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Microfluidic generation of PEG-b-PLA polymersomes containing alginate-based core hydrogel

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Herein, we demonstrate a novel method for the generation of monodisperse cell-like structures containing a biocompatible hydrogel matrix surrounded by a membrane responsive to chemical cues. Specifically, we employ droplet-based microfluidics to generate PEG-PLA polymersomes encapsulating alginate in liquid form. We investigate alginate core gelation by creating an osmotic pressure gradient across the polymeric membrane that, through expansion, allows the passage of calcium ions. The effects of calcium concentration on the core gelation are explored. © *2015 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4914112]

I. INTRODUCTION

Polymersomes are vesicle mimics made from amphiphilic synthetic block copolymers[1] having membrane thickness ranging from a few nanometers to a few tens of nanometers depending on the molecular weight of the chosen polymer.[2]

Over the last decade, these systems have found a diversity of applications as carriers of flavors and fragrances,[3] as drugs, DNA and protein delivery systems,[4–6] and as artificial cells[6–8] that mimic specific biological functions.[9]

Conventional approaches for producing vesicular structures, including hydration, electroformation, and extrusion methods, are limited in their ability to control polymersome size and encapsulation efficiency. Recently, it has been shown that microfluidic emulsification devices can be used to enhance polymersome monodispersity and control structural complexity.[10–12] Moreover, the microfluidic generation of polymersomes containing poly ethylene glycol diacrylate (PEGDA) networks demonstrated how a hydrogel core can increase polymersome stability and facilitate the release of an encapsulated payload.[13]

Despite PEGDA's excellent biocompatibility, the need for a photoinitiator and UV illumination to initiate gelation poses significant issues in applications requiring the encapsulation of biological entities prior to hydrogel polymerization. In this regard, alginate-based hydrogels are especially interesting since they do not require UV illumination and therefore represent an ideal environment for a diversity of cell-based studies and the formation of inner scaffolds for artificial cell facsimiles.

Previously microfluidic formats have been used to synthesize alginate spheres for use as substrates in cell culture,[14,15] spherical shells for reducing the initial burst of encapsulated drugs[16] and, in combination with other materials, capsules which can be used as supports for cell-free protein synthesis[17] and release.[18] In all these applications, alginate-based materials have been shown to be efficient in terms of biocompatibility and ease of gelation.[19]

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Herein we show, for the first time, the use of droplet-based microfluidics for the controlled generation of polymersomes containing an alginate core and, due to responsiveness of the membrane to external cues, we also explore the behavior of these systems when exposed to calcium chloride ($CaCl_2$) solutions.

The mechanism of alginate gelation in the presence of divalent ions[20] is well-known and has been widely exploited in food preparations and biology.[21] In a $CaCl_2$ solution, sodium alginate exchanges its monovalent sodium ions (Na^+) with divalent calcium ions (Ca^{2+}), with the alginate chains undergoing rapid crosslinking. To achieve alginate gelation within the polymersome core, Ca^{2+} (40.1 Da) must cross the polymeric membrane. The passage of ions through such a membrane can be achieved by imposing negative osmotic pressure which leads to an inflation of the polymersome and areal expansion of the membrane, thereby providing higher permeability of the loosened membrane (Fig. 1).

Due to the biocompatibility of the chosen materials, the responsive membrane properties to external chemical stimuli and the presence of a scaffold able to house biological material, these studies represent a significant step forward in the generation of artificial cell systems.[22]

II. EXPERIMENTAL

Polymersomes were prepared from double-emulsion droplets using glass capillary devices fabricated and chemically treated following established procedures.[11,13] Glass capillary diameters for injection of the inner phase and collection of double-emulsion droplets were 60 and 120 μm, respectively, and separated by approximately 60 μm.

Alginate-based solutions were prepared using 1% wt. of alginic acid sodium salt (180947, Sigma, USA) and 5% wt. of polyethylene glycol (MW 5000, Sigma, USA) dissolved in deionized (DI) water; to confirm gelation, 1 μm polystyrene beads were synthesized and dispersed in the solution of 0.5% wt. The middle oil phase was prepared as a mixture of chloroform (25693, Sigma, USA) and hexane (15671, Sigma, USA) with a volume ratio of 38:62 containing 5 mg/ml polyethylene glycol (MW 5000)-b-polylactic acid (MW 10 000) (PEG-b-PLA) (Polysciences Inc., USA). The continuous phase consisted of an aqueous solution of 10% wt. polyvinyl alcohol (PVA) (MW 13 000–23 000, Sigma-Aldrich, USA), while the collection phases were aqueous solutions of either 50 mM NaCl or $CaCl_2$ at three different concentrations of 3.33 mM, 33.3 mM, and 66.6 mM.

Osmolarities were measured using a freezing point depression osmometer (Osmomat 030-D, Gonotec, DE). Fluids were loaded into syringes controlled by precision syringe pumps

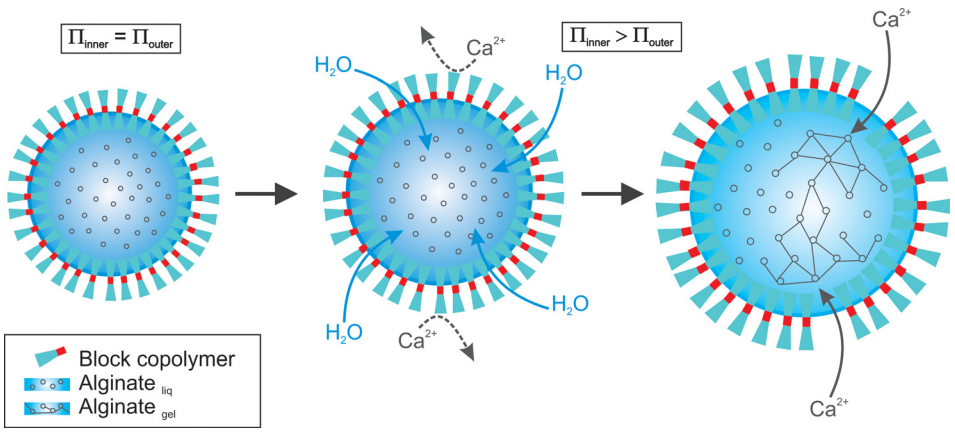

FIG. 1. Mechanism of polymersome core gelation through negative osmotic pressure. Cartoon showing a polymersome encapsulating sodium alginate that subsequently undergoes gelation through diffusion of Ca^{2+} across the bilayer formed by self-assembled block copolymer molecules. When the osmotic pressure of polymersome core (Π_{inner}) is larger than that of the surroundings (Π_{outer}), water molecules are pumped in through the bilayer membrane, leading to areal expansion of the membrane. Ca^{2+} is allowed to diffuse through the loosened membrane, enabling the gelation of alginate in the core. If the pressure difference is too large, the membrane will lose its integrity.

(Legato 100, KD Scientific, USA). Emulsion imaging was performed using a high-speed camera (Phantom Miro eX2, Vision Research).

III. RESULTS AND DISCUSSION

A. Osmolarity measurements

Like many membrane and vesicle-like systems, which allow the passage of small ions and water molecules across the membrane, polymersomes behave as semi-permeable membranes where the passage of water is driven by an osmotic pressure difference across the membrane. The pressure is proportional to osmoles of solute per liter of solution (Osm/l), known as osmolarity, which must be measured to calculate the pressure difference between an inner compartment and the surroundings. Unlike easily dissociable molecules, such as NaCl and $CaCl_2$, sodium alginate is composed of long polysaccharide chains that do not dissociate in water; accordingly the osmolarity of a concentrated alginate solution is difficult to measure.

We were able to measure the osmolarity of an alginic acid sodium salt solution whose concentration is as low as 0.1% wt., (5 mOsm/l). By assuming a linear relation between the concentration and osmolarity, we estimated that 1% wt. alginic acid solution has an osmolarity of 50 mOsm/l. The osmolarity of a 5% wt. PEG solution was measured to be 50 mOsm/l. Accordingly, the osmolarity of the innermost phase containing 1% wt. alginic acid sodium salt and 5% wt. PEG solution was initially estimated to be 100 mOsm/l. The osmolarity of the continuous phase, 10% wt. PVA, was measured at 100 mOsm/l, whereas osmolarities of the collection solutions were as follows: 100 mOsm/l (50 mM NaCl), 10 mOsm/l (3.3 mM $CaCl_2$), 100 mOsm/l (33.3 mM $CaCl_2$) and 200 mOsm/l (66.6 mM $CaCl_2$).

B. Formation of polymersomes from double-emulsion droplets

Inner, middle, and continuous phases were injected into the microfluidic device at flow rates of 400, 700, and 3000 μl/h, respectively. Operation at these flow rates produced double emulsion droplets with an average inner droplet diameter of 69.9 μm $\pm$ 1.5 μm and a coefficient of variation (CV) equal to 2.1% (Fig. 2(a)). Once generated, the emulsion was collected on a glass slide and inspected under a microscope. As expected and as described previously,[23] immediately after generation, different entities could be identified within the emulsion. These included double-emulsion droplets (denoted by "1" in Fig. 2(b)), double-emulsion droplets with the oil layer dewetting from the inner core ("2" in Fig. 2(b)), and polymersomes ("3" in Fig. 2(b)). Subsequently, the emulsion was collected in a vial containing 50 mM NaCl. Chloroform, a good solvent for PEG-b-PLA, rapidly diffuses from the middle layer to the surrounding layer, thereby concentrating hexane (a poor solvent) in the middle layer. This leads to the dewetting of the middle phase on the surface of the inner droplet, which leaves behind two overlapped interfaces with accumulated amphiphilic block-copolymers.[23] Accordingly, a polymersome with a unilamellar bilayer is formed once dewetting is complete, regardless of the concentration of PEG-b-PLA dissolved in middle layer; the excess polymer remains in the dewetted oil droplets.[24] We observed that the polymersomes localize at the bottom of the vial since the density of the inner core was higher than the collection liquid (Fig. 2(c)). Moreover, the density mismatch is further enhanced by the presence of PEG molecules within the core. Oil droplets separated from the cores have a high hexane concentration, which means they float on the surface of the collection liquid due to their lower density (Fig. 2(d)). Images of polymersomes collected in 50 mM NaCl solution were taken 10 min and 24 h after generation and show that, in comparison with the inner core diameter of the double emulsion at the generation site (69.9 $\pm$ 1.5 μm), polymersomes inflate in 10 min (83.6 $\pm$ 5.1 μm), and their diameters remain unchanged over the next 24 h (82.4 $\pm$ 4.1 μm); indicating that polymersomes are in chemical equilibrium with their surroundings. Inflation occurs due to an imbalance in osmotic pressure, which in turn causes an inward flux of water from the surroundings. Using the final diameter of inflated polymersomes, the osmolarity of initial cores could be estimated to be 164 mOsm/l. Accordingly, the osmolarity of 1% wt. alginic acid sodium salt was approximately equal to 114

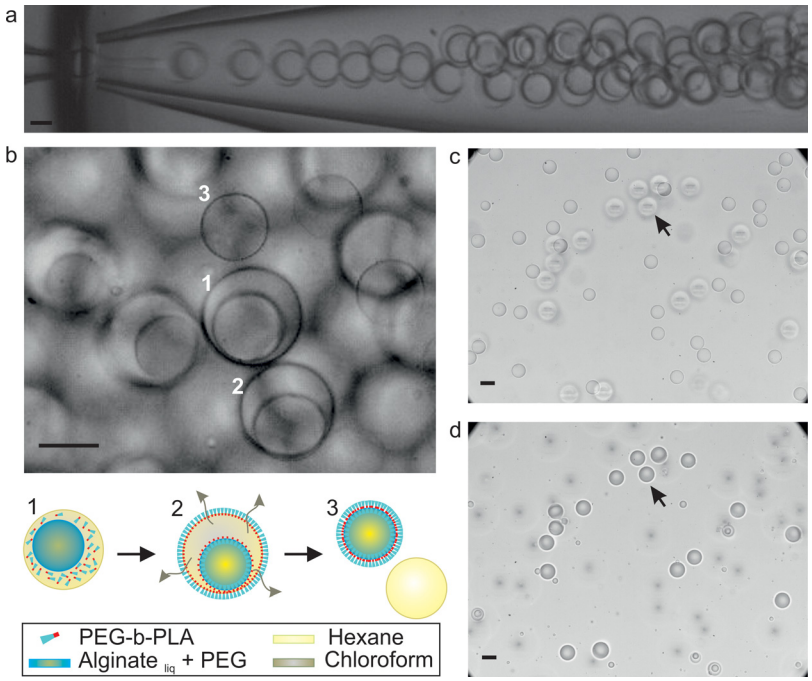

FIG. 2. Production of polymersomes from double-emulsion droplets. (a) Generation of double-emulsion droplets in a capillary device operating at flow rates equal to 400, 700, and 3000 μl/h for the inner, middle, and outer phases, respectively. The inner solution consists of DI water with 1% wt. of alginate and 5% wt. of PEG; the middle phase is a mixture of hexane and chloroform (38:62 in vol.) in which the PEG-b-PLA block copolymer is dissolved; the outer phase is aqueous solution of 10% wt. of PVA. (b) Double emulsion collected at the outlet of the device. It can be observed all the stages the emulsion go through to form polymersomes. Once a double emulsion droplet (1) is generated, the block copolymer molecules move at the interface while the chloroform evaporates. The oil layer de-wets (2) and separates from the inner droplet, which leaves behind a bilayer membrane (3). (c) and (d) The polymersomes collected in NaCl solution deposit at the bottom (c), whereas the separated oil droplets float to the surface of the solution (d). (c) and (d) Black arrows indicate the oil droplet out of focus and in focus. Scale bars are equal to 60 μm.

mOsm/l, a much higher value than that estimated from the measured value of the 0.1% wt. alginate solution.

C. Double-emulsion droplets collected in $CaCl_2$ solutions

Immediately after double-emulsion generation, droplets were collected in $CaCl_2$ solutions at three different concentrations and then inspected under a microscope both at collection and 24 h thereafter. Interestingly, once the double emulsion enters the $CaCl_2$ solution, opalescent regions appear within the collection phase, signaling the onset of gelation.

In 3.33 mM $CaCl_2$ solution (10 mOsm/l), a large population of "round" microgels (89% just after collection) was observed within the gel patches (Fig. 3(a)). The mean diameter of such microgels (70.3 ± 1.8 μm) was comparable to the diameters of the double-emulsion inner cores measured inside the capillary device and remained constant over 24 h (69.9 ± 4.9 μm). This indicates the absence of a polymersome membrane enclosing the microgels (otherwise the polymersomes would inflate due to an inward flux of water). The alginate in the core could be gelated by the rupture of double-emulsion droplets before the dewetting step, thereby producing microgels with sizes comparable to the cores of double-emulsion droplets. Inspection of polymersomes collected in 33.3 mM $CaCl_2$ solution (100 mOsm/l) instead indicated the presence of two distinct populations, namely, "round" and "tear"-shaped microgels, distributed in a roughly equal proportion (Fig. 3(b)); 54.5% the former, 45.5% the latter. The diameters of the "round" microgels (81.8 ± 1.0 μm) were comparable to those of polymersomes collected in the 50 mM NaCl solution (100 mOsm/L) and did not vary over time (83.2 ± 1.5 μm). This implies that polymersomes were formed and inflated by negative osmotic pressure in a manner similar to

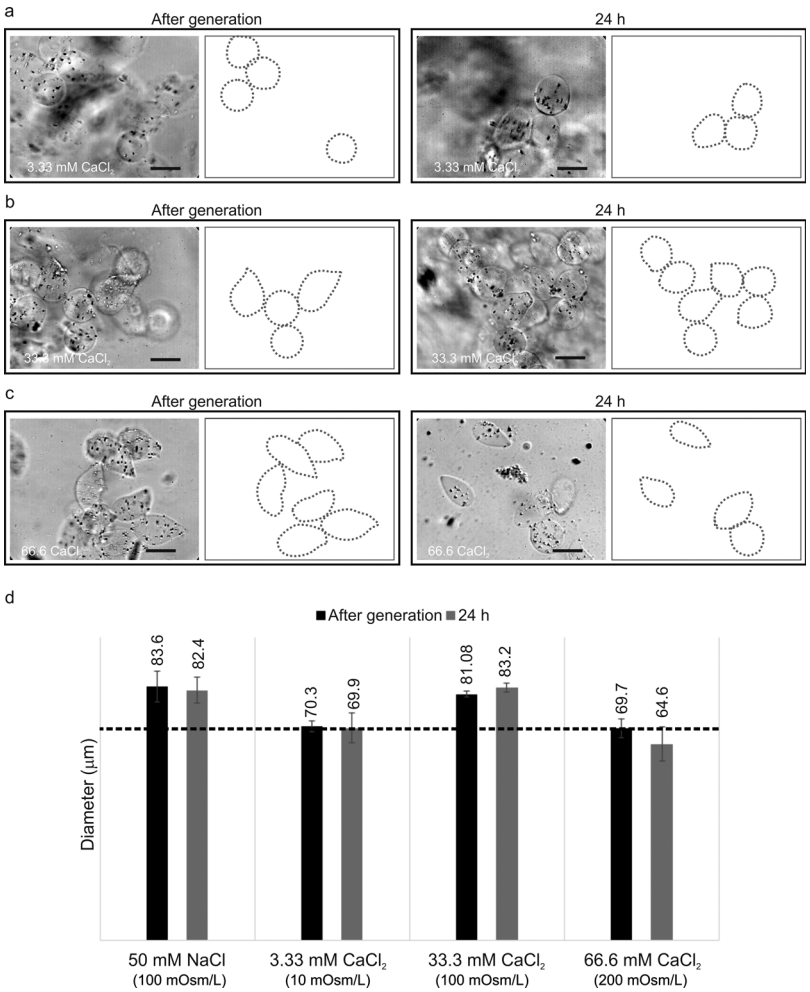

FIG. 3. Microgels formed in $CaCl_2$ solutions. (a) and (c) Contrast-enhanced pictures of microgels formed in $CaCl_2$ solutions from double-emulsion droplets and microgel extracted contours where three different concentrations of $CaCl_2$ are used as collection phase: (a) 3.33 mM $CaCl_2$ produced for the most part "round" microgels, (b) 33.3 mM $CaCl_2$ formed "round" and "tear-shaped" microgels in equal proportion, and (c) 66.6 mM $CaCl_2$ formed tear-shaped microgels with a small number of round ones. Black dots are polystyrene beads dispersed in the inner phase. Scale bars are equal to 70 μm. (d) Diameters of microgels or polymersomes formed in the different collection phases. The black dotted line indicates the inner droplet diameter immediately after double-emulsion generation, which is 69.9 μm $\pm$ 1.5 μm.

that observed for 50 mM NaCl (100 mOsm/l). Some of the inflated polymersomes rupture, forming "tear"-shaped microgels by fast gelation, whereas the cores of stable polymersomes are gelated by a slow flux of Ca^{2+} through the loosened bilayer membrane; this possibly creates microgels enclosed by polymeric bilayer membrane. By contrast, droplets collected in the 66.6 mM $CaCl_2$ solution (200 mOsm/l) become almost exclusively "tear-shaped" microgels (92.6% immediately after collection) with only few "round" microgels whose diameters were equal to 69.9 $\pm$ 3.1 μm just after collection and 64.6 $\pm$ 5.6 μm 24 h after collection (Fig. 3(c)). We attribute the "tear-shape" and small size of such microgels to positive osmotic pressure. The higher osmolarity of the surroundings makes the polymersomes deflate due to an outward flux of water, eventually causing them rupture. Accordingly, the core is suddenly exposed to the Ca^{2+} environment, forming "tear-shaped" microgels of smaller size than the cores of the double emulsion droplets. The microgels formed in 66.6 mM $CaCl_2$ (200 mOsm/l) were transferred into DI water and further investigated (Figs. 4(a) and 4(b). Both "round" and "tear-shaped" entities maintain their size in DI water (clearly visible in the enlargements in Fig. 4), indicating that there is no polymersome membrane and microgels are formed by rupture.

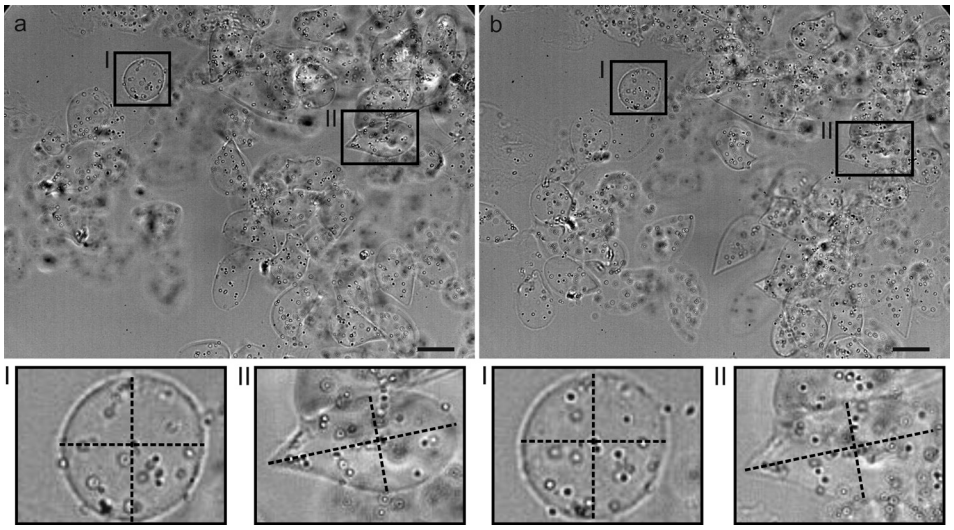

FIG. 4. Gel patches. Gel patches formed in 66.6 mM $CaCl_2$ solution and transferred into DI water using a pair of tweezers, where images are taken immediately after transfer (a) and 1 h after transfer (b). Sub-panels show magnified images of round and tear-shaped microgels. Scale bars are equal to 100 μm.

A comparison of the diameters of polymersomes and microgels formed in NaCl and $CaCl_2$ at different concentrations is presented in Fig. 3(d).

D. Addition of $CaCl_2$ on polymersomes in NaCl solution

To further investigate core gelation and the provision of stable gel core-bilayer shell structures, the core gelation step was separated from the membrane formation step. Polymersomes were produced in a 50 mM NaCl solution (100 mOsm/l), and then subjected to two different conditions of Ca^{2+} feeding. Aqueous solutions of either 1 M (3 Osm/l) or 3.33 mM $CaCl_2$ (10 mOsm/l) were gradually injected into the polymersomes. When the 1 M solution was added, a dramatic increase in the osmolarity of the outer solution causes the polymersomes to shrink and rupture. This produces small microgels by fast gelation, each of which possesses or releases a highly wrinkled polymer membrane (Fig. 5(a)). By contrast, a mild decrease in the osmolarity of the outer medium with introduction of 3.3 mM $CaCl_2$ slowly inflates the polymersomes and allows diffusion of Ca^{2+} through the loosened bilayer membrane. This results in well-defined spherical gel cores enclosed by a polymersome membrane (Fig. 5(b)). It is also noted that some membranes fail to keep their integrity due to a high degree of areal expansion during inflation. As a control, polymersomes collected in 50 mM NaCl solution were exposed to DI water and imaged before addition and 30 min after (Fig. 5(c)). As expected, due to positive osmotic pressure, polymersomes swell to 133% of their initial size with no evidence of gelation.

IV. CONCLUSIONS

We have shown for the first time the generation of PEG-PLA polymersomes with a 1% wt. alginate and 5% wt. PEG core. Leveraging the osmotic-pressure-responsive properties of the bilayer membrane, calcium ions could slowly diffuse into the core of polymersomes (which contain sodium alginate), thereby gelating the alginate within the polymersome. This results in gel core-bilayer shell structures, which are a good mimic of living cells containing responsive membranes. The $CaCl_2$ concentration is revealed to be a critical parameter in maintaining polymeric membrane integrity and determining the final shape of microgels. Interestingly, for polymersomes stored in NaCl solution, slow addition of $CaCl_2$ at low concentrations leads to inflation of the polymersome and an increase in membrane permeability, allowing the diffusion of calcium ions through the membrane and therefore facilitating gelation of the core. In addition, gel patches formed by direct incubation in $CaCl_2$ solution are easily manipulated and

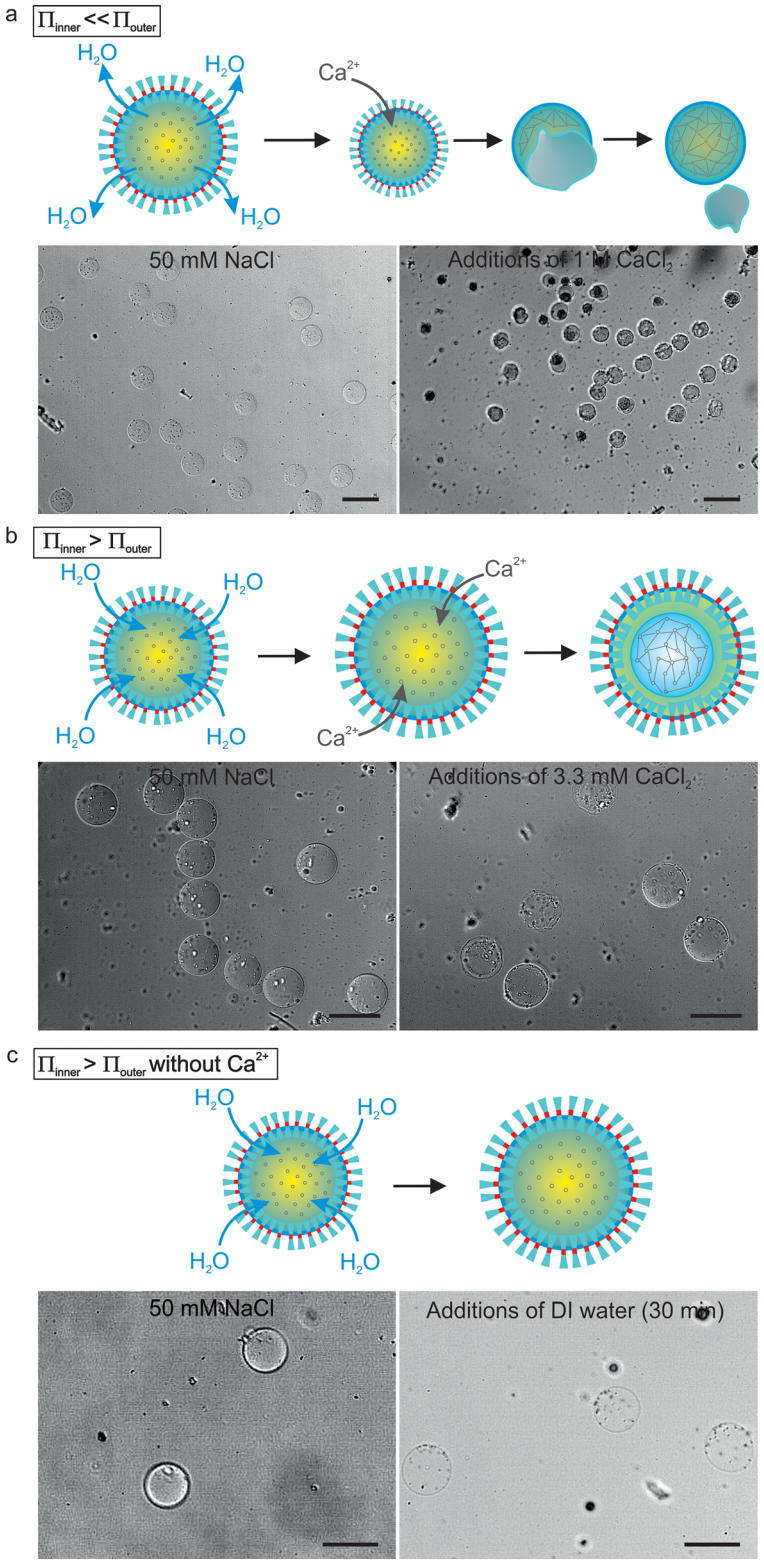

FIG. 5. Response of polymersomes under different conditions. (a) Polymersomes subjected to positive osmotic shock with 1 M $CaCl_2$ show rapid shrinkage and membrane rupture, forming small microgels and a highly wrinkled membrane patch. (b) Polymersomes exposed to mild negative pressure with introduction of 3.3 mM $CaCl_2$ show gentle expansion of membrane and gelation of cores through diffusion of Ca^{2+} through the loosen membrane. (c) Polymersomes exposed to negative pressure with introduction of DI water inflate up to 133% in diameter within 30 min without core gelation. Scale bars are equal to 100 μm.

resistant to mechanical stresses, providing an important feature for applications in cell culture and tissue engineering. Current investigations are focused on estimating membrane integrity and enriching the polymeric shell with active components such as proteins, acting as recognition sites, and magnetic particles, useful for hydrogel manipulation and localization.

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