Artificial Cells

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# Protein Expression, Aggregation, and Triggered Release from Polymersomes as Artificial Cell-like Structures**

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Artificial cells are synthetic compartments that mimic one or more properties of natural cells and provide useful platforms for the study of fundamental cellular functions.[1] Such structures should ideally consist of a robust shell that mimics the cell membrane, with an aqueous interior and exterior. This shell can also contain the machinery for the synthesis of biological molecules, including the production of proteins. Once the biological material has been produced there is also a significant interest in triggering its release, enabling delivery of the material.

Production of these artificial cell-like structures has, to date, proven difficult particularly if there is a need for triggered release. In this respect, the simplest form of artificial cells may be considered a water-in-oil (W/O) or water-in-oil-in-water (W/O/W) emulsion drop. However, while these emulsions provide the requisite spatial confinement of biological materials, the simple emulsions (W/O) lack the correct degree of biocompatibility (as the continuous phase is oil). Similarly, whilst double emulsions (W/O/W) place the artificial cell in an aqueous environment, they have a decreased membrane permeability because of the relatively thick "membrane", which may restrict the applications of such structures.[2–7]

As an alternative membrane for an artificial cell, liposomes or phospholipid bilayer vesicles, produced both in bulk reactors[8,9] and in microfluidic devices,[10–13] hold great promise as mimics for cells,[14] although these materials are highly susceptible to instability, breakage, and oxidation. It is also very difficult to efficiently encapsulate contents into such structures during their formation, although self-organizing pathways have improved this procedure.[15] Some of the limitations of using liposomes can potentially be overcome by creating highly functional polymersomes, where the membrane is formed using a bilayer structure of block copolymers. The enhanced stability and functionality of these structures could expand their applications beyond that of liposomes, such that they could be used as delivery vehicles for drugs and cosmetics.[16]

Polymersomes are therefore structures that hold much promise as artificial cells, and indeed they have already been used for biomimetic studies in which proteins, including both enzymes and transmembrane channels, have been introduced into their polymeric membranes.[17,18] However, conventional approaches to polymersome production (e.g. through electroformation) generate polymersomes with poor control of size and a low efficiency of encapsulation, which has restricted their use as artificial cells.

The limitations associated with the introduction of material within the polymersome shell and the uniformity of their size can both be overcome through the use of a microfluidic technique; herein, we show how such microfludic methods enable the fabrication of highly monodisperse polymersomes that can efficiently encapsulate active materials. These stable cell-like polymersome structures are biocompatible and biodegradable membranes. We also demonstrate encapsulation of the complete biological machinery required for protein expression. Furthermore, we achieve the cell-free expression of a membrane-related bacterial protein, MreB, with high yields in a few hours. During protein synthesis inside the polymersome, a small proportion of the protein migrates and anchors to the polymeric bilayer membrane, because of an interaction between a hydrophobic motif in the protein and the membrane. However, most of the protein remains suspended in the interior of the polymersomes. We also achieve the triggered release of the protein from the polymersomes through an osmotic shock, owing to the semipermeability of the membrane.

To achieve high efficiency of encapsulation of the biological machinery necessary for protein expression within the polymersomes, we use a capillary microfluidic device to produce W/O/W double-emulsion drops that serve as a template for the production of the polymersomes.[19] The microfluidic device consists of two tapered cylindrical capillaries inserted within a square capillary as shown in Figure 1a. The first cylindrical capillary is used for injection of the aqueous innermost phase and is silanized to provide a hydrophobic

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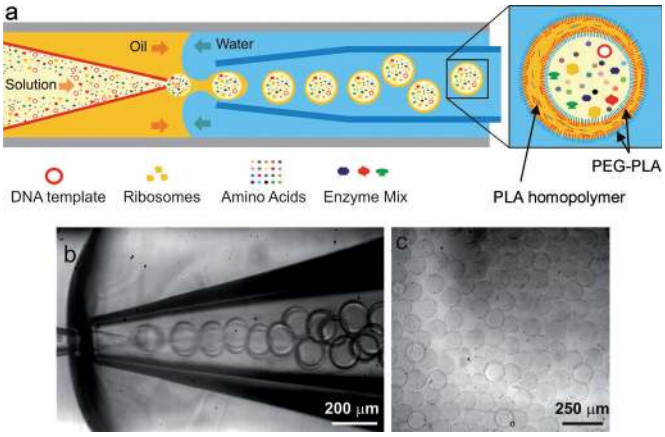


***Figure 1.*** a) Schematic illustrations of the microfluidic capillary device used for polymersome generation and the double-emulsion droplet just after generation. The droplet shows dewetting of amphiphile-laden oil phase on the surface of the innermost drops and separation of the oil, producing polymersomes with a PEG-*b*-PLA membrane; PLA homopolymers are included to strengthen the membrane. b) Optical micrograph showing the generation of W/O/W double-emulsion drops; the flow rates used for the delivery of biological solution, oil, and continuous phase were set respectively at 700, 800, and 3000 $\mu L\,h^{-1}$. c) Optical micrograph of monodisperse polymersomes, which have a mean diameter of 126 μm and a coefficient of variation (C.V.) of 2.7%.

surface; the second cylindrical capillary is used for collection of the double-emulsion drops and is silanized to produce a hydrophilic surface (see Experimental Section for details of the silanes used).

We use the first, hydrophobic capillary to inject an aqueous mixture of the *E. coli* ribosomal extract, required for protein expression, and the MreB DNA plasmid; this forms the innermost drop of the polymersome. As a middle phase, we use a mixture of chloroform and hexane at a volume ratio of 38:62, with 5 $mg\,mL^{-1}$ poly(ethylene glycol)-*block*-poly(lactic acid) (PEG-*b*-PLA; MW 5000 and 10000, respectively) and 2.5 $mg\,mL^{-1}$ PLA homopolymer (MW 15000). The PEG-*b*-PLA diblock copolymer serves as the amphiphile, while the PLA homopolymer is added to enhance the stability of the resultant polymersomes.[20] This mixture is injected through the interstices between the outer square capillary and the inner injection capillary. The continuous phase is an aqueous solution of 10 wt% poly(vinyl alcohol) (PVA, MW 13000–23000) and 0.1 M NaCl and is injected through the interstices between the outer square capillary and inner collection capillary, forming a counter-flow to the innermost and middle phases, as shown in Figure 1a.

Highly monodisperse double-emulsion droplets are produced in a dripping mode by one-step emulsification[20] (Figure 1b and Movie S1 in the Supporting Information). These double-emulsion droplets exhibit dewetting of the middle oil phase on the surface of the innermost droplets as the chloroform partitions out of the droplet; thus, the concentration of hexane, which is a poor solvent of PEG-*b*-PLA diblock copolymers, increases, resulting in expelling the oil layer from two monolayers of diblock copolymers at W/O and O/W interfaces. This dewetting induces a formation of unilamellar structures by overlapping the two monolayers.[19–21] In addition, the dewetted middle oil phase is separated from the innermost drop, producing polymersomes consisting of a bilayer membrane of PEG-*b*-PLA diblock copolymers, with an excess of PLA homopolymer in the inner, hydrophobic region of the bilayer. The dewetting and separation of oil droplets occurs in 5 minutes after droplet formation and because of this, the residual organic solvent in the membrane of the polymersomes becomes very small (below detection limits).

The resultant monodisperse polymersomes encapsulating the aqueous solution for protein expression are collected in a vessel containing 0.15 M aqueous solution of NaCl and incubated in an open vial at room temperature to let the organic solvent on top of the collection liquid evaporate; monodisperse polymersomes are shown in Figure 1c. To express prokaryotic cytoskeletal actin-like protein, MreB, the polymersomes are incubated at 32 °C, allowing the ribosomal machinery to work as schematically illustrated in Figure 2a. During the incubation two fundamental processes happen: DNA is transcribed into messenger RNA (mRNA) and mRNA is translated into polypeptides. The resultant MreB is a protein responsible for organizing the cell-wall synthesis machinery in rod-shaped bacteria and plays an important role in the maintenance of the bacteria's shape.[22–24]

To evaluate the protein expression in the polymersomes, we used a DNA plasmid for an MreB fusion protein with red

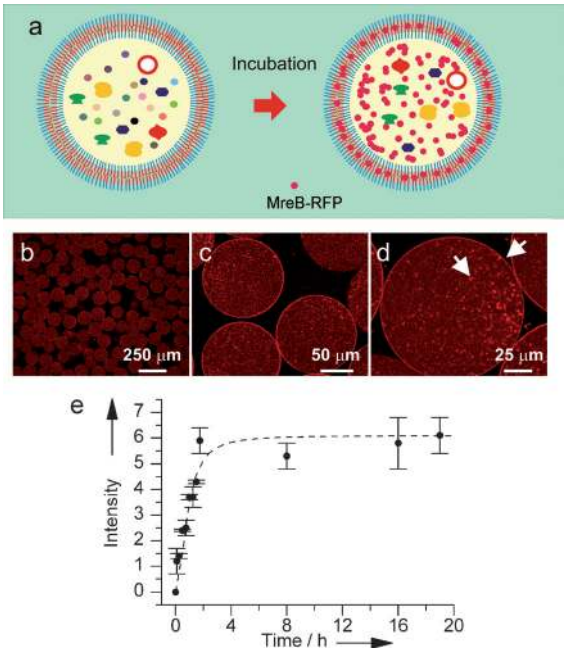


***Figure 2.*** MreB-RFP expression in polymersomes. a) Schematic illustration of a polymersome containing the cell-free protein expression solution. After two hours of incubation at 32 °C, the MreB-RFP protein is produced (red spots). b–d) Confocal microscope images at different magnifications of reinforced PEG-*b*-PLA polymersomes after 3 h of incubation. Arrows indicate the formation of polymerized MreB-RFP patches dispersed in the inner phase and the adhesion of the protein on the membrane. e) Fluorescence signal over time owing to protein expression in polymersomes.

fluorescent protein (MreB-RFP). The resultant MreB-RFP exhibits fluorescence, reaching a maximum after 2 h (Figure 2b,c). The MreB-RFP forms patches in the aqueous core of the polymersomes, with some protein attaching to the membrane (Figure 2d); to confirm that the fluorescence on the membrane is caused by the attachment of MreB-RFP, we observed the empty polymersomes with the confocal microscope under the same conditions as in Figure 2d (Figure S1, Supporting Information). Patch formation can be attributed to the propensity of the MreB to polymerize and form filaments. The adhesion of the proteins to the membrane is due to a helical hydrophobic motif in the MreB proteins, which interacts with the hydrophobic portion of the membrane; addition of PLA homopolymer to the bilayer increases the hydrophobicity of the membrane, thereby increasing the protein adhesion. By contrast, polymersomes composed of only PEG-*b*-PLA diblock copolymers without the additional PLA homopolymer do not exhibit any adhesion of the expressed protein to the membrane (Figure S2 in the Supporting Information). The adhesion of the protein to the hydrophobic membrane is consistent with recent evidence suggesting that the binding activity of MreB to the *E. coli* membrane relies on the presence of hydrophobic residues clustered on one side of an amphiphatic helix of MreB, facilitating interaction between the protein and the membrane.[22]

The kinetics of protein expression are detected by fluorescence measurements on several polymersomes over the time of incubation, where the core and membrane of the polymersomes are included in the measurement. Only ten polymersomes are considered in our measurments. Although not statistically relevant, all the considered polymersomes show almost the same level of fluorescent signal at the same incubation time. In the first hour, the fluorescence signal increases linearly and reaches a plateau after 2 h (Figure 2e). These data are consistent with other kinetic studies performed on protein formation in a cell-free system.[25,6] The polymersomes safely encapsulate the protein for 3 days after incubation. Although polymersomes are composed of biodegradable PLA, hydrolytic degradation does not affect the stability of the polymersomes over a period of two months.[20] Instead, attachment of MreB to the membrane can physically interrupt the bilayer, making them unstable after 3–4 days.

The membranes of the polymersomes are semipermeable, facilitating a technique by which we can trigger the release of the expressed proteins into the continuous phase using osmotic shock.[19,20,26] An osmotic pressure difference between the inner core and continuous phase causes a flux of water through the membrane until the osmotic pressure difference becomes insignificant; this changes the size of the polymersome, causing it to swell. We use negative osmotic shock to form a pore in the membrane as shown in Figure 3a; continuous inward water flux, driven by the large difference in osmolarity, causes an a real strain above the critical value, inducing formation of a pore at the weakest point on the membrane. Thus, when the polymersomes are transferred from 0.15 M aqueous solution of NaCl into deionized (DI) water, the diameter of polymersomes immediately increases, resulting in the formation of single pores in the membranes.

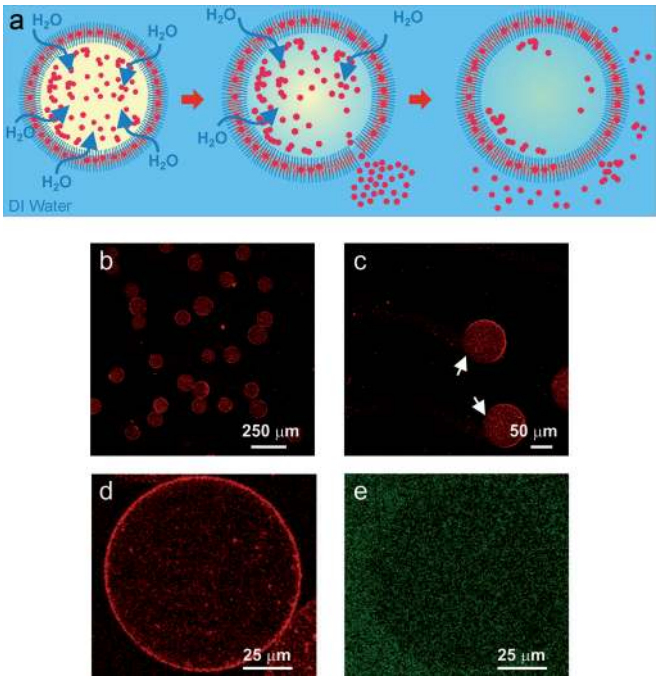


***Figure 3.*** Protein release under osmotic shock. a) Schematic illustration of protein release from polymersomes and subsequent self-sealing of the membrane in DI water. b,c) Confocal micrographs showing different magnifications of polymersomes immediately after their dispersion in DI water. Membrane rupture occurs in a localized region as denoted by arrows in (c). d,e) Dual-channel confocal micrographs of polymersomes after release of proteins. The red signal is from MreB-RFP fluorescence and the green signal is from fluorescein fluorescence.

As the bilayer polymer membrane is strengthened by PLA homopolymers, there is no disintegration of the overall structure. Instead, a pore is produced and the MreB is released into the DI water (Figure 3b,c; arrows denote the plume of protein released from the polymersomes). This release event is synchronized across many polymersomes, reflecting the uniformity in the size and structure of the polymersomes. After protein is released, the interior of the polymersome exhibits significantly reduced fluorescence intensity, whereas the fluorescence intensity at the membrane surface remains constant, owing to the adhesion of the protein on the inner interface of the membrane (Figure 3d).

The fact that some fluorescence remains in the interior implies that not all of the proteins have been released, suggesting that the pore heals as the osmotic pressures are equalized.[27] We confirm that the pore is actually formed in the membrane by adding green dye molecules, fluorescein (MW 332.31), to the DI water and observing that they enter the interior of the polymersome (Figure 3e). The release profile from the polymersomes is shown in Figure S4 in the Supporting Information.

In conclusion, we report the cell-free expression of actin-like structural proteins within polymersomes. The microfluidic technique based on formation of double-emulsion drops enables encapsulation of the protein expression machinery in monodisperse polymersomes without loss of material. The PLA homopolymer-reinforced polymersomes provide

  

a stable compartment for protein expression, while their semipermeability facilitates triggered release of expressed proteins through a negative osmotic shock. The stability, biocompatibility, and biodegradability of the materials provide new opportunities for in vivo drug delivery and regenerative medicine applications, in which in situ production and release of specific proteins may be required. Moreover, polymersome scaffolds are potentially useful as test systems for fundamental studies of systems biology.[28,29] Ultimately, a promising application of this work will be the construction of polymersomes which mimic aspects of the *E. coli* cytoskeleton. This can be achieved by introducing other components of the bacterial cytoskeleton, in combination with peptidoglycan deposits, into the polymersomes, thereby creating a protocell that has a reconstructed intracellular matrix and which might even be able to change shape. To achieve this, we will attempt to further increase the stability of the polymersomes and investigate the MreB-RFP interaction with the membrane using fluorescence recovery after photobleaching (FRAP) analysis.

## Experimental Section

Plasmid preparation: The gene for the fusion protein MreB-RFP with a glycine–serine (GS) linker was PCR-amplified from plasmid pSB1A2_BX(MreB-RFP) (prepared by Miss Yan Chen, Tianjin University) using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, UK) and primers MreB_NT_for CGCGGATCCATGTTGAAAAAATTTCGTGGCA and MreB_NT_rev AGGAAGCCATGCTACCGCTGCCGCTACCCTCTTCGCTGAACAGG. The general protocol for the polymerase was followed with an additional final 30 min extension step at 68 °C. After gel extraction (Qiagen, UK) the PCR products were used in pEXP5-NT/TOPO Cloning reactions (Invitrogen, UK) to obtain the plasmid pMreB-NT, which contained an N-terminal $His_6$-tagged fusion protein gene MreB-RFP with GS linker.

Preparation of solutions: The innermost aqueous solution for the cell-free protein expression was prepared by mixing 1 μg of plasmid pMreB-NT with the following components from Invitrogen cell-free expression kit (Expressway, Invitrogen, UK): *E. coli* extract (20 μL), T7 enzyme mix (1 μL), methionine (2 μL, 75 mM), amino acids (2.5 μL, 50 mM), reaction buffer (20 μL), feed buffer (25 μL), and RNase-free distilled water (29.5 μL). The solution was kept on ice until it was loaded into the syringe. The middle oil phase was a mixture of chloroform and hexane with a volume ratio of 32:68 with 5 mg mL$^{-1}$ of PEG-*b*-PLA (MW 5000 and 10000, respectively; Polyscience, Inc., US) and 2.5 mg mL$^{-1}$ of PLA (MW 15000; Polyscience, Inc., US). The continuous phase was an aqueous solution of 10 wt% PVA (Aldrich, US) and 0.1 M NaCl with 300 mOsm L$^{-1}$.

Formation of polymersomes and expression of MreB-RFP: Monodisperse polymersomes were prepared from double-emulsion drops using a glass capillary device. Two cylindrical glass capillaries of 1 mm in outer diameter (World Precision Instruments, Inc., 1B100-6) were tapered and assembled within a square capillary of 1.05 mm inner dimension (AIT glass) as shown in Figure 1a; prior to assembly, the cylindrical injection capillary was treated with n-octadecyltrimethoxyl silane (Aldrich, US) to make it hydrophobic while the cylindrical collection capillary was treated with 2-[methoxy(polyethyleneoxy)propyl]trimethoxyl silane (Gelest, Inc., US) to make it hydrophilic. The innermost, middle, and continuous phases were injected into the device at flow rates of 700, 800, and 3000 μL h$^{-1}$, respectively. The double-emulsion drops were prepared by one-step emulsification and collected in an aqueous solution of 0.15 M NaCl, resulting in polymersomes through dewetting and subsequent separation of the oil phase. The generation of double-emulsion drops was observed using a high-speed camera (Phantom V9, Vision Research, US). After generation of the polymersomes, the suspension was left at room temperature for 1 hour and then incubated for a few hours at 32 °C. The expression of MreB-RFP was evaluated by a confocal microscope (Leica, TCS SP5). For triggered release of proteins, small drops of polymersome suspension were transferred to DI water, thereby creating a negative osmotic shock, and the release behavior was monitored using confocal microscopy. All images were analysed using ImageJ software.

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