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Multiple Polymersomes for Programmed Release of Multiple Components

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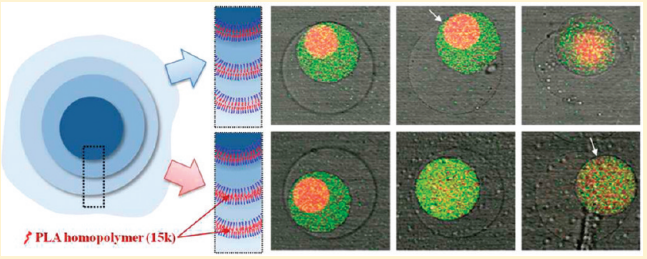
Supporting Information

ABSTRACT: Long-term storage and controlled release of multiple components while avoiding cross-contamination have potentially important applications for pharmaceuticals and cosmetics. Polymersomes are very promising delivery vehicles but cannot be used to encapsulate multiple independent components and release them in a controlled manner. Here, we report a microfluidic approach to produce multiple polymersomes, or polymersomes-in-polymersome by design, enabling encapsulation and programmed release of multiple components. Monodisperse polymersomes are prepared from templates of double-emulsion drops, which in turn are injected as the innermost phase to form the second level of double-emulsion drops, producing double polymersomes. Using the same strategy, higher-order polymersomes are also prepared. In addition, incorporation of hydrophobic homopolymer into the different bilayers of the multiple polymersomes enables controlled and sequential dissociation of the different bilayer membranes in a programmed fashion. The high encapsulation efficiency of this microfluidic approach, as well as its programmability and the biocompatibility of the materials used to form the polymersomes, will provide new opportunities for practical delivery systems of multiple components.



INTRODUCTION

Polymersomes are vesicles with membranes composed of bilayers of amphiphilic block-copolymers; they exhibit greatly enhanced stability and controllability by comparison to the more widely used liposomes whose membranes consist of bilayers of phospholipids.[1−3] Thus, polymersomes have great promise as capsules for drugs, cosmetics, and nutrients, enabling long-term storage and controlled release of the actives. However, conventional approaches to make vesicular structures, such as electroformation or bulk hydration of dried amphiphiles, achieve only limited control over size and low efficiency of encapsulation. However, recent advances in microfluidic devices have enabled production of monodisperse polymersomes with significantly improved size uniformity and encapsulation efficiency. For example, pulsed jetting of aqueous solution against a planar bilayer produces monodisperse giant vesicles.[4,5] Alternatively, sequential injection of water, amphiphile-laden oil, and water into small chambers and subsequent ejection of the liquids from the chamber produce monodisperse cell-sized vesicles.[6] Both approaches are limited to a production rate on the order of several vesicles per second. Polymersomes can also be fabricated using controlled water-in-oil-in-water double-emulsion drops as templates.[7−9] Through dewetting of the amphiphile-laden oil phase from the surface of the innermost water drop and the subsequent separation of the oil drop, unilamellar vesicle membranes can be efficiently fabricated. This approach increases the production rate of the vesicles to typically on the order of hundreds per second. Nonspherical polymersomes that encapsulate multiple distinct components can also be prepared using double-emulsion drops containing several core drops.[10,11] However, encapsulation of multiple distinct components, and their controlled sequential release while avoiding cross-contamination, remains an important challenge, as these would be very valuable in applications including, for example, growth-factor delivery and cancer therapy.[12,13] Liposomes with multiple compartments, or vesosomes, have been produced as vehicles capable of delivering multiple distinct drugs or other components; however, they are randomly structured and do not allow systematic incorporation of the different components into the compartments.[14] Thus, their applicability is severely limited, and new approaches to the controlled fabrication of such structures are highly desired.

In this paper, we report the use of microfluidic devices to fabricate multiple polymersomes, or polymersomes-in-polymersomes,

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which enable encapsulation and programmed release of multiple distinct components. Highly monodisperse polymersomes are prepared by templates of double-emulsion drops, which are then encapsulated in the innermost drop of the second level of double-emulsion drops using a capillary microfluidic device; this leads to formation of double polymersomes. In the double-emulsion generation, the number of inner polymersomes within each double polymersome can be controlled by adjusting the size of the orifice of the collection capillary relative to the size of the polymersomes. Moreover, triple polymersomes can also be prepared through a third step of encapsulation again using double-emulsion template, thereby adding a third bilayer membrane. Each membrane of the multiple polymersomes is composed of a bilayer of poly(ethylene glycol) (PEG)-b-poly(lactic acid) (PLA) diblock-copolymers; moreover, it is possible to incorporate in any of this bilayer additional PLA homopolymer, which enhances the stability of that bilayer and increases the critical stress for rupturing that membrane. This enables us to achieve programmed sequential rupturing of each membrane in the multiple polymersome structures.

RESULTS AND DISCUSSION

Preparation of Double Polymersomes. Monodisperse polymersomes whose membrane is composed of a PEG (M_w 5000)-b-PLA (M_w 10 000) bilayer are prepared from double-emulsion drops in a capillary microfluidic device as shown in Figure S1 of the Supporting Information.[15] We inject a 10 wt % aqueous solution of PEG (M_w 6000) with 100 mOsm/L through the injection capillary to form the core drop; red dye molecules, sulforhodamine B, are also dissolved in the PEG solution at a concentration of 10^{-5} M. We use a mixture of chloroform and hexane with a volume ratio of 38:62 with 5 mg/mL PEG-b-PLA to form middle phase and inject it through the interstices of the square and the injection capillaries. The continuous phase is 10 wt % aqueous solution of poly (vinyl alcohol) (PVA, M_w 13 000−23 000) with 100 mOsm/L; it is injected through the interstices between the square and collection capillaries, as shown in Figures S1a and b of the Supporting Information. The resultant monodisperse double-emulsion is collected into a bath containing an aqueous solution with 3 wt % PEG and 35 mM NaCl at an osmolarity of 100 mOsm/L; green dye molecules, 8-hydroxyl-1,3,6-pyrenetrisulfonic acid trisodium salt, are dissolved into the mixture at a concentration of 10^{-5} M. The PEG-b-PLA-laden oil layer dewets the surface of the innermost drop and detaches from it, leaving unilamellar polymersomes. These polymersomes have a higher density than that of collection phase but the osmolarities are the same; therefore, the polymersomes are collected at the bottom of the collection bath while the detached oil drops remain on top. In addition, the osmolarity of the collection liquid is adjusted to be the same as that of inner phase of polymersomes; therefore, there is negligible osmotic pressure difference and hence minimal water flux across the bilayer. The polymersomes are incubated in an open bath for a day at room temperature without mechanical stirring to ensure evaporation of all residual volatile oil from the bilayer prior to reinjection. Optical and confocal microscope images of monodisperse polymersomes are shown in Figures S1c and d, respectively (Supporting Information).

To form the multiple polymersomes, the monodisperse polymersomes dispersed in an aqueous solution of PEG and NaCl are reinjected into another capillary device as shown in Figure 1a. The 3 wt % PEG present in the polymersome suspension increases the density of the suspension, thereby causing sedimentation of the resultant double polymersomes. At the same time, PEG increases the viscosity of the suspension; this helps to prevent jamming of the injection capillary channel by lubricating polymersomes with respect to the neighboring polymersomes and to the capillary wall. The inner diameter of the orifice of the injection capillary is made larger than that of the polymersomes in the innermost phase to prevent their rupturing during reinjection.

The diameter of the orifice of the collection capillary relative to the diameter of the polymersomes determines the final structures of the double polymersomes. When the diameter of the orifice of the collection capillary is only slightly larger than that of the injected polymersomes, the breakup of the middle phase into the double-emulsion drops is triggered by the polymersomes themselves as they pass through the orifice;[16] thus, the resultant double-emulsion drops encapsulate only one inner polymersome per drop, as shown in Figure 1b and in Movie 1. Because the polymersomes in the innermost aqueous phase are comparable in size to the orifice in this breakup mode, they block the orifice and prevent further flow; this results in a narrow jet of the middle phase passing through the orifice. As pressure increases, a single polymersome in the innermost aqueous phase is injected in a pulse through the orifice; this produces a single polymersome in the innermost aqueous drop. The subsequent polymersome again blocks the entrance of the orifice, preventing the innermost aqueous phase from entering the orifice. This pulse-like injection of polymersomes induces the breakup of the middle jet at its trailing edge, resulting in double-emulsion drops, as shown in the series of images in Figure S2a in the Supporting Information. When the time interval between injections of two sequential polymersomes is relatively large, the breakup of the jet fist occurs at the leading edge, and subsequent breakup at the trailing edge of the jet produces satellite drops as shown in Figure S2b in the Supporting Information. The resultant double-emulsion drops contain a single polymersome in their innermost aqueous drop at every instance of the breakup of the middle jet. Double polymersomes, containing a single inner polymersome, are prepared after the dewetting and subsequent detachment of the middle phase of the outer double-emulsion drops, as shown in Figures 1c and d. Satellite drops are easily removed by exploiting the difference in density. The broader size distribution of the outer polymersomes by comparison to that of the inner polymersomes is attributed to inconsistent injection frequency of polymersomes into double-emulsion drops, leading to an inconsistent breakup frequency of the innermost aqueous drops.

By contrast, when the inner diameter of the orifice of the collection capillary is much larger than the diameter of the polymersomes, breakup of the middle jet is no longer triggered by the polymersomes but is instead triggered by the formation of the polymersome-containing innermost droplets, as shown in Figures 2a and b and in Movie 2. The relatively small polymersomes in the innermost aqueous phase flow through the orifice in a continuous manner rather than in a pulse-like flow. In this breakup mode, the innermost aqueous droplets grow at the tip of the injection capillary and are injected into the collection capillary in the form of coaxial interfaces with one consisting of the innermost aqueous phase and the middle oil phase and the second consisting of the middle oil phase and the continuous aqueous phase. The breakup of the inner interface is triggered by the pulse-like injection of the innermost aqueous drops whose diameter is comparable to that of orifice. The breakup of the

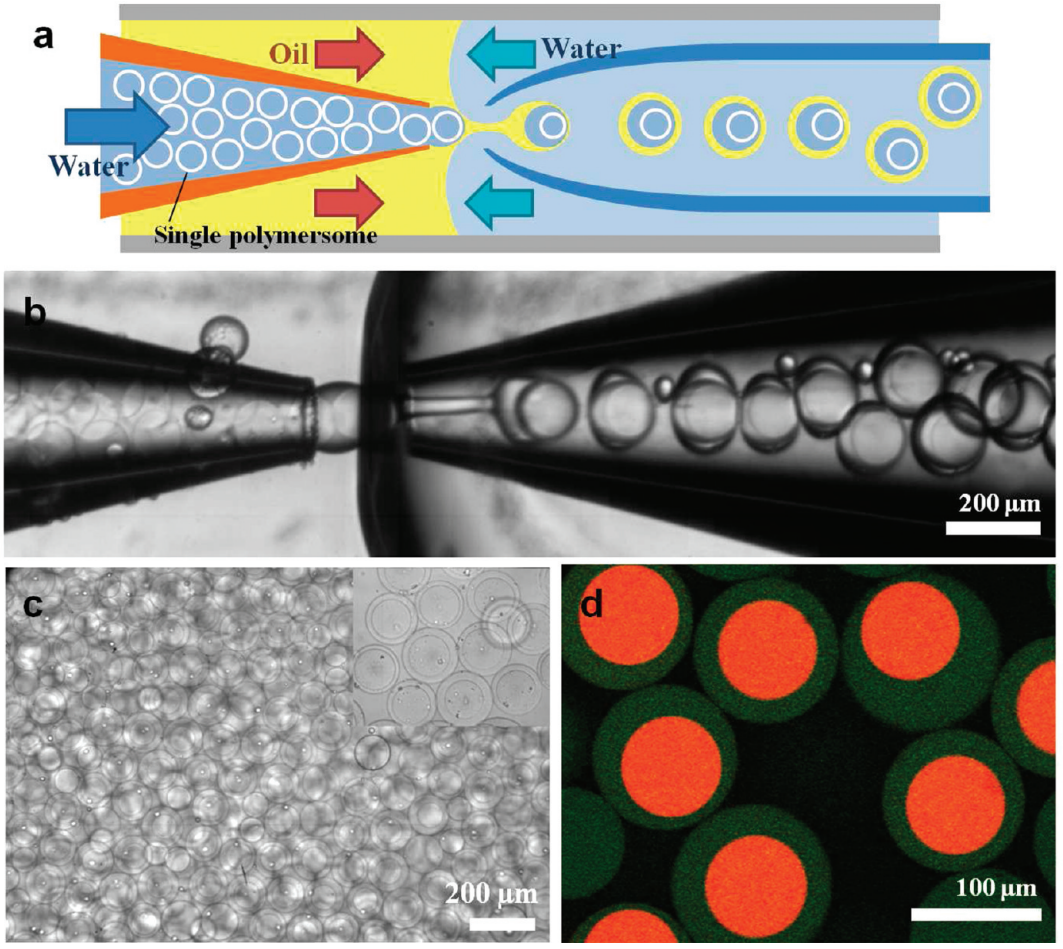

Figure 1. (a) Schematic illustration of the microfluidic device for preparation of double polymersomes containing a single inner polymersome. (b) Optical microscope image showing injection of polymersomes into the innermost drops of double-emulsion drops, where the breakup of middle phase is triggered by the polymersomes. (c) Optical microscope and (d) confocal microscope images of double polymersomes containing a single inner polymersome. Inset of (c) shows a diluted suspension of double polymersomes.

outer interface occurs at the leading edge of the middle jet, resulting in double-emulsion drops, as shown in Figure S3 in the Supporting Information. Subsequent breakup at the trailing edge of the jet produces satellite oil drops. Therefore, regardless of the number of polymersomes encapsulated in each double-emulsion drop, the breakup is determined by the flow rates of the innermost phase, which is an aqueous suspension of polymersomes; this leads to formation of monodisperse double polymersomes containing an uncontrolled number of the inner polymersomes. Using this approach, the outer polymersomes can contain several inner polymersomes encapsulating different ingredients. To demonstrate this feature, we prepare a mixture of three different polymersomes of the same size encapsulating a red dye, a green dye, and a mixture of red and green dyes respectively in the inner polymersomes, as shown in Figure 2c. The polymersomes containing both dyes appear yellow in an overlay confocal microscope image. The resultant double polymersomes contain a random combination of the three different inner polymersomes, as shown in Figure 2d.

Programmed Release from Double Polymersomes. The multiple polymersomes enable efficient encapsulation of multiple distinct components; fabrication with capillary microfluidic devices also provides considerable flexibility in creating structures where the release of encapsulated actives can be controlled in a programmable fashion. The simplest means of release is through mechanical strain, and even with this method, sequential rupturing of the bilayer membranes provides potential control of the order of release of the encapsulated materials; this enables use of these double polymersomes in cosmetic applications. For example, double polymersomes encapsulating two different cosmetic actives in the innermost and the outermost polymersomes respectively, can release the actives in the sequential fashion when they are spread on the surface of skin by rubbing; such sequential release of multiple actives is of potential value for cosmetics. When a double polymersome is forced to flow, at a rate of 500 μL/h, through an orifice whose diameter is larger than the innermost polymersome yet smaller than the outermost polymersome, the outermost polymersome is selectively ruptured due to the areal strain, as shown in Figure 3a and in Movie 3. The outermost polymersome must be deformed to pass through the orifice; thus, the surface area of the membrane must increase, and this can lead to rupture of the membrane. The bilayer can sustain its integrity up to a critical areal strain of approximately 0.2; this represent relative increase of surface area upon failure of the membrane. Similar behavior is observed when single polymersome with the same diameter is forced through this orifice at the same flow rate; the polymersome ruptures and all of the contents are released. By contrast, when a lower flow rate of 10 μL/h is

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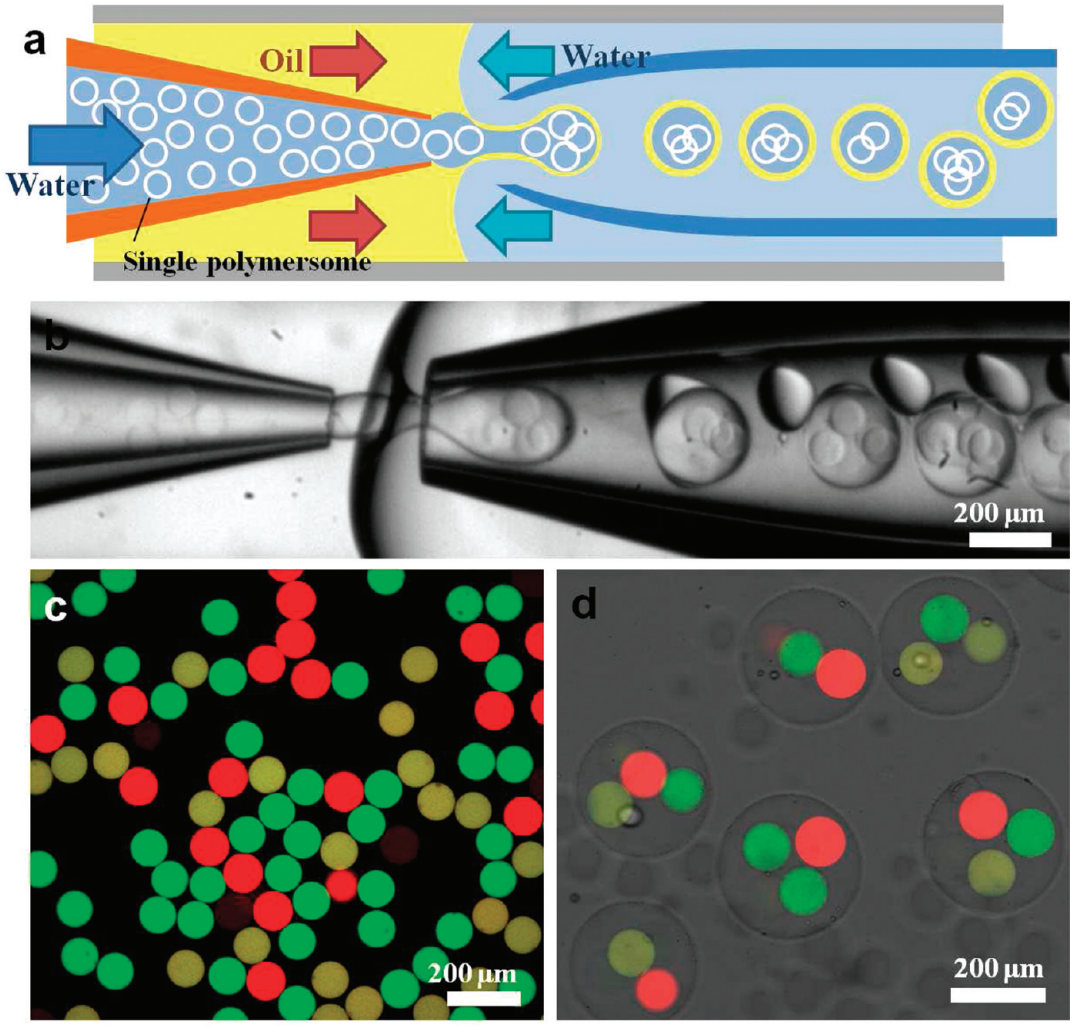

Figure 2. (a) Schematic illustration of the microfluidic device for preparation of double polymersomes containing several inner polymersomes. (b) Optical microscope image showing injection of polymersomes into the innermost drops of double-emulsion drops, where the breakup of the middle phase is triggered by the core drops of the aqueous suspension. (c) Confocal microscope image of a mixture of monodisperse single polymersomes encapsulating a red dye, a green dye, and a mixture of red and green dyes, respectively. (d) Confocal microscope image of double polymersomes containing several inner polymersomes with three different colors.

used for the injection, the single polymersome releases only some of the inner encapsulated ingredients as the membrane is stretched while passing through the orifice; however, the hole in the membrane recovers and the bilayer heals prior to complete release.[17,18] Interestingly, the deformation in the shape of the polymersome persists after it is ejected from the orifice, as shown in Figure S4 and in Movie 4. Similar behavior is observed for double polymersomes and the innermost polymersomes remain completely intact as they can pass through the orifice undeformed.

Apart from release of actives due to mechanical strain, even more controlled and indeed programmable release of actives can also be induced in nonaqueous environments by dissociation of bilayer membranes. When double polymersomes consisting of bilayers of PEG-b-PLA diblock-copolymers for both the inner and the outer membranes are dispersed in a mixture of water and ethanol in a volume ratio of 1:1, the outer membranes begin to dissociate, and the inner polymersomes are expelled to continuous phase. We illustrate this using polymersomes containing red dye in the innermost polymersome and green dye in the outer polymersome; the release of the green dye from the outer polymersome and the expulsion of the core polymersome containing red dye into the continuous phase of the mixture are shown in Figure 3c and in the first part of Movie 5. The PEG-b-PLA molecules favor the assembled membrane state of the bilayer in water as this reduces the exposed area of the PLA block to the water; by contrast, the molecules are disassembled and dispersed in the mixture of water and ethanol, thereby breaking the membrane. Upon release, the inner polymersomes remain stable for approximately 15 min, and then also become unstable and release the red dye.

To enable programmed release of actives, the stability of the bilayer membranes must be more finely controlled; this can be accomplished through the use of block-copolymers with different molecular weights.[19] Instead, here we use PLA homopolymers, which are incorporated into the hydrophobic region of the bilayer; this enhances the stability of the membrane. We prepare double-emulsion drops with a middle phase that contains 2.5 mg/mL PLA homopolymer (M_w 15 000) added to the original PEG-b-PLA solution. The resultant polymersome membranes show stronger mechanical and chemical stability. For example,

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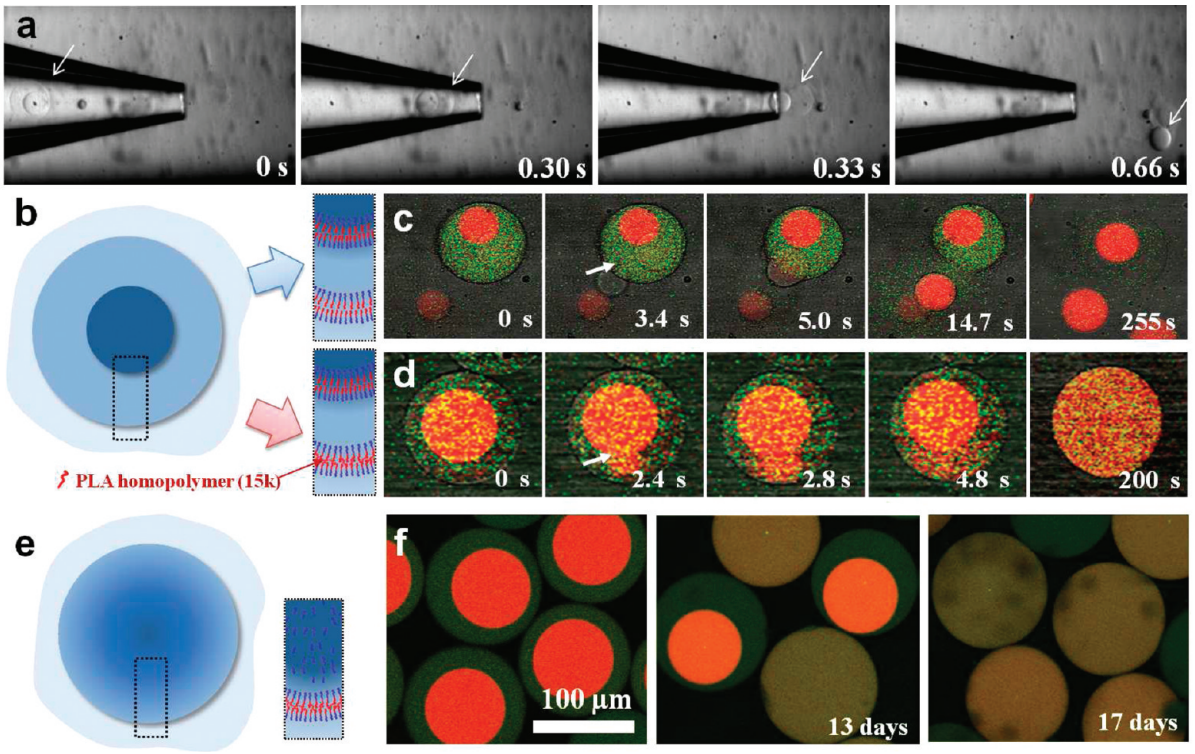

Figure 3. (a) Series of images showing selective rupture of the outer membrane of a double polymersome which passes through an orifice whose diameter is larger than the innermost polymersome yet smaller than the outermost polymersome. (b) Schematic illustration of a double polymersome showing bilayers with no internal homopolymer on the top right and one of the bilayers containing homopolymer on the bottom right. (c) Series of confocal images showing selective dissociation of the outer membrane of double polymersomes, consisting of a poly(lactic acid) (PLA)-homopolymer-free bilayer for both the inner and outer membranes, in a mixture of water and ethanol at a volume ratio of 1:1. (d) Series of confocal images showing selective dissociation of the inner membrane of double polymersomes, consisting of a PLA-homopolymer-free bilayer for the inner membrane and a PLA-homopolymer-loaded bilayer for the outer membrane, in the same mixture of water and ethanol. (e) Schematic illustration and (f) confocal microscope images showing selective dissociation of the inner membrane of double polymersomes in water consisting of a PLA-homopolymer-free bilayer for the innermost membrane and a PLA-homopolymer-loaded bilayer for the outer membrane. The dissociation proceeds through spontaneous degradation of the PLA.

polymersomes with a PLA-homopolymer-loaded bilayer do not exhibit membrane rupture even under high osmotic shock, with an aqueous solution of NaCl at 2 Osm/L, as shown in Figure S5 of the Supporting Information. Despite being deflated, the polymersomes do not burst but remain closed, and the red dye added in the aqueous solution of NaCl does not penetrate within the polymersomes, as shown in confocal microscope images in Figure S5b (Supporting Information); fluorescence observed in the vicinity of the capsules in 2D image is caused by severe folding of membrane, which makes difficult to distinguish the inside and the outside of the capsules in the same focal plane of confocal microscope. However, water is continuously extracted from the interior of the polymersomes by the high concentration of NaCl; as a result, the polymersomes shrink until the osmolarity of the core reaches that of the continuous phase. By contrast, the polymersomes without PLA homopolymers initially deflate faster, but subsequently begin to inflate to spherical shape under same conditions of the osmotic shock imposed by aqueous solution of NaCl at 2 Osm/L, as shown in Figure S6a of the Supporting Information; this inflation is attributed to the fast diffusion of sodium and chloride ions into the polymersomes during rupture of bilayer. Under high osmotic shock, the membrane is torn and the ions in the continuous phase diffuse quickly into the cores of the polymersomes through the tear; by contrast, the PEG molecules originally encapsulated within the core remain, due to their relatively low mobility. This results in a higher osmolarity in the core of the polymersomes. Due to their ability to spontaneously self-heal, as seen, for example, for the case of the small mechanical stress, the shrunken polymersomes are inflated by the inward flow of water from the continuous phase as a result of the increase in osmolarity in the polymersome cores. Therefore, the polymersomes recover their original spherical shape and their cores exhibit the fluorescence as a result of diffusion of red dye from the continuous phase into the polymersomes while they are ruptured, as shown in Figure S6b (Supporting Information).

By employing a PLA-homopolymer-loaded bilayer as the outer membrane of the double polymersomes, we can control the sequential rupturing of the membrane from the innermost to the outermost. The PLA-homopolymer-loaded bilayer is stable for approximately half an hour even in a mixture of water and ethanol; moreover, the bilayer is also permeable to ethanol. Therefore, when the double polymersomes are dispersed in this mixture, the inner polymersomes dissociate first and release their core materials into the outer polymersomes as shown in Figure 3d. In addition, the PLA-homopolymer-loaded bilayer exhibits long-term stability in water by comparison to the PLA-homopolymer-free bilayer. Because PLA is biodegradable due to the hydrolysis of the ester group in its chain, the bilayer of PEG-b-PLA diblock-copolymer dissociates slowly in water, even without

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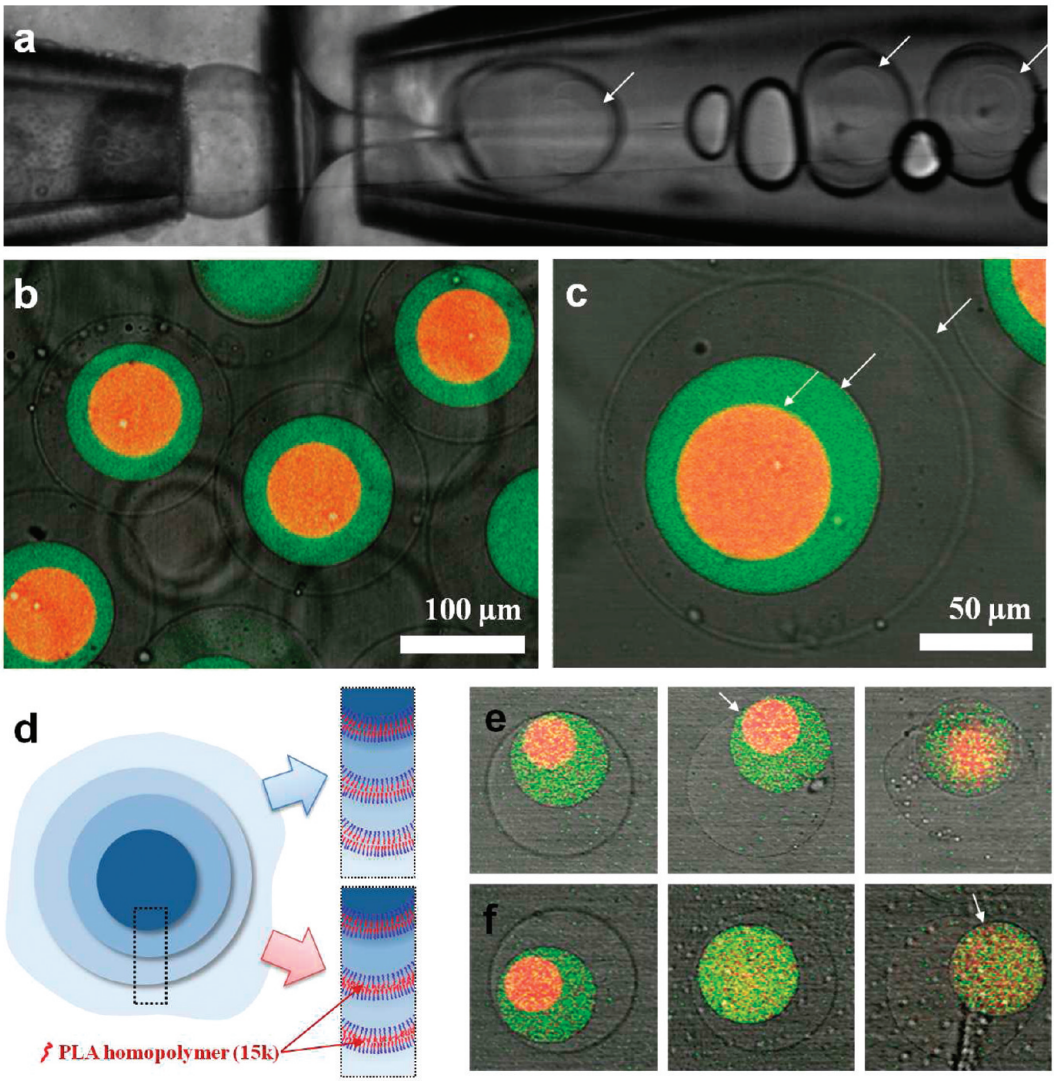

Figure 4. (a) Optical microscope image showing injection of double polymersomes into the innermost drops of double-emulsion drops. (b, c) Confocal microscope images showing triple polymersomes. The arrows denote three membranes of triple polymersomes. (d) Schematic illustration of a triple polymersome showing bilayers with no internal homopolymer on the top right and two of the bilayers containing homopolymer on the bottom right. (e) Series of confocal images showing sequential dissociation of membranes from the outermost to the middle of triple polymersome, consisting of a PLA-homopolymer-free bilayer for all three membranes, in a mixture of water and ethanol in a volume ratio of 1:1. (f) Series of confocal images showing sequential dissociation of membranes from the innermost to the outermost of triple polymersomes, consisting of a PLA-homopolymer-free bilayer for the innermost membrane and a PLA-homopolymer-loaded bilayer for the middle and outermost membranes, in the same mixture of water and ethanol.

a trigger.[20] The double polymersomes consisting of the PLA-homopolymer-free bilayer in the innermost membrane and the PLA-homopolymer-loaded bilayer in the outer membrane degrade by hydrolysis; for these, most inner polymersomes are ruptured by 17 days as shown in Figure 3e; by contrast, the outer membrane is stable for at least two months due to higher contents of PLA.

Triple Polymersomes and Programmed Release. Triple polymersomes can be prepared by injection of double polymersomes into the innermost drop of double-emulsion drops; an example of a device to accomplish this and some sample polymersomes injected into double emulsions are shown in Figure 4a. The white arrows highlight the double polymersomes inside double-emulsion drops. Examples of resultant triple polymersomes are shown in the confocal microscope images in Figures 4b and c; here, the inner and middle polymersomes contain red and green dyes, respectively. The sequence of the rupture of the membrane of the triple polymersomes is also controllable in the same manner as that of the double polymersomes. We use triple polymersomes, consisting of the PLA-homopolymer-free bilayer in all three membranes; when they are dispersed in a mixture of water and ethanol, they exhibit sequential rupturing from the outer membrane to the middle and inner membranes as shown in Figure 4e. By contrast, triple polymersomes that have no PLA homopolymer in the innermost membrane, but do contain the PLA homopolymer in the middle and outer membranes, exhibit sequential rupture of the membranes, beginning with the innermost then the outermost and finally the middle membrane, as shown in Figure 4f. As in the case of double polymersomes, selective rupture of the innermost polymersome of the triple polymersome can also be induced by the biodegradation of the polymer in water, as shown in Figure

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S7 of the Supporting Information. Our work shows that by tuning the stability of bilayer membranes in multiple polymersomes, programmed release of multiple distinct actives can be achieved.

CONCLUSIONS

In this work, we report biocompatible multiple polymersomes for controlled encapsulation and programmed releases of materials. The single and multiple polymersomes are produced using a double-emulsion-templated approach with capillary microfluidic devices. Moreover, we show that the membrane stability is enhanced by addition of hydrophobic homopolymers into the bilayer, which provides convenient means to achieve programmed rupture of the different membranes in multiple polymersomes. In this fashion, the multiple polymersomes allow sequential release of encapsulated materials from the outermost polymersome while at the same time avoiding cross-contamination; moreover, they can be used for reactions between separately encapsulated distinct reagents and subsequent release of the products of these reactions. In addition to programmable membrane rupture, the biocompatibility and the biodegradability of the PEG-b-PLA diblock-copolymers, as well as the high encapsulation efficiency of this double-emulsion approach, creates new opportunities to apply these novel polymersomes in practical biological delivery systems that require the encapsulation of multiple distinct active ingredients such as drugs, cosmetics, and nutrients. One promising application of these multiple polymersomes is the *in vivo* delivery of multiple growth factors for efficient tissue regeneration of blood vessels, nerves, and bones. For example, vascular endothelial growth factor (VEGF) can initiate generation of new branches of blood vessels, while platelet-derived growth factor (PDGF) promotes growth of the vessels.[12] Therefore, sequential addition of each growth factor to a damaged vessel enables more efficient recovery. This can be accomplished through the use of the double polymersomes, encapsulating VEGF in the outermost polymersome and PDGF in the innermost polymersome, and programming their release into the damaged vessel.

ASSOCIATED CONTENT

Supporting Information. Optical and confocal microscope images of single and multiple polymersomes are included to show their stability. This material is available free of charge via the Internet at http://pubs.acs.org.

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