}} = 5$ printed constructs, reported dead cell counts were the mean of the reported total cell death values from each well and error is expressed as ± 1 SD.

Following image analysis, the number of dead cells within each treated sample was quantified from the DRAQ7 stain (Figure 9.13). Treatment with media resulted in 5055 ± 887 dead cells per well, whilst CL treatment resulted in 5013 ± 2220 cells per well. Both media and CL liposomes exhibited similar toxicity, within error, although the CL control exhibited larger error. Treatment with Dasa liposomes exhibited the highest mean cell death quantity of 6943 ± 3098 cells per well, though the large experimental error meant this value was within the media and CL controls. In previous tissue culture studies within Chapter 9.2.3 large experimental error was typically indicative of higher cell death fractions, which may suggest the Dasa liposomes are inducing greater cell death. In summary, the large experimental error suggests the alive/ dead assay may not be scalable to bioprinted models, at least not in the current state. Although unsuccessful, this data set shows liposomal studies could be performed on bioprinted tissue constructs and enable quantification of the impact of a hydrogel ECM barrier on liposomal efficacy.

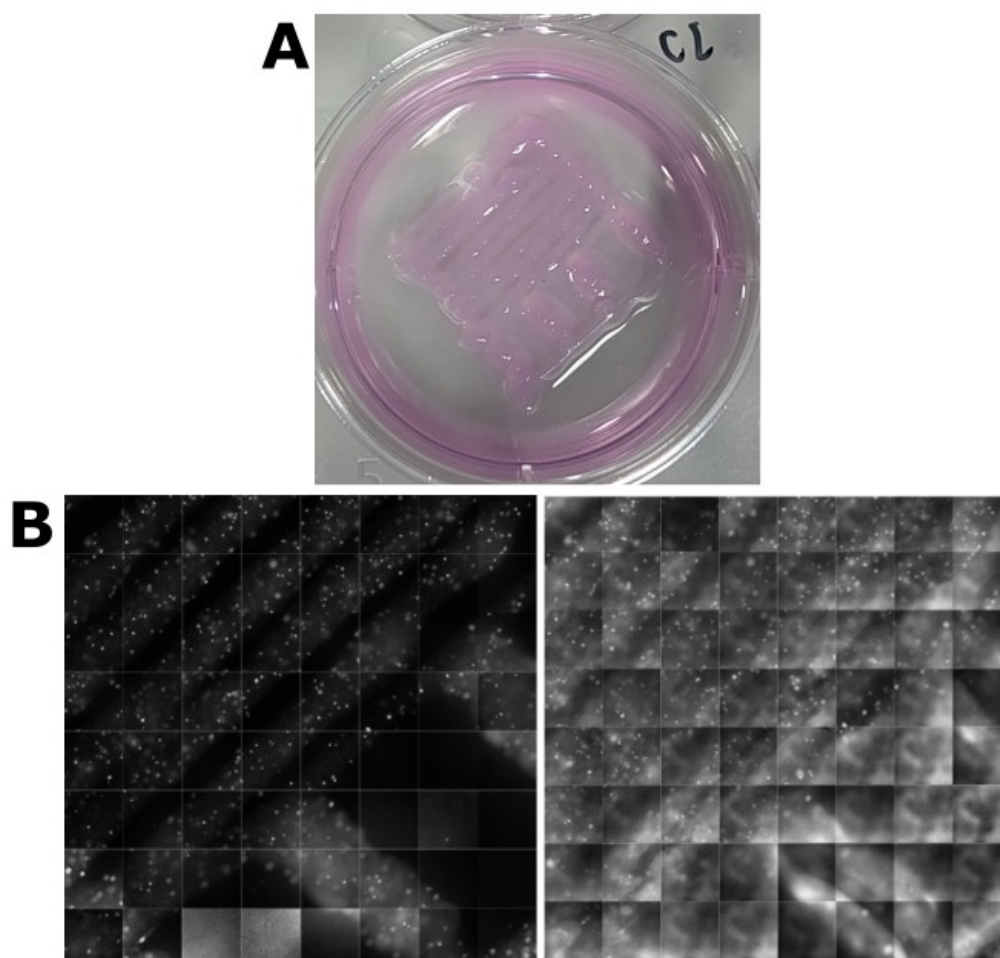


Figure 9.12) **A** Image at 5x magnification of bioprinted tissue construct within a well, showing the alginate grid structure submerged in media, print defects are shown to the bottom and relate to the imaged structures below **B** Tiled fluorescent microscopy images of the bioprinter tissue construct shown above in the DRAQ7 dead nuclei stain (Left) and Texas red stain indicating liposome accumulation (Right).

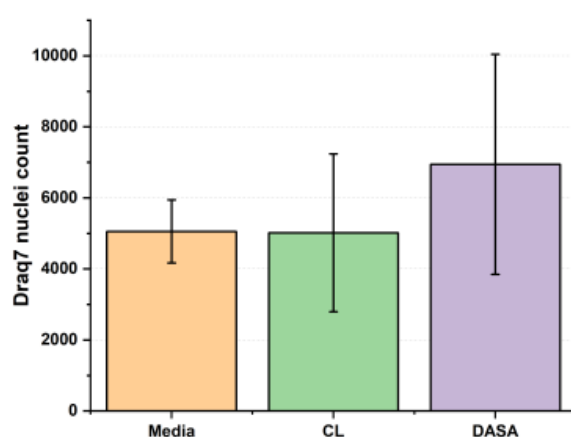


Figure 9.13) Draq7 positive nuclei count within H9C2 alginate bioprinted constructs incubated with media, control liposomes (70 μ M menadione) and Dasa liposomes (70 μ M menadione) for 3 hours.

9.7 Conclusion and future work

9.7.1 Conclusion

This Chapter presented an alive dead assay to determine the effects of free menadione solution on H9C2 cells. Menadione was shown to have a concentration and time dependent inhibitory effect on cell growth and viability. From the study, a concentration of 70 μM and incubation time of 3 hours was applied in future studies. At 70 μM and 3-hour incubation time, 30 – 40 % cell death was observed, this value was thought to be appropriate for alive dead study with liposomal systems. A low cell death (< 60 %) is preferable, as significant cell death (> 60 %) reduces the sample count resulting from cell detachment. Liposome dosing methods and concentration were then explored within H9C2 tissue culture, addition of 10 - 20 vol% control liposome volume over 3 hours was found to be ineffectual viability, within error (Table 8). Dialysis was used to explore the impact of residual ethanol, the process of dialysing liposomes before addition resulted insignificant change in viability, when considering the error. Dialysis results indicated residual ethanol exhibits minimal impact within the 3-hour time frame studied.

With the sample-based effects established, a preliminary study was performed using CL, Dasa liposomes and free menadione solution. The study established a low toxicity of CL encapsulated menadione (90.1 ± 5.0 %) and higher toxicity of Dasa menadione liposomes (41.6 ± 24.0 %), at 50 vol% concentration. The Dasa liposome toxicity was unexpectedly greater than the menadione stock (79.0 ± 4.9 %), at equivalent menadione concentration. Liposomal formulations encapsulating menadione have not been explored within the literature although the membrane activity of menadione added to liposomes has been assessed.²⁹⁹ Menadione is reported to exhibit favourable interactions with zwitterionic lipids and facilitate lipid peroxidation for poly unsaturated lipids.^{299–302} Despite the tendency of menadione to form non-lamellar phases, the direct encapsulation by VFF still produces apparent monomodal distributions by DLS, composed of liposomes around 100 nm in size (Chapter 8.4.3). The increased cell death of encapsulated menadione may result from the facilitated transfer of menadione to cell lipid structures producing an increase in the rate of cell death associated to lipid peroxidation.^{300,302}

To establish liposome localization within TC, liposomes were generated with 5 mol% DHPE-Texas red. In the study, DOPC liposomes were compared to the CL and Dasa formulations. The accumulation study showed liposomes accumulated near the cells, DOPC exhibited the highest intensity. This set demonstrated pegylated formulations likely accumulate less which is likely a result of the steric stabilization. Texas red accumulation was not observed within cell nuclei which suggests liposomes were either at the cell surface or up taken in the cytoplasm.

With cell localization confirmed, the toxicity of CL and Dasa liposomes containing menadione was explored using higher replicates. The CL liposomes were shown to exhibit low toxicity (84.4 ± 11.7 %) when compared to the media control (93.0 ± 6.5 %). Dasa menadione liposomes indicated high toxicity (36.3 ± 12.4 %) and remained within the value for menadione solution (52.5 ± 20.1 %), suggesting Dasa peptide could facilitate delivery of menadione *in-vitro*. The study was extended to multiple targeting liposome formulations by including the CHP peptide and scrambled CHP control. In the targeting study, all peptide linked liposomes were shown to exhibit potentially greater toxicity than the media and CL controls although large error in the experiment resulted in inconclusive results. Targeting menadione liposomes were shown to have a comparable effect on viability to the free menadione

solution. This targeting formulation study suggested that peptide sequence was non-specific to delivery efficacy as each targeting entity behaved similarly.

The alive dead assay was then applied within the bioprinted tissue construct. Viability could not be quantified as the Hoechst nuclear stain bound to the model ECM, making cell nucleus identification inaccurate. A DRAQ7 study was instead used to determine a dead nuclei count, whilst Texas red labelling of liposomes was used to show accumulation within cells and ECM. The DRAQ7 study provided preliminary data suggesting a greater number of dead cells are present in samples treated with Dasa menadione liposomes.

Our initial findings from studies with bioprinted H9C2 cell constructs suggest implementation into drug efficacy testing is feasible and our data demonstrates one of the first applications of liposomal delivery to 3D *in-vitro* cell models. Studying larger drug delivery systems (>20 nm) would likely require testing in conjunction with model ECM to fully optimise and screen interactions with the ECM to further improve cellular delivery. Overall, when combined with our high throughput VFF liposome production, this work details start to finish the lifecycle of liposomal development, formation and testing within an academic setting. Currently, limited examples currently exist of VFF production of liposomal drug delivery systems, especially with evidenced application, our work demonstrates successful application and efficacy study. Throughout the work, focus on scalability and accessibility ensures application to wider formulation exploration can be achieved to evaluate and hone new effective targeting modalities prior to *in-vivo* application. Development of *in-vitro* tissue models such as our bioprinted construct can replicate *in-vivo* topography and conceivably be implemented using patient donor cells to establish a new paradigm for both preclinical drug testing and personalized therapeutic treatment.^{92,94,95,303}

9.7.2 Future work

To further assay the performance of menadione *in-vitro* an alternative assay could be developed to study the production of cell reactive oxygen species using CellROX™, this could employ lower menadione dosage and reduce the decrease in sampling associated with cell death at high menadione concentration (>10 µM). To better characterise menadione loss within VFF, a potential limitation of the synthesis approach, a study measuring the absorption of a menadione solution before and after perfusion through the VFF device could possibly validate the approach further.¹⁴¹

Whilst the Texas red study indicated liposome localization to H9C2 cells, employing confocal microscopy could better aid visualization of the localization. Application of high-resolution confocal microscopy could potentially visualize liposome localization, this could be effectively achieved using larger (> 200 nm) liposomes, which could be representative of the ≈ 100 nm liposomes employed.

A potential substitute for Hoechst in the bioprinted assays could be Calcein-AM which is converted into fluorescent Calcein by intracellular esterase's.²⁹⁸ Calcein-AM could reduce the off-target fluorescence in the background and enable better image mask determination. Future work could employ confocal microscopy could improve data quality, a significant degree of cells were out of focus, confocal study would capture the full sample at the cost of longer data acquisition. Finally further work could focus on improving the biomicry of our system using ECM constituents found within cardiac tissue, this would further the model as a drug efficacy screening platform.

10 – Conclusions and outlook

In this thesis, the application of millifluidic generated drug delivery liposomes to both conventional cell culture and bio printed tissue models has been investigated. To achieve this, various fabrication methods such as photolithography, PDMS bonding and surface modification, PMMA bonding, 3D printing and 3D bioprinting have been employed. In the course of the work, many analytical methods were employed including DLS, Electron microscopy, Fluorescence spectroscopy, High content analysis and XRD. The overall aim is to establish a means to generate drug delivery liposomes in high throughput and evaluate their efficacy in conventional and engineered *in-vitro* models.

More precisely the aims summarized in Chapter 1.5 are:

- V. Achieve scalable production of therapeutically relevant liposomes through micro or millifluidics
- VI. Further understanding and attempting to address the limitations of the millifluidic, hydrodynamic flow focusing liposome synthesis method
- VII. Achieve liposomal drug encapsulation and evaluate the drug delivery liposomes
- VIII. Evaluate therapeutic liposomal systems on conventional cardiac (H9C2) cell culture and *in-vitro* tissue models using an effective assay

Within this chapter, the aims will be discussed by summary of the method chapters 3-9, this will be followed by the outlook for each aim.

10.1 Achieve scalable production of therapeutically relevant liposomes through micro or millifluidics

Chapters 3 and 4 addressed attempts to achieve microfluidic and millifluidic liposome production, respectively. Chapter 3 outlines the PDMS based fabrication of a MHF capable device, initial application within the untreated PDMS device was shown to produce broad liposome distributions unfit for therapeutic application. MHF was first reported by Jahn *et al*⁵⁹, where a cyclic olefin copolymer device was employed, this work used a PDMS based device and an adapted design. The PDMS device was investigated with a range of surface modifications focusing on improving the resultant liposome distributions. In the surface study, non-covalent modifications of hydrophilic PVA and hydrophobic RainX, were found to reduce MHF performance. The non-covalent modifications exhibited a change in resultant liposome distribution with extended use which implied the surface modification is altered during application. The fluorosilanes OTS and POTS were next employed as covalent hydrophobic surface modifications, these exhibited significantly lower distribution shift after multiple applications indicating the modifications were more robust. Each of the fluorosilane modifications resulted in therapeutically relevant liposomal distributions using pure DOPC (< 250 nm mean diameter, < 0.25 PDI). As POTS is less stable, with regards to storage and oxygen exposure, long-term application the OTS treatment was favoured over POTS. The surface modification study demonstrated the impact of surface chemistry within MHF on the resultant liposomal distributions formed.

Using the OTS modification, a FRR and TFR sweep was performed with the MHF device which demonstrated the expected trends outlined by Jahn *et al*^{59,61}. In this work, the device demonstrated a decrease in mean diameter with increasing FRR and an increase in PDI with increasing TFR. This data

set demonstrated the application of the OTS MHF device and reproducibility of MHF process in work by Jahn *et al*^{59,60}, despite employing a different device fabrication and material. In the work by Jahn *et al*^{59,60} channel structures were etched into silicon wafer using deep reactive ion etching and sealed using glass wafer, this thesis demonstrates MHF within a PDMS based devices and device surface optimisation. The MHF device was next used to explore binary lipid mixtures of DOPC:CHOL and DOPC:DOPG as well as ternary DOPC:DOPG:CHOL mixtures which demonstrates the first application of the charged lipid DOPG to the MHF process. Overall, DOPG inclusion was shown to have significant impact on the resultant liposome distributions, leading to bimodal distributions above 20 mol% in binary DOPC:DOPG mixtures. Within the ternary mixtures, both asymmetric and bimodal distributions were observed above 20 mol% DOPG. The formulation study evidenced lipid components within MHF impact the resultant liposomal distributions, in addition to FRR and TFR. Overall, the PDI values and throughput of MHF were deemed too low to facilitate large cell-based efficacy testing which led to the study of VFF within Chapter 4.

Chapter 4 first outlines an accessible and low cost PMMA based millifluidic manufacturing method. VFF was first reported by Hood *et al*⁶⁴ using a cyclic olefin copolymer device, in this work the fabrication for a PMMA device with high fluidic sealing and low warping, through application of a solvent assisted bonding method is presented. The application of PMMA based manufacture results in a highly accessible fabrication with a low cost per device, exhibiting great promise for the study of liposomes in academia. The PMMA device was optimised by study of resultant distributions and an effective run-through time and orientation was determined. The significance of orientation was first highlighted by Giorello *et al*¹⁷⁴ and this work demonstrated the practical significance for drug delivery liposome production, where remaining oriented against gravity resulted in the lowest mean diameter and PDI. With the optimisation achieved, VFF demonstrated therapeutically relevant liposome production using DOPC (< 250 nm mean diameter, < 0.25 PDI, 0.6 mL min⁻¹) whilst improving on the throughput of MHF (< 0.1 mL min⁻¹).

Building on the study within Chapter 3, the impact of lipid formulation on liposomal DLS distribution was explored within VFF at varied FRR and TFR. This formulation study supported the MHF study showing that differing lipid formulations impact the resultant liposomal distributions and certain trends can be established. The formulation study supported the idea that liposome formulation is dependent on both lipid formulation as well as FRR/TFR and that understanding the impact of specific lipids can aid improvement of resultant liposomal distributions and by relation, applications. The results demonstrate new insights into VFF for commonly applied liposome constituent's, the sterol CHOL, charged lipid DOPG and sterically bulky lipid DSPE-PEG₂₀₀₀.

Data from the formulation study was used to attempt to further understanding into the mechanism of MHF/VFF liposomal production, although it is clear significant work is required to accurately establish this. Addition of 58 mol% CHOL was shown to produce typically larger liposomes and sizes remained independent of FRR, this could be a result of CHOL ability to increase membrane rigidity.^{15,151,274,304–306} Higher membrane rigidity may slow the rate of closure of lipid intermediates into liposomes and allow for higher recruitment of lipid molecules thus increasing the size of resultant liposomes. Alternatively, rigid membranes could aid fusion of lipid/micellar intermediates analogously to the process of endocytosis, where membrane elasticity raises the barrier to endocytosis uptake.^{307,308} Similarly, 19 mol% and 38 mol% DOPG was also shown to drive formation of larger liposomes this could result by a similar mechanism as the CHOL example. Although, in the case of DOPG the electrostatic repulsion of charged headgroups would be the driving effect instead of membrane rigidity. Finally, 5.4 mol% and 11 mol% DSPE-PEG₂₀₀₀ was shown to drastically reduce size of the resultant liposomes, potentially favouring production of small liposomes or micelles. PEGylated

lipids are reported to form micelles preferentially at sufficiently high mol fraction.¹⁸³ The sterically bulky lipid could both favour rapid closure of lipid intermediates and reduce lipid intermediate fusion events. PEGylated lipids typically prefer higher curvature structures, so closure into highly curved micelles is thermodynamically favoured. PEG groups likely sterically stabilize micelles and small lipid intermediates preventing fusion and formation of liposomes at sufficient mol%.

10.1.1 Objective I. outlook

The results cover the significant impact of surface modification within MHF which is unreported for a single model design. Given access, study of MHF using simulation would greatly aid the discussion of surface impact as there are many surface modifications that could yet be employed. To avoid expensive and specialist clean room access the VFF device is designed with accessibility and cost efficacy in mind, whilst retaining effective performance as a drug delivery vesicle production method. Without surface modification the intrinsically hydrophilic PMMA produced < 0.2 PDI distributions, largely preventing the need for additional treatment. The accessibility and millifluidic nature of the device lowers the entry barrier for non-specialised users without a microfluidic background. The cost-effective fabrication could facilitate academic research for researchers operating with economic limitations, which is especially critical given the expense of lipid samples. Additionally, the layer-by-layer assembly provides significant promise for inclusion of further functionality within the device such as dialysis or lipid constituent mixing to enable one-step sample purification and large-scale lipid formulation study, respectively. More broadly, the application of MHF and VFF demonstrated an effective paradigm of liposome production with some significant advantages over the widely applied extrusion method. MHF/VFF offer far greater user control over liposomal size and subverts batch to batch variation in applied filters.

The exploration into lipid formulation within MHF/VFF uncovered potential trends that relate to the dynamic on-chip assembly of lipid molecules. Flow based mixing regimes are reported by Jahn *et al*⁶¹ for MHF, although formulation impact is currently unreported. The formulation trends signify a first study into significant numbers of different lipid formulations with varied FRR/TFR and the understanding could be used to improve lipid formulations when translating clinically viable formulations to MHF/VFF. The general trends observed could be applied to lipid mixtures with over 3 components to tune resultant liposome size and PDI. Future work to isolate or characterise the lipid structural intermediates within MHF/VFF would greatly aid field, several approaches could be undertaken to achieve this. Entrapping lipid intermediates using highly sterically hindered or charged lipids or the use of reactive lipids could entrap intermediates through photochemistry, this would enable study of stabilized intermediates by cryo-TEM. High speed (order of ms to ns) particle (< 10 nm) analysis, the mechanism could be further elucidated this could potentially be achieved using XRD through the VFF device at different points within the exit channel.³⁰⁹

10.2 Further understanding and attempting to address the limitations of the millifluidic, hydrodynamic flow focusing liposome synthesis method

Chapters 5 and 6 aimed to uncover limitations of the application of VFF producing drug delivery liposomes and employ a novel adaptation to address some of the potential shortfalls. Within Chapter 5, a fabrication method using fine adhesive backed PET films (62 μm) was employed to create VFF models with AR 161. The 161 AR devices exhibited poor performance at low FRR and TFR where bimodal distributions were observed, similar performance was observed above FRR 5, TFR 0.6 mL min^{-1} . Device simulation would aid in the understanding of the potential changes in flow regime AR can have. A drug delivery formulation (47:46:7 mol% DOPC:CHOL:DSPE-PEG₂₀₀₀) was then applied within the AR 50 device to demonstrate application to therapeutic liposomes. The technique of MADLS was then employed to characterise the concentration of liposomes produced from the drug delivery formulation and gave close agreement with the calculated value, suggesting minimal lipid is lost within the device. The MADLS data could be complemented with alternative particle concentration measurement such as nanoparticle tracking analysis or resonant mass measurement, to ensure validity.²⁰⁹

The latter section of Chapter 5 sought to evaluate the impact of ethanol on liposomes produced by MHF/VFF, which is currently not discussed within the field. At FRR 0.5 large (> 500 nm) structures are formed by VFF, and characterisation by cryo-EM suggested the high ethanol concentration resulted in structures that were either clustered liposomes/micelles or cubosomes. The TEM data demonstrates how essential FRR control is for drug delivery applications to favour liposomal formation over alternative structures. Ethanol impact was further characterised using BODIPY molecular rotors which enabled quantification of the bilayer micro-viscosity. Relative to extrusion, VFF liposomes were typically lower viscosity indicating ethanol is present within the bilayer. The data suggests both high FRR (> 10) and sample dialysis are required for resultant liposomes to closely match the micro-viscosity of ethanol free extruded liposomes. To remove residual ethanol on-chip, in a continuous fashion, a dialysis-on-chip design could be adapted from work by Hood *et al*¹²³. Dialysis on chip was attempted within the wide (10 mm) channel (Chapter 2.2.7) although the channel dimensions of VFF were impractical in conjunction with flexible dialysis membranes.

Within Chapter 6, a novel adaptation of MHF and VFF was achieved using Novec engineered fluid. Novec flow focusing was hypothesized to increase resultant liposome concentrations by employing an immiscible phase, this avoids the dilution that occurs with the fully miscible phases used in conventional flow focusing. Preliminary Novec MHF optimisation was achieved though resultant liposome distributions were typically too large and broad to see application in therapeutic delivery. Chemical incompatibility within the device and setup may be the reason for lack of Novec MHF success. To address this glass microfluidic devices could be developed which would also require Novec compatible fittings and tubing made from PEEK. By transferring to Novec VFF significant improvement was established by production of < 250 nm diameter liposomes with low PDI (0.034). The production of liposomes was confirmed using EM, signifying the feasibility and validity of the Novec flow focusing. Novec VFF exhibited promising results with low PDI and potentially higher resultant liposome concentrations as characterised by MADLS, for a given lipid concentration in ethanol.

10.2.1 Objective II. outlook

The comparison of AR across devices within this work demonstrates the robustness of VFF liposome generation and how device design can impact resultant liposome distribution. Exploring differing AR is important as it represents how subtle difference between applied designs can impact the resultant liposome distributions. Overall, the reduction in AR was beneficial for PDI of the liposomal peak although the reduction resulted in formation of bimodal distributions when using DOPC at certain FRR and TFR. The observation of bimodal distributions reinforces how essential understanding the flow and mixing regime is for the generation of therapeutic liposomes.

This work with BODIPY molecular rotors demonstrates the first example that quantifies the impact of ethanol on bilayer physical properties within VFF. Micro viscosity measurement could be an important paradigm within the field especially for application to drug delivery. Detecting ethanol could be critical to understanding potential liposomal cytotoxicity when combined with tissue culture studies. Through characterisation of micro viscosity, the parameters underpinning potential endocytosis mediated drug delivery could be provided. Rigid, higher viscosity membranes are reported to lower the energetic barrier for endocytosis.³⁰⁷ Finally, for multiple lipid mixtures within MHF/VFF rotor use could potentially indicate mixing success from analysis of micro viscosity between larger sample sizes.

The application of Novec flow focusing within this work demonstrates a major and novel adaptation of the flow focusing process, going beyond a single solvent change. Fluorinated oils are seeing ever more usage within microfluidics and the work demonstrates a potential new adaptation but also the challenges of applying fluorinated compounds in micro and millifluidic devices. In its current state Novec VFF requires further optimisation to reliably produce liposomes which could partially be achieved by employing Novec compatible fittings and tubing. The application of the range of available Novec solutions provide a further significant area of research for Novec flow focusing and would be benefitted greatly by simulation of Novec flow focusing, a non-trivial problem. Novec VFF exhibits a great deal of promise within academia and industry. Within academia, Novec could potentially result in significantly lower PDI liposomal suspensions and reduce the need for successive liposome concentrating processes. Highly monomodal distributions could aid study in conjunction with *in-vivo* studies, enabling study of specific liposome size applications. Industrially Novec could be utilised as an outer phase for the ethanol injection method, where Novec immiscibility could improve resultant liposomal concentrations. In an industrial setting ethanol enriched Novec potentially be recycled through mixture with DIW, effectively achieving dialysis with fluid contact, facilitating its reuse which could prove to be a greener solution reducing organic solution waste.

10.3 Achieve liposomal drug encapsulation and evaluate the drug delivery liposomes

Within Chapter 8 the intrinsic properties of drug delivery liposomes produced by VFF were investigated including the ability to encapsulate hydrophilic and hydrophobic molecules. Initial characterisation by XRD deemed the drug delivery formulation to be fluid lamellar (L_α) at 20 - 60°C. XRD data was used to inform temperature stability but also has connotations for MHF/VFF samples, the characterisation of fluid lamellar phase informed that the control liposome formulations will undergo lateral expansion and bilayer thinning in presence of ethanol. Direct encapsulation of CBF was then used to study the long-term (> 10 days) encapsulation of a hydrophilic moiety and compare with the extrusion method. The VFF process exhibited likely lower encapsulation efficiency for CBF than extrusion based on CBF release post lysis. The apparent longer-term stability at room temperature for VFF samples could stem from the ethanol presence within the bilayer acting to reduce bilayer permeability. Alternatively, the lower leakage rate could be the result of the lower encapsulation and by relation, concentration gradient between the liposome interior and exterior.

Next, encapsulation of the hydrophobic the antagonist menadione was explored as a molecule of interest for specific cardiotoxic delivery. Menadione encapsulation was first characterised using Uv-Vis spectroscopy and lysis, where a difference in absorption was shown after lysis with triton x-100. Lysis mediated release is a less valid measurement for hydrophobic moieties, given that lipid structures likely remain after lysis and could harbour menadione. Menadione encapsulation was further evaluated using DLS and MADLS. DLS study indicated a swelling, as a shift in peak maxima and broadening of liposomal distributions was observed upon encapsulation. DLS data supported menadione inclusion as menadione is expected to distribute within the liposomal bilayer, which would likely inducing a swelling effect. MADLS data demonstrated that within error, menadione inclusion exhibited minimal change on liposome concentration indicating the menadione concentration was insufficient to induce a phase change or significantly alter the liposome structure.

In the latter section of Chapter 8 the stability of drug delivery liposomes was assessed within H9C2 cell media. Osmolarity and pH was measured for the VFF buffer PBS and H9C2 media, both indicated close values signifying potential compatibility. By dilution in H9C2 cell media, the stability of liposomes was assessed by DLS to validate their application to H9C2 cell culture. When diluted in media, minimal shift in mean diameter and distribution was observed indicating compatibility over a 10-day time frame. Studying liposomes over prolonged periods (> 1 week) and 37°C temperature would further understanding around the stability. Additionally, adaptation of the CBF encapsulation assay using dye free H9C2 media dilution could shed further light on membrane integrity during incubation.

10.3.1 Objective III. outlook

Phase assignment could prove critical to optimising end therapeutic delivery systems as it aids description of stability and characterises potential limitations for encapsulation of hydrophobic or hydrophilic drugs. Lipid phase may also impact the facilitation of lipid and peptide endocytosis which has important connotations for both *in-vivo* functionality as well as therapeutic efficacy.³⁰⁸ Here, the encapsulation of CBF marks the first attempt at assessing and comparing encapsulation efficiency with the extrusion process. From this work it is clear VFF offers less effective encapsulation, a known limitation of direct encapsulation by flow focusing.^{62,123} Characterisation of encapsulation efficiency is a non-trivial measurement and further advances in the field could aid determination of accurate

encapsulation efficiencies. Application of active loading methods such as vincristine loading have been reported and could prove an essential means to achieve higher encapsulation efficiency by VFF.³¹⁰

Quantification of a true encapsulation efficiency is key for translating experimental screening data into feasible therapeutic formulations. Uncertainty around hydrophobic encapsulation is another important area for study, given the typically hydrophobic drug molecules such as doxorubicin employed within liposomes.^{1,2,19,311} Hydrophobic encapsulation could be especially critical for delivery of drug molecules with low solubility within aqueous environments and aid bioavailability *in-vivo*. This data marks the first example of menadione encapsulation in a one-step fashion and although promising, the true encapsulation efficiency is an area of uncertainty. Through application of BODIPY rotors it may be possible to pinpoint the menadione concentration within bilayers with comparison with extruded samples containing menadione. Menadione inclusion within the lipid leaflet likely impacts micro-viscosity which may be detectable by the rotor although an assumption will need to be made for the menadione quantity within the aqueous environment outside the liposomes.

Data within chapter 8 indicates VFF could be effective for application to cell-based studies, this is especially important for investigation significant formulation adaptations or simply larger scale cell-based validation. Within academia, extrusion is the current gold standard although the batch limited method makes significant formulation study an incredibly labour-intensive pursuit. The modular nature of the PMMA based VFF device ensures a lipid component mixing step can be added for the continuous formulation adaptation on-chip. Additionally, the high production rates of VFF are amenable to larger cell-based validation.

10.4 Evaluate therapeutic liposomal systems on conventional cardiac (H9C2) cell culture and *in-vitro* tissue models using an effective assay

Within Chapters 7 and 9, *in-vitro* models were first developed and used to evaluate specific drug delivery systems with comparison to conventional tissue culture. Chapter 7 initiated with the development of PDMS based TOC and highlighted the importance of biocompatible surface modification. Integration microgrooves into the TOC platform offered closer representation of *in-vivo* topology of cardiomyocytes, at the cost of lowering the effective cell capacity. The novel application of PVA as a non-covalently bound, biocompatible surface coating was the first of its kind, PVA is typically employed as a polymer substrate and not a surface coating. Within the micro grooved device, H9C2 cells were shown to adhere and elongate readily though were liable to detach under shear, when using a PVA surface. With further work, the PVA surface could prove to be a reliable treatment for static cell adhesion in PDMS devices. PVA offers the potential advantage of subverting naturally derived proteins utilised as surface coatings, which are prone to batch variation and typically higher cost. The data demonstrates the importance of covalently linked surface modifications for non-static cultures and evaluated a covalent collagen 1 bonding methods reported by Gunda *et al*¹²⁵. When evaluated by seeding density, covalently linked collagen 1 surfaces were compared to conventional tissue culture ware and the data demonstrated the low seeding densities for modified PDMS. Covalent protein surface modification was studied employing and APTES-collagen surface and an APTES-GA-collagen surface with the addition of a glutaraldehyde (GA) linker. Application of the GA linker yielded similar results to the APTES-collagen surface, given the extra fabrication step this meant glutaraldehyde use was unfavourable.

Chapter 7 concludes with the development and transfer to a 3D bioprinted cardiac tissue model. Bioprinted models were able to generate 3D ECM models, with potential for scalability through the process of printing into well plates. Bioprinting extrudes hydrogel to produce a fibre that attempts to capture the ECM structure of cardiomyocytes *in-vivo*. The bioprinted model was optimised for print quality, as such gelatine based bioink was avoided for the tendency to cause clogs. Alginate and nanocellulose based bioinks (Cellink) offered improved print quality and subsequent rapid curing to retain intended shapes, at the cost of lower biomimicry. Printed tissue constructs were initially designed for 96-well plates though low print adhesion limited the reliability and the design was adapted for a 6-well plate, with lower sample replicates.

Chapter 9 contained the application of targeting menadione liposomes, produced by VFF, to conventional tissue culture and bioprinted models. Menadione solutions were first used in conjunction with an alive/dead assay to study menadione toxicity on H9C2 cells and establish a treatment time for the menadione liposomes. Addition of non-targeting control liposomes was studied within tissue culture and low H9C2 toxicity was observed within the experimental period for dialysed and dialysed liposomes. Preliminary application of menadione containing liposomes with Dasa³⁹ targeting showed detectable differences in cell viability from the control indicating the alive/dead assay was suitable for screening efficacy of menadione delivery. Through application of Texas red fluorescently tagged liposomes, the presence of a binding interaction or internalization of liposomes within H9C2 cells was confirmed. This data is insufficient to distinguish whether the liposomes are bound, or internalized, future work could attempt to establish this using confocal microscopy.

Re-evaluation of Dasa liposomes in tissue culture demonstrated high toxicity, signifying successful menadione delivery at a comparable level to free menadione, at equivalent menadione concentration.

An EC₅₀ plot was used to show systematic differences in toxicity of control liposomes from Dasa liposomes, here the alive/dead assay is shown to effectively distinguish the effect of liposomal targeting. Although, when evaluated with other targeting peptides Dasa, cardiac homing peptide and scrambled cardiac homing peptide, the impact of menadione loaded liposomes on cell viability was indistinguishable, within error. This work demonstrated comparable performance between the peptides, and from the inclusion of the scrambled analogue may suggest the amino acid sequence is not specific for facilitating delivery. Development of a second alive/dead determination assay or measure of reactive oxygen species may aid further efficacy testing.

In the latter part of Chapter 9, liposome treatment was applied within the bioprinted tissue culture model. The preliminary liposome treatment data demonstrated the difficulty in translation of the alive/dead assay to 3D culture. In this work, alginate/cellulose-based inks were shown to retain unbound Hoechst after the washing stages and calcein-am is hypothesized to be a more suitable alternative, given the fluorescence following cell internalization. Through application of a dead cell count and liposome localization within the bioprinted tissue, preliminary data for the screening of menadione liposome toxicity was achieved. Dead nuclei count remained within error for the studied media, control liposome and dasa liposome treatment, within the focal plane imaged. Normalisation by total cell count would provide more conclusive data and facilitate comparison with the tissue culture measurement. Texas red accumulation indicated liposomes were retained within alginate/cellulose ECM and that liposomes were also able to perfuse through and interact with H9C2 cells.

10.4.1 Objective IV. outlook

Determination of therapeutic liposomal delivery within conventional tissue culture is a critical first stage in validation. However, there is significant difference in the delivery routes *in-vivo* for liposomal systems. This work demonstrates a novel approach at studying liposomal delivery to cells contained within a model cardiac ECM. The focus of liposomal diffusion in recent examples has employed purely hydrogel models, spheroid tumour samples and the study of transdermal diffusion.^{312–314} Liposome ECM interactions are unreported and could be a further area where liposomal drug delivery systems can be enhanced to better reach their target cell-type.³¹⁵ Through usage of biomimetic ECM, liposome formulations could potentially be adapted for specific tissues, improving their efficacy. Overall, this work demonstrates a full lifecycle of therapeutic liposomal generation to efficacy testing using approaches applicable to academic research. Combining VFF with bioprinting, the preliminary data suggests the combination could be employed to improve liposomal formulation and application *in vitro* more rapidly, providing necessary data towards preclinical drug trials. A focus was placed on scalable production, from near-continuous liposomal production and high replicate bioprinted *in vitro* models as this believed to be critical to achieve clarity in liposomal efficacy testing.

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12 – Supplementary

12.1 DLS raw data

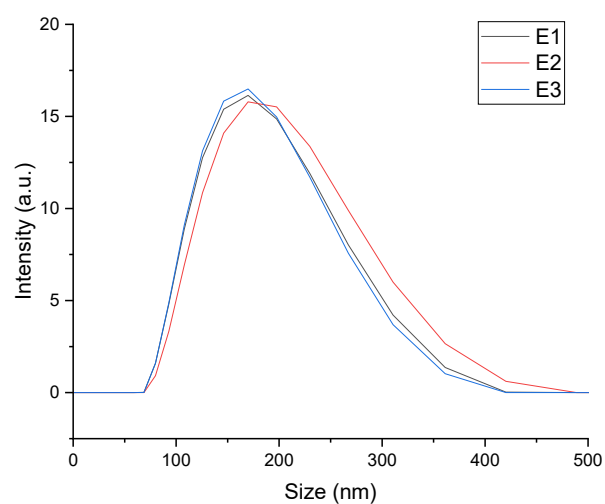


Figure S12.1A) Extrusion data from 200 nm extrusion of 1 mg mL⁻¹ DOPC in PBS

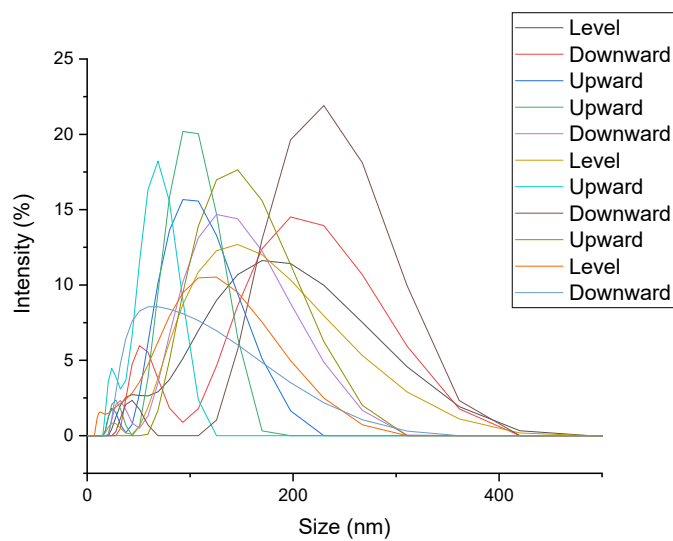
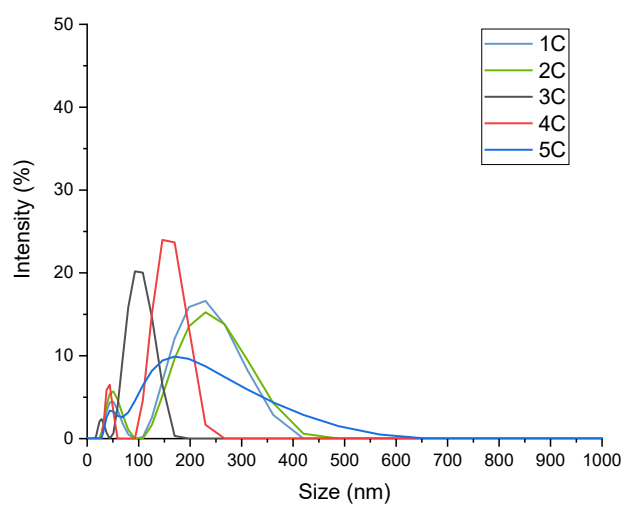
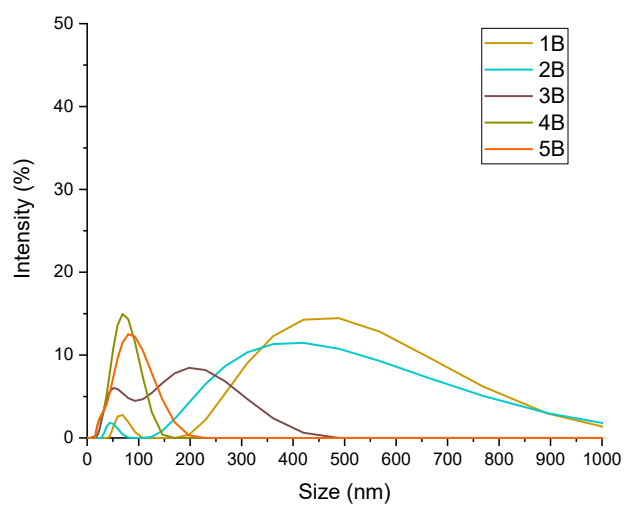
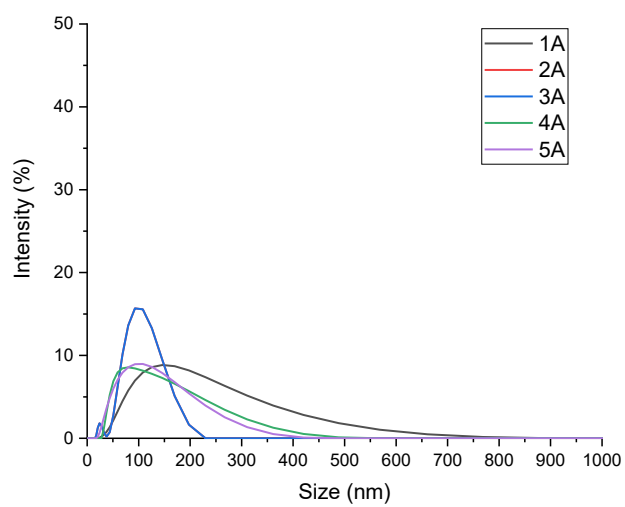


Figure S12.1B) Orientation study raw data from $n = 3$ using 0.1 mg mL DOPC at FRR 5 TFR 0.6 mL min⁻¹.



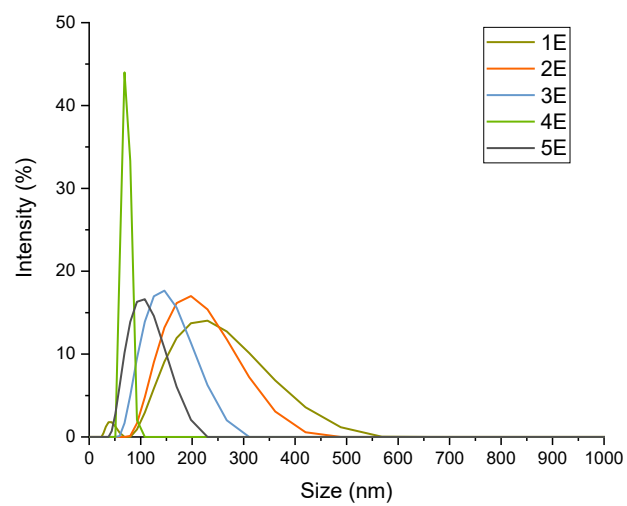
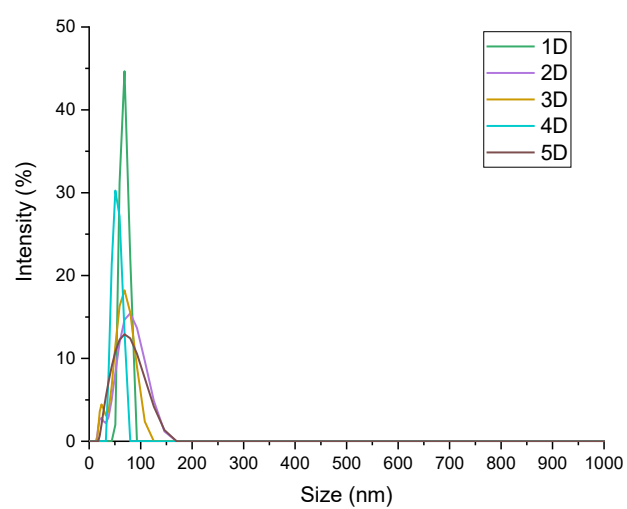
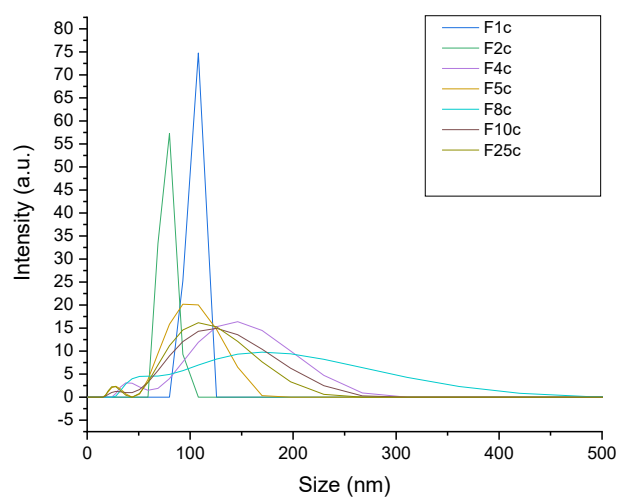
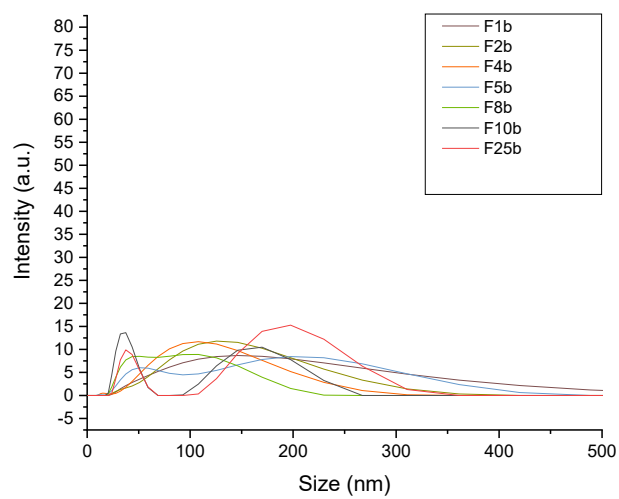
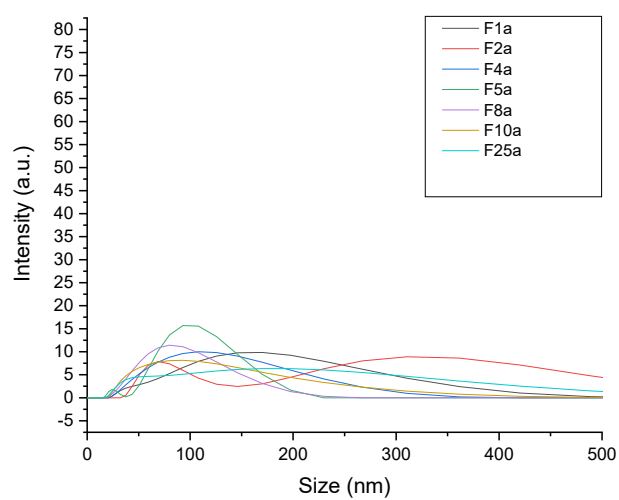


Figure S12.1C) Start up data raw from $n = 5$, 0.1 mg mL^{-1} DOPC samples at FRR 5 TFR 0.6 mL min^{-1} .



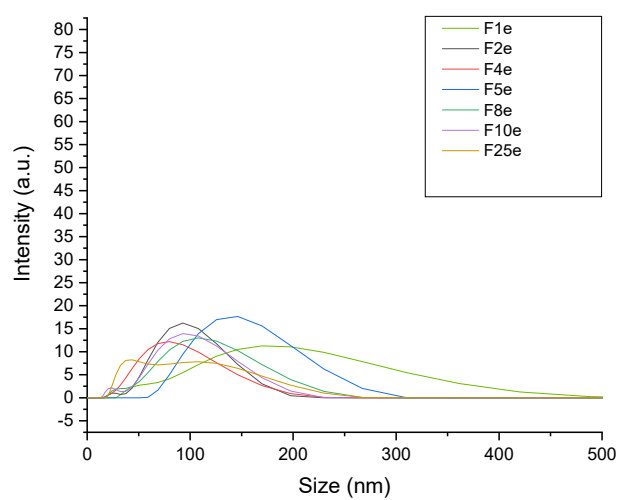
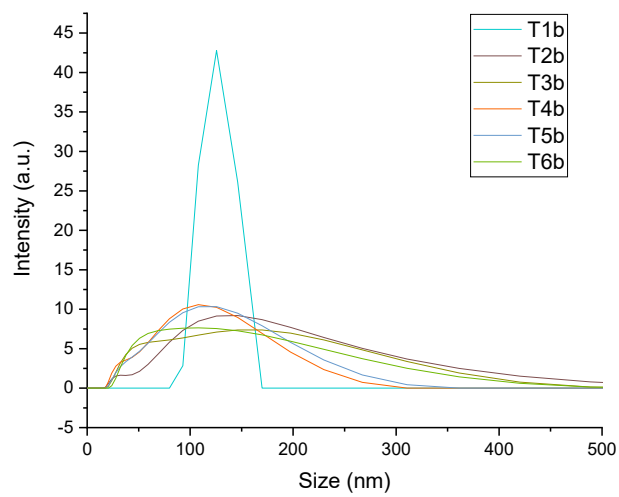
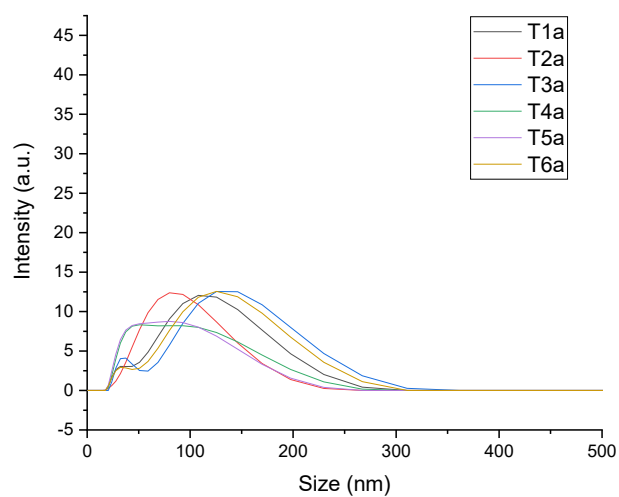


Figure S12.1D) FRR sweep 1-25 data raw from $n = 4$, 0.1 mg mL^{-1} DOPC samples at increasing TFR.



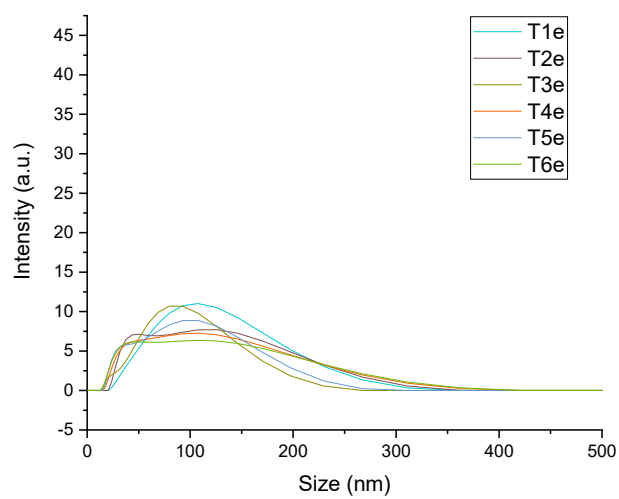
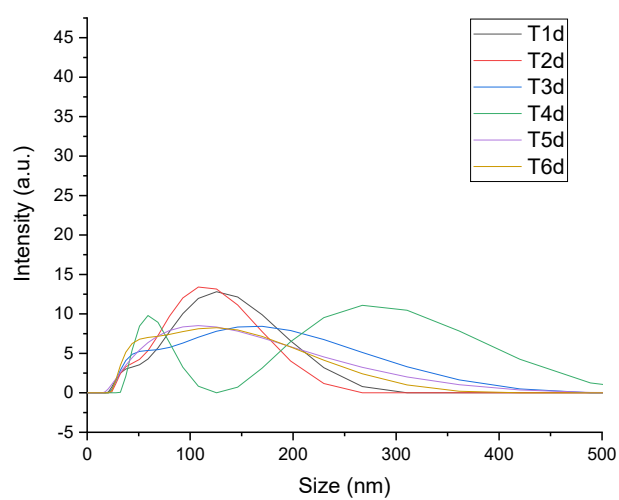


Figure S12.1E) TFR sweep data raw from $n = 4$, 0.1 mg mL^{-1} DOPC samples at FFR 5.

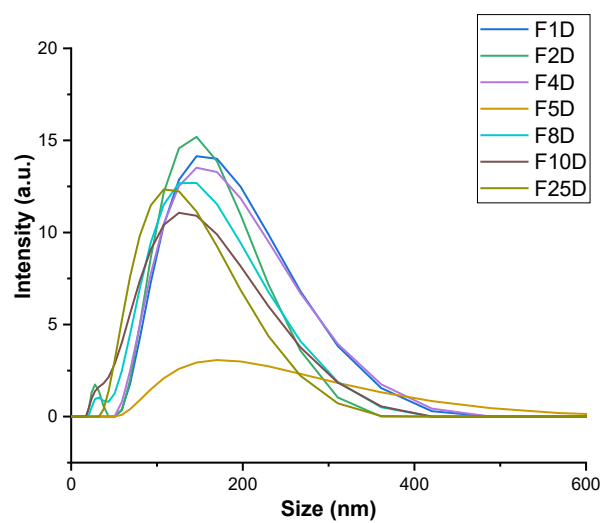
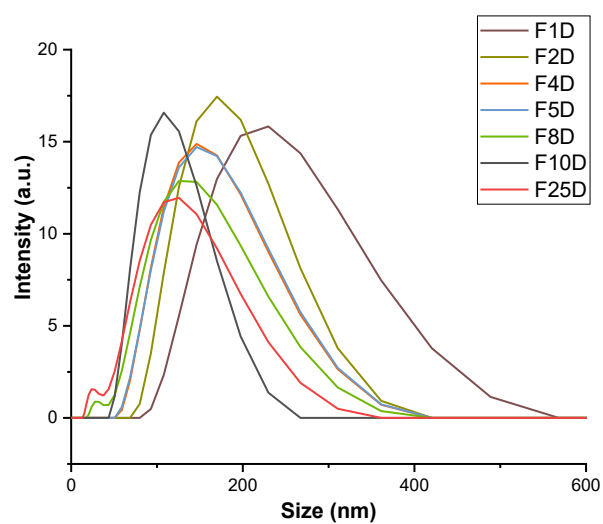
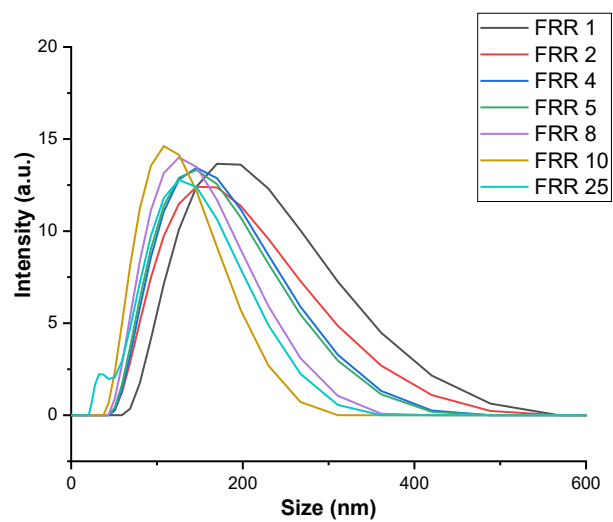
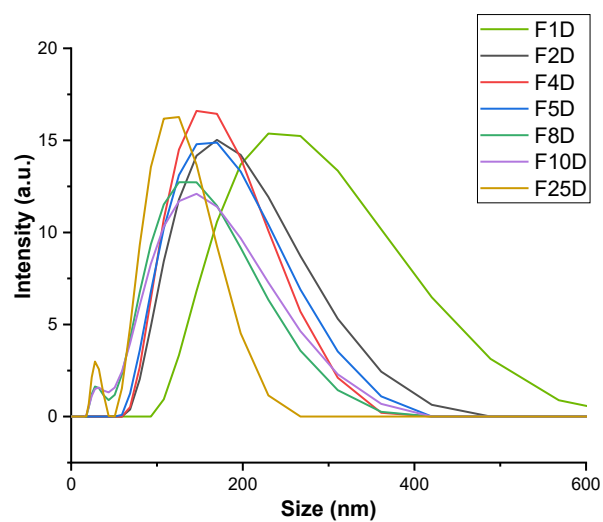
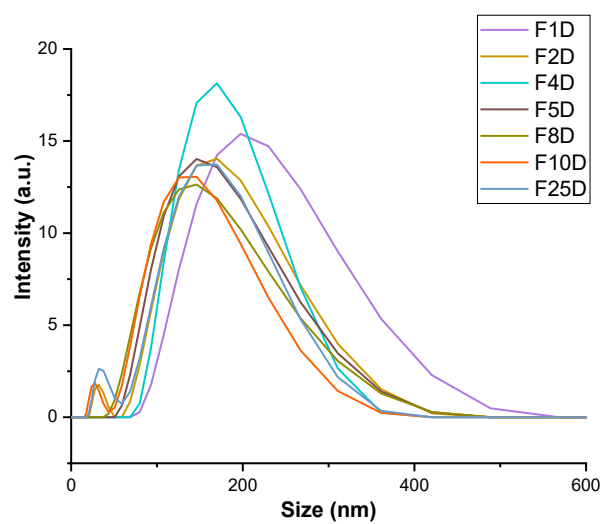


Figure S12.1F) FRR sweep 1-25 data raw from $n = 3$, 0.1 mg mL^{-1} DOPC, 10 mol% DOPG samples at increasing TFR.



DOPG TFR

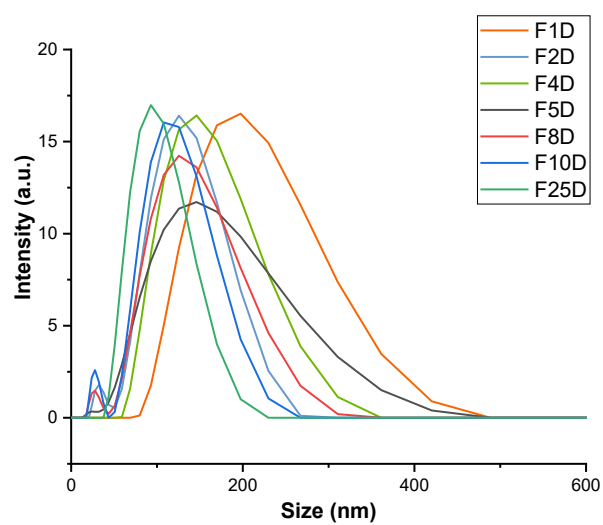


Figure S12.1G) FRR sweep 1-25 data raw from $n = 3$, 0.1 mg mL^{-1} DOPC, 20 mol% DOPG samples at increasing TFR.

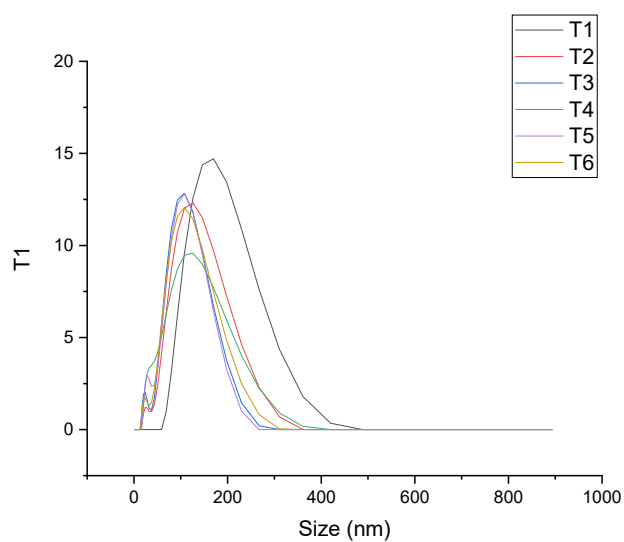
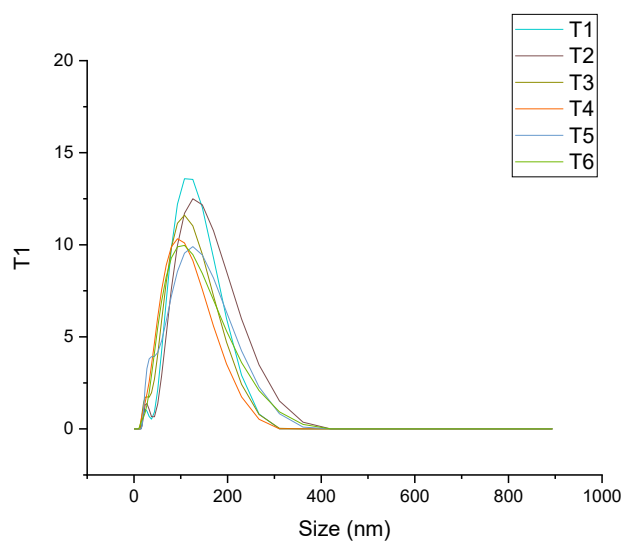
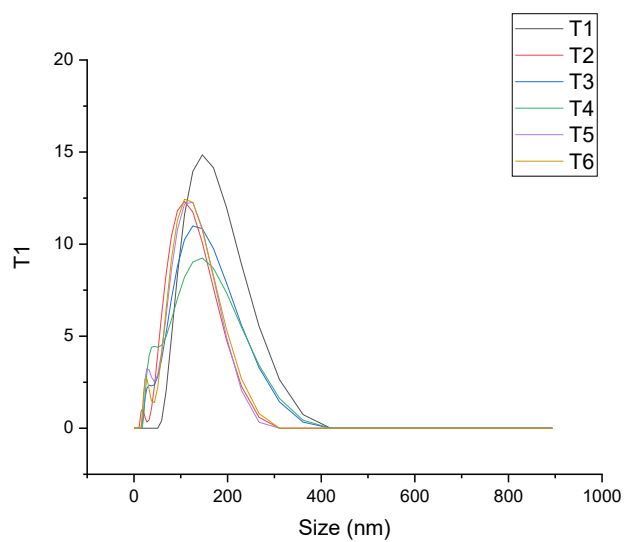


Figure S12.1H) TFR sweep data raw from $n = 3$, 0.1 mg mL^{-1} DOPC, 10 mol% DOPG samples at FRR 5.

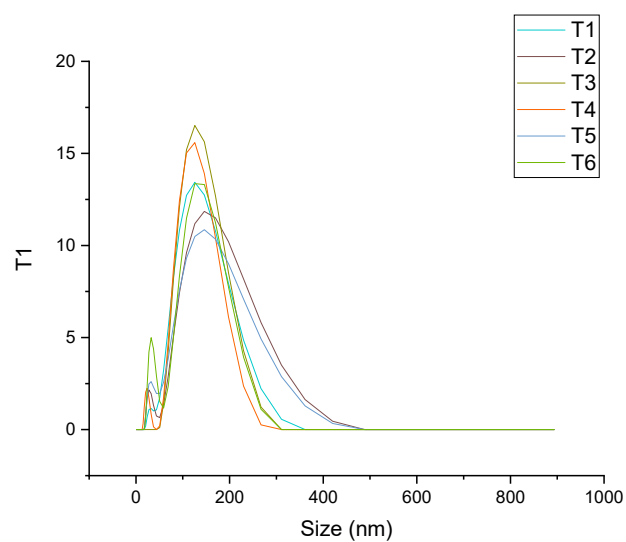
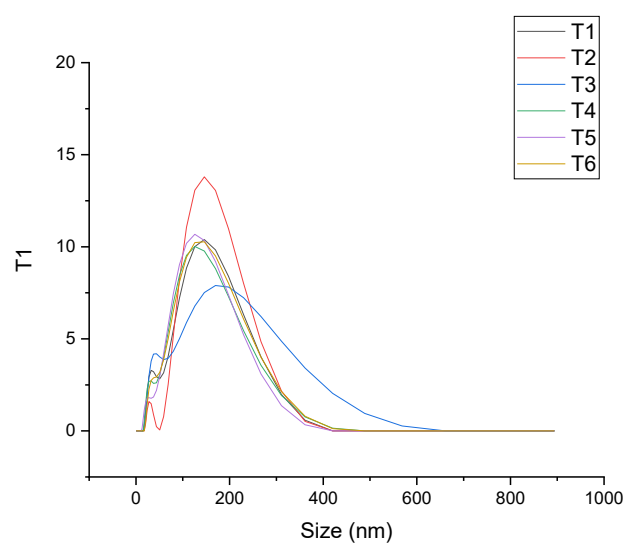
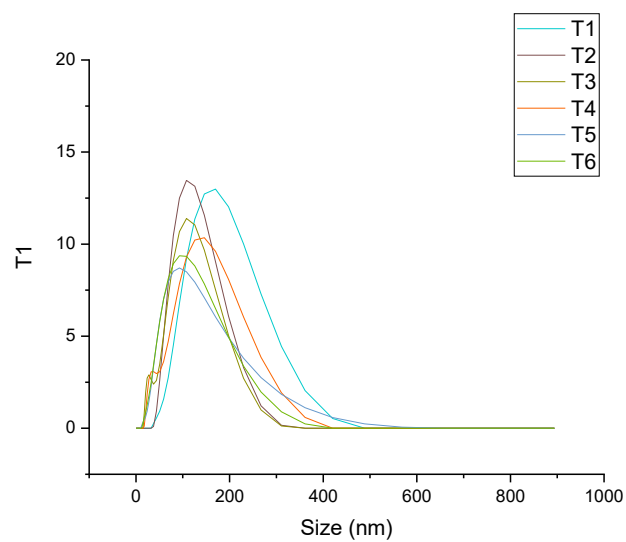


Figure S12.1I) TFR sweep data raw from $n = 3$, 0.1 mg mL^{-1} DOPC, 20 mol% DOPG samples at FRR 5.

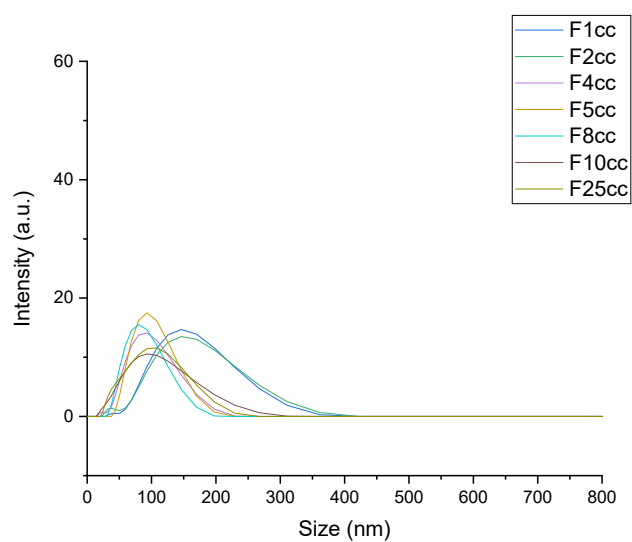
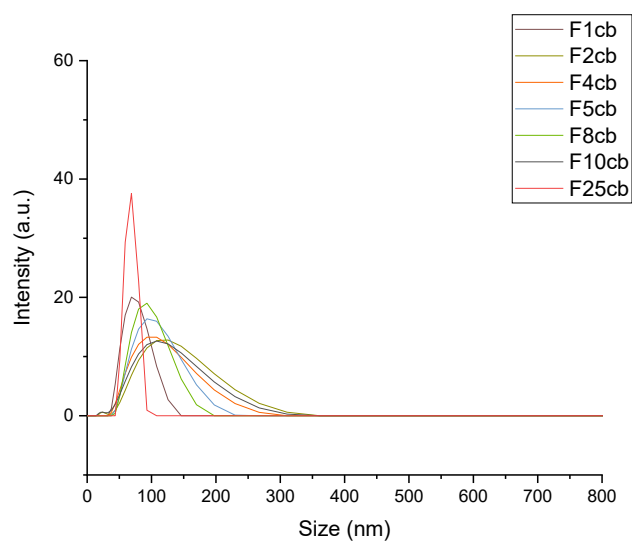
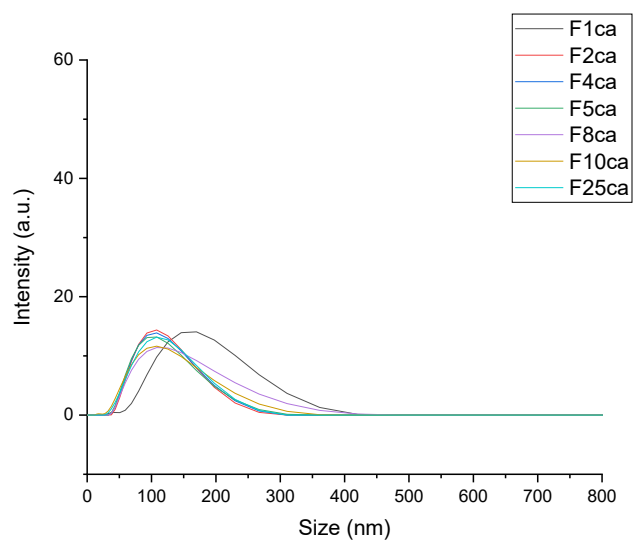


Figure S12.1J) FRR sweep 1-25 data raw from $n = 3$, 0.1 mg mL^{-1} DOPC, 10 mol% CHOL samples at increasing TFR.

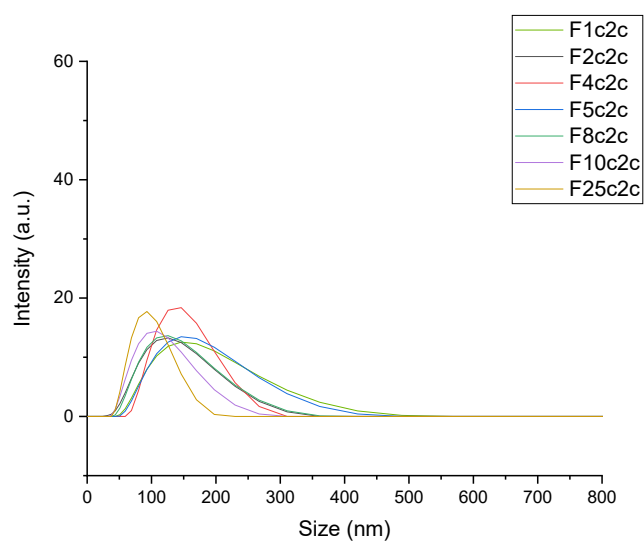
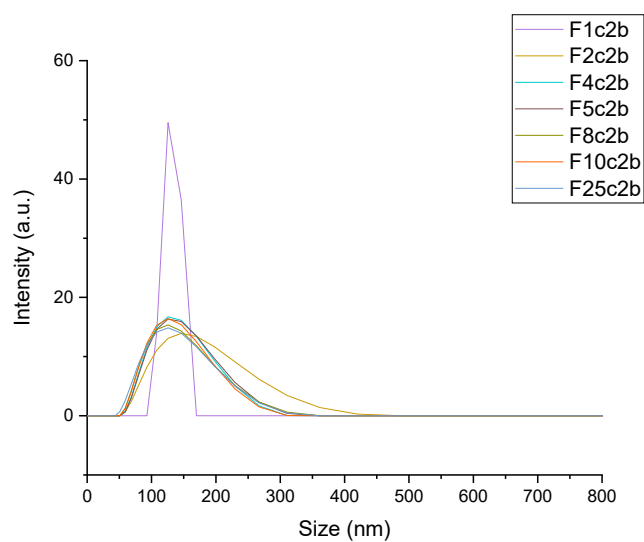
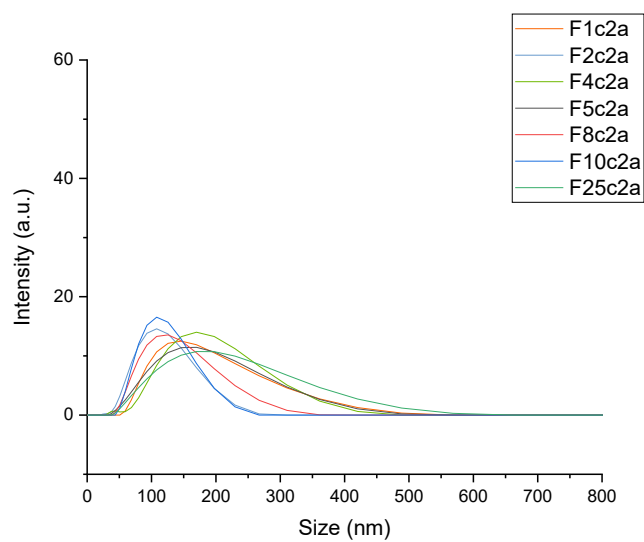


Figure S12.1K) FRR sweep 1-25 data raw from $n = 3$, 0.1 mg mL^{-1} DOPC, 20 mol% CHOL samples at increasing TFR.

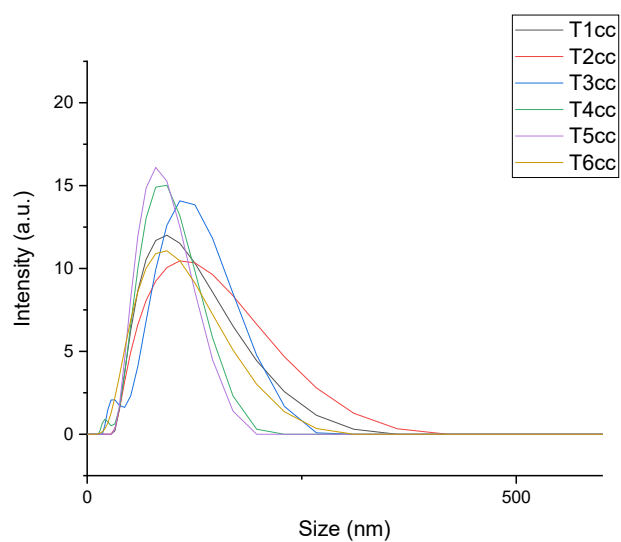
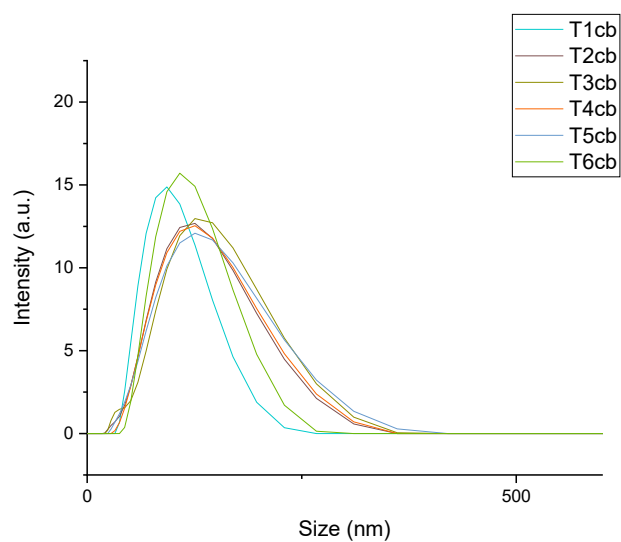
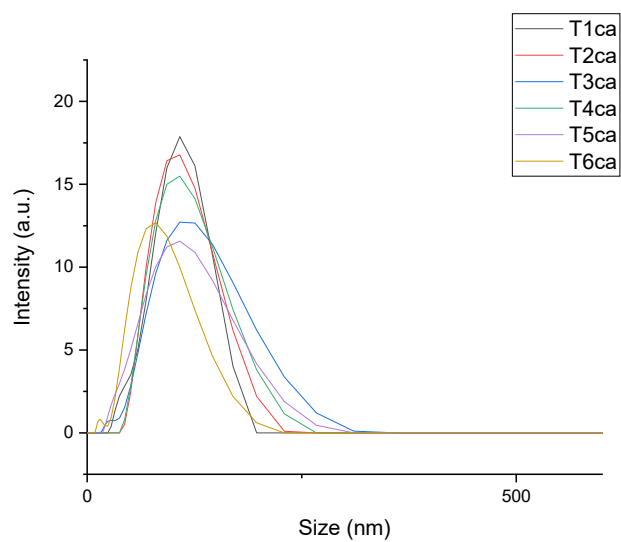


Figure S12.1L) TFR sweep data raw from $n = 3$, 0.1 mg mL^{-1} DOPC, 10 mol% CHOL samples at FRR 5.

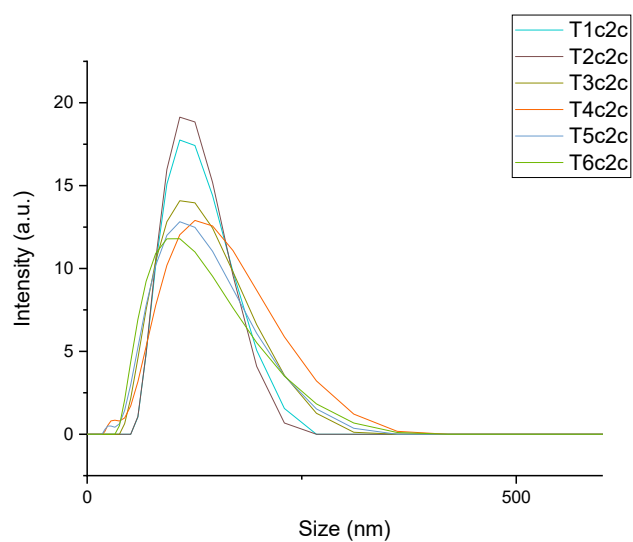
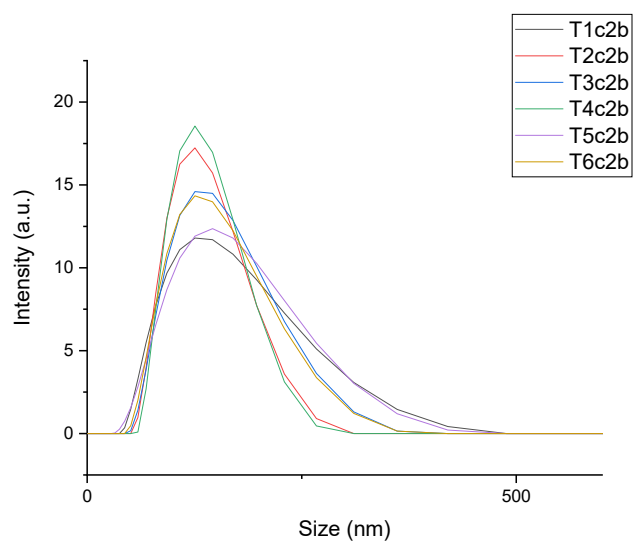
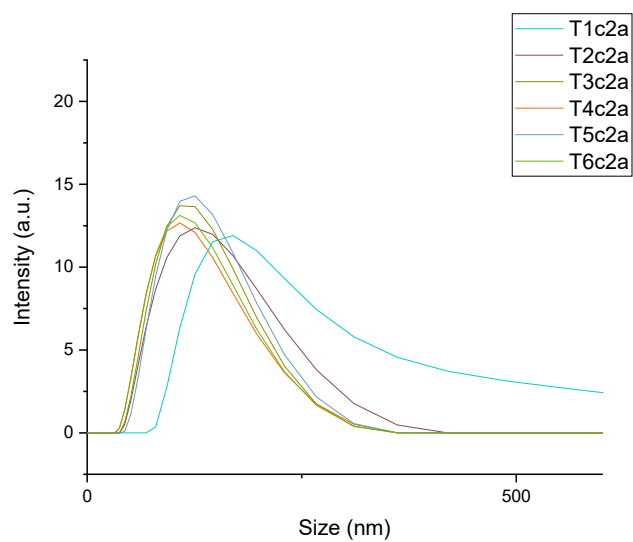


Figure S12.1M) TFR sweep data raw from $n = 3$, 0.1 mg mL^{-1} DOPC, 20 mol% CHOL samples at FRR 5.

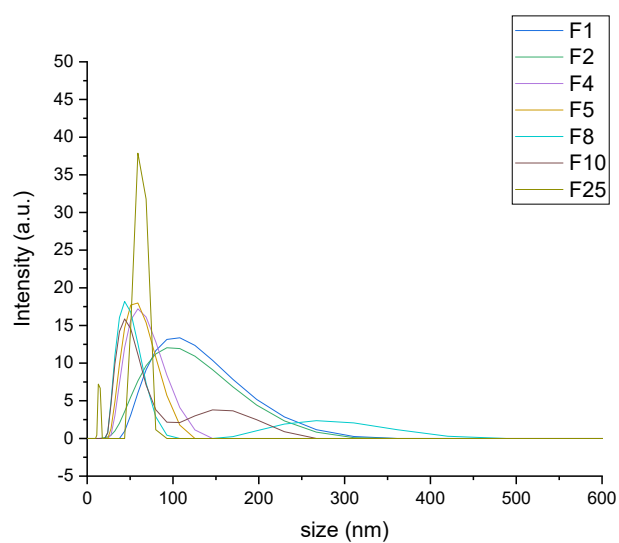
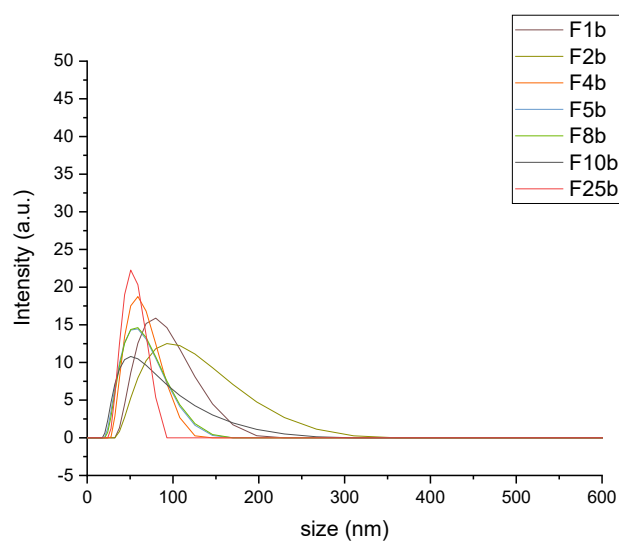
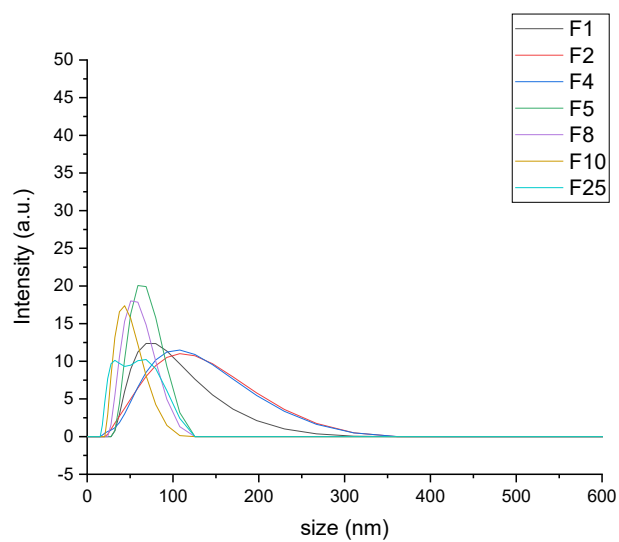


Figure S12.1N) FRR sweep 1-25 data raw from $n = 3$, 0.1 mg mL^{-1} DOPC, 10 mol% DSPEPEG samples at increasing TFR.

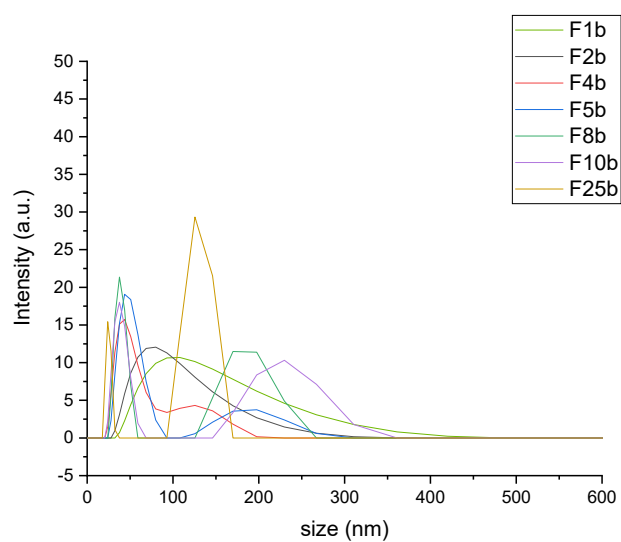
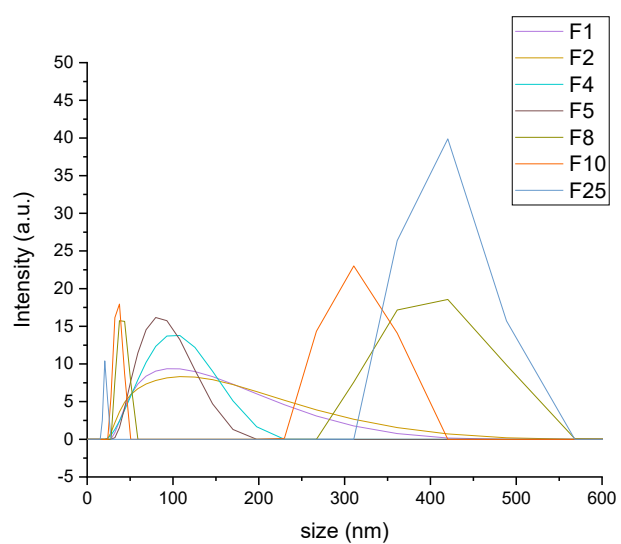
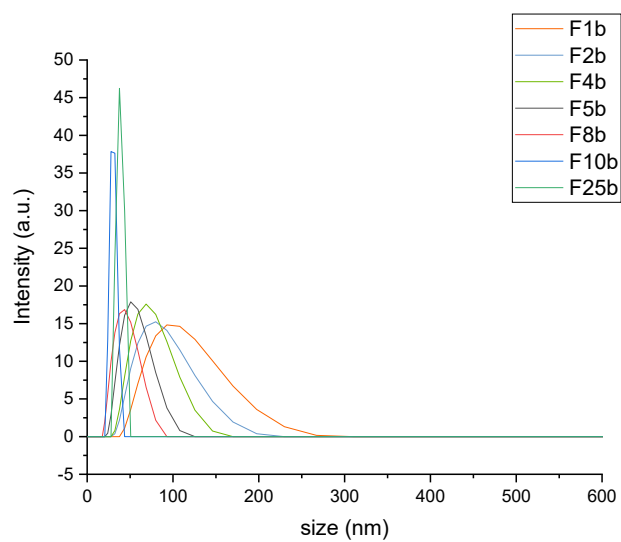


Figure S12.10) FRR sweep 1-25 data raw from $n = 3$, 0.1 mg mL^{-1} DOPC, 20 mol% DSPEPEG samples at increasing TFR.

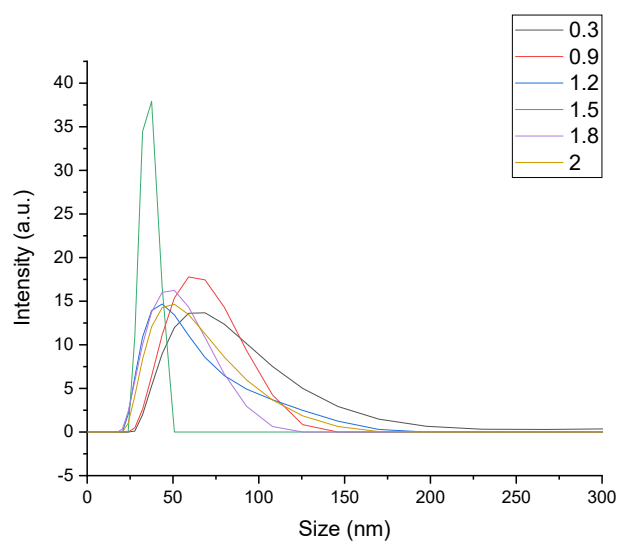
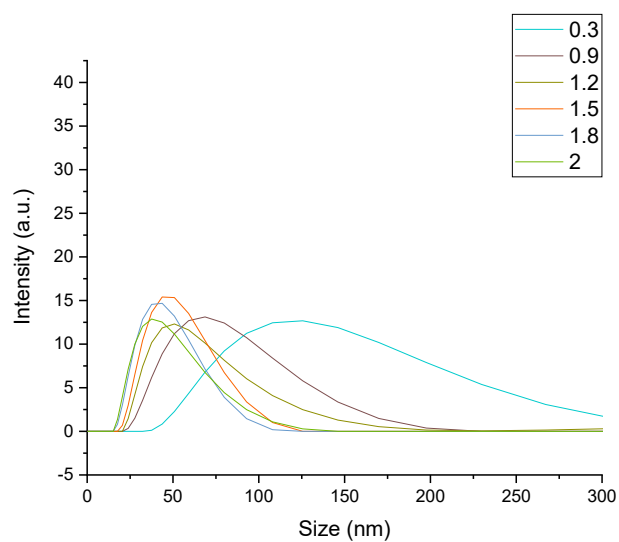
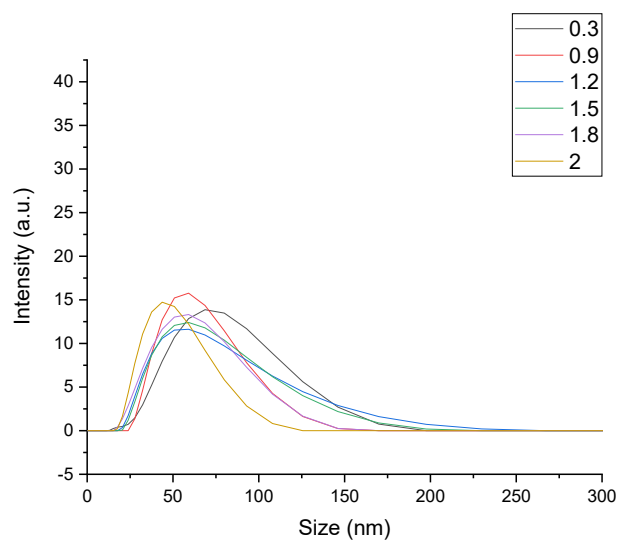


Figure S12.1P) TFR sweep data raw from $n = 3$, 0.1 mg mL^{-1} DOPC, 10 mol% DSPEPEG samples at FRR 5.

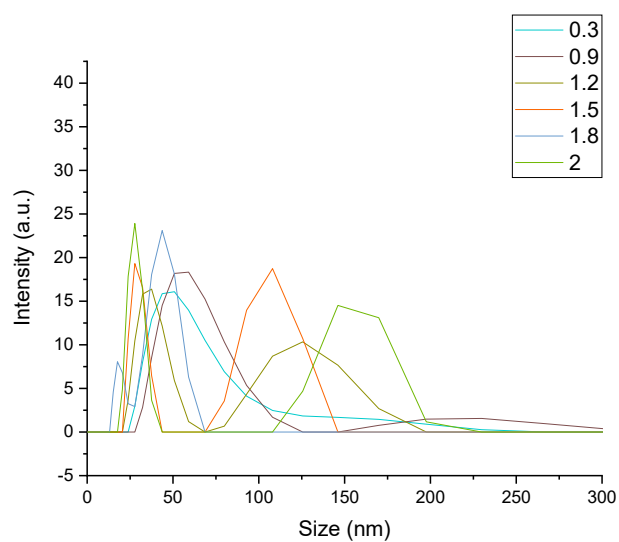
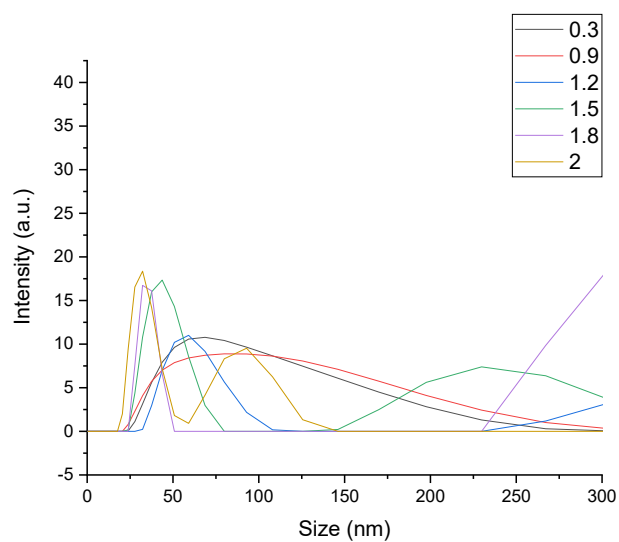
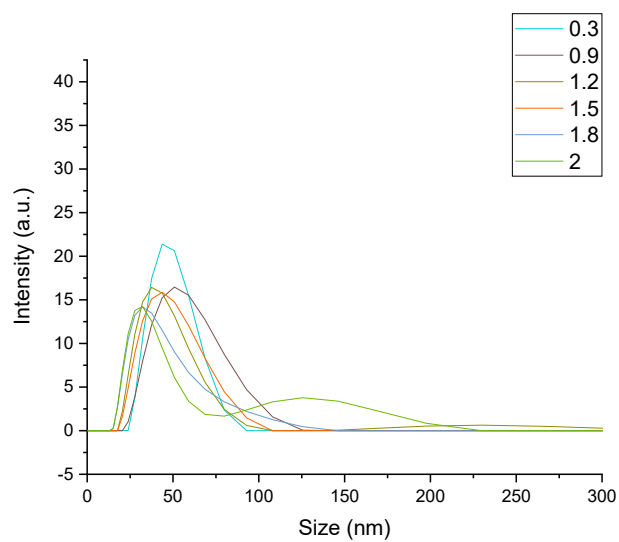


Figure S12.1Q) TFR sweep data raw from $n = 3$, 0.1 mg mL^{-1} DOPC, 10 mol% DSPEPEG samples at FRR 5.

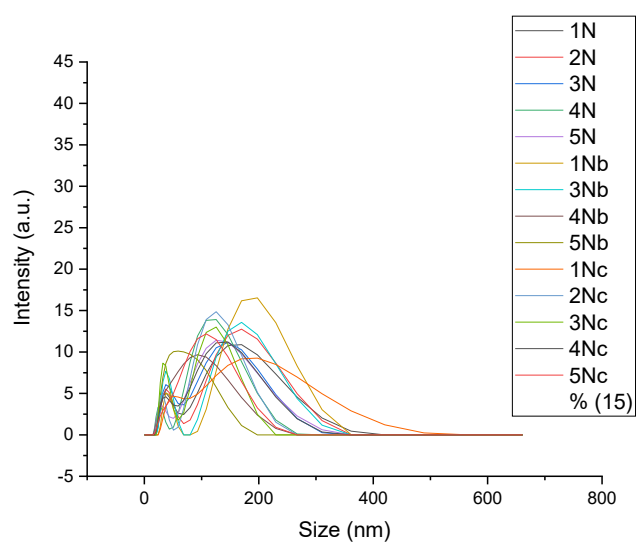
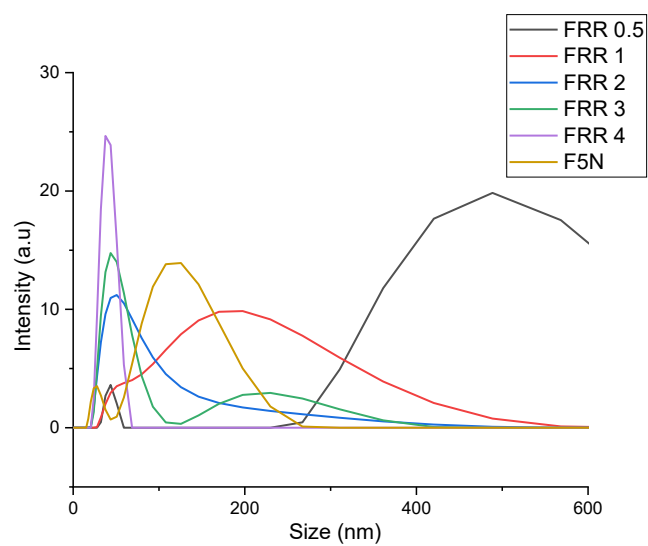


Figure S12.1R) Start up data raw from AR 161 device $n = 3$, 0.1 mg mL^{-1} DOPC samples at FRR 5 TFR 0.6 mL min^{-1} .



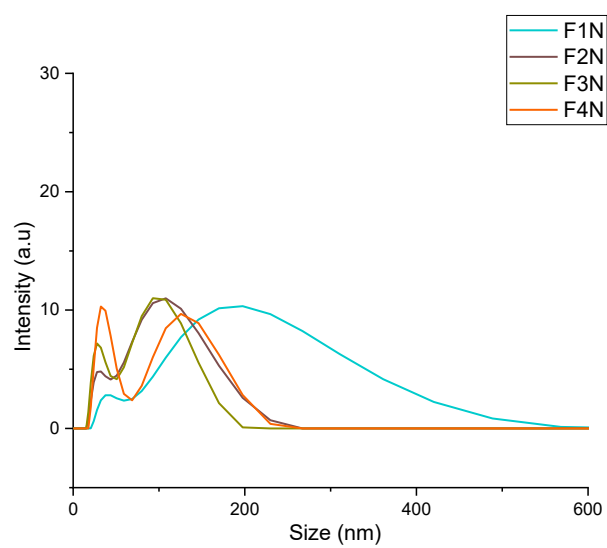
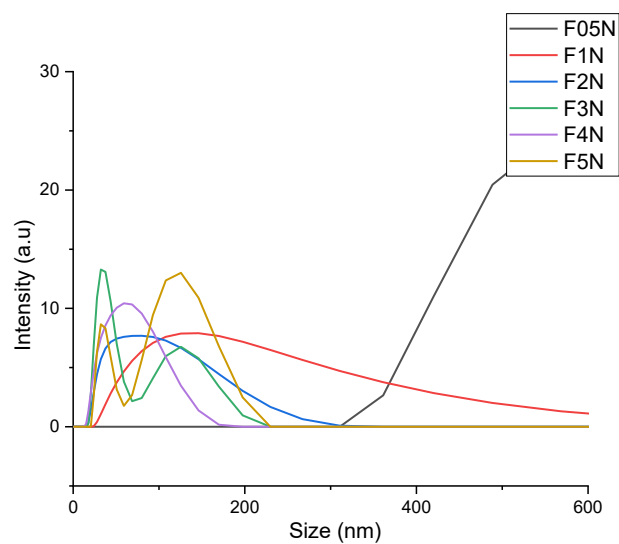
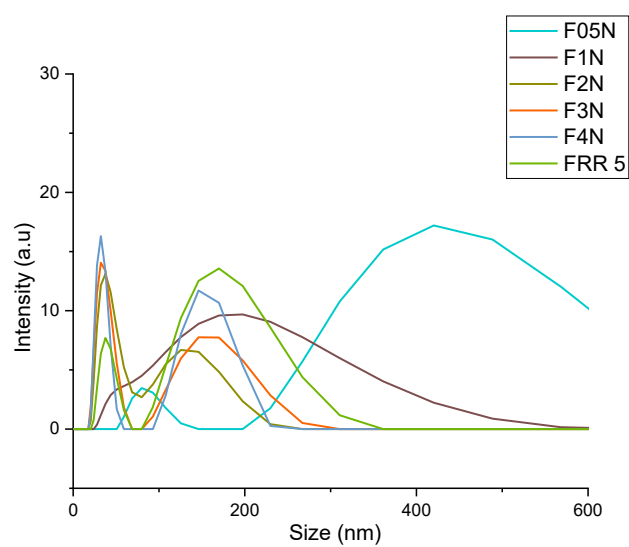
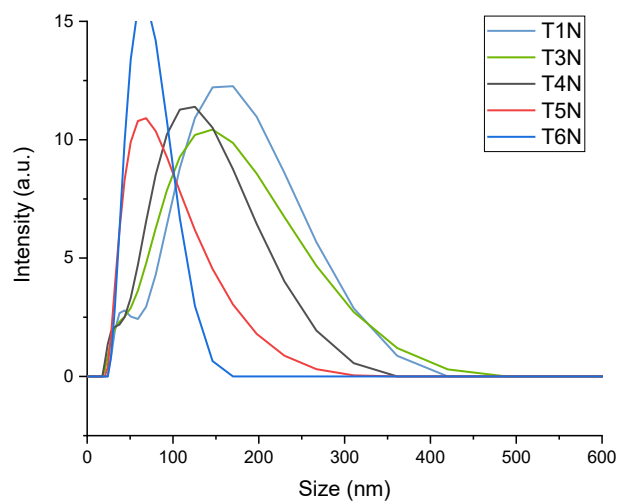
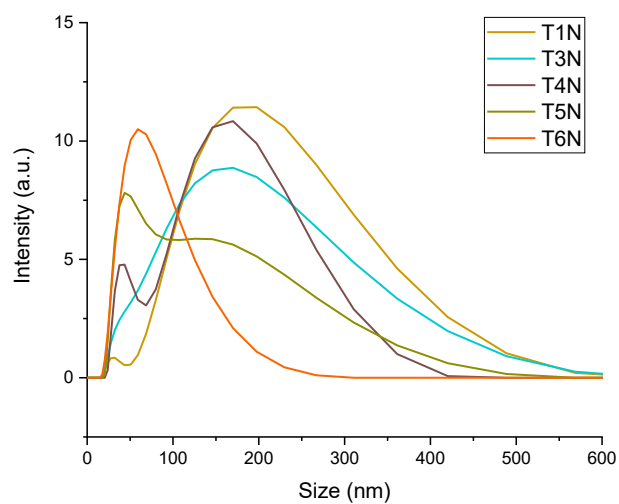
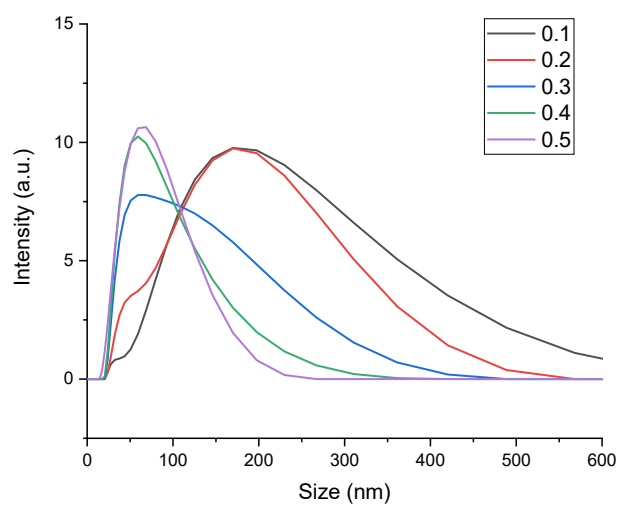


Figure S12.15) FRR sweep 0.5-5 data raw from $n = 4$, 0.1 mg mL^{-1} DOPC at increasing TFR.



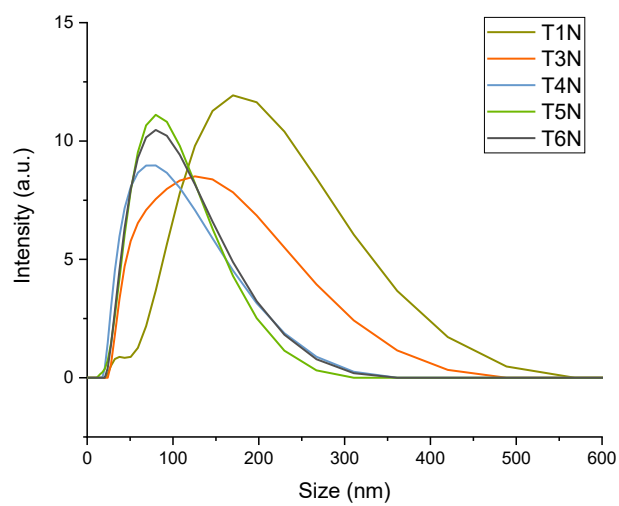
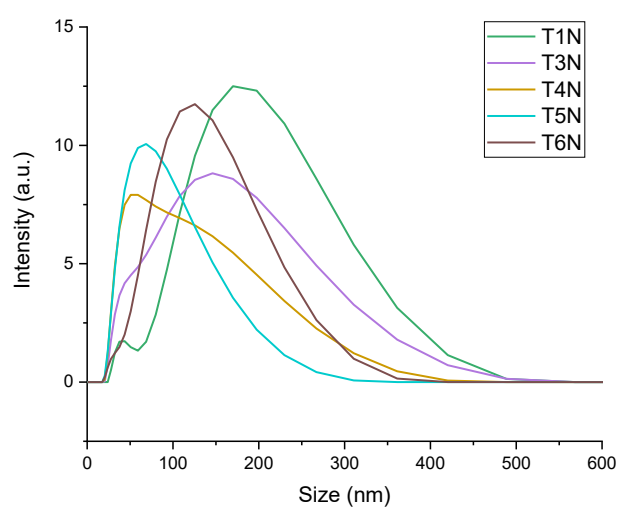
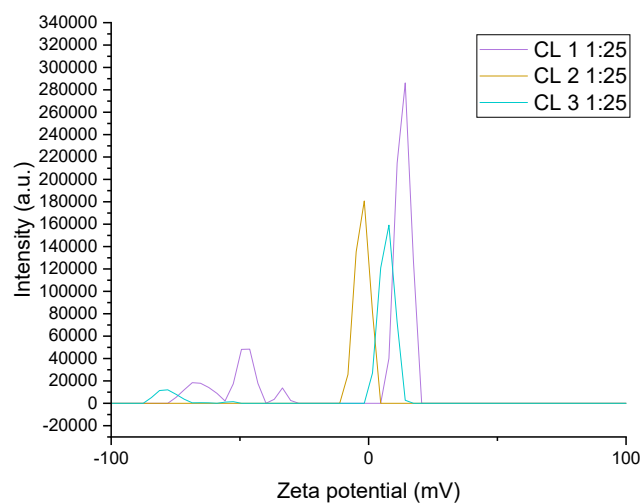
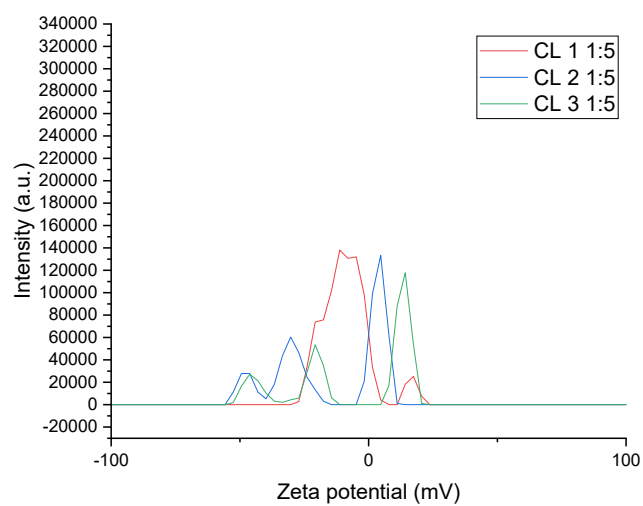


Figure S12.1T) TFR sweep data raw from $n = 4$, 0.1 mg mL^{-1} DOPC at FRR 1.



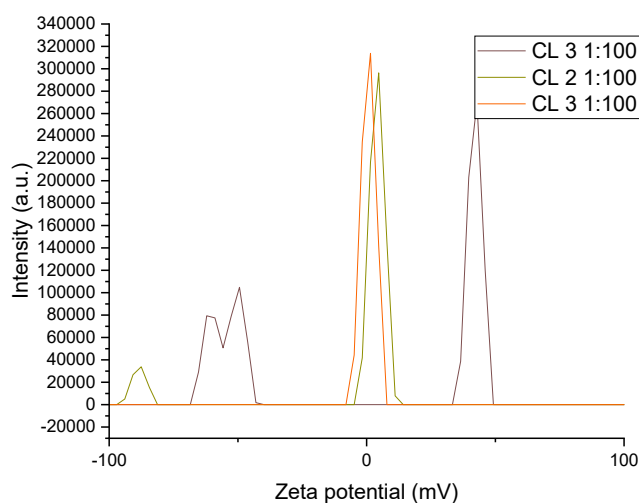


Figure S12.1U) Zeta dilution series 5 - 25 fold in DIW, for the CL formulation at 0.02 mg mL^{-1} to 0.004 mg mL^{-1} $n = 3$ samples.

12.2 HCS content screening

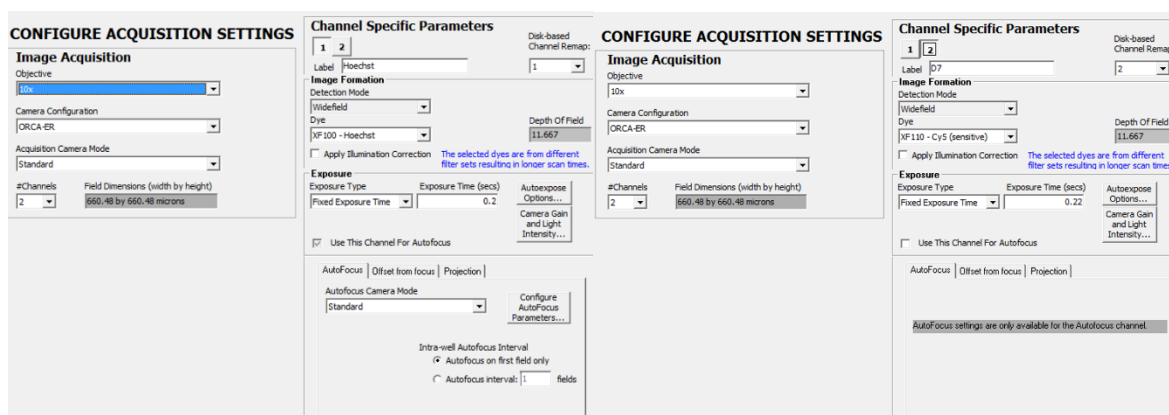


Figure S12.2A) HCS Cellomics settings 10x magnification, for Hoechst ((left) 0.2 s exposure) DRAQ7 ((right) 0.22 s exposure) assay.

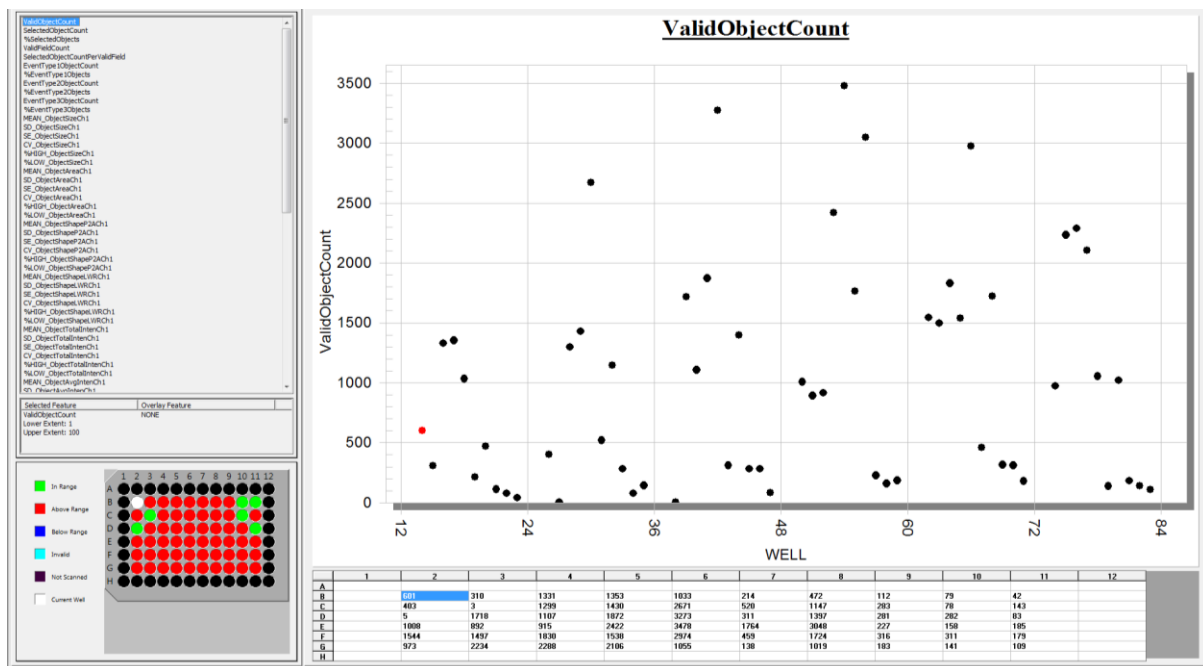


Figure S12.2B) Example valid object (Hoechst nuclei count) for a single plate, taken from Dasa EC₅₀ data set.

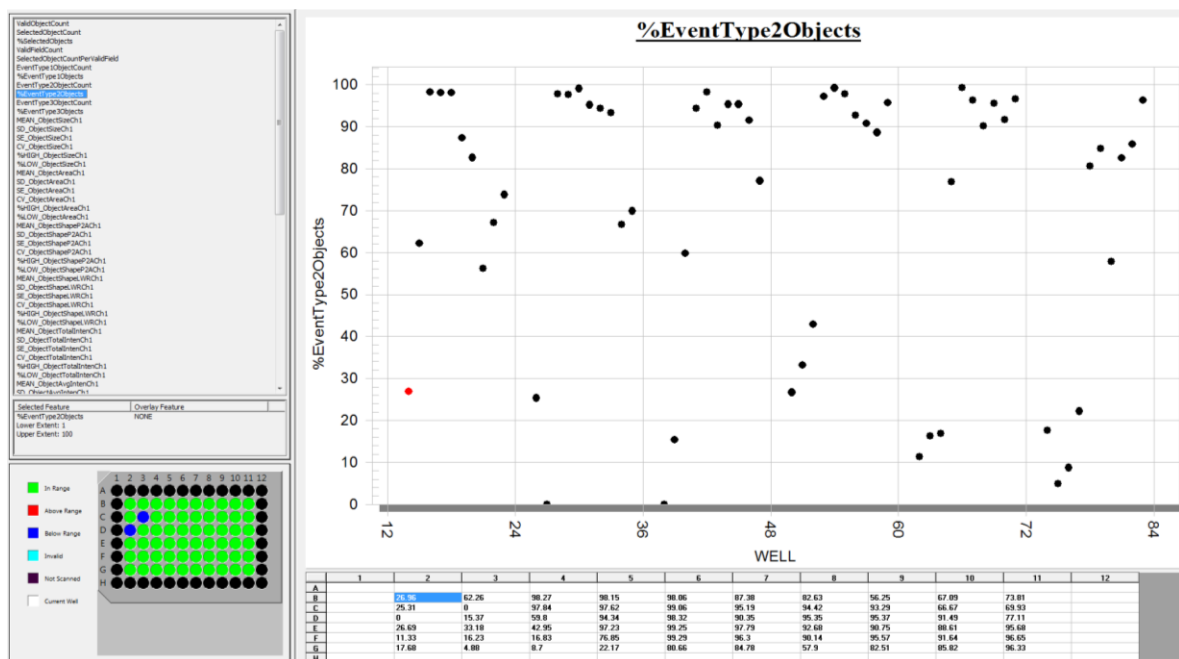


Figure S12.2C) Example object overlap (Hoechst nuclei/ DRAQ7 dead nuclei) for a single plate, taken from Dasa EC₅₀ data set.

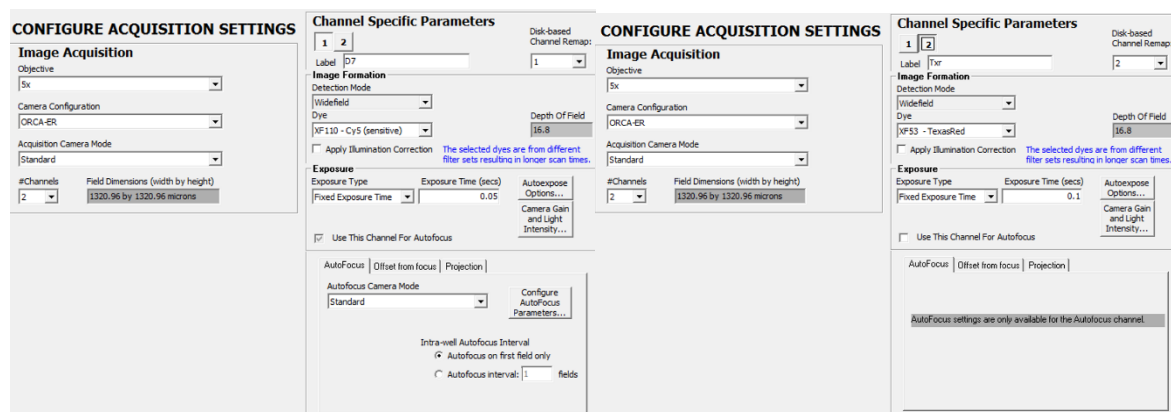


Figure S12.2D) HCS Cellomics settings 5x magnification, for DRAQ7 ((left) 0.05 s exposure) Texas red ((right) 0.1 s exposure) bioprinted tissue assay.

12.3 Flow cytometry cell size

H9C2 cell sizes

Avg. background		intensity
Avg. cells		/ image
Images		
Avg. circ.		
Avg. diam.		(microns)
Viable cells		/ml (x10 ⁶)
Total cells		/ml (x10 ⁶)
Viability		(%)
Viable cells		
Total cells		
Sample date		
Elapsed time		(hrs)
Viable cell		spot area (%)
Viable cell spot		brightness (%)
Cell		sharpness
Cell brightness		(%)
Dilution		factor
Max. diam.		(microns)
Min. diam.		(microns)
Cell type		
File name		
Sample ID		

204	204	204	204
1.9	1.9	1.6	1.7
50	50	50	50
0.51	0.51	0.52	0.60
20.64	19.34	19.33	18.36
0.099	0.10	0.083	0.089
0.10	0.11	0.088	0.097
95.7	95.9	94.9	92.0
89	93	75	80
93	97	79	87
6 Jul 2018 1-20-17 PM	6 Jul 2018 1-22-28 PM	6 Jul 2018 1-22-30 PM	6 Jul 2018 1-22-52 PM
5	5	5	5
75	75	75	75
100	100	100	100
85	85	85	85
1.0	1.0	1.0	1.0
50	50	50	50
5	5	5	5
Conc. Control 18c h9c2.txt	Conc. Control 19c h9c2.txt	Conc. Control 18m h9c2.txt	Conc. Control 19m h9c2.txt
18c h9c2	19c h9c2	18m h9c2	19m h9c2

Cell ink viability

Decluster	degree
Trypan blue	mixing cycles
Aspirate	cycles
Minimum	circularity
Comment	
Avg. background	intensity
Avg. cells	/ image
Images	
Avg. circ.	
Avg. diam.	(microns)
Viable cells	/ml (x10^6)
Total cells	/ml (x10^6)
Viability	(%)
Viable cells	
Total cells	
Sample date	
Elapsed time	(hrs)
Viable cell	spot area (%)
Viable cell spot	brightness (%)

Low	Low	Low
3	3	3
1	1	1
0.0	0.0	0.0
204	204	204
4.1	4.3	3.8
50	50	50
0.51	0.45	0.50
18.33	17.41	18.06
0.22	0.24	0.21
0.25	0.26	0.23
89.8	94.4	91.1
185	201	175
206	213	192
24 Nov 2024	24 Nov 2024	24 Nov 2024
5	5	5
75	75	75

12.4 Fibronectin/ collagen CA

PDMS APTES COL-1 surface contact angles

No.	IFT [mN/m] Theta(R)[deg]	Vol [ul] Theta(M)[deg]	Area [mm*2] Height [mm]	MethodMAG [pix/mm] Temp.[Cel.]	System Density	Theta(L)[deg] BD [mm]
0-0	8.55 20.00	16.08 -0.8500	CIR 3.345	107.96 Solvent 1a	84.7 84.7	84.7 1.524
0-1	8.54 20.00	16.07 -0.8500	CIR 3.344	107.96 Solvent 1a	84.7 84.7	84.7 1.523
0-2	8.54 20.00	16.07 -0.8500	CIR 3.344	107.96 Solvent 1a	84.6 84.6	84.6 1.523
0-3	7.51 20.00	14.80 -0.8500	CIR 3.288	107.96 Solvent 1a	81.5 81.5	81.5 1.418

0-4	7.51 14.81	CIR	107.96	Solvent 1a	81.5	81.5	81.5	1.417
20.00	-0.8500 3.290							
0-5	7.51 14.81	CIR	107.96	Solvent 1a	81.4	81.4	81.4	1.416
20.00	-0.8500 3.291							
0-6	7.47 15.70	CIR	107.96	Solvent 1a	62.5	62.5	62.5	1.160
20.00	-0.8500 3.822							
0-7	7.46 15.69	CIR	107.96	Solvent 1a	62.5	62.5	62.5	1.159
20.00	-0.8500 3.821							
0-8	7.45 15.68	CIR	107.96	Solvent 1a	62.5	62.5	62.5	1.158
20.00	-0.8500 3.820							
0-9	10.01 17.86	CIR	107.96	Solvent 1a	85.5	85.5	85.5	1.618
20.00	-0.8500 3.503							
0-10	10.01 17.85	CIR	107.96	Solvent 1a	85.4	85.4	85.4	1.617
20.00	-0.8500 3.504							
0-11	10.00 17.85	CIR	107.96	Solvent 1a	85.4	85.4	85.4	1.616
20.00	-0.8500 3.504							
0-12	6.34 14.02	CIR	107.96	Solvent 1a	63.2	63.2	63.2	1.107
20.00	-0.8500 3.599							
0-13	6.34 14.02	CIR	107.96	Solvent 1a	63.2	63.2	63.2	1.106
20.00	-0.8500 3.599							
0-14	6.33 14.01	CIR	107.96	Solvent 1a	63.1	63.1	63.1	1.106
20.00	-0.8500 3.599							
0-15	8.31 16.76	CIR	107.96	Solvent 1a	63.5	63.5	63.5	1.216
20.00	-0.8500 3.928							
0-16	8.30 16.75	CIR	107.96	Solvent 1a	63.5	63.5	63.5	1.215
20.00	-0.8500 3.926							
0-17	8.30 16.75	CIR	107.96	Solvent 1a	63.5	63.5	63.5	1.215
20.00	-0.8500 3.928							
0-18	9.66 17.80	CIR	107.96	Solvent 1a	73.7	73.7	73.7	1.427
20.00	-0.8500 3.811							
0-19	9.65 17.80	CIR	107.96	Solvent 1a	73.4	73.4	73.4	1.423
20.00	-0.8500 3.816							
0-20	9.68 17.85	CIR	107.96	Solvent 1a	73.3	73.3	73.3	1.422
20.00	-0.8500 3.826							
0-21	18.60 33.83	CIR	107.96	Solvent 1a	43.2	43.2	43.2	1.209
20.00	-0.8500 6.101							
0-22	18.54 33.73	CIR	107.96	Solvent 1a	43.3	43.3	43.3	1.209
20.00	-0.8500 6.091							

0-23	18.56	33.77	CIR	107.96	Solvent 1a	43.3	43.3	43.3	1.209
	20.00	-0.8500							
0-24	9.85	18.01	CIR	107.96	Solvent 1a	74.2	74.2	74.2	1.444
	20.00	-0.8500							
0-25	9.86	18.03	CIR	107.96	Solvent 1a	74.1	74.1	74.1	1.444
	20.00	-0.8500							
0-26	9.88	18.05	CIR	107.96	Solvent 1a	74.0	74.0	74.0	1.443
	20.00	-0.8500							
0-27	9.88	18.06	CIR	107.96	Solvent 1a	74.0	74.0	74.0	1.443
	20.00	-0.8500							
0-28	9.46	17.40	CIR	107.96	Solvent 1a	77.2	77.2	77.2	1.468
	20.00	-0.8500							
0-29	9.46	17.40	CIR	107.96	Solvent 1a	77.1	77.1	77.1	1.467
	20.00	-0.8500							
0-30	9.45	17.39	CIR	107.96	Solvent 1a	77.1	77.1	77.1	1.467
	20.00	-0.8500							
0-31	8.58	17.16	CIR	107.96	Solvent 1a	63.1	63.1	63.1	1.223
	20.00	-0.8500							
0-32	8.39	16.87	CIR	107.96	Solvent 1a	63.5	63.5	63.5	1.220
	20.00	-0.8500							
0-33	8.53	17.13	CIR	107.96	Solvent 1a	62.8	62.8	62.8	1.216
	20.00	-0.8500							
0-34	9.29	18.41	CIR	107.96	Solvent 1a	60.1	60.1	60.1	1.211
	20.00	-0.8500							
0-35	9.26	18.38	CIR	107.96	Solvent 1a	60.0	60.0	60.0	1.210
	20.00	-0.8500							
0-36	9.27	18.39	CIR	107.96	Solvent 1a	59.9	59.9	59.9	1.209
	20.00	-0.8500							
0-37	8.45	16.34	CIR	107.96	Solvent 1a	72.6	72.6	72.6	1.350
	20.00	-0.8500							
0-38	8.45	16.33	CIR	107.96	Solvent 1a	72.5	72.5	72.5	1.349
	20.00	-0.8500							
0-39	8.44	16.33	CIR	107.96	Solvent 1a	72.5	72.5	72.5	1.348
	20.00	-0.8500							
0-M	9.39	18.00	CIR		Solvent 1a	69.7	69.7	69.7	1.338
	3.901								

PDMS APTES GA COL-1 surface contact angles

No.	IFT [mN/m] Theta(R)[deg]	Vol [ul] Theta(M)[deg]	Area [mm*2] Height [mm]	MethodMAG [pix/mm] Temp.[Cel.]	System Density	Theta(L)[deg] BD [mm]
0-0	6.98 20.00	15.19 -0.8500	CIR 3.802	107.96 Solvent 1a 60.3	60.3	60.3 1.105
0-1	6.80 20.00	14.98 -0.8500	CIR 3.789	107.96 Solvent 1a 59.7	59.7	59.7 1.086
0-2	6.79 20.00	14.97 -0.8500	CIR 3.788	107.96 Solvent 1a 59.6	59.6	59.6 1.086
0-3	9.78 20.00	18.99 -0.8500	CIR 4.245	107.96 Solvent 1a 60.6	60.6	60.6 1.240
0-4	9.76 20.00	18.98 -0.8500	CIR 4.248	107.96 Solvent 1a 60.4	60.4	60.4 1.237
0-5	9.75 20.00	18.99 -0.8500	CIR 4.252	107.96 Solvent 1a 60.3	60.3	60.3 1.234
0-6	11.80 20.00	22.29 -0.8500	CIR 4.718	107.96 Solvent 1a 55.3	55.3	55.3 1.236
0-7	11.78 20.00	22.27 -0.8500	CIR 4.719	107.96 Solvent 1a 55.2	55.2	55.2 1.234
0-8	11.75 20.00	22.26 -0.8500	CIR 4.719	107.96 Solvent 1a 55.1	55.1	55.1 1.232
0-9	7.91 20.00	15.95 -0.8500	CIR 3.757	107.96 Solvent 1a 67.0	67.0	67.0 1.244
0-10	8.26 20.00	16.55 -0.8500	CIR 3.867	107.96 Solvent 1a 65.3	65.3	65.3 1.238
0-11	7.89 20.00	15.94 -0.8500	CIR 3.760	107.96 Solvent 1a 66.9	66.9	66.9 1.241
0-12	5.83 20.00	12.46 -0.8500	CIR 2.936	107.96 Solvent 1a 85.0	85.0	85.0 1.346
0-13	5.82 20.00	12.45 -0.8500	CIR 2.936	107.96 Solvent 1a 85.0	85.0	85.0 1.345
0-14	5.82 20.00	12.44 -0.8500	CIR 2.935	107.96 Solvent 1a 85.0	85.0	85.0 1.344
0-15	5.25 20.00	11.77 -0.8500	CIR 3.043	107.96 Solvent 1a 76.4	76.4	76.4 1.197
0-16	5.24 20.00	11.76 -0.8500	CIR 3.043	107.96 Solvent 1a 76.3	76.3	76.3 1.196
0-17	5.23 20.00	11.75 -0.8500	CIR 3.041	107.96 Solvent 1a 76.3	76.3	76.3 1.194

0-18	5.94	12.71	CIR	107.96	Solvent 1a	79.2	79.2	79.2	1.282
20.00	-0.8500	3.100							
0-19	5.93	12.70	CIR	107.96	Solvent 1a	79.2	79.2	79.2	1.281
20.00	-0.8500	3.099							
0-20	5.93	12.70	CIR	107.96	Solvent 1a	79.1	79.1	79.1	1.281
20.00	-0.8500	3.099							
0-21	9.88	17.67	CIR	107.96	Solvent 1a	89.7	89.7	89.7	1.673
20.00	-0.8500	3.363							
0-22	10.49	18.41	CIR	107.96	Solvent 1a	87.3	87.3	87.3	1.670
20.00	-0.8500	3.504							
0-23	10.50	18.42	CIR	107.96	Solvent 1a	87.2	87.2	87.2	1.670
20.00	-0.8500	3.507							
0-24	9.21	16.87	CIR	107.96	Solvent 1a	86.8	86.8	86.8	1.592
20.00	-0.8500	3.369							
0-25	9.20	16.86	CIR	107.96	Solvent 1a	86.8	86.8	86.8	1.591
20.00	-0.8500	3.368							
0-26	9.20	16.87	CIR	107.96	Solvent 1a	86.7	86.7	86.7	1.591
20.00	-0.8500	3.369							
0-27	8.67	16.23	CIR	107.96	Solvent 1a	84.8	84.8	84.8	1.532
20.00	-0.8500	3.358							
0-28	8.65	16.22	CIR	107.96	Solvent 1a	84.7	84.7	84.7	1.531
20.00	-0.8500	3.357							
0-29	8.64	16.20	CIR	107.96	Solvent 1a	84.7	84.7	84.7	1.530
20.00	-0.8500	3.355							
0-30	8.72	16.53	CIR	107.96	Solvent 1a	75.7	75.7	75.7	1.408
20.00	-0.8500	3.623							
0-31	8.71	16.53	CIR	107.96	Solvent 1a	75.7	75.7	75.7	1.407
20.00	-0.8500	3.623							
0-32	8.70	16.51	CIR	107.96	Solvent 1a	75.6	75.6	75.6	1.405
20.00	-0.8500	3.623							
0-33	5.23	11.86	CIR	107.96	Solvent 1a	72.9	72.9	72.9	1.154
20.00	-0.8500	3.126							
0-34	4.96	11.39	CIR	107.96	Solvent 1a	74.6	74.6	74.6	1.154
20.00	-0.8500	3.029							
0-35	8.66	16.32	CIR	107.96	Solvent 1a	80.1	80.1	80.1	1.467
20.00	-0.8500	3.489							
0-36	8.66	16.31	CIR	107.96	Solvent 1a	80.1	80.1	80.1	1.466
20.00	-0.8500	3.489							

0-37	8.66	16.31	CIR	107.96	Solvent 1a	80.0	80.0	80.0	1.465
	20.00	-0.8500	3.490						
0-M	8.08	15.88	CIR		Solvent 1a	74.8	74.8	74.8	1.347
	3.551								

PDMS Fibronectin surface contact angles

No.	Age [h:m:s:ms]	Theta(M)[deg]	IFT [mN/m]	Vol [ul]	Area [mm*2]	MethodMAG
[pix/mm]	BD [mm]	System	Theta(L)[deg]	Theta(R)[deg]	Height [mm]	Temp.[Cel.]
Density						
0-0	00:31:43:321	13.3	3.95	24.62	CIR	76.40
	13.3	13.3	0.324	20.00	1.0000	5.562
0-1	00:31:50:883	12.8	4.09	25.82	CIR	76.40
	12.8	12.8	0.319	20.00	1.0000	5.698
0-2	00:31:54:242	12.8	4.00	25.37	CIR	76.40
	12.8	12.8	0.318	20.00	1.0000	5.648
0-3	00:31:56:320	13.1	3.79	24.14	CIR	76.40
	13.1	13.1	0.317	20.00	1.0000	5.507
0-4	00:31:56:796	12.6	4.09	26.07	CIR	76.40
	12.6	12.6	0.316	20.00	1.0000	5.727
0-5	00:31:57:675	13.1	3.80	24.20	CIR	76.40
	13.1	13.1	0.316	20.00	1.0000	5.514
0-6	00:31:58:838	12.6	4.08	26.12	CIR	76.40
	12.6	12.6	0.315	20.00	1.0000	5.732
0-M	12.9		3.97	25.19	CIR	5.627
	12.9	12.9	0.318			Water (Chen) [HH]

12.5 Cell profiler pipeline

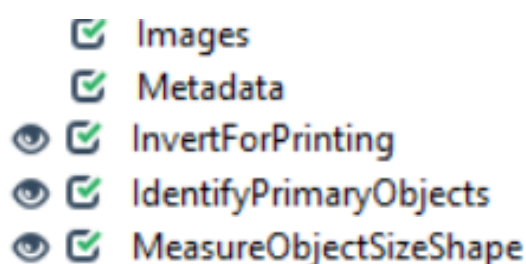


Figure S12.5) cell profiler pipeline used for 1) Pulling 'Images' 2) Inverting colour 'Invert for printing' 3) 'Identify primary objects', the cells found cells using Otsu adaptive thresholding, window was set to 5x cell size, thresholding was set to cell range 4) 'Measure object size shape' to output data on found cells

12.6 BODIPY rotor decay

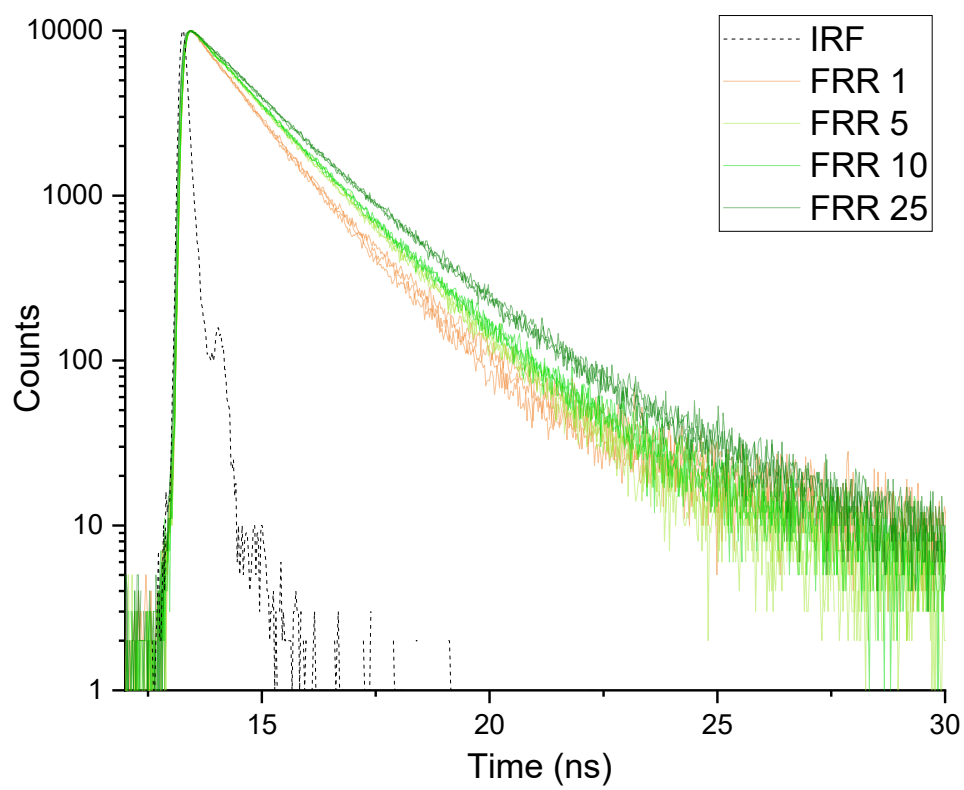


Figure S12.6 Example decay time resolved fluorescence for FRR BODIPY study where IRF is an internal reference.