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Polymersomes with Asymmetric Membranes Based on Readily Accessible Di- and Triblock Copolymers Synthesized via SET-LRP

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Supporting Information

ABSTRACT: One of the hallmarks of nature is compartmentalization, and natural cell membranes are often asymmetric in terms of the inner and outer side. This communication describes work toward synthesizing such an asymmetric membrane from the bottom-up. A family of amphiphilic di- and triblock copolymers were synthesized via Cu(0)-mediated single electron transfer-living radical polymerization with the aim to generate polymer vesicles, or polymersomes, with an asymmetric membrane. Self-assembly of these polymeric amphiphiles in aqueous media was investigated using asymmetric field-flow fractionation and cryo-electron microscopy. Utilizing mixtures of diblock copolymers with differing hydrophilic moieties resulted in the formation of vesicles with an asymmetric segregation of charge between the inner and outer leaflet, confirmed by zeta potential measurements. These polymers, synthesized in good yields and using a biologically compatible method to induce self-assembly, have a promising range of applications from nanomedicine to synthetic cell research.

Polymeric vesicles, or polymersomes, are a self-assembled structure resulting from the dispersion of polymeric amphiphiles in an aqueous environment, and are characterized by their spherical morphology and the presence of a membrane that separates the inner compartment from the outer medium. For this reason, polymersomes are of great interest in the fields of nanomedicine and synthetic cells, as they show structural resemblances to cells and organelles. However, natural cell membranes are extraordinarily complex self-assembled structures, with phenomena such as lipid raft formation and heterogeneity across the inner and outer side of the membrane. While several strategies exist to increase complexity, such as incorporating proteins in the membrane or labeling the exterior of the vesicle, polymersomes are generally rather simple, with symmetry in geometry (spherical structures) and chemistry (identical inner and outer leaflets).

Asymmetry across a polymer membrane can be generated by using a mixture of diblock copolymers with different sized hydrophilic blocks. The longer chains segregate into the outer leaflet of polymersomes, and the smaller chains display in the inner leaflet. The same principle applies with triblock copolymers, albeit using a single chain instead of a mixture of polymers. In support of these experimental examples, a Monte Carlo simulation of a mixed population of $A_2B_3/B_3C_3$ diblock copolymers found that at a mixing ratio of 2:3 small-to-large polymers, close to complete segregation was achieved. This communication describes the synthesis of a family of amphiphilic di- and triblock copolymers via Cu(0)-mediated single electron transfer-living radical polymerization (SET-LRP), and their self-assembly into structures with asymmetric membranes via direct hydration. The use of a low molecular weight PEG in the self-assembly process infers compatibility with sensitive biomacromolecules. SET-LRP allowed the rapid and precise synthesis of these polymers on a gram scale with quantitative conversion of the monomer and excellent agreement between planned and characterized structure. Block copolymers were synthesized by the subsequent addition of monomers to a growing polymer chain from either a PEG-based or PMA-based macroinitiator, as depicted in Scheme 1. For PEG-based polymers, two lengths of commercially available monomethyl ether PEG were reacted with bromoisobutyric acid via a Steglich esterification to yield PEG macoinitiators 1 and 2, with block masses of 750 and 2000 g/mol, respectively. The bromoisobutyrate functionality on the $ω$-terminus of the PEG chain acts as the source of initiation in the SET-LRP reaction. Methyl acrylate (MA) was polymerized via SET-LRP using initiators 1 or 2 to yield diblocks 1 and 2. A standard procedure was followed, using activated copper wire to polymerize MA in the presence of Me₄TREN and CuBr₂. As SET-LRP can be taken to complete monomer depletion with negligible detrimental effects such as bimolecular termination or loss of end group fidelity, the monomer/initiator feed ratio was calculated to the desired block mass.

The polymerization was commenced by the introduction of the activated copper wire to the monomer solution under argon, and reacted at 25 °C for 5 h. PEG-$b$-PMA diblock copolymers were purified by first diluting the reaction mixture with THF and passing the crude solution through a basic alumina column to remove copper. The eluate was concentrated in vacuo and precipitated into cold water to remove any...
traces of the DMSO solvent. Polymers were purified further via dialysis and dried to gummy, clear solids.

Triblock copolymers 1 and 2 were prepared by taking the crude PEG-b-PMA polymerization product from the previous step and performing a block extension via SET-LRP (Figure 1). The required amount of t-butyl acrylate (tBA) was added, sparged for another 30 min with argon, and a piece of freshly activated copper wire introduced under argon to initiate polymerization. It is indeed possible to add degassed monomer directly to the polymerization mixture to initiate chain extension, but in our experience this often lead to sluggish chain extension. This is in agreement with the findings of Haddleton et al. who found that block extension using SET-LRP was limited to three extensions unless fresh copper was used.18 Copper was removed from the reaction mixture by passing over an alumina column, and DMSO was removed by precipitation into water. For t-butyl deprotection, the polymer was dissolved in dichloromethane and trifluoroacetic acid was added. The deprotection was run overnight at ambient temperature, and the polymer was purified by dialysis to yield triblocks 1 and 2 as clear glassy solids.

PMA-b-PAA diblock copolymers 3 and 4 were synthesized in an analogous manner to the PEG-b-PMA diblocks, except ethyl bromoisobutyrate was used instead of a PEG-based macroinitiator. First, a methyl acrylate homopolymer was synthesized, which was then used to initiate the polymerization of tBA. After removal of DMSO by precipitation, the polymer was dissolved in CH₂Cl₂ and TFA was added to deprotect the t-butyl groups, yielding diblocks 3 and 4 as glassy clear solids after dialysis.

The structure and composition of the pure block copolymers was confirmed by GPC and 'H NMR, as summarized in Table S1. There is excellent agreement between the planned structure and the synthesized, and the synthesis of these polymeric amphiphiles by SET-LRP allowed grams of material to be generated with ease, which is important when the subsequent polymersome formation experiments require optimization and many repeats.

Self-assembly of these polymers in aqueous solution was induced via the direct hydration method, where the desired polymer is dissolved in a low molecular weight PEG, and the aqueous solution is added in gradually increasing aliquots.19 If encapsulation of a certain molecule is desired, it is included in the first aqueous aliquot. This method has several advantages over other polymersome formation techniques such as solvent switch8,20,21 or film rehydration22,23 in that it does not use any organic solvent, and self-assembled structures can be formed within minutes, forgoing the need to slowly add water over several hours or remove organic solvent by dialysis or evaporation. In this work, polymers were first dissolved in a low molecular weight PEG (550 g mol⁻¹) at a concentration of 1% (w/w). To initiate self-assembly, 50 μL of phosphate-buffered saline (PBS) was slowly added to 50 μL of the polymer solution. This solution was diluted using increasing amounts of PBS to a final polymer concentration of 0.1% (w/w). For the generation of asymmetric membranes, the ratio of inner/outer polymer was 2:3. For example, to induce the self-assembly of vesicles with a predominantly PAA exterior, 20 μL of polymer 1 stock was mixed with 30 μL of polymer 3 stock before PBS addition.

Characterization of the self-assembled structures was achieved using a suite of complementary techniques. Vesicular structure was directly confirmed using cryo-transmission electron microscopy (cryo-TEM), an imaging technique that allows nanoscale structures to be visualized in their native
aqueous environment by virtue of the rapid vitrification of the sample. However, this technique involves time-consuming sample preparation steps and is low throughput. Asymmetric field-flow fractionation coupled to a multiangle light scattering detector (FFF-MALS) was employed to characterize these systems in a high throughput manner. In this technique, particles are pushed to the bottom of a separation channel by a cross-flow perpendicular to the detector flow. As this cross-flow weakens, particles with a smaller density and cross section will rise up and interact more with the detector flow and, thus, elute first. The method developed for this work can rapidly and effectively discriminate micelles from vesicles. DLS was also used as a supporting sizing method.

The PEG-containing diblocks 1 and 2 both form vesicles, as they elute at 26 and 14 min, respectively, in the FFF-MALS data (Figure 2). This is confirmed by cryo-TEM with the size. On the other hand, the PAA-containing diblock copolymers form predominantly micellar structures, shown by their early elution time on FFF-MALS and the presence of small, solid spheres on cryo-EM.

When the diblocks 1 and 3 are combined to form hybrid 1/3 vesicles, a small amount of micelles are observed in FFF-MALS, but the predominant morphology is vesicles The smaller diameter of these vesicles compared to the pure diblock 1 system is likely due to the tighter packing arrangement allowed by the mixing of polymers. Similarly, with the mixed system of diblocks 2 and 4, no micelles are present in FFF-MALS, indicating complete stabilization of the polymers into hybrid 2/4 polymersomes. The vesicular structure of these hybrid systems was confirmed by cryo-TEM, and the segregation of polymers was determined by zeta potential measurements, shown below in Figure 4.

As a control, vesicles were formed with diblocks 1 and 2 in a 2:3 ratio, so that polymersomes would form with PEG on both the inner and outer leaflet. It has been shown that hydroxide ions preferentially absorb on PEG substrates explaining the observation of large spherical structures containing a clear membrane (Figure 3). It is interesting to note that diblock 1, with its smaller PEG fraction, assembles into much larger structures. The smaller size results in a decreased interfacial energy, as less PEG is present to crowd the exterior of the membrane, resulting in a larger radius of curvature and vesicle size.
slightly negative zeta potential observed for our PEG control and why it decreases with increasing pH. The hybrid 1/3 polymersomes, engineered to have PAA on the outer leaflet of the vesicle, have significant negative charge character compared to the PEG control vesicles, indicating that the polymer chains are oriented as desired. In contrast, the hybrid 2/4 polymersomes, engineered to have PAA on the inner leaflet, have a much reduced negative charge character. The PEG control and the hybrid 2/4 system do not superimpose due to the fact that the PMA-b-PAA diblock copolymers have a molar mass dispersity greater than 1, and hence, there will be some polymer chains longer than the average, which will segregate to the outer leaflet with some PAA in the outer leaflet. This behavior is inevitable with current polymerization techniques, and even if a dispersity of 1 was achieved, the computational model investigated by Cui et al. suggests that some amphiphiles will arrange in nonperfect orientations.\(^\text{15}\) Our results agree with this observation, demonstrating experimentally that while the majority of the diblocks behave as expected, a small amount of them do not.

Triblock polymers 1 and 2 have the same PEG/PMA/PAA block ratios as the hybrid 1/3 and 2/4, respectively, yet they largely self-assemble into micelles instead of polymersomes under the range of formation conditions attempted, evident from the main peak in FFF-MALS and cryo-TEM (Figures S1 and S2). A possible explanation for this is that the hydrophobic block is not large enough to support the formation of vesicular structures. As vesicle formation is achieved through the fusion of micelles, it is possible that the triblocks are “stuck” in a micellar morphology based on their larger overall hydrophilic volume fraction.

This publication reports the efficient and high yielding synthesis of a range of amphiphilic block copolymers via SET-LRP. These simple diblock copolymers self-assemble within minutes in aqueous solution to form vesicles with heterogeneity across the membrane reminiscent of the natural cell membrane. The use of the direct hydration method to achieve this avoids the use of any organic solvent, making this technique suitable for use with delicate biomacromolecules such as transgenes.

**REFERENCES**