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Giant Coacervate Vesicles as An Integrated Approach to Cytomimetic Modelling

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ABSTRACT: Although giant unilamellar vesicles (GUVs) have been extensively studied as synthetic cell-like micro-compartments, their applicability as cytomimetic models is severely compromised by low levels of membrane permeability, low encapsulation efficiencies and high physicochemical instability. Here, we develop an integrated cytomimetic model comprising a macromolecularly crowded interior with high sequestration efficiency and enclosed within a phospholipid membrane that is permeable to molecules below a molecular weight cut-off of *ca.* 4 kDa. The protocells are readily prepared by spontaneous assembly of a phospholipid membrane on the surface of preformed polynucleotide/polysaccharide coacervate micro-droplets and are designated as giant coacervate vesicles (GCVs). Partial anchoring of the GCV membrane to the underlying coacervate phase results in increased robustness, lower membrane fluidity and increased permeability compared with GUV counterparts. As a consequence, enzyme and ribozyme catalysis can be triggered in the molecularly crowded interior of the GCV but not inside the GUVs when small molecule substrates or inducers are present in the external environment. By integrating processes of membrane-mediated compartmentalization and liquid-liquid microphase separation, GCVs could offer substantial advantages as cytomimetic models, synthetic protocells and artificial biomolecular microreactors.

INTRODUCTION

Protocells are encapsulated soft microsystems capable of diverse biomimetic functions such as molecular compartmentalization, *in vitro* gene expression and proto-metabolism, and are therefore of special interest in studies on the origin of life, development of embodied constructs in synthetic biology and fabrication of small-scale devices in bioengineering and biomedicine.^{1, 2} Protocells can be assembled from lipids (vesicles),³ amphiphilic block copolymers (polymersomes),^{4, 5} inorganic nanoparticles (colloidosomes),^{6, 7} protein-polymer nano-conjugates (proteinosomes),^{8, 9} and polyelectrolytes undergoing liquid-liquid microphase separation (coacervation).¹⁰⁻¹⁵ Amongst these possibilities, lipid vesicles have been used extensively for studying membrane transport,¹⁶ macromolecular loading,¹⁷ DNA transcription,¹⁸ protein expression,¹⁹ molecular signaling²⁰ and the origin of life.²¹

Giant unilamellar vesicles (GUVs) are often employed as a cytomimetic model system due to the ease of tailoring the composition and structure of their phospholipid bilayer membrane as well as their size, shape, growth, fusion and attendant mechanical/chemical properties.^{22, 23} As a protocell model, GUVs are principally disadvantaged by the absence of a molecularly crowded chemically enriched interior, which is a hallmark of cellular organization.^{24, 25} In this respect, the spontaneous liquid-liquid phase separation and chemical enrichment in water of counter-charged polyelectrolytes into molecularly crowded microdroplets (coacervates) provides both a mechanism of prebiotic membrane-free compartmentalization^{26, 27} and a simple

model for the formation of certain organelles within cellular environments.^{28, 29} Recently, the spontaneous self-assembly of a continuous fatty-acid membrane at the surface of preformed coacervate microdroplets was used to develop a hybrid protocell model comprising both a continuous membrane boundary and molecularly crowded interior.^{30, 31} However, the single chain structure of fatty acids and their weak intermolecular interactions result in inefficient encapsulation of the coacervate phase and low levels of stability in the resulting membrane-bounded micro-compartments, thereby limiting the potential for development of these hybrid constructs in cytomimetic studies and applications. Other studies on the hierarchical self-assembly of copolymer membrane-stabilized coacervate protocells,^{32, 33} surfactant-encapsulated polyelectrolyte micelles,^{34, 35} and liposome-stabilized all-aqueous emulsions³⁶ have demonstrated increases in the robustness of synthetically prepared micro-compartments, but these models tend to have only a weak correspondence with the structure of living cells.

To address this cytomimetic challenge, here we develop a membrane-bounded macromolecularly crowded protocell model based on the spontaneous self-assembly of the two-chain zwitterionic phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) on the surface of preformed deoxyribonucleic acid/diethylaminoethyl-dextran (DNA/DEAE-dextran) membrane-free coacervate microdroplets. By using phospholipids instead of fatty acids our aim is to spontaneously assemble giant coacervate vesicles (GCVs) with increased robustness compared with

their water-filled counterparts, GUVs. We show that the GCV membrane exhibits a disordered bilayer arrangement with lower membrane fluidity and increased semi-permeability compared with GUVs prepared from the same phospholipid. In addition, we use an external glucose input to trigger an endogenous glucose oxidase (GOx) enzyme-mediated cascade within the coacervate interior of the GCVs, and exploit Mg^{2+} ion incursion to trigger a ribozyme cleavage reaction within the macromolecularly crowded protocell. Our approach opens the possibility of developing the GCVs as primitive cell-like constructs and provides a step towards new types of biomimetic micro-reactors for potential use in bioengineering, drug delivery, diagnostics and sensing.

RESULTS and DISCUSSION

Construction and characterization of giant coacervate vesicles. Preparation of GCVs involved a two-step procedure (Figure 1A). Turbid aqueous suspensions of coacervate microdroplets were prepared at pH 7.0-9.0 by direct mixing of aqueous solutions of DNA (low molecular weight, salmon sperm) and DEAE-dextran (Mw 500 kDa) under a range of polyelectrolyte weight ratios (Figure 1B). Zeta potential measurements indicated that positive, neutral and negative charged droplets could be produced by changes in the charge ratio (Figure 1C), which also corresponded to modifications in the average droplet size (Figure 1D). Typically, droplets with a mean diameter of $9.3 \pm 3.4 \mu m$ were produced under close to charge neutral conditions. For GCV assembly, we used positively charged

Figure 1. Design and fabrication GCV-based protocells. (A) Mixing of anionic DNA and cationic DEAE-dextran gives rise to liquid-liquid phase separation and the formation of coacervate microdroplets (step 1). Subsequent addition of a DPPC ethanol solution leads to encapsulation of the coacervate droplets and formation of a population of phospholipid-coated molecularly crowded GCVs (step 2). (B) Partial phase diagram for DNA/DEAE-dextran liquid-liquid phase separation based on optical transmittance (Trans., 420 nm) measurements recorded at different polyelectrolyte concentrations. A concentration-dependent contour line is shown. Coacervation (minimal transmission) was predominant at charge ratios close to the isoelectric point curve (data points, h-j; DNA/DEAE-dextran weight ratio = 0.47). (C, D) Zeta potentials (C) and corresponding mean hydrodynamic diameters (light scattering) (D) of DNA/DEAE-dextran coacervate microdroplets produced at different polyelectrolyte weight ratios (0.3-0.6, w/w). (E) Bright field (left) and corresponding fluorescence microscopy image (right) of positively charged coacervate microdroplets used for GCV construction. Droplets are stained with Hoechst dye. (DNA, 5.6 mg·mL⁻¹, 1 mL; DEAE-dextran, 14 mg·mL⁻¹, 1 mL; DNA: DEAE-dextran = 0.4 w/w; +10.3 $\pm$ 2.7 mV; see data point c in B-D). Scale bars, 25 μm . (F) Plot of zeta potentials for GCVs prepared at various

DPPC content (φ = DPPC: coacervate, w/w) showing reversal of the droplet surface charge with increasing DPPC content. (G) Bright field (left) and corresponding fluorescent microscopy image (right) of DNA/DEAE-dextran coacervate droplets after addition of DPPC (5.0 wt%) and staining with dye Dil showing a continuous phospholipid outer membrane (red fluorescence). Scale bars, 5 μm . (H) Bright field (left) and corresponding 2D (centre) and reconstructed 3D (right) fluorescence confocal microscopy images of a single GCV. Scale bars, 5 μm .

DNA/DEAE-dextran droplets (zeta potential = $+10.3 \pm 2.7$ mV) prepared at a DNA: DEAE-dextran weight ratio of 0.4 to facilitate assembly of DPPC at the droplet surface. Bright field and fluorescence microscopy images showed discrete spherical droplets with mean size of 6.0 ± 4.3 μm that exhibited homogeneous blue fluorescence when stained with a Hoechst dye (Figure 1E). Addition of a DPPC ethanol solution to the freshly prepared coacervate suspension, followed by aging for 12 h in a refrigerator, resulted in a progressive reduction in the positive surface charge of the microdroplets that ultimately led to charge reversal with a zeta potential of approximately -8 mV at a DPPC content of 5 wt% (Figure 1F). We attributed the change in surface charge to electrostatically mediated adsorption of the zwitterionic DPPC molecules on the droplet surface. This was in agreement with confocal microscopy images of DPPC-treated droplets that revealed a bright red fluorescence outer shell when stained with the lipophilic lipid bilayer probe Dil (Figure 1G). 2D and reconstructed 3D fluorescence images on single droplets indicated that the phospholipid coating was uniform and continuous (Figure

1H), confirming encapsulation of the coacervate phase and spontaneous reconfiguration of the droplets into GCVs.

Small angle X-ray scattering (SAXS) studies on the uncoated DNA/DEAE-dextran coacervate phase showed no evidence for long-range mesostructural order (Figure S1A). In contrast, SAXS profiles from the DPPC-encapsulated microdroplets showed two Bragg reflection peaks at 0.11 and 0.21 $\text{\AA}^{-1}$ that were assigned to first and second order reflection peaks of a lamellar phase with a d spacing of 5.7 nm, consistent with the formation of a phospholipid bilayer membrane.³⁷ This was also in agreement with transmission microscopy images of the coated droplets that revealed a dense interior enclosed by a membrane with a thickness of ca. 6.3 ± 0.8 nm (Figure S1B).

In general, spontaneous assembly of the GCVs was dependent on the adsorption of phospholipid monomers from an ethanolic DPPC solution onto the surface of the positively charged coacervate droplets. Use of the ethanolic solution provided relatively high concentrations of DPPC monomers that facilitated interactions with the coacervate polyelectrolytes. In contrast, attempts to prepare GCVs from DPPC aqueous solutions at concentrations above the

Figure 2. FRAP measurements of membrane fluidity. (A, B) Time series of confocal fluorescence images of a single GCV membrane doped with NBD-PE (A) and corresponding time-dependent changes in membrane fluorescence intensity (B) recorded before (left), 10 s after the onset of photo-bleaching (middle, high laser power) and 20 min after recovery (right, low laser power). The photo-

bleached area is delineated by the red box in (A) and corresponds to the red plot in (B). The blue box and blue plot shown in (A) and (B) respectively, correspond to a control region of the membrane (un-bleached). Scale bars, 5 μm . (C, D) As for (A, B) but for a single GUV; note the considerably shorter recovery time due to increased membrane fluidity. Scale bars, 5 μm . (E, F) Comparison between membrane diffusion coefficients (D) (E, FRAP data) and fluorescence polarization values (F) for GCV and GUV membranes. (G) Schematic illustration of vesicle organization and influence on membrane fluidity in GCV (left hemisphere, low mobility) and GUV (right hemisphere, high mobility) cytomimetic models.

critical micelle concentration (4.6×10^{-10} M) were not successful and resulted in the formation of liposomes that subsequently adsorbed onto the coacervate droplet surface (Figure S2). Other experiments indicated that low yields of GCVs could be prepared using ethanolic DPPC and negatively charged (-10.0 mV) DNA/DEAE-dextran coacervate droplets but the vesicles were often only partially coated and contained coacervate aggregates (Figure S3). This suggested that interactions between DEAE-dextran and the negatively charged diphosphoester group of zwitterionic DPPC were primarily responsible for high fidelity membrane assembly at the coacervate/water interface.

Membrane fluidity and permeability of giant coacervate vesicles. We performed fluorescence recovery after photobleaching (FRAP) experiments using the membrane fluorophore 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) to determine the fluidity of the GCV membrane. Photobleaching a small section of the DPPC membrane of a single GCV gave rise to a relatively slow recovery of fluorescence intensity compared with similar measurements recorded for the DPPC membrane of a water-filled GUV (Figures 2A-D). The GCV membrane fluorescence intensity slowly increased to 65% of its original value over a period of 20 min while the same recovery level required 25 s for the photobleached GUV

Figure 3. Molecular permeability studies. (A) Confocal fluorescence microscopy images of single GCVs (top row) and GUVs (bottom row) incubated in aqueous solutions of Nile red (lipophilic/hydrophilic, red fluorescence), propidium iodide (PI, cationic, DNA intercalation, pink fluorescence) or calcein (anionic, DEAE-dextran binding, green fluorescence). The three solutes penetrate the GCV membrane and are sequestered in the DNA/DEAE-dextran coacervate interior but remain excluded from the GUV lumen. Nile red binds to both the GCV and GUV lipid membranes. Scale bars, 2 μm . (B) Time-dependent changes in the intra-GCV PI concentration after addition of PI to a suspension of uncoated DNA/DEAE-dextran coacervate droplets ([DPPC], $\phi = 0$ wt%) or DPCC-coated

coacervate droplets prepared at different lipid concentrations ($\phi = 1.0$ and 5.0 wt%). Inset shows initial rates of PI influx. DNA-binding enhancement of PI fluorescence is used to determine the rate of molecular transport rate. (C) As for A but after exposure to FITC-dextran (green fluorescence) with average molecular weights of 4 (left), 40 (middle), and 120 kDa (right); in each case, the solutes are impermeable to the GCV and GUV membranes. Scale bars, $2\ \mu\text{m}$. (D) Plot of guest partition coefficients (ratio of fluorescence intensity inside GCV to fluorescence intensity outside GCV) against guest molecular weight showing membrane transport cut-off at *ca.* 4 kDa. Data points are for Nile red, PI, calcein, FITC-dextran (4 kDa), FITC-dextran (40 kDa), and FITC-dextran (120 kDa) and collected on single GCVs ($n = 3$). (E) Schematic illustration of vesicle organization and influence on membrane permeability in GCV (left hemisphere, high permeability) and GUV (right hemisphere, low permeability) cell-like models. Modelling the FRAP curves with Fick's second law in a two-dimensional plane (Figure S4), gave estimated diffusion coefficients (D) of $1.3\ \mu\text{m}^2\cdot\text{s}^{-1}$ and $7.6\ \mu\text{m}^2\cdot\text{s}^{-1}$ for fluorescent NBD-PE molecules present in the GCV and GUV, respectively (Figure 2E), indicating that lipid mobility was greatly inhibited in the GCV membrane compared with the free-standing GUV counterpart. This was consistent with fluorescence polarization (FP) experiments in which 1,6-diphenyl-1,3,5-hexatriene (DPH) was inserted into the hydrophobic lipid membrane and used as a FP probe of the membrane fluidity.³⁸ Corresponding FP values for the GCV and GUV membranes were 0.19 and 0.09 , respectively, (Figure 2F), consistent with decreased fluidity of the GCV membrane. We speculate that the decrease in membrane fluidity arises from charge and polar interactions between DPPC molecules present on the inner leaflet of the membrane and DNA/DEAE-dextran complexes at the surface of the encapsulated coacervate phase that result in anchoring of the lipid molecules to the molecularly crowded interior (Figure 2G). Similar decreases in diffusion coefficients have been reported for planar-supported lipid membranes in comparison to free-standing GUVs.³⁹⁻⁴¹

Confocal fluorescence microscopy was used to assess the permeability of the GCV membrane to guest molecules of different charge and size. Incubation of the GCVs in solutions of Nile red (neutral), propidium iodide (PI, cationic) or calcein (anionic) resulted in high levels of fluorescence throughout in the DNA/DEAE-dextran coacervate core, consistent with passive uptake of the small molecules across the DPPC shell (Figure 3A). The results were consistent with observations obtained for the membrane-free coacervate micro-compartments (Figure S5) except that Nile red was also bound to the membrane of the GCVs (Figure 3A). In contrast, the GUV membrane was essentially impermeable to the three dyes with Nile red strongly bound to the phospholipid bilayer (Figure 3A). Progressive decreases in the rate of PI influx (R) were observed as the amount of DPPC used to prepare the GCVs was increased between 0 and 5 wt% ($R = 0.60$ and $0.48\ \text{mM}\cdot\text{s}^{-1}$, respectively), consistent with an increasing sealing of the coacervate droplets with the phospholipid membrane (Figure 3B and Figure S6). In contrast to the above results, FITC-dextran of different molecular weights (4 , 40 or 120 kDa) was largely excluded from the interior of both the GCVs and GUVs (Figure 3C), although the polysaccharides were readily sequestered in the membrane-free DNA/DEAE-dextran coacervate droplets (Figure S5).

Taken together, the results indicated that the GCVs were enclosed by a semi-permeable membrane with a molecular cut-off of around 4 kDa (Figure 3D) and were therefore significant more permeable to small molecules compared

with the GUVs. One possibility is that anchoring of the DPPC molecules to the underlying coacervate phase results in increased numbers of structural defects in the phospholipid membrane that remain unannealed because of the relatively strong membrane-matrix interactions (Figure 3E). Given these observations, dynamic and reversible transfer of guest molecules between different GCVs populations could be achieved by juxtaposing single populations of PI-containing GCVs and non-fluorescent GCVs and monitoring the movement of the small molecular dye through the non-fluorescent population as PI was released and then re-captured (Figure S7). In contrast, minimal levels of macromolecular transfer occurred between adjacent populations with or without sequestered FITC-dextran (40 kDa) (Figure S8).

Enzyme cascade reactions in GCV microreactors. Cytomimetic micro-reactors capable of compartmentalized biomolecular transformations were developed by exploiting the membrane semi-permeability and internal molecular crowding of the GCVs for the selective mass exchange and accumulation of components used in enzyme and RNA-mediated catalysis. As proof of principle, we co-sequestered glucose oxidase (GOx), horseradish peroxidase (HRP) and Amplex red within the DNA/DEAE-dextran coacervate micro-droplets prior to DPPC membrane encapsulation and triggered the production of hydrogen peroxide within the GCVs by addition of glucose to the external environment (Figure 4A). Diffusion of glucose across the GCV membrane resulted in an almost immediate increase in the fluorescence intensity recorded at $590\ \text{nm}$ due to the HRP-mediated formation of resorufin (Figure 4B). Typically, approximately $0.8\ \mu\text{M}$ of Amplex red was converted into resorufin within $1200\ \text{s}$ of adding $20\ \text{mM}$ glucose but this could be attenuated by *in situ* decomposition of the released hydrogen peroxide by co-sequestration of catalase within the GCVs (Figure 4B). In contrast, no apparent fluorescence enhancement was observed for GUVs prepared under the same conditions due to the low permeability of glucose across the phospholipid bilayer of the water-filled vesicles (Figure S9). Localization of the cascade reaction specifically within the interior of the GCVs and retention of the reaction product within the protocell were confirmed by time-dependent confocal laser scanning microscopy (CLSM) (Figure 4C). Corresponding line profiles of the fluorescence intensity across single GCVs at different times after addition of glucose showed a homogeneous distribution of resorufin in the coacervate phase that increased progressively over $1200\ \text{s}$ (Figure 4D). A sharp cut-off in the fluorescence intensity was observed at the surface of the GCV. Measurements of the mean fluorescence intensity inside and outside the GCVs

confirmed that minimal amounts of resorufin were released into the external environment (Figure 4E). Steady state kinetic analyses in the presence of varying glucose concentrations yielded a series of enzyme activity plots (Figure S10), which were fitted to the Michaelis-Menten model with values for the Michaelis constant (K_m) and turnover number (k_{cat}) of 70 mM, and 1180 s^{-1} , respectively (Figure 4F). In contrast, the values of K_m and k_{cat} determined in bulk PBS buffer were 40 mM and 1710 s^{-1} , respectively,

indicating that the activity of the cascade reaction was attenuated inside the GCVs possibly due to a decrease in the glucose binding efficiency or partial deactivation of the enzymes in the presence of the high polyelectrolyte concentrations associated with the coacervate phase. In part, these deleterious effects were alleviated by the high partitioning constants for the enzymes within the DNA/DEAE-dextran coacervate ($K(GOx) = 109$; $K(HRP) = 210$).

Figure 4. Enzyme cascade reactions in GCV microreactors. (A) Schematic illustration of a GCV containing encapsulated GOx and HRP along with the non-fluorescent substrate Amplex red. Uptake of glucose through the semi-permeable membrane triggers the cascade reaction within the coacervate interior to produce red fluorescent resorufin via a hydrogen peroxide (H_2O_2) intermediate. D-(+)-Gluconic acid δ -lactone (GDL) is also produced. Co-encapsulation of catalase (CAT) in the GCV consumes H_2O_2 and inhibits the GOx/HRP cascade. (B) Time-dependent fluorescence response (resorufin concentration) of a GOx/HRP-containing GCV suspension (9.8 mg mL^{-1}) after addition of glucose. GOx $1.0 \text{ } \mu\text{g}\cdot\text{mL}^{-1}$, HRP $0.5 \text{ } \mu\text{g}\cdot\text{mL}^{-1}$, Amplex red $2 \text{ } \mu\text{M}$. Blank (black): absence of glucose as control; Glu(+): 10 mM glucose (blue); Glu(++): 20 mM glucose (grey); Glu(++)/CAT, 20 mM glucose, $0.05 \text{ } \mu\text{g}\cdot\text{mL}^{-1}$ catalase (red). Insets: Photographs of a GCV suspensions before (bottom) and after (top) addition of glucose showing oxidation of Amplex red to resorufin (pink color). (C) Time-dependent CLSM fluorescence images of a single GCV showing increase in red fluorescence throughout the coacervate interior of the protocell over a period of 1200 s. Conditions as in (B) with [glucose] = 20 mM. The reaction product (resorufin) accumulates in the molecularly crowded coacervate interior of the GCV. Scale bar, 5 μm . (D, E) Time-dependent fluorescence intensity (grey values) profiles (D) and plot of changes in mean fluorescence recorded inside and outside (E) of the GCV that is displayed in (C). (F) Plot of initial rates against glucose concentration for GOx/HRP cascade reactions within GCVs (red plot) or in bulk PBS buffer (black plot). GOx $1.0 \text{ } \mu\text{g}\cdot\text{mL}^{-1}$; HRP $0.5 \text{ } \mu\text{g}\cdot\text{mL}^{-1}$; Amplex red $2 \text{ } \mu\text{M}$. 10 mM pH=7.5 PBS. In both cases, Michaelis-Menten kinetics are used to fit the data.

Ribozyme activity in GCV microreactors. Given the above observations, we developed the GCVs as cell-like

micro-reactors capable of undertaking ribozyme-mediated cleavage reactions within the molecularly crowded

DNA/DEAE-dextran coacervate interior (Figure 5A). To achieve this, we co-sequestered a tetramethyl rhodamine (TAMRA)-labelled hammerhead ribozyme (HHRib) and a cognate fluorescein (FAM)-labelled RNA strand substrate into the coacervate droplets to produce a donor-acceptor FRET complex that was then encapsulated within the GCVs after membrane assembly. As HHRib catalyzes the cleavage of the substrate strand in the hybridized complex in the presence of Mg^{2+} (Figure S11)⁴²⁻⁴⁴ we initiated ribozyme activity in the GCVs by adding Mg^{2+} ions to the continuous aqueous phase and monitoring changes in FAM-donor fluorescence at 518 nm (Figure 5B). Significant increases in the FAM-labelled RNA substrate fluorescence were observed within a few minutes after addition of Mg^{2+} ions at concentrations between 5-20 mM. The fluorescence enhancement was Mg^{2+} concentration-dependent and at 20 mM was approximately 4.5-fold higher than in the absence of Mg^{2+} ions (Figure 5B). Corresponding confocal fluorescence microscopy images indicated that the addition

of Mg^{2+} ions elicited a bright green fluorescence specifically within the coacervate phase, confirming ribozyme activity within the GCVs (Figure 5C). In contrast, no changes in FAM-donor fluorescence were observed in control experiments undertaken with GUVs containing the HHRib/RNA complex due to the impermeability of the vesicle membrane (Figure S12).

Plots of initial reaction rates against Mg^{2+} concentration indicated that the ribozyme activity was enhanced within the GCVs compared with catalysis in free buffer solution (Figure 5D) with turnover numbers (k_{cat}) of $1.7 \times 10^{-6} s^{-1}$ and $1.2 \times 10^{-6} s^{-1}$, respectively. The enhancement in ribozyme activity within the GCVs was confirmed by semi-quantitative polyacrylamide gel electrophoresis (PAGE) which showed well-delineated bands for the RNA substrate before and after cleavage in the presence of Mg^{2+} ions (Figure 5E). Time-dependent PAGE profiles of the reaction undertaken inside the GCVs in the presence of 20 mM Mg^{2+} showed a clear band for the cleavage strand after 10 min

Figure 5. Ribozyme activity in GCV microreactors. (A) Schematic illustration of a Mg^{2+} -triggered ribozyme-mediated strand cleavage reaction within the coacervate interior of a single GCV. A hybridized HHRib/RNA complex is encapsulated within the GCV as a TAMRA (acceptor, red)/FAM (donor, green) FRET pair and Mg^{2+} ions are added to the external environment (left). Uptake of Mg^{2+} results in folding of the complex to initiate cleavage of the substrate and dissociation of the TAMRA/FAM FRET pair within the GCV. Cleavage is monitored by measuring the time-dependent changes in FAM-donor fluorescence enhancement at 518 nm. Sequences for HHRib and the RNA substrate are shown. (B) Fluorescence spectra and time-dependent increases in FAM-labelled cleavage strand (inset) after addition of different amounts of Mg^{2+} (0-20 mM) to a suspension of GCVs ($9.8 \text{ mg} \cdot \text{mL}^{-1}$) containing a FRET-paired TAMRA-HHRib/FAM-RNA complex. A low level of green fluorescence is observed in the absence of Mg^{2+} due to intrinsic ribozyme activity. (C) Fluorescence confocal microscopy

images of a population of TAMRA-HHRib/FAM-RNA-containing GCVs in the absence of Mg^{2+} after 60 min (left) and 60 min after addition of Mg^{2+} (20 mM) (right) showing homogeneous distribution of high intensity FAM fluorescence specifically within the protocells. Weak green fluorescence is observed in the absence of Mg^{2+} (left) due to intrinsic ribozyme activity. (D) Plot of initial rates of strand cleavage against Mg^{2+} concentration for HHRib ribozyme activity reaction within GCVs (10 mM, pH=7.4, PBS buffer or in bulk PBS buffer. HHRib 1.0 μ M, substrate strand 3.0 μ M, Mg^{2+} ions 0-20 mM. (E) Denatured PAGE analysis of ribozyme activity in a population of GCVs in the absence (left column) or presence of Mg^{2+} ions (20 mM, after 50 min, right column). Top and bottom rows show native RNA substrate and cleavage strand, respectively. (F, G) Non-denatured PAGE gels showing time-dependent changes in the concentrations of the native (top row) and cleaved (bottom row) substrate for HHRib catalysis in GCVs (F) or bulk PBS buffer (G). Reactions undertaken for 50 min with 20 mM Mg^{2+} ions. (H) Plots of fraction of cleaved substrate produced with time for ribozyme reactions conducted in GCVs (black line), and in bulk PBS buffer (red line). Substrate fractions were determined from PAGE analysis.

that increased in intensity progressively over 50 min (Figure 5F). This was accompanied by a concomitant decrease in the intensity of the uncleaved substrate band, which reached a negligible level after 40 min. In contrast, PAGE analysis of ribozyme activity in PBS under the same conditions showed a slower increase in the intensity of the cleavage strand with significant amounts of the uncleaved RNA remaining after 50 min (Figure 5G). Quantitative analysis revealed that over 90% of the substrate was cleaved in the GCVs after 50 min while only 42% was observed in free buffer over the same time period (Figure 5H). This corresponded to approximately a 2.2fold increase in ribozyme activity within the molecularly crowded interior of the GCVs.

Taken together, the results indicate that diffusion of Mg^{2+} ions across the GCV membrane is facile and Mg^{2+} binding to the HHRib/RNA complex is effective in the DNA/DEAE-dextran coacervate medium. More specifically, the changes in folding required for ribozyme-mediated cleavage and dissociation of the FRET pair remain accessible even in the molecularly crowded environment of the GCV interior. Moreover, ribozyme activity within the GCV is accelerated in the coacervate phase, in agreement with previous studies on molecular crowded systems.⁴⁵⁻⁴⁸ It seems feasible therefore that molecular crowding in the GCV interior increases the stability of compact conformations of the ribozyme/substrate complex, which in the case of HHRib corresponds to the active state.⁴⁶⁻⁴⁸

CONCLUSION

In conclusion, a new protocell model with selective membrane permeability and macromolecular crowded interior is demonstrated based on the spontaneous coating of polynucleotide/polysaccharide coacervate micro-droplets with a thin phospholipid shell. The hybrid micro-compartments are designated as “giant coacervate vesicles” and their cytomimetic properties are compared and contrasted with water-filled GUV counterparts. The GCV membrane exhibits lower fluidity and increased permeability compared with GUVs, which we attribute to anchoring of the DPPC membrane to the underlying coacervate medium and associated structural defects and discontinuities in the outer shell. As a consequence, the GCVs are responsive to small molecules in the external environment such that enzyme and ribozyme catalysis can be triggered in the molecularly crowded compartments but not inside the GUVs. Moreover, the high sequestration

potential of the coacervate interior facilitates high levels of molecular and macromolecular (MW < 4 kDa) uptake and retention in the GCVs, which is not possible with the GUV protocell model. This enhanced partitioning could offer substantial advantages in bioreactor construction, particularly for cytomimetic systems where macromolecular crowding in nearly identical internal microenvironments is desirable across a population of bioreactors. We also note that preparation of the GCVs is highly reproducible and facile, and that the hybrid protocells are considerably more mechanically robust compared with GUVs that are notoriously unstable. Thus, we expect GCVs to be useful in a range of bioengineering applications such as prototissue construction, design of artificial intracellular organelles and fabrication of reversible swelling micro-devices.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge via the Internet at <http://pubs.acs.org>.

Further experimental details, methods, and figures.

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