Generation of Polymerosomes from Double-Emulsions

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Diblock copolymers are known to spontaneously organize into polymer vesicles. Typically, this is achieved through the techniques of film rehydration or electroformation. We present a new method for generating polymer vesicles from double emulsions. We generate precision water-in-oil-in-water double emulsions from the breakup of concentric fluid streams; the hydrophobic fluid is a volatile mixture of organic solvent that contains dissolved diblock copolymers. We collect the double emulsions and slowly evaporate the organic solvent, which ultimately directs the self-assembly of the dissolved diblock copolymers into vesicular structures. Independent control over all three fluid streams enables precision assembly of polymer vesicles and provides for highly efficient encapsulation of active ingredients within the polymerosomes. We also use double emulsions with several internal drops to form new polymerosome structures.

The use of diblock copolymers to generate polymer vesicles is an attractive strategy to create new structures for encapsulation. These structures are called polymerosomes, and like more traditional vesicles formed from bilayers of phospholipids, they can encapsulate nano- to picoliter volumes of fluid. In addition, the low toxicity of certain diblock copolymers makes these structures promising for drug delivery applications. Depending on the length and chemical nature of the copolymers, the resultant polymerosome can be much more robust than liposomes of comparable sizes.[1,2] The flexibility afforded by the use of diblock copolymers significantly increases the control over the properties of polymerosomes; the character of each block of the diblocks can be tuned to fit the desired application. For example, the membrane thickness can be controlled by varying the degree of polymerization of the individual diblock molecules, whereas fluidity and permeability of the membrane can be adjusted by changing the glass transition temperature of the hydrophobic block.[3−5] Similarly, control over the nature of the individual polymer blocks can lead to alternative mechanisms to trigger release.[6]

Polymerosomes can be spontaneously formed by precipitating the block copolymers by adding a poor solvent for one of the diblocks.[7] Alternatively, they can be formed by rehydrating a dried film of the copolymers;[5,8] this is a standard technique to form lipid vesicles from dried phospholipid films. Rehydrating a lamellar structure of diblocks causes them to assemble into layers that pucker and fuse to form vesicles among other ordered structures such as micelles or wormlike micelles; however, the resultant polymerosomes are highly polydisperse.

Encapsulation and film rehydration can be combined into one step by rehydrating the dried films with an aqueous solution that contains the desired material to be encapsulated; as the polymersomes form, they trap some of the surrounding fluid within. Alternatively, the polymerosomes can be filled after they are formed by osmotically driving the desired materials inside. In either case, the encapsulation efficiency is low. Furthermore, both encapsulation techniques present difficulties when encapsulating hydrophobic materials or materials that cannot be driven through the membrane. Thus, efficient use of polymerosomes in different encapsulation technologies requires new fabrication methodologies.

In this paper, we describe a new method to create highly uniform polymerosomes using a one-step process where the inner and outer fluids are maintained as completely separate streams; this ensures highly efficient encapsulation. We use a microfluidic technique to generate uniform double emulsions consisting of water droplets surrounded by a layer of organic solvent;[9] these droplet-in-drop or core−shell structures are dispersed in a continuous water phase. The diblock copolymers are dissolved in the organic solvent; these self-assemble on the concentric interfaces of the double emulsions. Polymerosomes are then formed by completely evaporating the organic solvent from the

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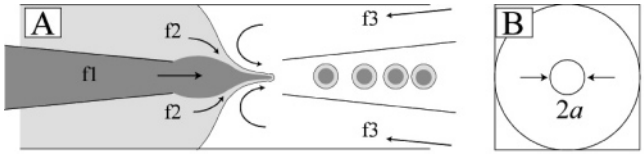

Figure 1. (A) Side and (B) front view of the device geometry. Two tapered glass capillary tubes are nested within a square capillary tube. Three different fluids denoted as f1, f2, and f3 are pumped into the device and forced to flow through the constriction with a radius a.

shell. This technique allows us to control the size of the polymerosome and to maintain complete separation of the internal fluids from the external fluid throughout the entire process, providing for highly efficient encapsulation.

To form the double emulsions, we use a microfluidic device, which consists of two round glass capillary tubes nested within an outer square tube; these round tubes are tapered at the ends as shown in Figure 1. The inner dimension of the outer square tube is equal to the outer dimension of the inner round tubes; this simplifies the alignment. The outer diameter of the round tubes is typically 1 mm, whereas the radii of the tapered orifices are typically between 20 and 100 μm. Three different fluids are simultaneously pumped into the system at controlled flow rates. The innermost fluid is pumped through the inner tube, whereas the middle fluid is pumped through the outer square capillary in the same direction (Figure 1). This produces a coaxial flow at the exit of the first tapered capillary tube. The outermost fluid is pumped from the opposite direction through the outer square capillary; it acts to hydrodynamically focus the middle and inner fluids through the second round capillary tube, as shown in Figure 1. The coaxial flow of the inner fluids is maintained through the second orifice where surface tension causes the fluid stream to break into drops; surprisingly, the coaxial geometry is maintained and double emulsions are formed with no leakage of the inner fluid to the outer fluid.

The position of drop formation depends on the flow rate of the outermost fluid. At low outer flow rates, double emulsion drops are formed within one orifice diameter of the entrance; by contrast at higher flow rates, the neck is stretched into a coaxial thread that breaks further downstream. We call the formation of drops close to the orifice dripping, whereas we call the formation of drops at the end of the long thread jetting. Drops formed through the dripping mechanism are highly monodisperse, whereas drops formed through the jetting mechanism have a larger polydispersity. Typically, drops formed through the dripping mechanism have very low polydispersities, less than 3%, as observed in Figure 2A. The number of small aqueous droplets contained within the larger oil drops depends on the relative frequency of drop formation for the innermost and middle fluids. If the innermost fluid breaks into droplets more rapidly than the middle fluid, we obtain large oil drops that contain many small aqueous droplets. However, if the rate of drop formation is the same, double emulsions with a single inner droplet are formed. This technique maintains complete separation of the internal and external fluids, making it extremely useful for generating capsule geometries such as polymerosomes. The overall frequency of drop production in the dripping regime can range from 100 to 7000 Hz, which enables formation of up to about 10^7 double emulsion drops per hour.

To generate polymerosomes, we use a new strategy that exploits the core−shell structures of the double emulsion. We generate water-in-oil-in-water double emulsions with a diblock copolymer dissolved in the intermediate hydrophobic fluid. The inner aqueous phase is distilled water (Millipore, MA), and outer phase is a mixture of 80% (v/v) glycerol in distilled water. We add glycerol to the outer fluid to increase its viscosity, which improves the efficiency of the flow focusing which is required to make the double emulsions. The intermediate phase is a volatile organic solvent; the evaporation of the organic solvent causes the amphiphilic block copolymers to self-assemble, forming the polymerosome. In our experiments, we use the diblock copolymers poly(normal-butyl acrylate)-poly(acrylic acid) (PBA-PAA).[10,11] The PBA is the hydrophobic block and has a molecular weight (MW) of 4K, whereas the PAA is the hydrophilic block and has a MW of 1.5K. It is critical that the dissolved diblocks are mostly unimers as opposed to larger aggregates because such aggregates do not efficiently stabilize the inner droplet against coalescence with the outer phase, and thus double emulsion break-up might occur. Tetrahydrofuran (THF) is a very good solvent for both the PBA and the PAA blocks and the diblocks dissolve as unimers in it; however, since THF is highly miscible with water, it cannot be used alone as the organic solvent because the drop formation requires an interfacial tension between the middle fluid and the two others fluids. Therefore, we use a cosolvent mixture of THF and toluene; this mixture is still a good solvent for the diblock while simultaneously having sufficiently large surface tension to enable drop formation. We use cosolvent mixtures of toluene and THF varying from 50−50 wt % to 80−20 wt %, and add between 0.1 and 5 wt % PBA−PAA. Despite the addition of the large amount of toluene, which decreases the polar character of the organic mixture, the diblocks retain their amphiphilic nature and stabilize the double emulsion as shown in Figure 2A which is a microscope image of the double emulsion formation in the capillaries, acquired with a high-speed video camera. In control experiments, we confirm that the stability imparted to the double emulsions by the diblocks is crucial; without them, the inner water droplet does not maintain its integrity and simply breaks through the intermediate hydrophobic phase as shown in Figure 2B.

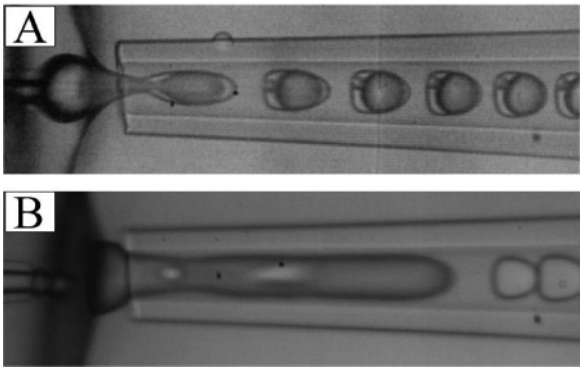

Figure 2. Influence of the diblock copolymers on double emulsion stability. In panel A, 0.8 wt % of the copolymer was added to the cosolvent solution f2. In panel B, no diblocks were added to the cosolvent mixture, f2. Stable double emulsions are generated in A, whereas in B, the inner phase breaks through the organic phase; we observe only simple emulsions of the middle fluid in the continuous aqueous phase. The radius of the constriction is 70 μm for both A and B.

We follow the formation of the PBA-PAA polymersomes from double emulsions by monitoring the evaporation of the cosolvent mixture from the middle phase, as shown by the microscope images in Figure 3. We clearly see the

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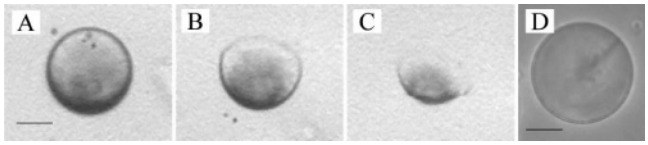

Figure 3. Evaporation and dissolution of a THF-toluene mixture from the oil phase of a double emulsion. Evaporation of the organic solvents allows the PBA-PAA diblock to organize into a vesicle. (A) The middle fluid layer is clearly visible. (B and C) Index matching of the inner and outer fluids causes the drop to fade in bright field microscopy as the solvent leaves the middle layer. Panels A−C are separated by 3 min. The scale bar in A represents 40 μm. (D) Polymersome imaged with phase contrast microscopy. The scale bar in D represents 30 μm.

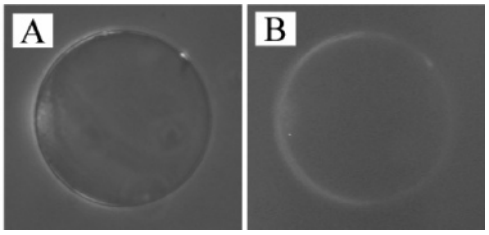

Figure 4. Encapsulation of hydrophobic quantum dots in a polymer vesicle. (A) Phase contrast microscopy image of the 70 micron vesicle. (B) The quantum dots trapped in the membrane are imaged in fluorescence at an excitation wavelength of 488 nm.

two interfaces of the double emulsion separated by the thin shell of organic fluid (Figure 3A). As the organic solvents dissolve in the surrounding water and ultimately evaporate, the shell gradually becomes thinner and the interfaces tend to disappear; the bottom part vanishes after the upper part due to inhomogeneity in the thickness of the organic fluid shell (Figure 3, panels B and C). Using bright field microscopy, the thin wall of the vesicle formed after the solvents have evaporated cannot be clearly seen because the refractive indices of the inner and outer fluids are nearly equal. Instead, we use phase contrast microscopy to enhance the index-of-refraction mismatch between the membrane and the surrounding fluid, as shown in Figure 3D.

To characterize the nature of the membrane of the polymersome, we add a small quantity of CdSe quantum dots to the inner fluid before the formation of the double emulsion; these serve as fluorescent markers that are trapped on the inner side of the walls of the polymersome, providing a means of visualizing the membrane. A phase contrast image is shown in Figure 4A, and for comparison, a fluorescence image, with excitation at 488 nm, is shown in Figure 4B; the ring of fluorescing quantum dots trapped in the wall of the membrane is clearly visible. The initial thickness of the shell was about 30 μm, and the initial diblock concentration was 0.5 wt %; since the radius of the shell remains the same, the final thickness would be about 1.5 μm if all of the solvent evaporates. This is consistent with the measured membrane thickness of a few microns; however, we cannot exclude the possibility that a small amount of solvent remains, albeit only a small amount. It is clear, however, that the membrane is not unilamellar; the expected thickness of a bilayer of these diblocks is about 40 nm, suggesting that the shell is at least on the order of 100 layers thick in this case. However, the thickness of the wall can be controlled by adjusting the initial concentration of the diblock copolymers in the cosolvent mixture. We observed no leakage of the fluorescent particles through the walls of the polymersome over at least several days, confirming that the self-assembled membrane is stable.

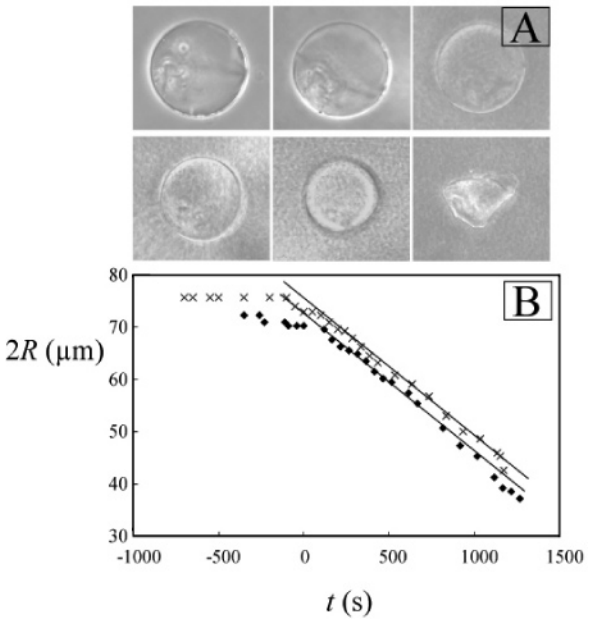

Figure 5. Evolution of polymerosomes under osmotic pressure shock. We subject the polymersomes to an osmotic shock by introducing glucose to the continuous aqueous phase at a time taken as the origin. The final concentration of sucrose is approximately 100 mM. Before the addition of sucrose ($t < 0$), the vesicle radius is constant. After the addition of sucrose ($t > 0$), the resultant osmotic pressure pumps water out of the polymersome and the vesicles shrink due to the osmotic pressure difference between the external and internal environments. (A) Pictures showing the evolution of a 70 μm diameter vesicle under an osmotic pressure shock. The interval between two successive images is 60 s except for the last two images, which are separated by 5 min. (B) Radius of two polymerosomes of roughly the same initial radius displayed as a function of time. The two polymerosomes are submitted to an osmotic pressure shock by placing them in a 100 mM glucose solution. The permeability of the polymerosomes is deduced from the slope of the linear regime that is observed for the two vesicles using eq 1.

In our synthesis technique, it is essential to have an interfacial tension between the middle fluid and both the inner and outer fluids to drive the Rayleigh-Plateau instability that forms the double emulsions. However, a polymersome should have a vanishing surface tension if the membrane is to be truly flexible. In our case this is possible as poly(butyl acrylate) has a glass transition temperature of −35 °C, so the vesicle membrane is fluid. This can only occur if all of the solvent in the shell evaporates, leaving a membrane comprised only of diblock copolymers. To test whether this occurs, we subject our polymersomes to an osmotic shock by introducing glucose to the outer, continuous phase, at a final concentration of approximately 100 mM. The resultant osmotic pressure pumps water out of the polymersome causing the membrane to collapse as shown in the sequence of images in Figure 5A. The interval between two successive images is 60 s except for the last two images, which are separated by 5 min. The deflation and collapse of the polymerosome confirms that there is no remaining surface tension and that virtually all of the organic solvent has evaporated. However, liposomes and polymerosomes are known to undergo well-defined shape transformations as the osmotic pressure in the environment is varied;[12] the collapsed morphology of the last image has never been reported.

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This might be an indication of the membrane heterogeneity or incomplete drying of the membrane.

We can determine the permeability of the membrane, P, from the initial rate of change in the polymersome radius, R

$$\frac{dR}{dt} = -\alpha P \Delta c \quad (1)$$

where α is the molar volume of water ($\alpha = 18 \times 10^{-3}$ L/mol) and Δc (mol/L) is the difference in concentration of glucose between the inner and outer solutions. We find that R decreases linearly in time as shown in Figure 5B for two different vesicles. From the slope we determine $P \approx 7 \pm 1 \mu$m/s, where the error reflects our uncertainty in the glucose concentration. This value of permeability is comparable to that measured for others polymerosomes,[1] $P \sim 10$ μm/s, and is about 10 times smaller than that measured for phospholipid vesicles,[1] $P \sim 15-150$ μm/s. In addition, these results suggest that the membrane thickness of our polymerosomes is probably not excessively large because the permeability is comparable to that of others polymerosomes. Nevertheless, we suspect that the membranes are heterogeneous; we notice regions of nonuniform thickness in some polymerosomes. Moreover, it is still possible that some solvent remains trapped in the membrane, which might alter the permeability.

Our synthesis technique is not restricted to simple polymerosomes. We can also form double emulsion drops that contain more than a single internal water droplet. These can be used to form a new class of polymersome structures. We show an example of such a double emulsion drop with many internal water droplets in Figure 6A, where we have again labeled the organic phase with quantum dots to allow visualization by fluorescence microscopy. Surprisingly, the interior water droplets retain their integrity as the organic fluid evaporates. This results in the formation of a foamlike structure, with the final polymersome consisting of many internal compartments as shown by the phase contrast image in Figure 6B and the fluorescence image in Figure 6C. These structures confirm that the internal water droplets act as a template for creating the polymersome in our synthesis technique.

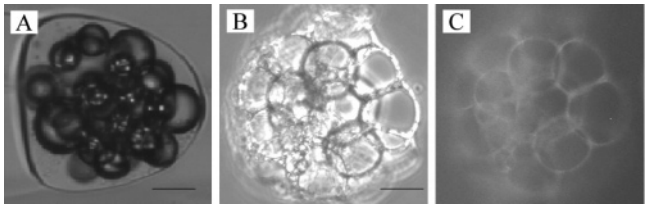

Figure 6. Transition from a double emulsion that contains many small aqueous droplets to a multi-compartmentalized vesicular structure. (A) Double emulsion template. (B and C) Phase contrast and fluorescent images of the multi-compartment vesicle obtained after the evaporation of the solvent. The scale bars in panels A and B are 30 μm.

Moreover the flexibility afforded by this method allows the creation of new classes of structures which may find additional uses as encapsulation structures.

In this paper, we demonstrate a new method to fabricate polymerosomes from templates formed from double emulsions. Diblock copolymers are dissolved in a volatile organic solvent which forms the intermediate fluid of the double emulsion drops. The polymers adsorb to the interface, and when the solvent evaporates, a polymersome is generated. We use a microcapillary device to form highly controlled double emulsions with a core−shell structure to produce the polymerosome. We have also fabricated new multicompartment polymersomes by using a multiple emulsion with many internal droplets as the template. With our fabrication technique the internal fluid phase remains completely isolated from the external phase providing for highly efficient encapsulation. Moreover each of the fluid streams is completely separate at all phases of the fabrication process; this affords great flexibility in the choice of materials and the structures that can be produced.

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