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Biodegradable hybrid block copolymer – lipid vesicles as potential drug delivery systems

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ABSTRACT

The anticipated benefits of nano-formulations for drug delivery are well known: for nanomedicines to achieve this potential, new materials are required with predictive and tuneable properties. Excretion of excipients following delivery is advantageous to minimise the possibility of adverse effects; biodegradability to non-toxic products is therefore desirable. With this in mind, we aim to develop tuneable hybrid lipid-block copolymer vesicle formulations where the hydrophilic polymer block is polyethylene glycol (PEG), which has accepted biocompatibility, and the hydrophobic block of the polymer is biodegradable: polycaprolactone (PCL) or polylactide (PLA). We investigate five different block copolymers for the formation of 1:1 phospholipid:polymer hybrid vesicles, compare their properties to the appropriate unitary liposome (POPC) and polymersome systems and assess their potential for future development as nanomedicine formulations. The PEG-PCL polymers under investigation do not form polymersomes and exhibit poor colloidal and/or encapsulation stability in hybrid formulations with lipids. The properties of PEG-PLA hybrid vesicles are found to be more encouraging: they have much enhanced passive loading of a hydrophilic small molecule (carboxyfluorescein) compared to their respective polymersomes and reduces serum induced lysis of the vesicle compared to the liposome. Significantly, burst release from hybrid vesicles can be substantially reduced by making the polymer components of the hybrid vesicle a mixture containing 10 mol% of PEG_{15}-PLA_{25} that is intermediate in size between the phospholipid and larger PEG_{45}-PLA_{54} components. We conclude that hybrid lipid/PEG-PLA vesicles warrant further assessment and development as candidate drug delivery systems.

INTRODUCTION

Formulation of drugs within nanoscale particles has been the focus of intensive investigation driven by the promise of enhanced pharmacokinetics through a range of mechanisms that improve the location and timing of *in vivo* drug release.[1, 2] Within the vanguard of clinical nanomedicines are hollow carriers composed of lipids (liposomes),[3, 4] which mimic natural transport vesicles that ferry biomolecular cargos through intracellular or extracellular environments. The advantage of using natural lipid membrane systems like liposomes is their biocompatibility, however the use of liposomal formulations is limited due to their low stability in serum. A surface coating of hydrophilic polymers on these lipid membranes has been shown to increase liposome stability in biological media for drug delivery: the most common example of this is the surface functionalisation of liposomes with membrane-anchored polyethylene glycol (PEG) as a strategy to provide steric-shields that protect the lipid membrane and increase stability[5] resulting in increased circulation time in humans[6]. Despite numerous successful examples of liposomal drugs reaching the clinic, relatively few have received clinical approval when considered against the benefits these nanoformulations are expected to bring[7]. Therefore, liposomes have failed to reach their full potential and are often criticised for poor stability as well as limited tuneability and environmental responsiveness.

A more recent analogue to liposome carriers with the potential to surmount the aforementioned weaknesses is the polymersome.[8] Composed of amphiphilic block copolymers, the broad scope for variation of the molecular size, architecture, flexibility and chemical composition of their constituent building blocks provides generous capacity to engineer the material properties of these vesicular particles, including membrane thickness and permeability.[9] While no polymersome nanomedicine has yet successfully reached the clinic to deliver patient benefit, wide-ranging examples of their development for medical therapies exist in the academic literature.[10, 11, 12, 13, 14, 15] The fact that many polymersomes under investigation for nanomedicine applications are composed of inherently PEGylated polymers means that they have a naturally high PEG density at their surface compared to PEGylated liposomes, which are typically formulated with ~5-10 mol% PEGylated lipid:[16, 17, 18] high PEGylated lipid densities disrupt vesicle stability.[19] Polymersomes have been reported to have up to double the circulation half-life of PEGylated liposomes,[20] but polymersomes have been reported to have high levels of hepatic uptake.[21]

Despite the conceived advantages of block copolymers, they lack the natural biocompatibility and biofunctionality of phospholipids. In recent years, this has led to an interest in developing hybrid lipid – block copolymer systems for numerous vesicle technologies with the aim of synergistically combining the benefits of each constituent (Fig. 1).[22, 23, 24, 25, 26, 27, 28, 29, 30] By blending two components, this increases the degrees of freedom available to tune vesicle properties through varying their relative mixing ratio. Hybrid vesicles can either form well mixed membranes or phase-separate into polymer-rich and lipid-rich domains, depending on the chemical nature of the two species and their relative compositions.[23, 24, 29, 31, 32, 33, 34]

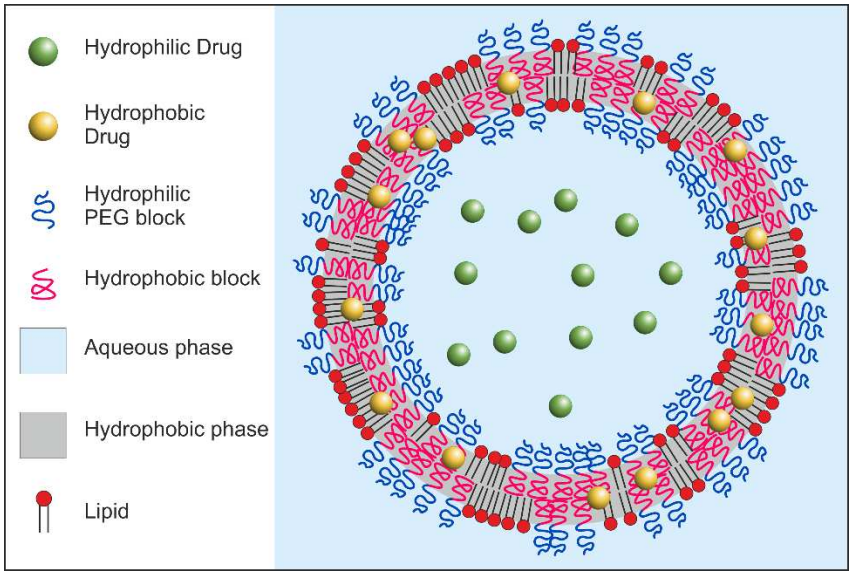


Figure 1: Cartoon structure of a hybrid lipid – block copolymer vesicle and their potential for encapsulation of hydrophilic and hydrophobic drug molecules.

Most commonly, hybrid vesicles have been reported with either polydimethylsiloxane or polybutadiene as the hydrophobic block of the polymer.[23, 24, 27, 31, 32, 35, 36, 37, 38, 39] However, for *in vivo* drug delivery, it is highly desirable that the polymers are biodegradable, which neither of these are.[40, 41] Biodegradability enhances clearance of excipients following delivery of the drug, minimising risk of accumulation and adverse associated toxicities that might limit drug dosing. Common biodegradable polymers that have been reported for formation of pure polymersome systems include polylactide and polycaprolactone hydrophobic blocks.[42, 43, 44, 45, 46, 47] A non-fouling vesicle surface is also required to inhibit opsonisation by *in vivo* protein absorption, marking these particles for removal by the reticuloendothelial system. As discussed above, polyethylene glycol (PEG) is a well-established stealth coating for nanomedicines that meets these criteria.[48] Hybrid vesicles offer the prospect of greater tuneability of the PEGylated surface between the ~10 mol% PEG of stealth liposomes and the 100 mol% PEG of many polymersome formulations, which could allow improved optimisation of *in vivo* circulation times and biodistribution. Benefits of the hybrid vesicle approach have already been reported for targeted delivery, where lower surface PEG density reduced steric frustration of cell-surface targeting of the vesicles.[35]

Very limited examples exist of biodegradable block copolymers being incorporated within hybrid membranes with phospholipids. Up to 10 mol% of polycaprolactone-block-polyethylene oxide (PCL-PEG) copolymers have been investigated in DPPC liposomes, a similar compositional range used for pegylated lipids in current stealth liposome formulations.[49, 50] These were comparatively large polymers with molecular masses in the range 7.5 – 10.6 kDa (10x or more the mass of the phospholipid) with between 30-53% of the polymer mass composed of PCL. These polymers reduced the size of DPPC vesicles and did not significantly shift the melting temperature of DPPC, although the larger polymer did considerably broaden the observed transition width. Exploration of the full compositional space from 0 to 100% polymer is yet to be reported for hybrid vesicles with a biodegradable block copolymer.

Towards understanding the potential and viability of lipid – biodegradable block copolymer hybrid vesicles for parenteral nanomedicine technologies, we investigate the formation of vesicles composed of the phospholipid POPC and a range of different biodegradable, amphiphilic diblock copolymers. We will aim to make vesicles that are approximately 100 nm in diameter as this falls within the size-range that is advantageous for tumour targeting by the

enhanced permeability and retention (EPR) effect.[51, 52] Where viable hybrid vesicles form, we further investigate some of their physicochemical properties relevant to drug delivery applications and compare them to their respective unitary liposome and polymersome counterparts.

MATERIALS AND METHODS

Materials

The phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, 16:0-18:1 PC, 850457) and the fluorescently labelled lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-PE, 18:1 Liss Rhod PE, 810150) were purchased from Avanti Polar Lipids. Polyethylene glycol – polycaprolactone (PEG-PCL) and polyethylene oxide – polylactide (PEG-PLA, DL form) block copolymers were purchased from Polymer Source Inc., Canada (see table 1). The molecular weight, block ratios and polydispersities for these polymers were taken from the manufacturer data sheets. Stock solutions of each block copolymer were prepared at 33 mM in chloroform. 5(6)-Carboxyfluorescein was purchased from ACROS Organics™ (CAS No. 72088-94-9).

Vesicle formation

For the preparation of vesicles, 100 µL of stock solution in chloroform, where lipids and polymers were dissolved in the desired molar ratio (to a total of 6.58 µmoles), along with added 0.5 mol% of the rhodamine labelled lipid (Rh-PE) to help track the sample. The lipid/polymer chloroform solution was briefly mixed using a vortex mixer before drying to a film in a glass vial under continuous vacuum in a desiccator for ~2 h. 1.0 ml of HEPES buffer (20 mM HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid), 137 mM NaCl, pH 7.4) was added to the dried lipid/polymer films and vortexed for 30 s. This was followed by bath sonication at 55 °C for 10 min to ensure full resuspension of lipid/polymer into the buffer. The resultant suspension was put through five freeze-thaw-vortex cycles using liquid nitrogen and a warm water bath. Next, the samples were extruded 11 times at 55°C through 100 nm pore size polycarbonate membrane filters using an Avanti® Mini-Extruder to form nanoscale vesicles. Samples were stored at 4 °C prior to further characterisation.

Vesicle characterisation

Vesicle size distributions were analysed using Dynamic light scattering (DLS) experiments performed using a Malvern Zetasizer Nano ZSP. Three measurements for each sample were performed at 25 °C (run duration of 10 s with 12 runs per measurement; angle of detection set to 173°) and vesicle size is reported as the average of these three measurements with respect to intensity and % volume. The raw intensity autocorrelation functions are also reported in the supplementary information.

Carboxyfluorescein encapsulation and release

Carboxyfluorescein (CF) was encapsulated in vesicles at self-quenching concentrations. The fluorescence of CF is >95% self-quenched at concentrations >100 mM.[53] The buffer used to hydrate the dried lipid/polymer film in the above vesicle preparation protocol was switched to CF HEPES Buffer (20 mM HEPES, 137 mM NaCl and 125 mM 5(6)-Carboxyfluorescein (CF), pH 7.4. Following extrusion, vesicles were run through a Sephadex G50 size exclusion column (diameter = 1.5 cm, height = 15 cm) to separate them from unencapsulated CF. Concentration of the sample was determined via a standard phosphorus assay to determine phospholipid concentration [54, 55], or the trace lipid (Rh-PE; 0.5 mol%) was used to determine the

concentration of polymer only samples via UV-vis spectroscopy and comparison to a standard curve.

To determine the passive encapsulation efficiency of CF in vesicles, the collected fractions were diluted by a factor of 10^4 with the HEPES buffer (without CF) in a cuvette up to a total of 3 ml (so that the total amphiphile concentration is 0.165 µM) and the background fluorescence emission was recorded. All fluorescence measurements were conducted using a Horiba Fluoromax-3. The fluorescence excitation wavelength (λ_{ex}) of CF is at 492nm and the wavelength of fluorescence emission (λ_{em}) is 517 nm. To destabilise the vesicles and release encapsulated CF, 50 µl of 10% Triton X-100 (w/v) was added to the 3 ml of the diluted sample and the fluorescence emission was again recorded. A semi-quantitative measure of encapsulation was thus obtained from the difference of the release CF following background subtraction.

To determine the CF release kinetics from vesicles, samples were again diluted by a factor of 10^4, following chromatographic separation, with the HEPES buffer on the day of sample preparation (Day 0): the background fluorescence intensity of these samples was measured (I_0). The fluorescence intensity ($I_x(t)$) of these samples was measured at appropriate time intervals, t. The total content of dye inside the vesicle (I_{max}) was measured by destabilising the vesicles with the addition of 50 µl of 10% Triton X-100 (w/v); this was done for each individual time point measurement.

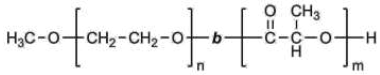
Thermal analysis (DSC)

Phase transitions in constituent hybrid vesicles were characterised by differential scanning calorimetry measurements, conducted using a MicroCal VP-DSC (Temperature range = 4°C to 80°C, scan rate = 90 °C/hr, filtering period = 2 s). We used one heating cycle and one subsequent cooling cycle for each sample).

DSC analysis of dry bulk polymers was performed using a TA Instruments Q2000 with refrigerated cooling system. The DSC cell was purged with nitrogen. Samples were analysed in hermetically sealed aluminium pans. A small amount of solid (~ 2- 10 mg) was added to the hermetic pan and sealed. A modulated DSC method was used. Samples were heated from 25 to 80'C, 80'C to -80'C and back to 80'C using the following modulated parameters – 3'C/min underlying heating rate, amplitude +/- 1'C every 40 seconds.

RESULTS AND DISCUSSION

Five distinct block copolymers were selected to test for vesicle formation in blends with phospholipids (Table 1). All polymers have a polyethylene oxide (PEG) hydrophilic block and either a polycaprolactone (PCL) or polylactide (PLA) biodegradable hydrophobic block with total polymer molecular weights ranging between 1.65 to 6.8 kDa. The polymers were also selected based on the published concept that the volume fraction of the PEG block (f_{EO}) should be in the range 0.20 to 0.42 for polymersome formation.[42]

Block-copolymers		M_n / kDa EO-*b*-X	Transition Temperatures / °C			f_{EO}
			T_m	T_c	T_g	
 Poly(ethylene oxide -b- lactide)	PEG_{15}-*b*-PLA_{25} (DL form)	0.7-*b*-1.8	--	--	-34.9	0.27
	PEG_{45}-*b*-PLA_{54} (DL form)	2.0-*b*-3.9	--	--	43.9	0.33

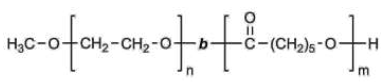
 Poly(ethylene oxide-b-caprolactone)	PEG_{12}-*b*-PCL_{10}	0.55-*b*-1.1	43.1	13.6	No T_g	0.34
	PEG_{45}-*b*-PCL_{25}	2.0-*b*-2.8	50.1	26.9	No T_g	0.42
	PEG_{45}-*b*-PCL_{42}	2.0-*b*-4.8	47.8	17.1	No T_g	0.30

Table 1. Block-copolymers used in this work. The polymers are given in notation that represents the number of monomers in each block and the number averaged molecular weights (M_n) of each block are given where X represents the hydrophobic block (either LA or CL). Transition temperatures were obtained from solid-phase DSC (supplementary information Figure S1) and were consistent with the manufacturer's data: melting (T_m), crystallisation (T_c) and glass (T_g). The hydrophilic PEG volume fraction (f_{EO}) of these polymers is calculated using homopolymer melt densities of 1.13, 1.09 and 1.14 g cm^{-3} for PEG, PLA and PCL respectively.[42]

To verify the formation of vesicular structures the samples were analysed using dynamic light scattering (DLS) to ascertain particles formed within the expected size range: the unfitted intensity autocorrelation functions are presented in the supplementary information (Fig. S2). CF encapsulation will also be reported below to demonstrate the particles contained an aqueous lumen that is able to sequester hydrophilic molecules.

Initially the block-copolymers were tested to determine whether they would spontaneously form polymersomes in an aqueous environment. Polymer suspensions were prepared according to our vesicle preparation protocol at a concentration of 6.6 mM in HEPES buffer. The size distributions (Figure 2A) show that both PEG-PLA polymers form monomodal vesicle-sized structures at ~100 nm, consistent with extrusion through a 100 nm pore-size polycarbonate membrane. The polydispersity index (PDI) for both PEG-PLA polymers was relatively low (in the range 0.067 - 0.133) and consistent with typical PDIs for lipid vesicles.

In contrast to PEG-PLA polymers, the size distribution profiles for the pure PEG-PCL polymer systems show that all three failed to form vesicle-sized structures. PEG_{45}-*b*-PCL_{25} and PEG_{45}-*b*-PCL_{42} showed single peaks (CONTIN) corresponding to a hydrodynamic size range typical for spherical polymer micelles (28.21 nm and 24.36 nm respectively). A different behaviour was observed for PEG_{12}-*b*-PCL_{10}: a typical size distribution profile showed 3 peaks (shown at 106 nm, 955 nm and 5560 nm in Fig. 2A). A plot of the autocorrelation coefficient function for this sample (Fig. S2) shows multiple relaxation modes in the data, consistent with the multiple distinct size populations of different polymer assemblies extracted in the CONTIN fit. Aqueous dispersions of this polymer were also notably more viscous than the other samples, therefore it is hypothesised that this could be indicative of the formation of worm-like micelles. It has previously been reported that block copolymers with an ethylene oxide volume fraction in the range of $0.20<f_{EO}<0.42$ form vesicles.[42] This informed our rationale for initially selecting the three PEG-PCL polymers reported above, which has an f_{EO} either within or very close to this range. However a 2013 study investigating parameters for successful PEG-PCL polymersome formation concluded that the molecular weight of the PEG block must be in the range 1.1 - 3 kDa and $0.14<f_{EO}<0.21$.[44] Therefore this is consistent with the formation of non-vesicular structures by our chosen PEG-PCL polymers.

To confirm formation of vesicle structures with an inner aqueous volume with these polymers, CF encapsulation experiments were conducted to test passive encapsulation of hydrophilic small molecules, which would only be expected for structures having an enclosed lumen (Figure 2B). Only PEG_{45}-*b*-PLA_{54} showed any appreciable CF encapsulation with the others showing negligible dye content. However, CF encapsulation in PEG_{45}-*b*-PLA_{54} polymersomes

is found to be comparatively poor: these polymersomes have a tenfold lower encapsulation when compared to POPC liposomes. The PEG_{15}-*b*-PLA_{25} polymer sample was determined to be size stable at ~100 nm via DLS, however it shows very little appreciable CF encapsulation. This poor encapsulation of the CF dye could be due to the fact that polymer membranes have a much higher lysis strain than liposomes and the freeze-thaw performed during vesicle formation step is not as effective at improving loading through repeated rupture and resealing. Poor encapsulation of small molecule probes in polymersomes is also consistent with other reports in the literature, where the efficiency of encapsulation is also strongly dependent on the vesicle formation process.[56]

Next, 50% hybrid polymer-lipid systems were formed by mixing POPC and polymers in a 1:1 molar ratio and conducting protocols for vesicle formation by extrusion. While some of our polymers (PEG-PCL variants) do not form vesicles as unitary systems, membrane-forming lipids are likely to assist their arrangement with vesicle structures, at least over some range of relative composition. The size distribution profiles (Figure 2C) show that expected vesicle-size (~100 nm) structures form for both PEG-PLA polymers and the smaller PEG-PCL (PEG_{12}-$_b$-PCL_{10}). The PEG-PLA hybrids exhibited single peaks with relatively low polydispersity: 0.085 and 0.137 for PEG_{15}-*b*-PLA_{25} and PEG_{45}-*b*-PLA_{54} respectively. However the size distribution profile for the PEG_{12}-*b*-PCL_{10}:POPC blend shows a vesicle-sized peak at 75.2 nm, coexisting aggregates are also observed in the 1-10 μm range giving a relatively high polydispersity of 0.300. This suggests that the supposed hybrid vesicles that have formed for this polymer may not be colloidally stable, leading to aggregation. Finally the remaining two PCL-PEG polymers (PEG_{45}-*b*-PCL_{25} and PEG_{45}-*b*-PCL_{42}) show single peaks at 26 nm and 20 nm respectively indicating micellar structures still dominate for these hybrid samples. However, their PDIs are large (0.256 and 0.298) due to a broad shoulder in the distribution towards larger structure sizes, suggestive that some vesicle-like structures may have formed: this is most apparent in the intensity-weighted size distributions, which are most sensitive to the largest structures due to the scattering intensity scaling with the 6^{th} power of particle size.

Hybrid vesicles had much enhanced passive encapsulation compared to the unitary polymersome systems for all polymers studied (Figure 2D). While CF encapsulation for pure polymer systems ranged between 0-10% that of liposomes, hybrid vesicles encapsulated 33-54% of CF compared to POPC lipids, at least a 4-fold improvement compared to the polymersomes (Fig. 2B). PEG_{12}-*b*-PCL_{10}:POPC hybrids vesicles exhibiting the highest encapsulation and the monomodal-sized PEG-PLA hybrids had encapsulation efficiencies of ~37% compared to POPC liposomes.

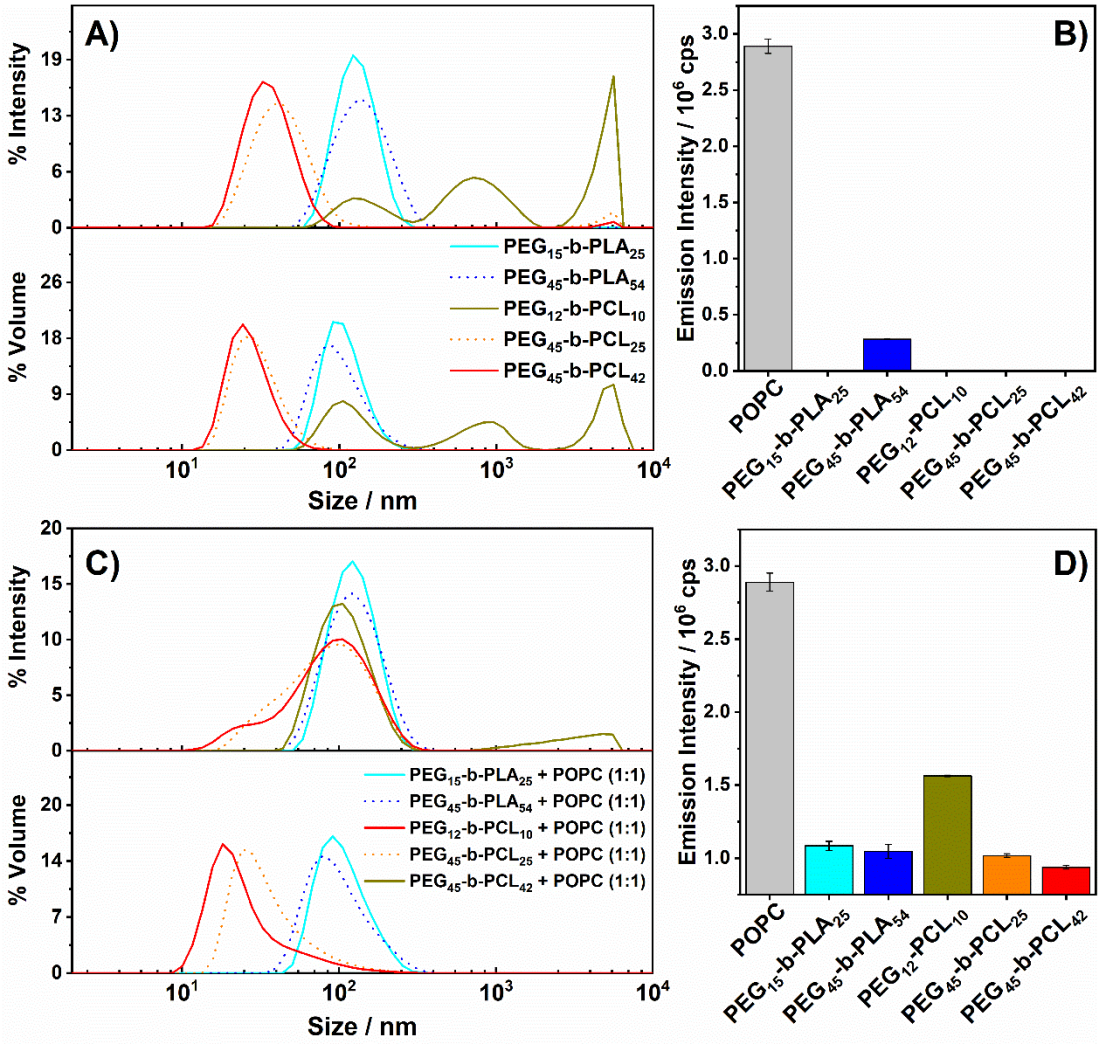


Figure 2: A) Particle-size distribution with respect to Intensity and % volume of polymer suspensions, B) CF dye encapsulation in polymer samples, C) Particle-size distribution with respect to Intensity and % volume for samples of 50% hybrid vesicles (containing 1:1 molar ratio of polymer and POPC lipid) and D) CF dye encapsulation in 50% hybrid vesicles. All samples prepared via the vesicle preparation method described. Error bars represent mean ± SD (n= 3).

Table 2: Summary of PSD and CF encapsulation data shown in Figure 2. The Z-average particle size of the global size distribution and polydispersity index (PDI) are obtained from a second order cumulant fit to the intensity autocorrelation function, in contrast to the size distributions in figure 2, which are obtained from a CONTIN fit. Values are mean +/- SD (n=3).

Lipid:Polymer composition	**% Polymer**		**Size distribution profile**		**% CF encapsulation normalised to POPC total encapsulation**
	mol %	weight %	z-average size / nm	PDI	
POPC	0	0	114 ± 2	0.051	100.0 ± 2.2
PEG_{15}-*b*-PLA_{25}	100	100.0	123 ± 2	0.067	0.1 ± 2.2
PEG_{45}-*b*-PLA_{54}	100	100.0	125 ± 2	0.133	9.8 ± 0.5
PEG_{12}-*b*-PCL_{10}	100	100.0	840 ± 200	1.000	N/A
PEG_{45}-*b*-PCL_{25}	100	100.0	44 ± 1	0.264	0.2 ± 1.7
PEG_{45}-*b*-PCL_{42}	100	100.0	34 ± 1	0.167	0.1 ± 3.5
1:1 POPC:PEG_{15}-*b*-PLA_{25}	50	76.7	122 ± 3	0.085	37.5 ± 2.9
1:1 POPC:PEG_{45}-*b*-PLA_{54}	50	88.6	118 ± 3	0.137	36.2 ± 4.6
1:1 POPC:PEG_{12}-*b*-PCL_{10}	50	68.5	115 ± 12	0.300	54.1 ± 0.3
1:1 POPC:PEG_{45}-*b*-PCL_{25}	50	86.3	66 ± 2	0.258	35.2 ± 1.2
1:1 POPC:PEG_{45}-*b*-PCL_{42}	50	89.9	62 ± 1	0.298	32.4 ± 1.3

With the exception of PEG_{15}-*b*-PLA_{25}, the hydrophobic blocks of all our polymers exhibit transition temperatures in the range of or close to 5 to 50 °C, which may impact behaviour during refrigerator storage (4 °C) and during *in vivo* delivery (37 °C). Therefore it is informative to characterise any modulation of these phase transitions in hybrid vesicles. Note that POPC has a chain ordering transition at -2 °C, outside of this range. Differential scanning calorimetry (DSC) was utilized to elucidate the thermal transitions of 50% hybrid vesicles (Figure 3). The thermograms for PEG_{15}-*b*-PLA_{25} and PEG_{45}-*b*-PLA_{54} show some subtle features but no clear phase transition from what is known about the phase behaviour of the individual components: no baseline shift is seen in Fig. 3B consistent with the glass transition of PEG_{45}-*b*-PLA_{54} at ~44 °C.

PEG_{12}-*b*-PCL_{10} (Fig. 3C) shows distinct transitions: in the heating curve an event indicating loss of structure akin to a melting transition is measured at 45 °C with a distinct shoulder at 39 °C. The product data sheet suggests two melts at 40°C and 46°C but we experimentally only observe a single melt at ~ 43°C (Fig S1A). The DSC of unhydrated polymer (Fig. S1) does not correspond to that of the hydrated polymer suspensions (Fig. 3) due to effects of solvation i.e. polymer is suspended in water. Hysteresis in this transition is seen in the cooling scan with an event indicating increase in structure akin to crystallisation transition at 28 °C, which is not in agreement with the crystallisation transition observed with the single component polymer in bulk (T_c = 13°C). This could be attributed to the solvation of the PEG block of the copolymer in the aqueous buffer and thus the hydrophobic interactions between the PCL blocks becomes the major driving force for the polymer-polymer/polymer-lipid interactions. These transition temperatures were also observed in single component PEG_{12}-*b*-PCL_{10} samples (Fig. 3C), indicating that these phase transitions are not significantly affected by the presence of the lipid and therefore indicative of poor mixing of these two components in aqueous dispersions. On visual inspection, it was noted that when PEG_{12}-*b*-PCL_{10} suspension was heated above the melting temperature, the polymer aggregated and precipitated out of solution.

Thermograms of PEG_{45}-*b*-PCL_{25} and PEG_{45}-*b*-PCL_{42} hybrid systems (Fig. 3D,E) show subtle features consistent with their melting transitions. PEG_{45}-*b*-PCL_{25} showed a transition at 35 °C compared to the single component T_m of 50 °C (Table 1). PEG_{45}-*b*-PCL_{42} is expected to have a double melting transition in the unmixed state of 40 and 46 °C (Table 1), shows two distinct transitions at 37 and 46 °C in a 1:1 mixture with POPC. Note that POPC does not have any phase transitions in the temperature range under investigation and is in its fluid phase (T_m = -2°C).[57] Upon cooling of the PEG_{45}-*b*-PCL_{25} and PEG_{45}-*b*-PCL_{42} hybrid samples, no crystallisation transition was observed. We hypothesised that this was due to very slow re-crystallisation kinetics for these larger polymers. This is consistent with the melting transitions not being observable when we conducted an immediate second heating cycle; however, incubation of the samples for several hours following the first heat-cool cycle was sufficient to facilitate recovery of these transition peaks in a subsequent heating scan.

Long-term colloidal stability of 1:1 polymer:lipid hybrid compositions was monitored over a period of three weeks (Figure 4). PEG_{15}-*b*-PLA_{25} and PEG_{45}-*b*-PLA_{54} hybrid vesicles are considered to be stable during this timeframe: while the z-averaged size decreases very slowly and the PDI increases slightly in the first 10-15 days, these average effects are very small and not considered very significant when considering the error bars for these measurements. The sample which showed the largest polydispersity increase was PEG_{12}-*b*-PCL_{10}:POPC hybrids vesicles which had an initial PDI of 0.30 on day 0 and 0.48 by day 21. The average size of assemblies in these samples also increase from ~100 to ~400 nm in this timeframe. This suggests that POPC:PEG_{12}-*b*-PCL_{10} hybrid samples do not form stable assemblies at this composition with significant aggregation or sample ripening over time. PEG_{45}-*b*-PCL_{42} hybrid samples showed a moderate increase in average structure size and polydispersity over this time scale, whereas PEG_{45}-*b*-PCL_{25} samples were reasonably stable in size and polydispersity over this time frame. However these two compositions start with high PDIs in the range 0.25 – 0.30, which could indicate significant structural heterogeneity that is undesirable for drug formulations and may render these samples more prone to slow ripening processes during storage. Therefore PEG-PLA hybrids show considerably superior homogeneity and colloidal stability when compared to the PEG-PCL hybrid samples.

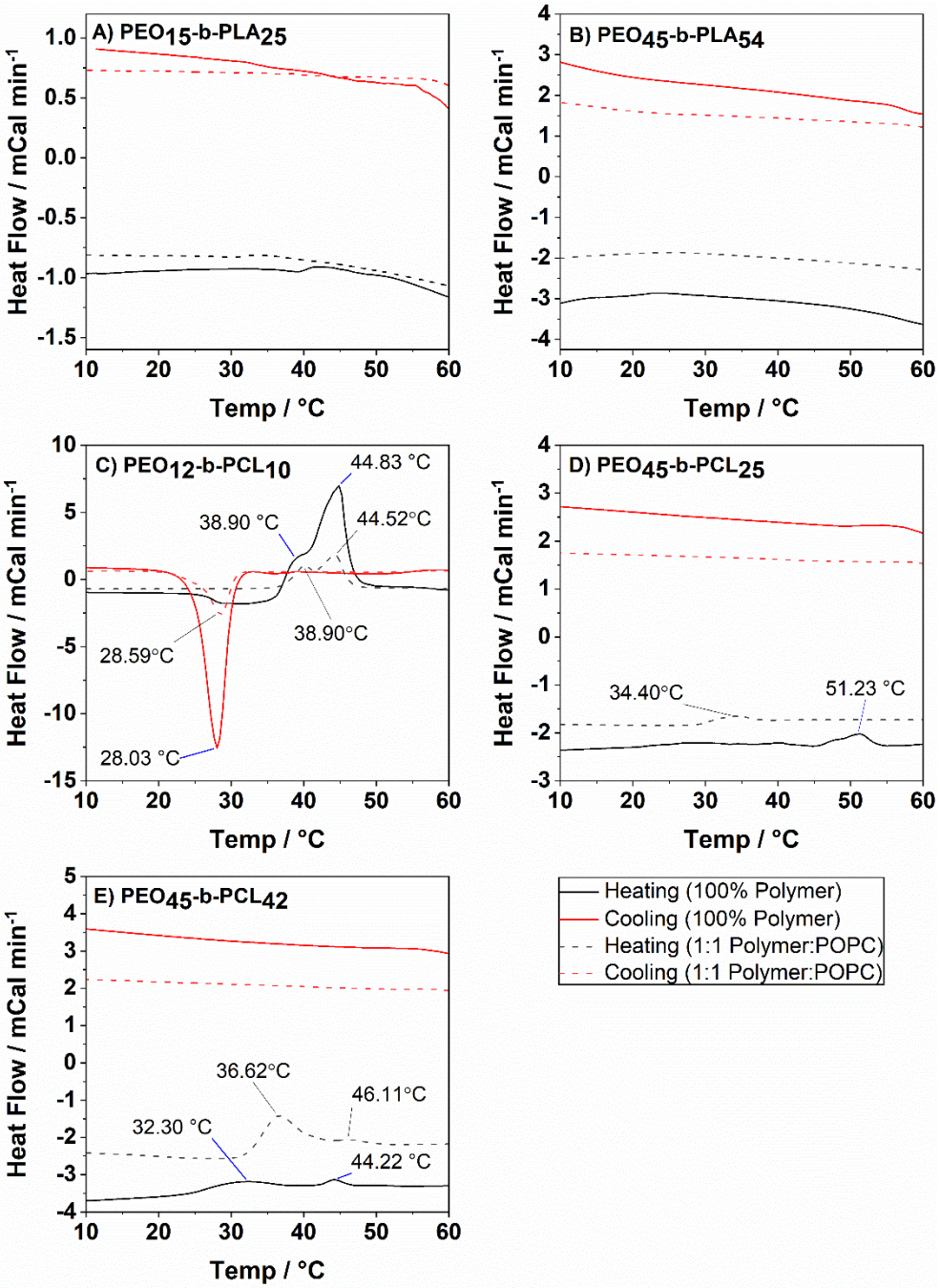


Figure 3: Differential scanning calorimetry (DSC) of; A) PEG_{15}-*b*-PLA_{25}; B) PEG_{45}-*b*-PLA_{54}; C) PEG_{12}-*b*-PCL_{10}; D) PEG_{45}-*b*-PCL_{25}; E) PEG_{45}-*b*-PCL_{42} as 100% polymer suspensions and 50 mol% preparation with POPC in PBS. Plot of differential power (DP / mCal min^{-1}) against temperature (Temp / °C).

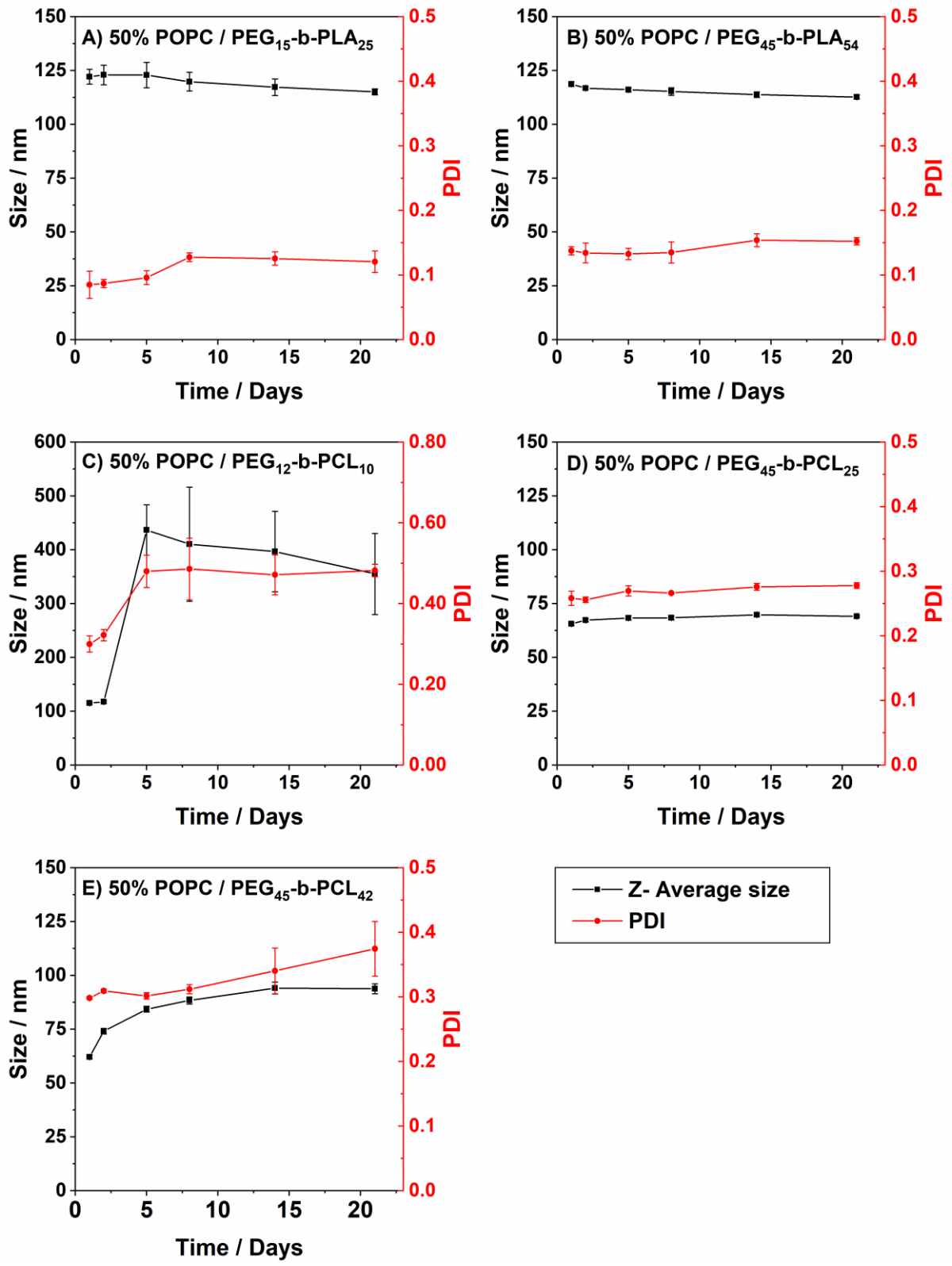


Figure 4: 1:1 Hybrid vesicle size stability at 4°C. Samples are monitored by DLS over a period of 3 weeks. The z-averaged size and PDI are reported. A) PEG_{15}-*b*-PLA_{25}; B) PEG_{45}-*b*-PLA_{54}; C) PEG_{12}-*b*-PCL_{10}; D) PEG_{45}-*b*-PCL_{25}; and E) PEG_{45}-*b*-PCL_{42}. Trend lines are shown to guide the eye. Error bars represent mean ± SD (n= 3).

Encapsulation stability and release rates of these formulations are also essential to their potential development for drug formulation and delivery. CF was encapsulated in hybrid vesicles at self-quenching concentrations and release was measured through an increase in fluorescence due to dequenching when liberated from vesicle encapsulation resulting from dilution within the extravesicular medium (Figure 5). Initially these experiments were conducted at 4 °C (cold storage) and body temperature (37 °C). POPC shows a more variable release profile at 4 °C in all of the triplicate repeats: we hypothesize that this is due to the storage temperature being close to the melting transition of POPC (-2°C) such that small temperature fluctuations may cause significant variation in membrane permeability during the course of the experiment. We find that, while hybrid vesicles containing 50% PEG_{45}-*b*-PCL_{25} and 50% PEG_{45}-*b*-PCL_{42} can initially encapsulate the hydrophilic dye, these vesicles are not stable and release 50% of the encapsulated dye in under 2 days while stored at 4°C and 37°C. The 50% PEG_{15}-b-PLA_{25}/POPC (Fig. 5) has a release profile very similar to that of POPC liposomes indicating that at this ratio POPC is dominating the release properties at this composition. The PEG_{45}-PLA_{54} polymer however has a stable release profile, as well as good colloidal stability, and thus remains a viable candidate for hybrid vesicle formulation with POPC.

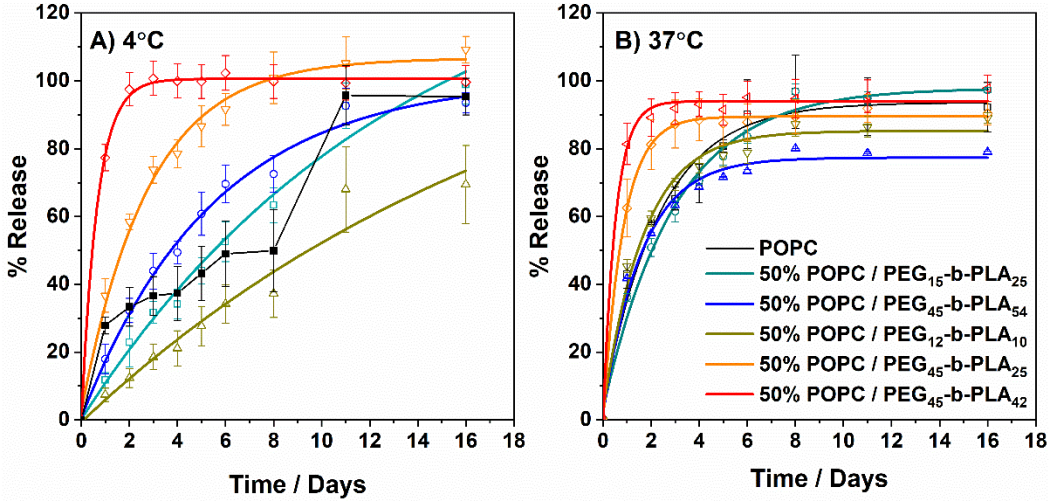


Figure 5: CF release in HEPES saline buffer. Shows an increase in % CF release over time, while samples were stored at A) 4°C, and B) 37°C. Trend lines are fits to a first order exponential release, with the exception of POPC at 4°C, which is a trend line to guide the eye. Error bars represent mean ± SD (n= 3).

Encapsulation of CF within PEG_{45}-*b*-PCL_{25}/POPC and PEG_{45}-*b*-PCL_{42}/POPC mixtures are particularly unstable with significantly faster release kinetics than the other formulations. These samples have a broad distribution of particle sizes and micellar sized structures dominate in their corresponding pure polymer systems. We hypothesize that coexistence of micelles and vesicles disrupts the stability of vesicle encapsulation. To test this, we investigate the release of CF from POPC liposomes in the presence of PEG-PCL micelles with a 1:1 lipid:polymer molar ratio at ambient temperature (Fig. 6). An increased rate of CF release from liposomes was observed in the presence of polymer micelles (PEG_{45}-*b*-PCL_{25}/POPC, $t_{1/2}$ = 1.7 days; PEG_{45}-*b*-PCL_{42}/POPC, $t_{1/2}$ = 1.1 days) compared to POPC liposomes alone ($t_{1/2}$ = 3.4

days), but not as fast as the respective 1:1 lipid:polymer blends ($t_{1/2}$ =0.68 days and $t_{1/2}$ = 0.40 days for 1:1 hybrid vesicles composed of PEG_{45}-*b*-PCL_{25}:POPC and PEG_{45}-*b*-PCL_{42}:POPC respectively). This demonstrates that PEG-PCL micelles perturb the integrity of vesicle membranes, but initially mixed lipid-polymer blends exhibit poorer encapsulation stability than this, likely in part due to these mixed membranes being intrinsically more porous than a pure lipid bilayer. For the purpose of creating nanomedicine formulations encapsulating water soluble compounds, it is clear that PEG_{45}-*b*-PCL_{25} and PEG_{45}-*b*-PCL_{42} block copolymers are not suitable for forming useful hybrid vesicles under these conditions.

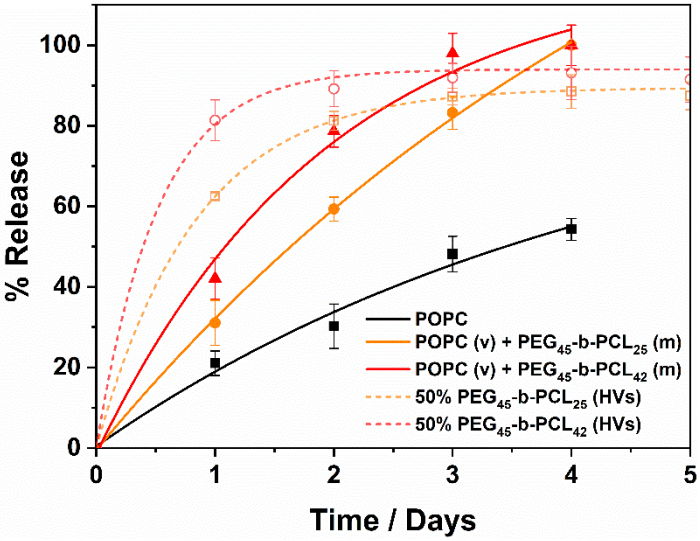


Figure 6: CF release from POPC vesicles with and without the presence of PEG-PCL micelles. These are compared to the release profiles of hybrid 1:1 POPC:PEG-PCL vesicles. Trend lines are fits to a first order exponential release. Error bars represent mean ± SD (n= 3).

Based on the results we have presented so far, we now focus on PEG_{45}-*b*-PLA_{54} as our preferred polymer for biodegradable hybrid vesicles. These hybrids form monomodal vesicle distributions that have good colloidal stability and passive encapsulation as well as release kinetics comparable with liposomes. However, it will be important for nanomedicine applications to be able to tune release rates and hence drug pharmacokinetics in complex biological environments such as serum. Towards this tuneability, we vary the lipid:polymer mixing ratio in POPC:PEG_{45}-*b*-PLA_{54} hybrid vesicles. We first examine the effect of hybrid vesicle composition on the passive encapsulation (Figure 7). This shows a trade-off between polymer content and CF encapsulation: increasing polymer content decreases the loading of CF into these vesicles. The DLS size distributions for these hybrid samples are presented in Figure S3.

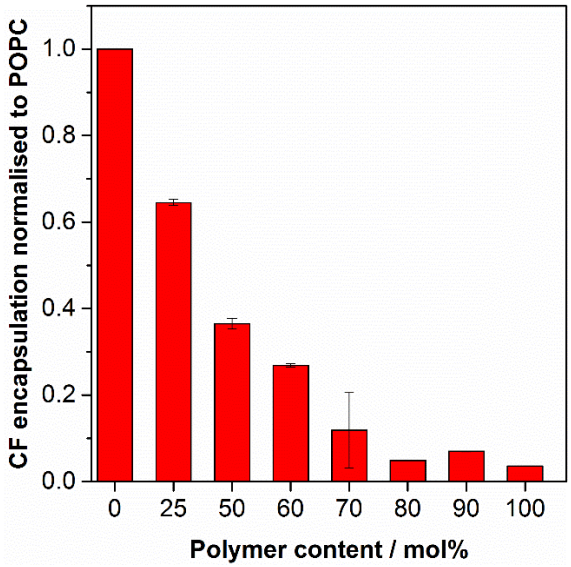


Figure 7: CF dye encapsulation in POPC/PEG$_{45}$-*b*-PLA$_{54}$ hybrid vesicles, with increasing polymer mol% content, relative to POPC. Error bars represent mean ± SD (n= 3).

Towards understanding the release profiles of these formulations in a biological context, we measure the release rates of POPC/PEG-PLA hybrid vesicle formulations in 10% serum at 37 °C (Figure 8). For these measurements the release of CF was continuously monitored for the first hour with constant stirring of the sample (while incubating at 37°C), then removed from the instrument and moved to a separate incubator (also set at 37°C) before further measurements were taken at intermittent intervals up to 36 h. From this data, two phases of release could be distinguished: an initial rapid "burst" release within 0.4 h followed by a slower secondary release rate.

Hybrid block co-polymer:POPC vesicles increase serum stability compared to POPC liposomes, which have very poor encapsulation stability in serum (~60% contents lost in the initial burst release). Increasing block copolymer content decreases the extent of the initial burst release, down to approximately 30% burst release for 70% PEG$_{45}$-*b*-PLA$_{54}$/POPC hybrid vesicles (76.9 wt% polymer).

Interestingly we found a significant enhancement in serum stability for polymer hybrid vesicles where the polymer component is a 5:1:4 mix of PEG$_{45}$-*b*-PLA$_{54}$:PEG$_{15}$-*b*-PLA$_{25}$:POPC (wt% = 30.5 wt% PEG$_{45}$-*b*-PLA$_{54}$, 64.6 wt% PEG$_{15}$-*b*-PLA$_{25}$ and 4.9 wt% POPC). The size distribution and passive CF encapsulation of this vesicle formulation is presented in Figure S4. We hypothesise that the shorter polymer acts to compensate the hydrophobic mismatch between the phospholipid and larger PEG$_{45}$-*b*-PLA$_{54}$ block copolymer, enhancing the stability and decreasing the permeability of the hybrid membranes. This points to a promising route to further optimisation of these biodegradable hybrid vesicles when considering bespoke formulation of specific active pharmaceutical ingredients designed for treatment of a particular clinical indication.

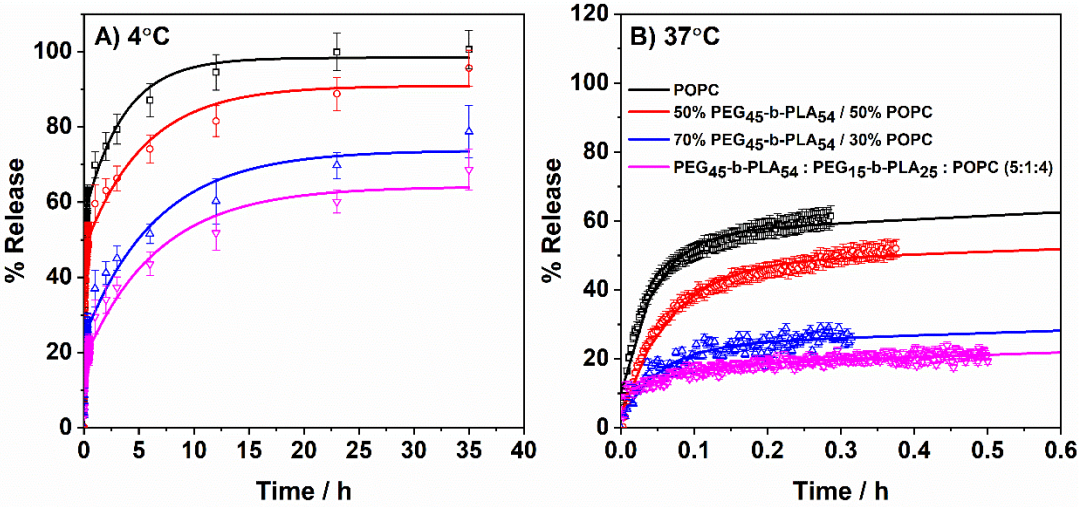


Figure 8: (A) % CF release over time from POPC/PEG-PLA Hybrid vesicles with varying mol% polymer at 37°C in 10% Foetal Bovine Serum. (B) Expanded view of the initial burst release kinetics for the data in part (A). Trend lines are fits to a double exponential release accounting for the initial rapid burst release and the long term extended release phase of these formulations. Error bars represent mean ± SD (n= 3).

CONCLUSIONS

Here we have presented biodegradable hybrid vesicles as a potential alternative to liposomal drug delivery systems. We investigated five different block copolymers with biodegradable hydrophobic blocks in 1:1 molar blends with the phospholipid POPC and compared their properties to the respective unitary polymersome and liposome systems. The PCL-PEG polymers formed micelles and as hybrids formed a polydisperse system hypothesised to be polymer-rich micelles and lipid-rich vesicles. These hybrid PCL-PEG:POPC systems could encapsulate a hydrophilic dye but PCL-PEG:POPC hybrids had poor encapsulation stability and/or colloidal stability, making them unsuitable for further consideration as candidate materials for nanomedicine. Recent literature suggests that larger PEG-PCL block copolymers with a smaller ethylene oxide volume fraction ($0.14<f_{EO}<0.21$) should be investigated if a polycaprolactone hydrophobic block is specifically desired.[44] However, in our investigations, PEG-PLA block copolymers were much more promising for development as drug delivery formulations.

The PEG-PLA block copolymers that we have investigated both formed monomodal vesicle distributions as pure polymersomes and 1:1 hybrid vesicles with POPC. While these polymersomes have negligible to poor passive loading of CF, their hybrid vesicles with phospholipid significantly improved small molecule encapsulation to make them more comparable to liposomes, which are known to have good passive loading properties. PEG-PLA hybrids also show good colloidal stability over 3 weeks and similar release kinetics to liposomes in HEPES buffered saline at 4 °C and 37 °C. Where the PEG-PLA hybrids outperform liposomes is in their release kinetics in the presence of serum: increasing block copolymer content significantly reduces initial burst release of encapsulated CF and slows down the long-term release rate. Interestingly, we find that blending 10 mol% of PEG_{15}-*b*-PLA_{25}, intermediate in molecular weight between phospholipids and the larger PEG_{45}-*b*-PLA_{54}

block copolymer in a 1:1 lipid:polymer hybrid stabilises the vesicle by significantly reduces the rate of CF release compared to a 1:1 hybrid where all the polymer is made up of the larger PEG_{45}-*b*-PLA_{54}.

Hybrid vesicles have previously been shown to synergistically combine advantages of unitary lipid and polymer vesicle systems. In the case of biodegradable hybrids presented in this study, these systems combined the higher passive encapsulation of liposomes with the enhanced serum stability of polymersomes. The wide tuneability of vesicle properties obtainable through varying the block copolymer and its relative mixing ratio with the phospholipid will allow formulations to be tailored to the requirements of the desired application. During this early exploration of the self-assembly and material properties of biodegradable hybrid vesicles we have used a water soluble dye as a model drug for encapsulation and release studies. However these properties are strongly dependent on the physicochemical properties of the active compound being formulated. The synergistic advantages we have uncovered for biodegradable hybrid vesicles are encouraging for further investigation of the available parameter space to design clinically viable nanomedicines.

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GRAPHICAL ABSTRACT