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COMMUNICATION

Micropipette aspiration of double emulsion-templated polymersomes†

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We measured the materials properties of polymersomes templated by double emulsions. Using micropipette aspiration, we verified the unilamellarity of fluid membranes consisting of PEO_{30}-*b*-PBD_{46} diblock copolymers. In addition, we used micropipette aspiration to both track and verify solvent removal from double emulsion-templated polymersomes.

Polymersomes,[1] bilayer vesicles made from amphiphilic diblock copolymers, are useful for applications ranging from the construction of biomimetic systems[2] to the development of soft materials with novel mechanical and physical properties.[3] Microfluidic techniques are becoming a preferred method to generate polymer and lipid vesicles, as they offer increased monodispersity, high encapsulation efficiency, and the ability to generate asymmetric membranes, compared to the conventional method of film hydration.[4–7] In particular, microfluidic devices can be used to prepare monodisperse water-in-oil-in-water (W/O/W) double emulsions, which can be used as templates to generate polymersomes with controlled composition and size.[4,8–10] In this method, an amphiphilic diblock copolymer is dissolved in the middle phase of W/O/W double emulsions, and vesicles are subsequently formed upon removal of the solvent. Although it has been suggested that this approach results in the formation of bilayer membranes,[9,11] little is known about the structure of the double-emulsion templated vesicles. In addition, previous results indicate the possibility of residual solvent altering the properties of polymer vesicles formed from double emulsions.[8] Verifying both the lack of residual solvent in these polymersomes and the unilamellarity of their membranes is important in order to ascertain their biological compatibility and rheological properties.

Common methods to characterize membranes include membrane uptake of α-hemolysin[6] and osmotic stress tests.[8] While easily accessible, these techniques do not provide sufficiently sensitive information about membrane elasticity and structure. In addition, these methods cannot distinguish between unilamellar and multilamellar vesicles[12] and furthermore, are unable to detect residual solvent in the membrane. Micropipette aspiration, in contrast, is a sensitive and versatile method to study membrane properties and structure in cellular and synthetic membranes on the micron scale.[13–16] In fact, the first polymersomes were characterized by micropipette aspiration, in which the elastic modulus and bending rigidity of polymersome membranes were measured and used to determine the unilamellarity and fluidity of the bilayer vesicles.[1,17] In this study, we use micropipette aspiration to characterize the unilamellarity and complete solvent removal in double-emulsion templated polymersomes.

We prepared polymersomes from microfluidic-generated double emulsions that contain the amphiphilic diblock copolymer, PEO_{30}(1300)-*b*-PBD_{46}(2500). A sucrose solution, toluene and chloroform mixture, and phosphate buffered saline (PBS) made up the inner, middle, and outer phases, respectively, of the double emulsions. During formation of the double emulsion templates, stabilizers such as PVA, SDS, and dextran are typically used to prevent coalescence of double emulsions.[4,8,9,18,19] We were, however, able to generate polymersomes without the use of such stabilizers by incorporating salt (by way of PBS) in the continuous phase and by adopting a very slow evaporation rate. This slow evaporation rate was achieved by (i) encapsulating a dense inner phase solution that caused double emulsions to sink away from the air interface, (ii) keeping the double emulsions loosely covered after collection and (iii) adjusting the ratio of chloroform: toluene in the oil phase. These techniques improved stability of the double emulsions to allow for membrane assembly. A porphyrin-based dye[20] was mixed with polymer in the middle phase during double emulsion formation in order to visualize solvent removal and membrane formation over the course of two weeks (Fig. 1A). The oil layer of the emulsion gradually thins as the toluene and chloroform dissolve into the surrounding aqueous solution, eventually evaporating. All subsequent observation of polymersome formation and micropipette aspiration was conducted with polymersomes that did not contain any membrane dye. We observed that excess polymer was either expelled (Fig. S1) or invaginated to form an interior vesicle (Fig. S2). Applying oscillatory shear by placing double emulsion samples on a rotator aided in detachment of the vesicle from excess polymer. These polymersomes remained stable for more than one month at 25 °C.

To evaluate the materials properties of the vesicle membranes, we used micropipette aspiration (Fig. 2A). Briefly, this technique works by imposing a controlled deformation upon a vesicle by applying a large applied suction pressure that pressurizes the vesicle interior and creates a uniform tension on the membrane, determined through the law of Laplace. This applied membrane tension subsequently causes the membrane to stretch, monitored by the areal strain $\alpha = \Delta A/A_0$, where A_0 is the initial membrane area and ΔA is the change

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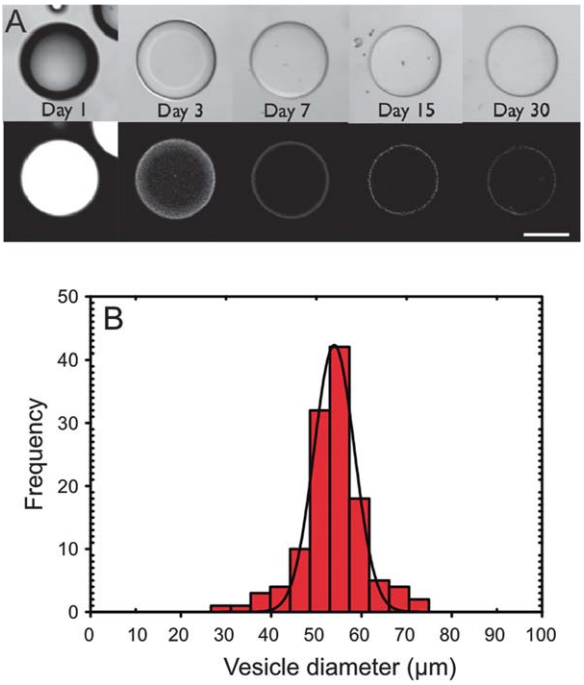

Fig. 1 Vesicle formation through solvent evaporation. (A) Evaporation of a toluene: chloroform mixture from the organic phase of double emulsions leads to assembly of PEO-*b*-PBD diblock copolymers into a vesicle. (Top) DIC images of representative vesicles at different time points (Bottom) Fluorescent images of the porphyrin dye in the organic phase of the emulsions shown in the top panel. (B) Distribution of vesicle size at 1 month (n = 122, mean =53.7 μm, CV = 13%). Scale bar is 25 μm.

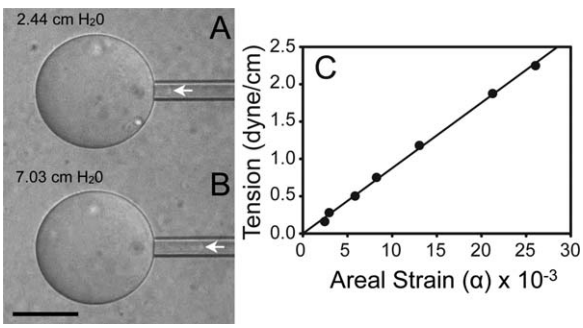

Fig. 2 Micropipette aspiration to measure vesicle elastic modulus. (A) Projection of the vesicle membrane upon aspiration into a pipette. (B) The extension of the membrane increases in displacement as aspiration pressure is increased. White arrows indicate the end of the extension. (C) Membrane tension *versus* area dilation for a polymersome measured during micropipette aspiration. The slope of this line determines the elastic modulus. $R^2 = 0.99$. Scale bar is 25 μm. (n=15 polymersomes for all K_a values reported in this study)

in area. Fluid bilayer vesicles demonstrate linear responses in areal strain to changes in membrane tension[21] and the slope of the membrane tension *versus* area change defines the area elastic modulus (K_a). The K_a of polymersomes has previously been shown to be independent of the molecular weight of the polymer that constitutes the membrane.[17] Vesicles with single or multiple bilayers can also be distinguished from one another since the elastic moduli of multi-bilayer membranes fall at approximately integer multiples of the elastic modulus of a single bilayer.[22,23] Previous measurements of the area elastic modulus for this polymer provide a reference value to assess the membrane elasticity of our annealed structures.

Bilayer membranes assembled from PEO(1300)-*b*-PBD(2500) block copolymers are expected to be fluid at room temperature. We first determined that polymersomes, generated through double emulsion templates and allowed to anneal over a one-month period, could be aspirated (Fig. 2A,B). The linear response in membrane areal expansion with applied tension confirmed the fluidity of the membrane (Fig. 2C).

Unilamellarity of the polymersome membrane, formed through the double emulsion template, was verified by determining the vesicle area elastic modulus. The K_a was found to be 92 ± 12 dyne cm^{-1} one month after vesicle formation from double emulsions. As shown in Fig. 3A, the mean value K_a of double-emulsion generated polymersomes agrees with the K_a (94 ± 12 dyne cm^{-1}) of unilamellar PEO_{30}-*b*-PBD_{46} polymersomes formed through thin film hydration[24] and with the K_a reported for various other molecular weight PEO-*b*-PBD polymersomes ($K_a = 102 \pm 10$ dyne cm^{-1}).[17] Taking advantage of the ability to precisely control the size of polymersomes, we find that the elastic moduli of different sized polymersomes prepared was independent of vesicle size (Fig. 3B). The consistent mechanical behaviour of double emulsion-templated polymersomes, as well as the similar

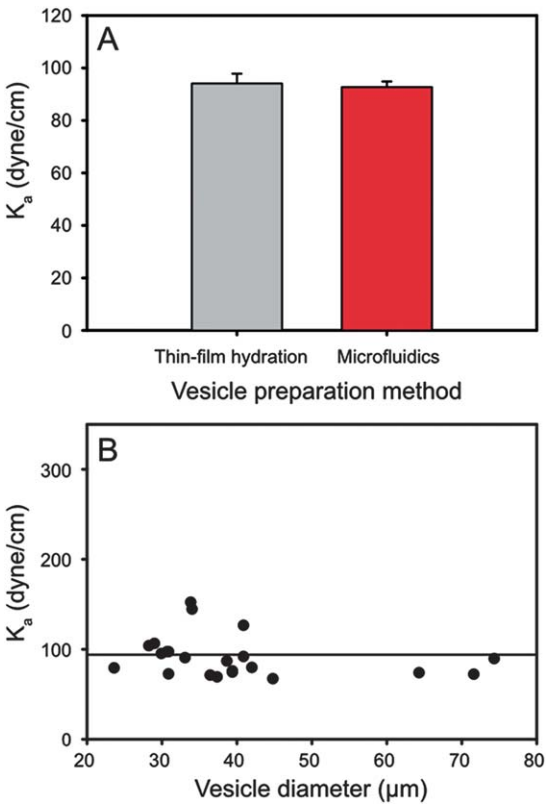

Fig. 3 Elastic modulus of double emulsion-templated polymersomes. (A) Elastic modulus of polymersomes made from double emulsion templates *vs.* thin-film hydration. Error bars represent standard error of the mean (SEM) ($p > 0.05$). (B) Relationship between the elastic modulus and the diameter of the polymersomes.

K_a to previously determined unilamellar polymersomes, indicates the membranes are unilamellar. This result, we believe, is the first verification of the unilamellarity of double-emulsion templated polymersomes.

The concern that organic solvent remains in polymersome membranes after production limits the use of these polymersomes for biological applications. The K_a, determined by micropipette aspiration, provides a straightforward approach to monitor solvent removal. Primarily related to the interfacial tension of the membrane (γ), the K_a reflects the chemical composition at the interface between the membrane's PEO chains and hydrophobic PBD core. The area expansion modulus and interfacial tension are directly related, with the relationship $K_a = 4\gamma$.[17] Thus, changes to the interfacial tension, which depends on the composition of the membrane, can be tracked by monitoring changes in the membrane K_a.

At three days post double emulsion production, vesicles display a low K_a (~44 dyne/cm), consistent with the presence of toluene and chloroform in the membrane (Fig. 4). Over time, the K_a increases as solvent continues to be removed from the middle phase until it finally plateaus at 95 dyne cm^{-1}. Remarkably, the complete removal of solvent is not reached until between one to two weeks after the generation of double emulsions. The final K_a matches the K_a of solvent-free vesicles prepared through thin-film hydration. Thus the progressive increase in K_a indirectly allows monitoring of the evaporation of organic solvent from the membrane.

The effect of the concentration of diblock copolymer in the middle phase of the emulsion on vesicle stability is another important parameter to study. For the solvent removal study, vesicles were prepared from a typical double emulsion size that contained an inner radius of ~36 μm and an outer radius of ~66 μm. From these dimensions, we can calculate the expected area per polymer chain in the double emulsion for a given wt% polymer in the middle phase. The expected interfacial area per polymer chain for PEO_{30}-*b*-PBD_{46} diblock copolymers in bilayer polymersomes is 1 nm^2/chain.[25] In the current study, this interfacial area per chain requires a middle phase polymer concentration of approximately 0.6 mg mL^{-1} to sufficiently stabilize the double emulsion initially. We find that when we increase this concentration to 2.4 mg mL^{-1}, so that there are additional polymer chains per surface area, the emulsion forms a cohesive membrane at a faster rate indicated by the increased K_a at each time point before the membrane stabilizes (Fig. 4, black curve). We hypothesize that the excess polymer in polymersomes made at 2.4 mg mL^{-1} is expelled or invaginated, since the resulting K_a is consistent with that of a single bilayer membrane.

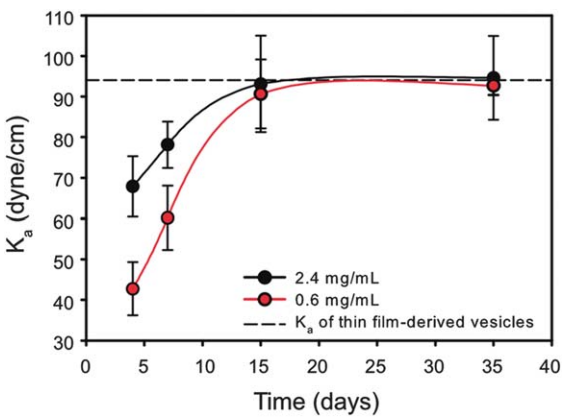

Fig. 4 Evolution of the areal expansion modulus of polymersomes made by double emulsion templating. The areal expansion modulus (K_a) of double emulsions increases overtime as solvent in the membrane evaporates and approaches the K_a of polymersomes prepared by thin-film rehydration. Increasing polymer concentration in the middle phase of double emulsions increases the rate at which solvent is removed.

In summary, we confirmed that double emulsion-derived polymersomes are unilamellar and exhibit fluid membrane behavior after an extended period of solvent removal. The time-scale for solvent removal would depend on polymer type, concentration and solvent type, and should be verified for each composition. Our results indicated that the complete removal of solvent from double emulsions takes a significant time (up to two weeks). This fact should be carefully considered in using double emulsion-templated polymersomes in biomedical applications, and future studies to accelerate the complete removal of solvent from double emulsion-templated polymersomes could greatly benefit numerous applications. The current study also demonstrates how micropipette techniques can be used to determine the final composition and structure of an extended array of giant polymersomes (hundreds to several microns in size) formed through organic solvent-based methods. With the growing popularity and progress of microfluidic methods for preparation of polymer particles, we expect there is a broad need for characterizing vesicles with this sensitive technique.

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