Extending the self-assembly of coiled-coil hybrids

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DETERGENT AIDED POLYMERSOME PREPARATION

Until now, most preparative methods used to form polymeric vesicles involve either organic cosolvents and/or sonication. In this Chapter a detergent aided method to produce polymersomes is demonstrate for the first time. 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 cholate 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 ~ 300 nm were observed, as well as discs with average diameters of ~ 100 nm. This technique will be particularly useful when delicate biomacromolecules such as (membrane) proteins, peptides, or nucleic acids are to be encapsulated in the polymersomes, as the detergent used is 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 to viruses: to carry, protect, target, and release cargo.[1] This cargo is increasingly biological substances such as proteins, peptides or nucleic acids, intended for biomedical activity in the body.[2] 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 preparations: solvent free, and with organic solvents.[3] In the first class the block copolymer is hydrated to form polymersomes. Some block copolymers require no agitation during hydration, while others require stirring, vortexing, extrusion, electric current or sonication. Other block copolymers are too hydrophobic to undergo controlled aggregation in aqueous solution, and need to be first dissolved in an organic solvent which is then mixed with/exchanged for an aqueous solution.

In the growing number of cases in which biological molecules – macromolecules 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 cannot always be used as they would degrade the activity of the cargo. Thus, a dilemma remains: for polymersomes that are intended to incorporate sensitive biological substances, but are unable to form vesicles directly in water, or cannot be sonicated, there is currently no suitable method available.

However, a third class of vesicle formation has been used for nearly forty years to create liposomes: the detergent removal technique.[4] This has been the preferred preparation method for liposomes incorporating biological substances as it does not diminish the biological activity.[5]

The first step of this method is to solubilize the water insoluble phospholipid that is to constitute the liposomes in a detergent (water soluble surfactant). Low molecular weight detergents typically have a large hydrophilic section in comparison to 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 to 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, at a concentration higher than the critical micelle concentration (c.m.c.) of the detergent, mixed micelles result – micelles composed of the detergent, with the bilayer-forming lipid as an 'impurity'. 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.[5] The exchange rate of the lipid between aggregates is dramatically lower than that of the detergent, on the order of seconds to hours,[6-8] due to the poor solubility of the larger hydrophobic block in water. The second step is to alter conditions such that the morphology of self-assembled particles is no longer directed by the molecular properties of the detergent, but rather by those of the phospholipid. The mixed micelles are slowly diluted below the c.m.c. 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 c.m.c. of the detergent close to eliminate hydrophobic edge exposure to water, culminating in vesicles.[9]

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 Chapter the detergent removal method is adapted for the first time to block copolymers to create polymersomes. The polymer specific adaptations are explained, making this biomacromolecule-friendly technique readily applicable to create biomacromolecule-containing polymersomes in the future.

RESULTS AND DISCUSSION

While phospholipids are typically ~ 2 nm long and somewhat flexible, the amphiphilic block copolymer $PBLG_{36}$-E used in this study has an average length of ~ 8 nm and is relatively rigid. Both the poly(γ-benzyl L-glutamate) block, denoted $PBLG_{36}$, and the peptide E (amino acid sequence $G(EIAALEK)_3$) adopt an α-helical conformation. The hydrophobic $PBLG_{36}$ block has an average length of 4.5 nm, and peptide E is water soluble and ~ 3.5 nm long. In Chapter 4 it was shown that $PBLG_{36}$-E forms bilayered vesicles in aqueous solution. Due to the large hydrophobic block size none of the common solvent free polymersome preparation methods, i.e. bulk/film hydration, sonication etc., which all require hydration of a macroscopic phase of the block copolymer, have been successful. In this Chapter sodium cholate is used as the detergent to solubilize the block copolymer. Sodium cholate is a low molecular weight rigid disc-like anionic detergent with a c.m.c. of ~ 10 mM in 100 mM NaCl aqueous solution at 25 °C[10] that is often used to immobilize and/or encapsulate proteins in liposomes.[5] The relative sizes of cholate, a phospholipid typically used to prepare liposomes by the detergent removal method, and $PBLG_{36}$-E are illustrated in Figure 1. From the size disparity between the block copolymer and the phospholipid and detergent it can be readily 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.

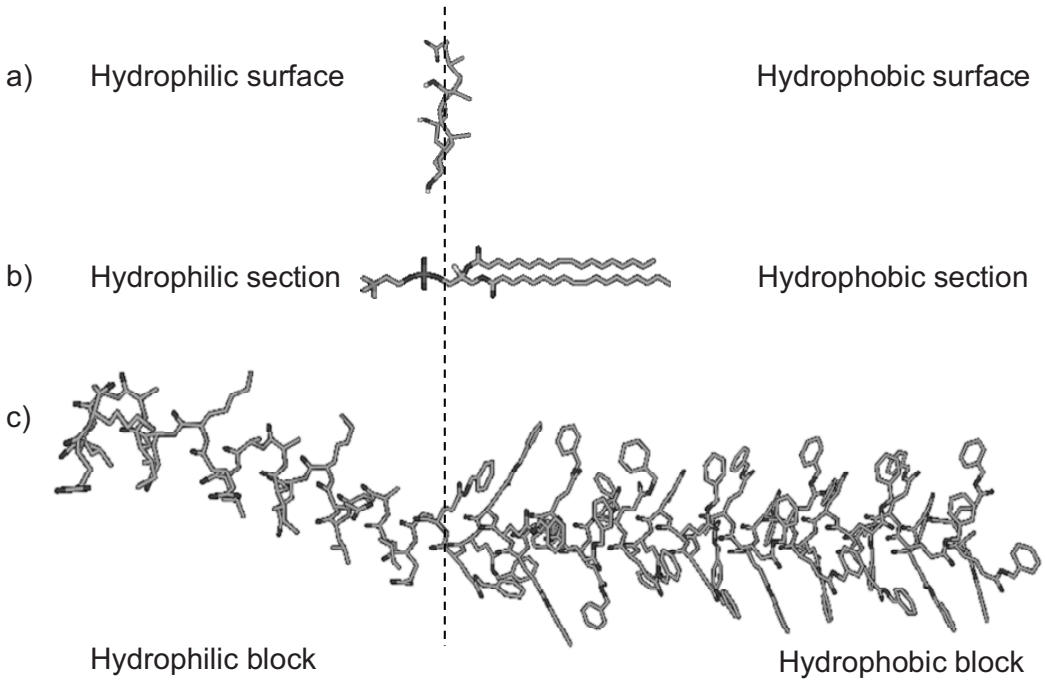

Figure 1. The molecular shape, amphiphilic nature, and relative size of cholate, DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), and $PBLG_{36}$-E are illustrated in a), b) and c) respectively. The molecules are depicted approximately to scale, with the hydrophilic sections of the molecules aligned on the left side of the dashed line and the hydrophobic sections on the right.

The process of making polymersomes starts with forming mixed micelles of the detergent molecule and the block copolymer from a $PBLG_{36}$-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.[10-12] Cholate micelles (200 mM in PBS, 25 °C) were determined by dynamic light scattering (DLS) to have an average hydrodynamic diameter of 2 nm, in agreement with reported values,[13] and a size range of about 1 – 5 nm (Figure 2a). Using the detergent dilution method to produce liposomes, lipid:cholate molar ratios on the order of 1:2 are typically employed.[14,15] In comparison to lipids, the block copolymer has a much larger surface area to be encapsulated and therefore a $PBLG_{36}$-E:cholate molar ratio of 1:4000 (0.05 mM $PBLG_{36}$:200 mM cholate) was chosen. Thin films of $PBLG_{36}$-E were hydrated in aqueous cholate solutions for 24 hours with occasional gentle agitation to solubilize the block copolymer. Since many cholate molecules are required to shield the large hydrophobic PBLG block from the aqueous solution, a departure from pure cholate micellar morphology is expected.[15,17] This was observed by transmission electron microscopy (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 in 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 2). The size distributions of mixed micelle solutions were stable for at least 4 days as determined by DLS.

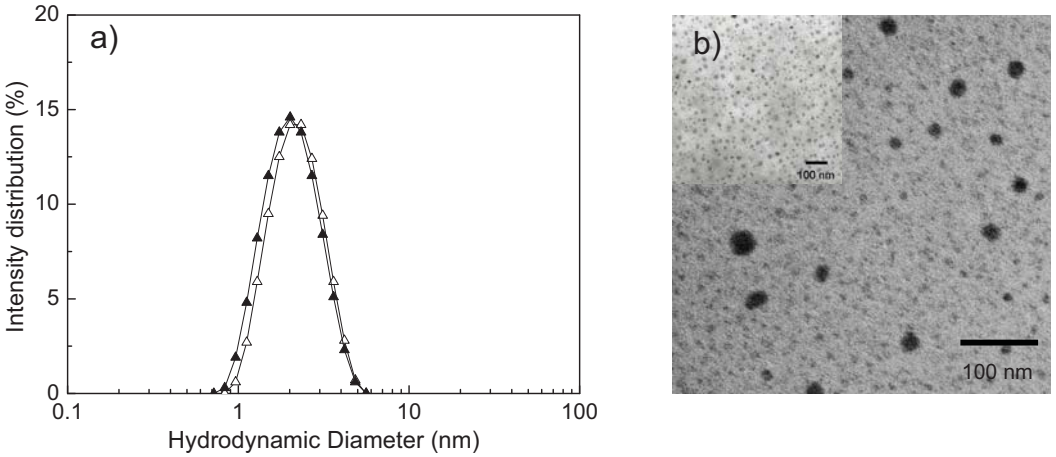

Figure 2. a) DLS intensity distributions of sodium cholate (Δ) and sodium cholate:$PBLG_{36}$-E 1:4000 (▲). b) PTA stained TEM image of a sodium cholate:$PBLG_{36}$-E 1:4000 mixture showing micelles and mixed micelles. Inset: PTA stained TEM image of pure cholate micelles. Conditions: 200 mM cholate, 0.05 mM $PBLG_{36}$-E in PBS, 25 °C.

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 self-assembling 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 c.m.c., to 2 mM, below the c.m.c.. The solution was

stirred rapidly during detergent dilution in order to prevent uncontrolled aggregation, similar to the preparation of liposomes using the detergent dilution method.[18] Moreover, well-defined size distributions were observed with DLS only when the aqueous solution was added gradually (over 30 minutes or longer). After this dilution step the particle sizes had increased from 2 nm to larger structures with a bimodal distribution as observed with DLS. The average D_h of the predominant structure was ~ 350 nm, and the D_h of the second population averaged ~ 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 ($PBLG_{\geq 22, \leq 46}$, PDI = 1.1[19]).

For detergent/phospholipid systems the initial mixed micelles increase in dimension upon dilution and finally form liposomes around the c.m.c. of the detergent.[9] This means that the intrinsic self-assembly of the lipid only fully emerges, and liposomes assemble, when the detergent concentration becomes too low to form micelles. The energetic determinants of supramolecular vesicle formation are different for block copolymers as compared to lipids, and it is not clear if the spatial and temporal route from micelles to vesicles is the same. DLS was therefore conducted during detergent dilution to gain insight into the route to vesicle formation. PBS was added incrementally to mixed micelles (200 mM cholate) and the development of the size distributions was monitored. From the first PBS addition (170 mM cholate) the transition to the final polymersome size was already observed (Figure 3b), which is in marked contrast to the temporal pathway of liposome formation. As more PBS was added the number of vesicles gradually increased and there was a simultaneous decrease in the number of micelles. As the detergent concentration passed below the c.m.c. of cholate (10 mM), micelles were no longer detected.[20] The cholate concentration at which the first polymersomes are detected is approximately 15 x its c.m.c., implying that the determining factor in the micelle-to-vesicle transition for this polymer is not the dispersion of the micelles at the c.m.c. of the detergent.

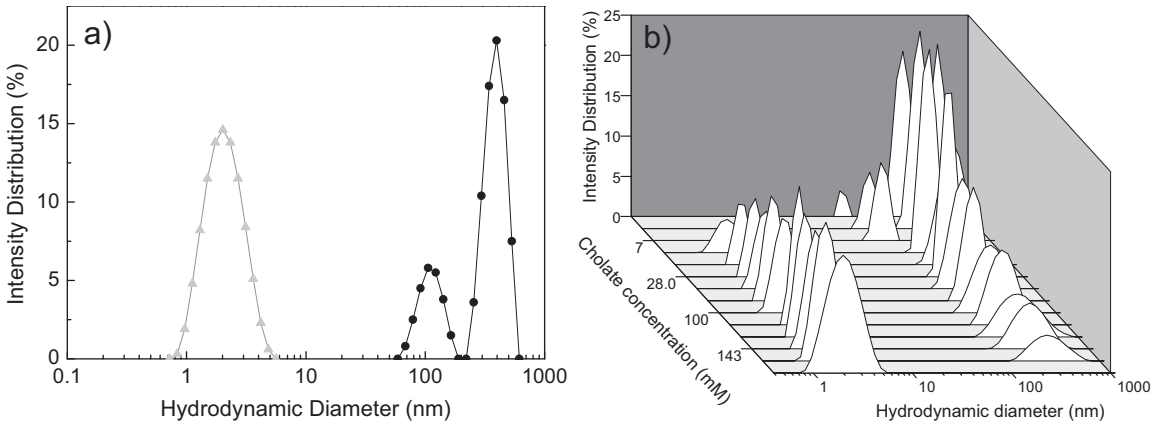

Figure 3. a) DLS intensity distributions during vesicle preparation: (▲) cholate:$PBLG_{36}$-E mixed micelles, (●) polymersomes formed after diluting the mixed micelles to 2 mM detergent. b) Evolution of micelle/polymersome diameters as a function of cholate concentration during dilution from 200 mM to 2 mM. Initial conditions: 200 mM cholate, 0.05 mM $PBLG_{36}$-E in PBS, 25 °C.

In fact, it was not necessary to dilute the samples below the c.m.c. of the micelles as the vesicle population was stable before all micelles (many of which would be pure cholate micelles) had dispersed, as seen in Figure 3b. In order to avoid unnecessary dilution of the polymersome 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 c.m.c. of the detergent (Figure 4a). After detergent dilution to 20 mM, TEM revealed polymersomes with diameters matching the DLS distribution, and membrane thicknesses of ~ 15 – 20 nm, which is in close agreement with the calculated average bilayer thickness of 18 nm (Figure 4b). In addition to polymersomes, another bilayered structure, discs, were observed. The diameter of the of discs was ~ 100 nm, which is also consistent with the DLS results.

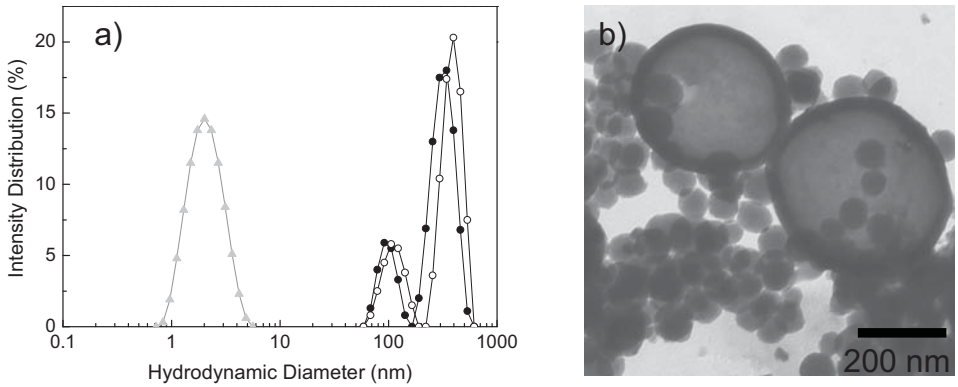

Figure 4. a) DLS intensity distributions during vesicle preparation: (▲) cholate:$PBLG_{36}$-E mixed micelles, (○) polymersomes formed after diluting the mixed micelles to 2 mM detergent, (●) polymersomes formed after diluting the mixed micelles to 20 mM detergent. b) OsO_4 stained TEM image of polymersomes and polymer discs after diluting the mixed micelles to 20 mM. Initial conditions: 200 mM cholate, 0.05 mM $PBLG_{36}$-E in PBS, 25 °C.

These results show that the relative influence and function of the detergent on the vesicle self-assembly process is clearly different for phospholipids and these block copolymers. In order to verify that it is dilution, i.e. removal of detergent from the mixed micelles, not only stirring that induces self-assembly of the block copolymer a preparation with 200 mM cholate was stirred without dilution. A population of larger particles does emerge (Figure 5), although the detected size distribution is quite variable and the rate of formation is reduced at least four-fold, with the large particles still forming after two hours, whereas with dilution and stirring all of the polymer has assembled into bilayered structures within half an hour. 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 structural conversion.

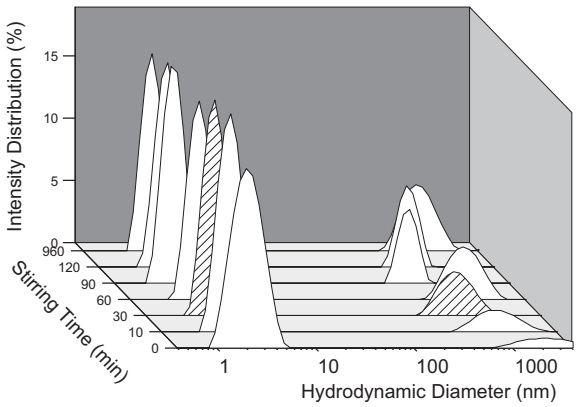

Figure 5. Evolution of micelle/polymersome diameters as a function of time, without dilution. The size distribution after the standard duration of stirring is thatched. Conditions: 200 mM cholate, 0.05 mM $PBLG_{36}$-E in PBS, 25 °C, stirring at 700 rpm. D_h intensity distributions determined by DLS.

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 disc-like cholate molecules.[12] Due to 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. Due to its large hydrophobic block, $PBLG_{36}$-E exhibits very strong phase separation in comparison to phospholipids in aqueous solution, with similar block copolymers having exchange rates between pure micelles ranging from hours to non-detectable.[23-25] Once a number of $PBLG_{36}$-E molecules self-assemble, it is unlikely that the reverse process would occur. As a control experiment, $PBLG_{36}$-E polymersomes were prepared and cholate was added to a final concentration of 200 mM. The polymersome/micelle suspension was stirred for 30 minutes (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 completely solubilize the block copolymer, and to accelerate the structural conversion the mixed micelles should be diluted until the transformation of the block copolymer into vesicles is complete, with the precise detergent concentration dependent on the block copolymer used.

Because detergents may interact with other molecules in the environment to which the polymersomes are applied, in some instances detergent removal may be desired after detergent dilution. Therefore dialysis was used to reduce the detergent concentration

outside the polymersomes from 20 mM to ~ 0.1 μM. The size distribution did not change significantly during dialysis (Figure 6a), and was stable for 9 days at 4 °C. After this time aggregation was apparent by DLS, with the limited stability presumably arising from residual cholate trapped within the polymersomes. 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. Thus, the rate of detergent removal depends on how readily the detergent diffuses through the vesicle membrane, and the rate of amphiphile flip-flop between the bilayers.[5] These polymersomes have relatively thick and rigid bilayers, and the rate of flip-flop is expected to be insignificant; hence the rate detergent removal depends almost entirely on the diffusion of entrapped cholate through the $PBLG_{36}$-E membrane, and it is expected to be more difficult to remove residual detergent from polymersomes than from liposomes.[26] Following dialysis for 48 hours the 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 $PBLG_{36}$-E after dialysis was nearly quantitative (80%).

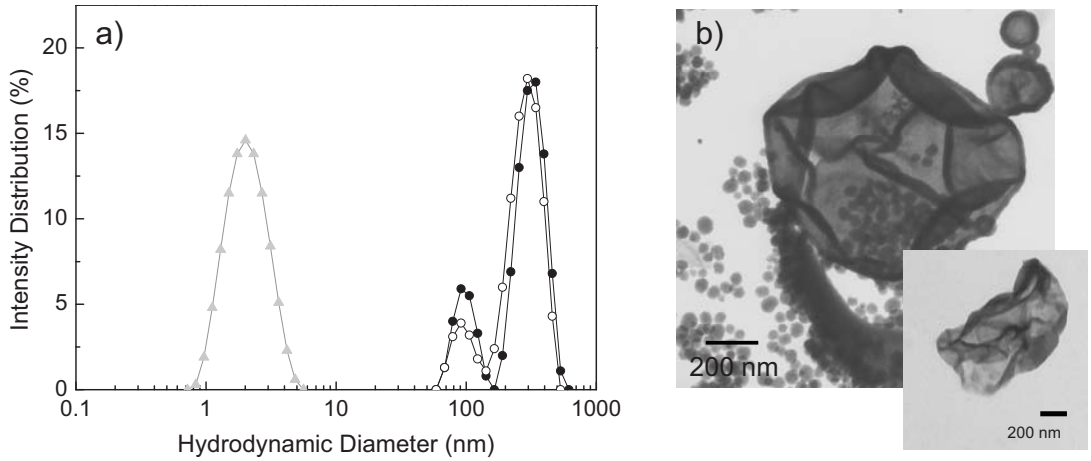

Figure 5. a) DLS intensity distributions during vesicle preparation: (▲) cholate:$PBLG_{36}$-E mixed micelles, (●) polymersomes formed after diluting the mixed micelles to 20 mM detergent, (○) polymersomes after detergent removal by dialysis. b) OsO_4 stained TEM images of polymersomes and micelles after diluting the mixed micelles to 20 mM, inset: polymersomes after detergent removal by dialysis. Initial conditions: 200 mM cholate, 0.05 mM $PBLG_{36}$-E in PBS, 25 °C.

CONCLUSIONS

It was demonstrated that the detergent removal technique, which has been used to produce liposomes for three decades[27], can also be used to produce polymersomes. The dilution of cholate/$PBLG_{36}$-E mixed micelles leads to a controlled transition from micelles to polymersomes with average diameters of ~ 350 nm, as well as polymers discs with average diameters of ~ 100 nm. While in the case of liposome formation the micelle-to-vesicle transition is controlled by the break-up of detergent micelles, this block copolymer dictates the self-assembled structures of the two-component system more forcibly, and the micelle-to-vesicle transition is determined by the self-assembly of predominantly detergent covered hydrophobic polymer blocks well above the c.m.c. 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 precipitation that occurs without a shielding layer. As 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 the possible applications of polymersomes as it does not require high energy input (for example sonication) or possibly damaging organic solvents, 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 is it possible to control the liposome size and homogeneity. This can be achieved by varying the rate of detergent dilution,[9,18] using different classes of detergent[15,28,29] or vesicle-forming lipid,[18,27] varying the initial detergent:lipid ratios[15] and concentrations,[9] and by changing the pH[27] and ionic strength[30] of the aqueous solution. The effect of these parameters on polymersome properties, the structural evolution during the formation of vesicles, and the applicability of this method to other block copolymers will be the subject of future investigation.

EXPERIMENTAL SECTION

Materials and Methods

$PBLG_{36}$-E was synthesized as described in Chapter 4. The amino acid sequence of the E block was $G(EIAALEK)_3$-NH_2. The average molecular weight of $PBLG_{36}$-E was 10230 g mol^{-1}, and the PDI was 1.1. Sodium cholate was obtained from Fluka, and tetrahydrofuran was from Biosolve. Phosphate buffered saline, PBS: 8 mM $Na_2HPO_4.2H_2O$, 20 mM KH_2PO_4, 30 mM KCl, 137 mM NaCl, pH 7.2.

Preparation of Polymersome Suspensions

Preparation of mixed micelles

0.01 μmol of $PBLG_{36}$-E was used to create a uniform polymer film in a 100 mL round-bottomed flask (100 μL of a 1 mg ml^{-1} $PBLG_{36}$-E stock solution in THF, with the THF was subsequently removed on a rotary evaporator under reduced pressure). 200 μL of a 200 mM sodium cholate solution in PBS was added to the round-bottomed flask, which was then gently agitated until the polymer film was solubilized and the suspension was homogenous.

Detergent Dilution

The mixed micelle suspension was diluted ten-fold by the addition of 1.8 mL of 15 mM PBS over ½ hour using a syringe pump (NE-300, just infusion, Prosense B.V.), while stirring at 700 rpm.

Removing Detergent

The majority of the detergent was removed by means of dialysis (MWCO 7000 g mol^{-1} Slide-A-Lyser dialysis cassette from Therm Scientific), thoroughly rinsed with water then PBS. Samples were dialyzed against PBS for at least 48 hours with two changes of buffer).

$PBLG_{36}$-E and cholate Quantification

After dialysis (against water) the amount of $PBLG_{36}$-E and cholate in the samples was quantified by NMR spectroscopy (^{1}H-NMR spectra were recorded on a Bruker AV-400 spectrometer, in 7:3 (v/v) DMF-d_7:TFA to prevent block copolymer aggregation. A residual DMF proton resonance was used for ppm calibration, and HMDS as an internal calibrant to quantify the amount of $PBLG_{36}$-E and cholate). It was found that 80% of the original polymer material was present after dialysis, and no cholate could be detected (sensitivity ~ 0.25 μmol).

Characterization of Polymersome Suspensions

Dynamic Light Scattering

Dynamic light scattering was conducted as detailed in the experimental section of Chapter 3.

Transmission Electron Microscopy

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 each solution on carbon-coated copper grids. After ~ 5 minutes the droplet was removed from the edge of the grid. 5 μL of 2% PTA stain or 2% OsO_4 stain was applied and the excess was removed after 2 minutes.

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