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Detergent-aided polymersome preparation

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Abstract

Until now, most preparative methods used to form polymeric vesicles involve either organic cosolvents or sonication. In this communication, we demonstrate for the first time a detergent-aided method to produce polymersomes. Peptidic polymersomes were formed from the rod-rod block copolymer PBLG$_{36}$-E, where PBLG is hydrophobic poly(γ-benzyl L-glutamate) and E is a hydrophilic designed peptide. The block copolymer was first solubilized by detergent micelles in aqueous buffer, after which the concentration of detergent was reduced by dilution, transforming the particle morphology in solution from mixed micelles to polymersomes. The polymersome formation was monitored with dynamic light scattering and confirmed with transmission electron microscopy. Polymersomes with average diameters of approximately 300 nm were obtained as well as discs with average diameters of approximately 100 nm. This detergent-based method can be used to create polymersomes with a range of properties, as verified by its application to another biocompatible block copolymer, the flexible polybutadiene$_{46}$-b-poly(ethylene glycol)$_{30}$. The technique will be particularly useful when delicate biomacromolecules such as (membrane) proteins, peptides, or nucleic acids are to be encapsulated in the polymersomes because the detergents used are compatible with these compounds, and the possible denaturing effect of sonication or organic solvents on the biological activity of the molecule of interest is avoided.
Introduction

Polymersomes are structurally similar to viral capsids in many ways and are now being designed to perform in a similar way as viruses: to carry, protect, target, and release cargo. Biological cargo, such as proteins, peptides, or nucleic acids, is becoming increasingly common, intended for biomedical activity in the body. The advantage of polymersomes over the traditional nanocapsules, liposomes, is that their membranes are more stable, leading to an enhanced ability to carry and protect cargo. The targeting and release properties of polymersomes also have more potential to be tailored to the intended application than liposomes, owing to the wide range of block copolymers available.

There are currently two classes of polymersome preparation: solvent free and with organic solvents. In the first class, the block copolymer is hydrated to form polymersomes. Some block copolymers require no agitation during hydration, whereas others require stirring, vortexing, extrusion, electric current, or sonication. Other block copolymers are too hydrophobic to undergo controlled aggregation in aqueous solution and first need to be dissolved in an organic solvent, which is then mixed with/ exchanged for an aqueous solution.

In the growing number of cases in which biomacromolecules, whose functions depend on intra- and intermolecular structures, are to be incorporated into the polymersome membrane or aqueous interior, organic solvents or high energy input (that is, sonication) cannot always be used because they would degrade the activity of the cargo. Therefore, a dilemma remains: for polymersomes that are intended to incorporate sensitive biomacromolecules but are unable to form vesicles directly in water or cannot be sonicated, there is currently no suitable method available. However, a third method for vesicle formation has been used for nearly 40 years to create liposomes: the detergent removal technique. This has been the preferred preparation method for liposomes incorporating sensitive membrane proteins with preserved structure and function. The first step of this method is to solubilize the water insoluble phospholipid that is going to constitute the liposomes in a detergent (water-soluble surfactant). Low-molecular-weight detergents typically have a large hydrophilic section in comparison with the hydrophobic section and form micellar structures with highly curved interfaces. Amphiphiles such as phospholipids and certain block copolymers have a larger hydrophobic component in comparison with the polar section and form lamellar assemblies, such as vesicles. When bilayer-forming phospholipids are solubilized by a large excess of high curvature detergent molecules above the critical micelle concentration (cmc) of the detergent, mixed micelles are formed composed of the detergent and the phospholipid. The detergent in the micelles is in equilibrium with the detergent monomers in the aqueous phase, with the exchange rate in the microsecond range for medium chain detergent molecules. The exchange rate of the lipid between
aggregates is dramatically lower than that of the detergent, on the order of seconds to hours,[8-10] because of the poor solubility of the larger hydrophobic block in water. The second step is to alter conditions such that the morphology of the self-assembled particles is no longer directed by the molecular properties of the detergent but rather by the phospholipid. The mixed micelles are slowly diluted below the cmc of the detergent by adding aqueous solution, and as the micellar-monomer equilibrium is maintained, the amount of detergent in the micelles is reduced. As the proportion of bilayer-forming molecules in the mixed micelles increases, new, lower curvature structures evolve. With lipids, the departure from high curvature micelles passes through sheets, which near the cmc of the detergent close to eliminate exposure of the hydrophobic edge to water, culminating in vesicles.[11]

Although certain phospholipids and block copolymers share molecular characteristics such that they each assemble into vesicles, their interaction parameters between the hydrophilic and hydrophobic components and the aqueous solvent are very different, which affects the self-assembly process.[8] In this article, we adapt the detergent removal method to block copolymers for the first time to create polymersomes. The polymer specific adaptations are explained, making this technique readily applicable to the creation of biomacromolecule-containing polymersomes in the future.

Fig.: Molecular shape, amphiphilic nature, and relative size of (A) cholate, (B) DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), and (C) PBLG_{36-E} [12] are illustrated. The molecules are depicted approximately to scale, with the hydrophilic sections of the molecules aligned on the left side of the dashed line and the hydrophobic sections on the right.
Experimental section

**Block Copolymers.** PBLG\textsubscript{36}-E was synthesized as previously described.\cite{12} The amino acid sequence of the E block was G(EIAALEK)\textsubscript{3}-NH\textsubscript{2}. The average molecular weight of PBLG\textsubscript{36}-E was 10230 g.mol\textsuperscript{-1}, and the polydispersity index (PDI) was 1.1. Polybutadiene\textsubscript{46}-b-poly(ethylene glycol)\textsubscript{30} (PB46-b-PEG30, Polymer Source Inc.) had an average molecular weight of 3800 g.mol\textsuperscript{-1} and a PDI of 1.05. The polybutadiene was polymerized by 1,2-addition.

**Preparation of Mixed Micelles.** A uniform polymer film was created in a 100 mL round-bottomed flask using 0.01 μmol of block copolymer (100 μL of a 0.1 mM block copolymer stock solution in tetrahydrofuran was added to the round-bottomed flask, and the solvent was removed by rotary evaporation under reduced pressure). Detergent (40 μmol) was added to the round-bottomed flask (200 μL of 200 mM sodium cholate or octyl glucoside in phosphate buffered saline (PBS, 10 mM phosphate, 137 mM NaCl, 3.35 mM KCl, pH 7.4 buffer)). The flask was then gently agitated until the polymer film was solubilized and the suspension was homogeneous.

**Detergent Dilution.** The mixed micelle suspension was diluted by the addition of PBS over 1/2 h using a syringe pump (NE-300, just infusion, Prosense B.V.) with stirring at 700 rpm.

**Detergent Removal.** The majority of the detergent was removed by means of dialysis. Slide-A-Lyser dialysis cassettes (Therm Scientific) with a molecular weight cutoff of 3000 g.mol\textsuperscript{-1} were used. The cassettes were thoroughly rinsed with water and then PBS. Samples were dialyzed against PBS for at least 48 h with two changes of buffer.

**PBLG\textsubscript{36}-E and Cholate Quantification.** After dialysis (against water), the amount of PBLG\textsubscript{36}-E and cholate in the samples was quantified by nuclear magnetic resonance spectroscopy. \textsuperscript{1}H NMR spectra were recorded on a Bruker AV-400 spectrometer, in 7:3 (v/v) dimethylformamide-d\textsubscript{7}/trifluoroacetic acid to prevent block copolymer aggregation. A residual dimethylformamide proton resonance was used to calibrate the chemical shifts, and dioxane was used as an internal calibrant to quantify the amount of PBLG\textsubscript{36}-E and cholate. It was found that 80% of the original polymer material was present after dialysis, and no cholate could be detected (sensitivity ∼0.1 μmol).

**Characterization.** Experimental diffusion coefficients, D, were measured at 25 °C by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS equipped with
a Peltier-controlled thermostatic cell holder. The laser wavelength was 633 nm and the scattering angle was 173°. The Stokes-Einstein relationship \( D = \frac{k_b T}{3\pi \eta d_h} \) was used to estimate the hydrodynamic diameter, \( d_h \). Here \( k_b \) is the Boltzmann constant and \( \eta \) is the solvent viscosity.

Transmission electron microscopy (TEM) was conducted on a JEOL 1010 instrument with an accelerating voltage of 60 kV. Samples for TEM were prepared by placing 5 μL of solution on carbon-coated copper grids. After ~5 min, the droplet was removed from the edge of the grid. A drop of 2% phosphotungstic acid (PTA) or 2% osmium tetroxide (OsO4) stain was applied and removed after 2 min.

Results and Discussion

Whereas phospholipids are typically ~2 nm long and somewhat flexible, the amphiphilic block copolymer PBLG36-E used in this study has an average length of ~8 nm and is relatively rigid.[12] Both the poly(γ-benzyl L-glutamate) block, denoted PBLG36, and the peptide E (amino acid sequence G(EIAALEK)3) can adopt an R-helical conformation. In this conformation, the hydrophobic PBLG36 block has an average length of 4.5 nm, whereas the water-soluble peptide E block is ~3.5 nm long. Recently, we have shown that PBLG36-E forms bilayered vesicles in aqueous solution.[12] Because of the large hydrophobic block size, none of the common solvent-free polymersome preparation methods, that is, bulk/film hydration, sonication, and so on, which all require hydration of a macroscopic phase of the block copolymer, have been successful. In this communication, we use sodium cholate as the detergent to solubilize PBLG36-E. Sodium cholate is often used to incorporate proteins in liposomes.[7] It is a low-molecular weight, rigid, disk-like anionic detergent with a cmc of ~10 mM in 100 mM NaCl aqueous solution at 25 °C.[13] The relative sizes of cholate, a phospholipid typically used to prepare liposomes by the detergent removal method, and PBLG36-E are illustrated in Figure 1. From the size disparity between the block copolymer and both the phospholipid and detergent, it can readily be appreciated that the balance of self-assembling forces between the micelle- and vesicle-forming molecules is very different when using this method to prepare liposomes or polymersomes. The process of making polymersomes starts with forming mixed micelles of the detergent molecule and the block copolymer from a PBLG36-E film and an aqueous solution of cholate micelles. The aggregation number of cholate micelles is quite variable, with micelles containing between 2 and 30 molecules.[13-15] Cholate micelles (200 mM in PBS, 25 °C) were determined by DLS to have an average hydrodynamic diameter (dh) of 2 nm, in agreement with reported values,[16] and a size range of about 1-5 nm (Figure2A). Using the detergent dilution method to produce liposomes, lipid/cholate molar ratios on the order of 1:2 are typically employed.[17,18] In comparison with lipids, the block copolymer has a much larger surface area to be encapsulated;
therefore, a PBLG36-E/cholate molar ratio of 1:4000 (0.05 mM PBLG36:200 mM cholate) was chosen. Thin films of PBLG36-E were hydrated in aqueous cholate solutions for 24 h with occasional gentle agitation to solubilize the block copolymer. Because many cholate molecules are required to shield the large hydrophobic PBLG block from the aqueous solution, a departure from the morphology of pure cholate micelles is expected.[19,18] This was observed by TEM, with images of the mixed micelle stage containing a majority of pure cholate micelles and some larger particles between 5 and 20 nm in diameter (Figure 2B). With the low block copolymer/cholate ratio employed under the current preparation conditions, the size distribution of the mixed micelle population as determined by DLS does not vary significantly from that of pure cholate micelles (Figure 2A). The size distributions of mixed micelle solutions were stable for at least 4 days, as determined by DLS.

The second step in the formation of vesicles is the dilution of the mixed micelles such that detergent molecules are gradually removed from the micelles and the morphology of the structures shifts from being dominated by the selfassembling properties of the detergent to those of the block copolymer. This was achieved by diluting the mixed micelle solution from 200 mM cholate, well above the cmc, to 2 mM, which is well below the cmc. The solution was stirred rapidly during detergent dilution to prevent uncontrolled aggregation, similar to the preparation of liposomes using the detergent dilution method.[20] Moreover, well-defined size distributions were only observed when the aqueous solution was gradually added (over 30 min or longer).

After this dilution step, the particle sizes as observed with DLS had increased from 2 nm to larger structures with a bimodal distribution. The average d\textsubscript{h} of the
predominant structure was \( \sim 350 \) nm, and the average \( d_h \) of the second population was \( \sim 100 \) nm (Figure 3A). This size distribution is most likely due to the range of molecular lengths and self-assembling characteristics of the block copolymer (PBLG\( _{22,\leq 46} \), PDI = 1.1).

For detergent/phospholipid systems, the initial mixed micelles increase in dimension upon dilution and finally form liposomes around the cmc of the detergent.[11] This means that the intrinsic self-assembly of the lipids only fully emerges and liposomes assemble when the detergent concentration becomes too low to form micelles. The energetic determinants of vesicle formation are different for block copolymers and lipids; therefore, it may be expected that aspects of the micelle to vesicle transition also differ.

![Figure 3](image-url)

**Fig. 3:** (A) DLS intensity distributions of cholate/ PBLG\(_{36}\)-E mixed micelles (▲) and of polymersomes formed after diluting the mixed micelles to 2 mM detergent (●), (B) Evolution of micelle/polymersome diameters as a function of cholate concentration during dilution from 200 to 2 mM. Initial conditions: 200 mM cholate, 0.05 mM PBLG\(_{36}\)-E in PBS, 25 °C.

DLS was conducted during the detergent dilution step to gain insight into the route of vesicle formation. PBS was added incrementally to mixed micelles (200 mM cholate, 0.05 mM PBLG\(_{36}\)-E), and the size distribution was monitored after each dilution step. Upon the first PBS addition (170 mM cholate), a transition from micelles to large structures was observed (Figure 3B), which is in marked contrast with the temporal pathway of liposome formation. As more PBS was added, the amount of large structures gradually increased, and there was a simultaneous decrease in the amount of micelles. As the detergent concentration passed below the cmc of cholate (10 mM), micelles were no longer detected. It should be noted, however, that because DLS is intrinsically biased toward the detection of large particles, it is expected that micelles are present beyond the detection limit of DLS. The cholate concentration at which the large structures are first detected is approximately 15 times its cmc, implying that the determining factor in the micelle-to-vesicle transition for this polymer is not the dispersion of the micelles at the cmc of the detergent.
In fact, it was not necessary to dilute the samples below the cmc of the detergent because the 350 nm population was stable before all micelles (many of which would be pure cholate micelles) had dispersed, as seen in Figure 3B. To avoid unnecessary dilution of the suspensions, it was preferred to dilute the cholate from 200 to 20 mM, with the vesicle size distribution not significantly different from samples that had been diluted below the cmc of the detergent (Figure 4A). After dilution to 20 mM cholate, TEM revealed polymersomes with diameters matching the DLS distribution and with membrane thicknesses of ~15-20 nm, which is in close agreement with the calculated average thickness of 18 nm[12] (Figure 4B). In addition to polymersomes, another bilayered structure, discs, was observed. The diameter of the discs was ~100 nm, which is also consistent with the DLS results. If polymersomes are required as the exclusive morphology, parameters such as the dilution rate or the concentrations of the detergent and block copolymer should be adjusted, or an alternative block copolymer/detergent should be used.

These results show that the relative influence and function of the detergent on the vesicle self-assembly process is clearly different for phospholipids and this block copolymer. To verify that it is dilution, that is, removal of detergent from the mixed micelles, not only stirring that induces self-assembly of the block copolymer, a sample with 200 mM cholate and 0.05 mM PBLG$_{36}$E was stirred without dilution. A population of particles did emerge, although the detected size distribution varied haphazardly during stirring, with the average $d_h$ ranging between 300 and 1000 nm. Additionally, the rate of formation was reduced at least four-fold, with large particles still forming after 2 h. In contrast, when samples are diluted and stirred, the entire polymer population assembles into stable polymersomes within 0.5 h. This shows that with a PBLG$_{36}$E/cholate ratio of 1:4000, each polymer is effectively
isolated from one another, and removal of cholate molecules from the mixed micelles facilitates complete conversion to well-defined vesicles.

A possible explanation of the observed results is as follows. In the mixed micelle stage, the large hydrophobic PBLG block is shielded from the aqueous environment by a layer of disk-like cholate molecules. [15] Because of the high exchange rate of cholate between micelles and solution,[21,22] detergent depleted “sticky patches” temporarily appear, allowing the block copolymer to exert its native self-assembling propensities and leading to coalescence between detergent-coated block copolymers. Because of its large hydrophobic block, PBLG\textsubscript{36}-E exhibits very strong phase separation in comparison with phospholipids in aqueous solution, with similar block copolymers having exchange rates ranging from hours to being nondetectable.[23-25] Once a number of PBLG\textsubscript{36}-E molecules self-assembles, it is unlikely that the reverse process would occur. As a control experiment, PBLG\textsubscript{36}-E polymersomes were prepared, and cholate was added to a final concentration of 200 mM. The polymersome/micelle suspension was stirred for 30 min (the standard duration of dilution), and no significant changes in the polymersome population were observed with DLS. In essence, for this polymersome assembly process, the important aspect of the detergent is that it provides a means of solubilizing the block copolymer and dampening its strong aggregation tendency en route to polymersomes. The micelle, to monomer transition of the detergent does not induce polymersome formation. In more general terms, the initial detergent concentration should be high enough to solubilize completely the block copolymer, and to trigger the structural conversion the mixed micelles should be diluted until all of the block copolymer has assembled into vesicles, with the precise detergent concentration dependent on the block copolymer and detergent used.

The polymersome preparation method was also applied to the flexible, unstructured, neutral block copolymer polybutadiene\textsubscript{46}-b-poly(ethylene glycol)\textsubscript{30}. Although PB\textsubscript{46}-b-PEG\textsubscript{30} and PBLG\textsubscript{36}-E have very different physical properties, in comparison with lipids they both contain very large hydrophobic blocks that aggregate strongly in aqueous solution. Mixed micelles of PB\textsubscript{46}-b-PEG\textsubscript{30} and the detergent octyl glucoside were diluted, generating polymersomes above the cmc of the detergent (Figure S1 of the Supporting Information). These results reveal the generality of this detergent based method and indicate that it is applicable to a diverse range of block copolymers and detergents.

Because detergents may interact with other molecules in the environment to which the polymersomes are applied, in some instances, detergent removal may be desired. Therefore, dialysis was used to reduce the detergent concentration outside the PB\textsubscript{46}-b-PEG\textsubscript{30} and PBLG\textsubscript{36}-E polymersomes from 20 mM to \(\sim 0.1 \mu M\). The distributions of polymersome diameters did not change significantly during dialysis (e.g., Figure 5A), and the polymersomes were stable for at least 1 week at 4 °C.

Because detergents may interact with other molecules in the environment to which the polymersomes are applied, in some instances, detergent removal may be desired. Therefore, dialysis was used to reduce the detergent concentration outside
the PB₄₆-b-PEG₃₀ and PBLG₃₆-E polymersomes from 20 mM to ~0.1 μM. The distributions of polymersome diameters did not change significantly during dialysis (e.g., Figure 5A), and the polymersomes were stable for at least 1 week at 4 °C. After vesicles have formed in solution, the enclosed detergent will not diffuse out of the assembly as readily as from micellar or lamellar sheet morphologies. Therefore, the rate of detergent removal depends on how readily the detergent diffuses through the vesicle membrane and the rate of flip-flop of the amphiphile between the bilayers.[7] Polymersomes have relatively thick and rigid bilayers, and the rate of flip-flop is expected to be insignificant; therefore, the rate of detergent removal depends almost entirely on the diffusion of entrapped detergent through the block copolymer membrane, and it is expected to be more difficult to remove residual detergent from polymersomes than from liposomes. Following dialysis for 48 h, PBLG₃₆-E polymersomes were solubilized, and it was observed with NMR spectroscopy that <0.5% of the cholate remained after dialysis. From the NMR spectra, it was also seen that the recovery of PBLG₃₆-E after dialysis was nearly quantitative (80%).

**Fig. 5:** (A) DLS intensity distributions of cholate/PBLG₃₆-E mixed micelles (▲), polymersomes formed after diluting the mixed micelles to 20 mM detergent (○), and polymersomes after detergent removal by dialysis (●). (B) TEM image of OsO₄-stained sample after diluting the mixed micelles to 20 mM. Insert: after dialysis. Initial conditions: 200 mM cholate, 0.05 mM PBLG₃₆-E in PBS, 25 °C.

**Conclusions**

We have demonstrated that the detergent removal technique, which has been used to produce liposomes for four decades,[4,27] can also be used to produce polymersomes. The dilution of cholate/ PBLG₃₆-E or octyl glucoside/PB₄₆-b-PEG₃₀ mixed micelles leads to a controlled transition from micelles to polymersomes. Whereas in the case of liposome formation, the micelle-to-vesicle transition is controlled by the breakup of detergent micelles, these block copolymers dictate the self-assembled structures of the two-component systems more forcibly, and the
micelle-to-vesicle transition is determined by the self-assembly of predominantly detergent-covered hydrophobic polymer blocks well above the cmc of the detergent. The detergent is an agent to modulate the force of the phase separation such that well-ordered nanophase separation can occur in aqueous solution rather than the uncontrolled aggregation that occurs without a shielding layer. In this respect, the role of the detergent is reminiscent of the role of organic solvent in the commonly used solvent-replacement technique. [28-30] Because the utility of the detergent is restricted to its ability to solubilize the polymer, the method is termed “detergent-aided polymersome preparation”. This new pathway to produce polymersomes increases their possible applications because it does not require high energy input (e.g., sonication) or possibly damaging organic solvents, and thus it is compatible with labile biomacromolecules. Other than the benign nature of the detergent removal method, another advantage of this route has traditionally been that it is possible to control the liposome size and homogeneity. This can be achieved by varying the rate of detergent dilution [20,11] using different classes of detergent [31,32,18] or vesicle-forming lipid, [20,27] varying the initial detergent/lipid ratios [18] and concentrations, [11] and by changing the pH [27] and ionic strength [33] of the aqueous solution. The effect of these parameters on the properties of the polymersomes and the structural evolution during the formation of vesicles will be the subject of future publications.
References
