Polymersomes

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Salt, Shake, Fuse—Giant Hybrid Polymer/Lipid Vesicles through Mechanically Activated Fusion**

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Abstract: *Large (200 nm) poly(ethylene oxide)-b-poly(butadiene) polymer vesicles fuse into giant (> 1 μm) vesicles with mild agitation in dilute aqueous NaCl solutions. This unusual effect is attributed to the salt-induced contraction of the poly(ethylene oxide) corona, reducing steric resistance between vesicles and, with agitation, increasing the probability of contact between the hydrophobic cores of adjacent membranes. In addition, NaCl and agitation facilitated the creation of giant hybrid vesicles from much smaller homogeneous polymersomes and liposomes. Whereas lipid vesicles do not readily fuse with each other under the same circumstances, they did fuse with polymersomes to produce hybrid polymer/lipid vesicles.*

Biomimetic vesicles are integral to a wide range of technical applications, including nanoscale reactors[1] and drug delivery vehicles.[2] Synthetic, vesicle-based structures also have the potential to form engineered systems that duplicate and rival some functions of cellular organelles, such as energy transduction and dynamic, responsive sensors.[3] Engineering dynamic, reconfigurable vesicle systems requires control over the structural reorganization of bilayer membranes, including processes of membrane budding, fission, and fusion.

Fusion processes in lipid-based vesicle systems have been explored in great detail,[4] yet the fusion of synthetic polymer-based vesicle systems, or polymersomes, has been relatively unexplored. Polymersomes have distinct advantages over lipid-based systems for creating dynamic, reconfigurable soft systems: they generally exhibit greater chemical and mechanical stabilities, and a boundless range of modification chemistries.[5,11] A few studies have explored polymersome fusion using techniques such as adding poly(ethylene oxide) (PEO) homopolymer[6] or surfactants,[7] and sonicating vesicles composed of a class of branched-polymers,[8] yet there are no reports of which we are aware that examine the effects of simple salts on the aggregation (or fusion) behavior of polymersomes.

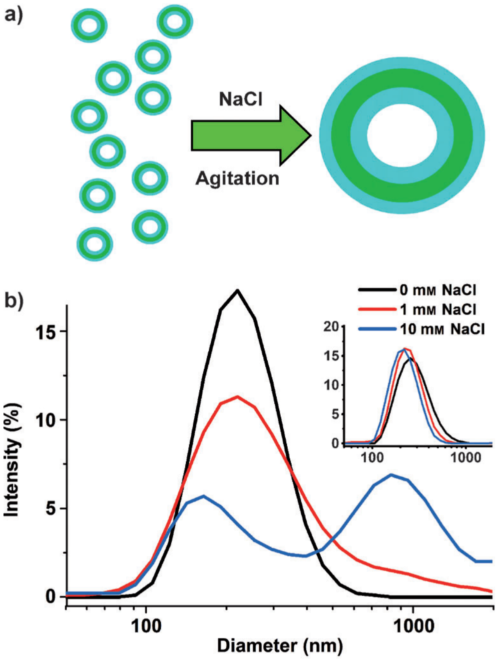

Figure 1. a) Visual representation of vesicle fusion of polymersomes composed of poly(ethylene oxide) (light blue)-*b*-poly(butadiene) (green). b) Intensity distributions from dynamic light scattering for 200 nm vesicles (PEO_{21}-PBd_{32}) in sucrose (100 mM) and in the presence of 0, 1, and 10 mM NaCl before (inset) and after (main plot) agitation on a sample vortexer at 20 Hz for 1 h.

The fusion of liposomes in the presence of divalent salts[4a] and their reversible aggregation in the presence of high concentrations (> 100 mM) of monovalent salts[9] are well documented. When added to suspensions of block copolymers containing PEO in polar solvents, monovalent salts have been shown to change the morphology of polymer amphiphile chains, as well as the morphology of the resulting aggregates.[10] The ability to modulate the sterically stabilizing PEO block in amphiphilic copolymers encouraged our notion that added salt could affect polymersome aggregation dynamics with the potential for facilitating vesicle fusion.

In this study, we investigated the ability of NaCl to facilitate the fusion of poly(ethylene oxide)-*b*-poly(butadiene) (PEO-PBd) vesicles during agitation. Additionally, we have created hybrid vesicles by fusion of lipid and polymer vesicles using salt and agitation and characterized these structures using dye co-localization and Förster resonance energy transfer (FRET).

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Figure 1 shows a visual representation of the fusion process along with dynamic light scattering (DLS) data for extruded polymersomes with the addition of NaCl (0, 1, and 10 mM) before and after agitation. Simple addition of NaCl (without agitation) did not induce a change in intensity distribution of the vesicles (Figure 1, inset). Likewise, agitation alone in the absence of NaCl effected no change. Under agitation conditions, the addition of NaCl resulted in a slight broadening of the intensity distribution at 1 mM of NaCl and a shift to a bimodal intensity distribution at 10 mM of NaCl. A smaller peak centered on 160 nm shows a small but notable decrease in size from the unagitated and unsalted samples, whereas a second peak centered on about 700 nm, and its shoulder extending into the several μm range, acknowledges the presence of much larger structures. Although DLS cannot distinguish between aggregated and fused structures, fluorescence microscopy (Figure 2) revealed that polymersome samples (containing polymer chains functionalized with rhodamine 110) agitated in the presence of 10 mM salt were comprised of a range of structures, including a large fraction of giant vesicles that arise from the fusion of much smaller extruded vesicles.

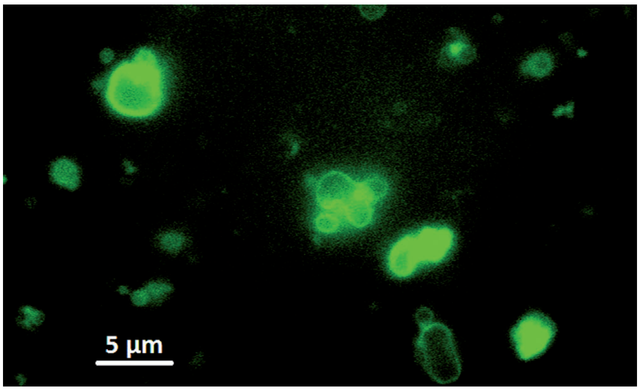

Figure 2. Fluorescence microscopy image of giant poly(ethylene oxide)-*b*-poly(butadiene) (PEO_{21}-PBd_{32}) polymersomes labeled with covalently attached rhodamine-110, prepared by adding NaCl (10 mM) to a suspension of 200 nm polymersomes in sucrose (100 mM) and agitating at 20 Hz for 1 h.

The fusion of polymer vesicles observed in our experiments was surprising. Polymer vesicles are thermodynamically very stable with critical aggregation concentrations and amphiphile exchange rates between aggregates that scale exponentially with the length of the hydrophobic block of individual polymer chains.[11] As a result, membrane reorganizations that facilitate contact between the hydrophobic layers of adjacent vesicles (events required for fusion) are expected to be highly disfavored for the polymersomes used in our experiments. Nevertheless, the fusion of polymer vesicles does occur with the addition of millimolar concentrations of monovalent salts under mild agitation.

The addition of salt introduces two significant changes to the system. First, it substantially reduces the Debye screening length, which stabilizes suspensions of vesicles, including neutral vesicles (e.g. containing PEO) that may develop charges at aqueous interfaces.[12] More importantly for neutral polymersomes, the addition of salt contracts the PEO corona presented at polymersome surfaces. Viscosity measurements have previously shown[13] that high salt concentrations (> 100 mM) cause contraction of PEO coils in aqueous solutions. At much lower concentrations (0.1–1 mM), monovalent salts demonstrably alter[10a] the morphology of PEO-polystyrene chains and their resulting aggregate structures in *N*,*N*-dimethylformamide, although these changes were not fusion related. We attribute the fusion observed in our experiments primarily to a similar effect, that is, the salt concentration causes the PEO corona of the vesicles to contract, introducing morphology changes in the sterically stabilizing hydrophilic block that permit the initiation of fusion. Whereas osmotic gradients of > 150 mosM are known to induce the fusion of lipid-based bilayers,[14] it is important to note that the NaCl gradient across the membrane in our experiments (ca. 20 mosM) was not a factor in their fusion. In fact, polymer vesicles with NaCl (10 mM) present both internally and in the external bulk solution (i.e., zero gradient) behaved similarly upon agitation to the vesicles with NaCl added only to the bulk solution (see the Supporting Information). Salt-induced PEO contraction alone is not enough to cause major morphological changes in suspended vesicles, as shown by our non-agitated control samples (Figure 1, inset). Nevertheless, PEO contraction could increase the probability (i.e. reduce the activation barrier) of contact between hydrophobic membrane cores of adjacent polymersomes and allow polymer chains to, with gentle agitation, intermingle and initiate fusion.

We investigated two additional parameters that may play a role in the fusion process: vesicle size and molecular weight of the polymeric amphiphiles. Polymer vesicles of various sizes were prepared by extrusion through polycarbonate membranes with 400, 200, 100, or 50 nm nominal pore sizes. All of the extruded suspensions subject to salt (10 mM) and agitation exhibited fusion (Figure 3), with no apparent differences in efficiency, regardless of the starting vesicle size. Although not strictly quantitative, the volume distribution estimates that roughly half of the volume of suspended objects is occupied by vesicles (and other aggregates) larger than 1 μm, a substantial increase over the size of the unagitated vesicles.

Whereas vesicle size had little effect on fusion efficiency, polymer molecular weight, perhaps unsurprisingly, played a significant role. We prepared extruded polymersomes (200 nm pores) composed of polymers with a range of molecular weights in NaCl (10 mM) and measured their intensity distributions before and after agitation under the same conditions (10 mM NaCl, 20 Hz agitation, for 1 h; Figure 4). Increasing either the PEO (from 23–34 repeat units) or PBd blocks (from 37–46 repeat units) noticeably decreased the efficiency of fusion, whereas PEO_{89}-PBd_{111} exhibited no observable fusion at all. These results show that both the PEO and PBd block lengths have a strong effect on the efficiency of mechanically activated fusion.

In addition to investigating the effect of initial vesicle size and polymer molecular weight, we also probed whether or not the internal contents of the vesicles are preserved during

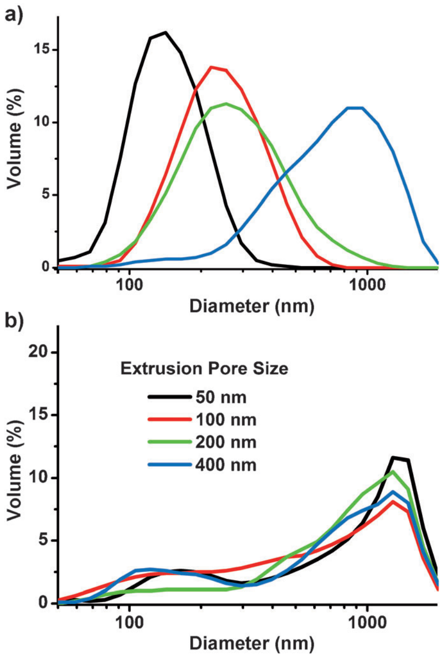

Figure 3. Volume distributions from dynamic light scattering of PEO_{21}-PBd_{32} vesicles extruded through membranes with pores of various sizes in sucrose/NaCl (100 mM/10 mM) before (a) and after (b) agitation on a sample vortexer at 20 Hz for 1 h.

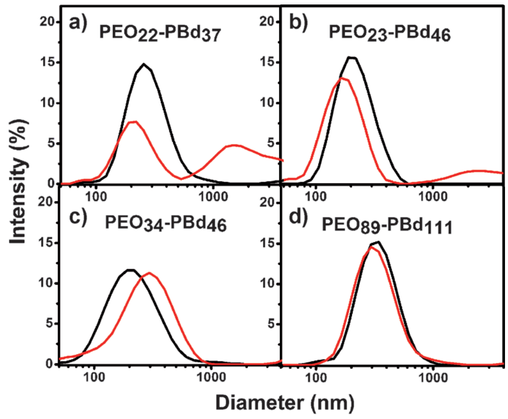

Figure 4. Intensity distributions from dynamic light scattering before (black) and after (red) mechanically activated fusion at 20 Hz for 1 h of extruded vesicles composed of different molecular weight PEO-PBd polymers in sucrose/NaCl (100 mM/10 mM), demonstrating the effect of increasing the PBd block (a vs. b), increasing the PEO block (b vs. a), or both (d).

fusion using a fluorescent probe, 8-hydroxy-pyrene-1,3,6-trisulfonate (HPTS) and a suitable quencher *p*-xylene-bis(*N*-pyridinium bromide) (DPX). Polymer films were rehydrated in HPTS/sucrose (1 mM/100 mM), extruded (200 nm pore size), and dialyzed for several days against an isotonic sucrose solution. The remaining unencapsulated HPTS was quenched with DPX (10 mM), which also served as the salt. The comparison of intensity distributions before and after agitation (20 Hz, 1 h), and optical microscopy confirmed fusion. Fluorescence intensities before and after agitation, however, showed a large decrease in fluorescence, which indicated intermixing of the bulk media containing the quencher and the internal solution containing the fluorophore (see the Supporting Information).

We have shown that agitating polymersome suspensions in the presence of salt causes vesicle fusion. Liposomes, however, do not fuse under the same conditions (see the Supporting Information). High concentrations (> 150 mM) of monovalent salts are known to cause liposome aggregation,[9,15] but we could find no instance in which neutral vesicles, lipid- or polymer-based, were fused by agitation or with the addition of monovalent salts. In order to test the combined effects of salt and agitation on liposome suspensions, we rehydrated and extruded 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) vesicles in sucrose (100 mM); NaCl (10 mM final concentration) was added after extrusion. Agitating under conditions identical to polymersome suspensions yielded no evidence of liposome fusion, only a slight broadening of the size distribution by DLS (see the Supporting Information). Interestingly, when equal amounts of extruded liposomes and polymer vesicles (0.5 mg of each species in water (1 mL) were combined in 100 mM each of sucrose and NaCl and agitated, larger structures were observed.

To determine if the lipid and polymer vesicles were actually fusing together, we labeled each component and used a dye co-localization assay. The PEO-PBd copolymer was functionalized with rhodamine 110 and added to the solution used to prepare the films from which the polymers were rehydrated to give 1.3 % functionalized polymer. Texas Red DHPE was added to the films from which the lipid vesicles were rehydrated, giving liposomes in which 1.5 percent of the lipids were dye-labeled. Both sets of extruded vesicles were mixed in NaCl (10 mM), agitated, and characterized by fluorescence microscopy. The resulting giant hybrid vesicles exhibited co-localization of the lipid and polymer dyes, giving a distinct yellow color when the normalized red and green channels are overlaid (Figure 5) which confirmed the fusion of liposomes with polymersomes. In addition to giant hybrid vesicles, small unfused liposomes (red) and polymersomes (green) were also observed in the fluorescence microscopy images (see the Supporting Information). Although polymer/lipid hybrid vesicles have previously been prepared by rehydration of polymer/lipid films,[16] the mechanical assembly described here is unique in that disimilar vesicles may be prepared independently and, subsequently, their interactions with each other may be “activated” with appropriate mechanical stimuli.

Despite the definite occurrence of combinatory fusion between lipid and polymer vesicles, the light scattering data shows fewer large vesicles than occurs with polymersome fusion (see the Supporting Information). Given that the lipid vesicles do not fuse with each other, it is not surprising that their incorporation into the polymer fusion process is less efficient than the fusion of polymersomes alone. It would also account for the multitude of unfused pure-lipid vesicles present in the microscopy images, which outnumber unfused polymer vesicles by a large margin.

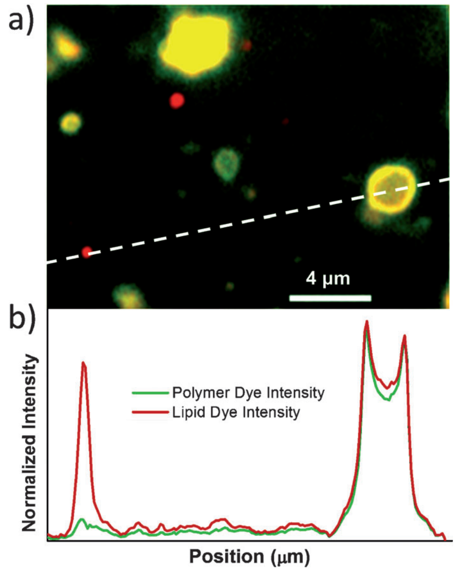

Figure 5. Hybrid polymer/lipid vesicles characterized by dye co-localization. a) Overlay of normalized red and green channels of fluorescence micrographs of the hybrid vesicles (yellow) and unfused lipid vesicles (red). b) Intensity profile across the white dotted line section in the micrograph, indicating the co-localization of the Texas Red DHPE and rhodamine 110 PEO-PBd dyes.

In addition to co-localization studies, Förster Resonance Energy Transfer (FRET) was used to probe the homogeneity of the fused vesicles. The hybrid vesicles generated a FRET signal with an efficiency of 0.18 ± 0.04. This value is not significantly different from the FRET efficiency of vesicles that were rehydrated from a polymer-lipid film of the same ratio of starting materials as the fused hybrid vesicles (0.15 ± 0.03). These results suggest that the fused hybrid vesicles share the homogenous distribution of polymers and lipids found in vesicles rehydrated from a polymer/lipid mixture.[16a]

In conclusion, we have studied the fusion of extruded polymersomes of ($\mathrm{PEO_{21}}$-$\mathrm{PBd_{32}}$) made possible by a combination of mild agitation and a 10 mM solution of NaCl. We also created hybrid polymer/lipid vesicles using the fusogenic capabilities of the polymer vesicles through mechanical activation. Our approach to mechanically activating fusion, with necessary optimizations, would allow stimuli-triggered combination of chemical bearing vesicles, resulting in the formation of mechanically responsive nanoreactors. Nevertheless, vesicles that release their contents upon mechanical activation, as the polymersomes described here do, have recently been highlighted in a novel strategy for targeting the delivery of therapeutics to constricted arteries that cause substantially elevated shear stresses.[17] Additionally, the combination of lipid and polymer vesicles may have important implications for drug delivery vesicles that contain stabilizing PEO chains, as native lipid bilayers and high salt concentrations are present throughout the human body and high PEO loadings might encourage vesicle fusion under physiological conditions. We intend to more fully investigate the effects of polymer and solution properties, such as monomer composition, and different salts, on the mechanically activated fusion of polymersomes.

Experimental Section

Polymer vesicles were prepared by rehydrating polymer amphiphile (5 mg; $\mathrm{PEO_{21}}$-$\mathrm{PBd_{32}}$; from Polymer Source) with 1 mL of sucrose solution (100 mM, with 0.01 % $\mathrm{NaN_3}$ as a preservative; to minimize osmotic gradients), and the resulting suspension was extruded 11 times through the appropriate pore-size membranes (Whatman). Vesicle suspensions were diluted to 1 mg mL^{-1} polymer with an isotonic sucrose solution to which NaCl was added to give a solution that was 10 mM in NaCl. The slight osmotic gradient across the membrane (120 mosM outside versus 100 mosM inside) was not found to be an important factor in our experiments. Samples were agitated in polystyrene cuvettes (1 mL) at 20 Hz for 1 h. Vesicle suspensions were characterized by dynamic light scattering and fluorescence microscopy. Retention of dye inside the vesicles was characterized by fluorimetry.

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