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Article

Architecting Multicompartmentalized, Giant Vesicles with Recombinant Fusion Proteins

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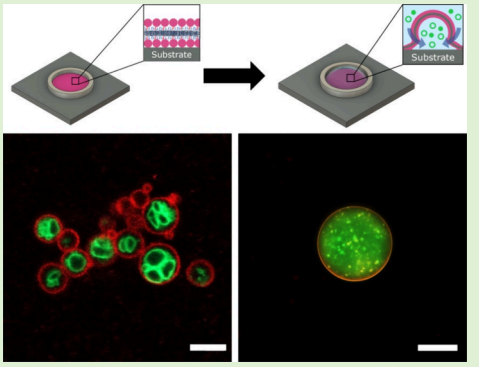
ABSTRACT: We present a straightforward strategy for constructing giant, multicompartmentalized vesicles using recombinant fusion proteins. Our method leverages the self-assembly of globule-zipper-elastin-like polypeptide fusion protein complexes in aqueous conditions, eliminating the need for organic solvents and chemical conjugation. By employing the thin-film rehydration method, we have successfully encapsulated a diverse range of bioactive macromolecules and engineered organelle-like compartments—ranging from soluble proteins and coacervate droplets to vesicles—within these protein-assembled giant vesicles. This approach also facilitates the integration of water-soluble block copolymers, enhancing the structural stability and functional versatility of the vesicles. Our results suggest that these multicompartment giant protein vesicles not only mimic the complex architecture of living cells but also support biochemically distinct reactions regulated by functionally folded proteins, providing a robust model for studying cellular processes and designing microreactor systems. This work highlights the transformative potential of self-assembling recombinant fusion proteins in artificial cell design.



INTRODUCTION

To obtain a deeper understanding of the intricate mechanisms of life and utilize the specific capabilities of cells such as sensing, cargo transportation, transcription, and translation for diverse scientific applications, the creation of cell-like structures has emerged as a critical research area over several decades.[1,2] These cell-like structures are referred to as artificial cells. In the field of artificial cells, numerous platforms have been actively explored, each offering a unique set of advantages and applications. These platforms include coacervates,[3] emulsions,[4] or vesicles.[5,6] Among these potential platforms, synthetic vesicles have emerged as one of the most promising options due to their biomimicry characteristics.[7]

One of the key challenges in designing artificial cells is mimicking the complex but highly ordered structures of biological cells. The structural features of living cells, such as their outer membrane, crowded interior, and various internal organelles, fundamentally affect their functions by regulating the access of molecules, controlling the diffusion rate of reactants within the cell, and performing specific functions.[8−11] Particularly, giant vesicles, which are larger than 10 μm in diameter, have captured the attention of scientists and engineers for artificial cell platforms due to their resemblance in size to biological cells in size. Furthermore, giant vesicles have also been explored for investigating aspects of biological cell membranes such as lateral heterogeneities, membrane budding or fusion, and activities of reconstituted membrane proteins.[12,13] Additionally, multicompartmentalization is one of the most essential features of cells. Several compartments enable the precise control of systematic enzyme reactions through spatial and temporal confinement of incompatible enzymes and substrates.[14,15]

Engineering the structural properties of synthetic vesicles toward artificial cell models has been actively pursued through various methods, including microfluidics devices[16] or emulsion phase transfer.[17] However, these methods have primarily been employed with traditional amphiphilic building blocks, such as lipids and synthetic polymers, which dissolve in organic solvents. The use of organic solvents could hamper the biological activity and restrict the biocompatibility.[18] Consequently, the utilization of these methods with functional and water-soluble proteins or molecules is difficult to apply in all-aqueous systems. To overcome these challenges, researchers have endeavored to develop engineering strategies to produce synthetic vesicles in aqueous solution to employ water-soluble proteins as building blocks.[19] However, constructing multicompartment synthetic vesicles using only proteins and polypeptides in an organic solvent-free environment remains challenging.

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Proteins, as representative biomacromolecules in living organisms, have emerged as promising building blocks for the construction of synthetic vesicles owing to their biological properties, including specific functions and biocompatibility. Particularly, incorporating proteins into synthetic vesicle membranes is pivotal for the development of artificial cells, offering various functionalities crucial for their biomimetic properties. Several techniques like detergent-mediated reconstitution,[20] conjugation,[21] and direct incorporation in cell-free systems[22] have been developed and employed effectively for membrane protein integration. However, the incorporation of water-soluble, full-sized globular proteins into synthetic vesicles is still challenging due to their intricately folded structures and hydrophilic surfaces. Researchers have developed techniques for incorporating water-soluble globular proteins to unlock the full spectrum of capabilities of proteins.

Champion group first introduced globular protein vesicles (GPVs) through self-assembly of recombinant fusion proteins.[23−25] GPVs are constructed with a complex of two recombinant fusion proteins consisting of globular proteins and elastin-like polypeptides (ELPs). A complementary leucine zipper pair (Z_E/Z_R), which shows a high binding affinity ($K_d \approx 10^{-15}$ M),[26] is genetically fused with globular protein and ELP (globule-Z_E and Z_R-ELP, respectively) to form fusion protein complexes. The driving force behind vesicle assembly is the lower critical solution temperature (LCST) behavior of ELP. Due to the conformational change of the ELP chain, ELPs become hydrophobic above the transition temperature, resulting in vesicle formation in the aqueous phase. GPVs stand out as one of the most promising artificial cell platforms since incorporation of functional full-sized protein is possible in benign conditions without the use of organic solvents and chemical conjugation, which are known as hampering protein activity.[18,27] Furthermore, the advent of DNA recombinant technologies has opened a new era of protein and polypeptide engineering, enabling more precise manipulation of recombinant proteins and versatile applications.[28] However, it has remained a challenge to utilize GPVs for artificial cell applications due to their limited size of a few micrometers and low stability in a couple of days.

In this study, we utilize a water-based thin film rehydration method for the formation of giant, multicompartment GPVs from recombinant fusion proteins. These methods enable the construction of artificial cells with cell-like structures and composition by encapsulating cytosol mimicking crowding agents and internal compartments. Poly(ethylene glycol) (PEG) and dextran are used as crowding agents to mimic crowded cytosolic environments. For the internal compartment, small-sized self-assembled GPVs and protein coacervates are introduced into the giant GPVs for model artificial organelles. Furthermore, a triblock copolymer comprising hydrophilic poly(propylene oxide) (PPO) and poly(glycerol monomethacrylate) (PGM) blocks is employed to decorate the surface of GPVs, resulting in the formation of stable protein−polymer hybrid vesicles. The produced giant vesicles are investigated for further development of an artificial cell model. To the best of our knowledge, this is the first report on architecting multicompartment GPVs under organic solvent-free conditions. The construction of protein-based multicompartment giant synthetic vesicles will suggest a new era of artificial cell research, leveraging the biocompatibility and functions of proteins.

EXPERIMENTAL SECTION

Materials. Three different types of recombinant fusion proteins were used in this work: Z_R-ELP, mCherry-Z_E, and eGFP-Z_E. Z_R-ELP is composed of an arginine-rich leucine zipper (Z_R) fused with an elastin-like polypeptide (ELP, $[(VPGVG)_2(VPGFG)(VPGVG)_2]_5$) and globular, fluorescent mCherry and eGFP proteins fused with a glutamic acid-rich leucine zipper (Z_E). Fluorescein isothiocyanate poly(ethylene glycol) [FITC-PEG, ($\langle M_n \rangle$ = 5000 g/mol)] is purchased from nanocs. FITC-dextran (10 and 250 kDa) is purchased from Sigma-Aldrich. Methacryloyl chloride, copper(I) bromide (CuBr), and 2-bromoisobutyryl bromide were obtained from Sigma-Aldrich and used as received. Solketal, triethylamine (Et_3N), poly(propylene oxide) (PPO_{34}), 2,2′-bipyridine (Bpy), rhodamine B (RhB), *N*,*N*′-dicyclohexylcarbodiimide (DCC), and 4-dimethylaminopyridine (DMAP) were purchased from TCI and used directly unless otherwise mentioned. NMR solvents such as chloroform-*d* ($CDCl_3$) and dimethyl sulfoxide-d_6 (DMSO-d_6) were purchased from Cambridge Isotope Laboratories. All other chemicals and solvents used in this study were acquired from VWR.

Protein Expression and Purification. Preparation of plasmids for mCherry-Z_E, eGFP-Z_E, and Z_R-ELP (pQE60-mCherry-Z_E, pQE60-eGFP-Z_E, and pQE60-His_6Z_E/Z_R-ELP) was described in a previous work.[23] All plasmids are transformed into *Escherichia coli* strain BL21 for expression. All cell cultures were grown at 37 °C in LB broth media containing ampicillin (200 mg/L) and chloramphenicol (32 mg/L). Isopropylβ-thiogalactoside (1.0 mM, IPTG) was injected to the cell culture to induce protein expression when the optical density at 600 nm of cell culture was between 0.8 and 1.0. After 5 h of expression at 37 °C, cells were collected by centrifugation (4000*g*, 30 min). The purification of Z_R-ELP was conducted under denaturing conditions. All denaturing condition buffer contained 8 M urea, 10 mM Tris, and 100 mM Na_2HPO_4 (pH 8: lysis buffer, pH 6.3: washing buffer). Cell lysates including both coexpressed His_6Z_E and Z_R-ELP were incubated with Ni-NTA resin for 1 h. After the washing step, Z_R-ELP was collected from 6 M guanidine hydrochloride-based elution buffer (pH 8) by detaching Z_R-ELP from His_6Z_E. Purified proteins were dialyzed into DI water. mCherry-Z_E and eGFP-Z_E were purified under native conditions. All native condition buffer contained 50 mM Na_2HPO_4, 300 mM NaCl, and 10−250 mM imidazole (10 mM: lysis, 20 mM: washing, 250 mM: elution). The purified protein was dialyzed into 1× PBS.

Synthesis and Characterization of the Water-Soluble Triblock Copolymer. The detailed synthetic procedures of the water-soluble triblock copolymer, PGM_{15}-*b*-PPO_{34}-*b*-PGM_{15}, are provided in the Supporting Information in detail with supplementary figures (Scheme S1 and Figures S3−S8). Briefly, the monomer solketal methacrylate (SMA) was synthesized from methacryloyl chloride and solketal, and the macroinitiator (Br-PPO_{34}−Br) was synthesized from hydroxy-capped PPO_{34} and 2-bromoisobutyryl bromide and then polymerized via atom-transfer radical polymerization (ATRP) to form $PSMA_{15}$-*b*-PPO_{34}-*b*-$PSMA_{15}$, which was converted to PGM_{15}-*b*-PPO_{34}-*b*-PGM_{15}. The polymers were labeled with rhodamine B through Steglich esterification, and the final fluorescent RhB@PGM_{15}-*b*-PPO_{34}-*b*-PGM_{15} was purified by dialysis and freeze-drying. Dichloromethane (DCM) was dried over CaH_2 following the standard protocol prior to use. ^1H and ^{13}C NMR spectra were recorded with a Bruker 500 MHz spectrometer at room temperature. Chemical shifts (δ) are given in parts per million (ppm) and referenced to residual solvent peaks ($CDCl_3$= δ^1H 7.26 ppm; DMSO-d_6 = δ^1H 2.50 ppm). Size exclusion chromatography (SEC) was performed on an Agilent-Wyatt combination system calibrated with polystyrene standards (d*n*/d*c* = 0.1850 mL/g). The system was equipped with a degasser, an autosampler, three Agilent PLgel Mixed-C columns, and an Agilent 1260 infinity series pump. Measurements were carried out in THF at a flow rate of 1.0 mL/min using an Optilab rEx refractive index (RI) detector. Astra software was used for molecular weight (M_n) and dispersity ($Đ$) analysis.

Formation of a Well for Thin-Film Rehydration. To make a well for thin-film formation, a poly(dimethylsiloxane) (PDMS)

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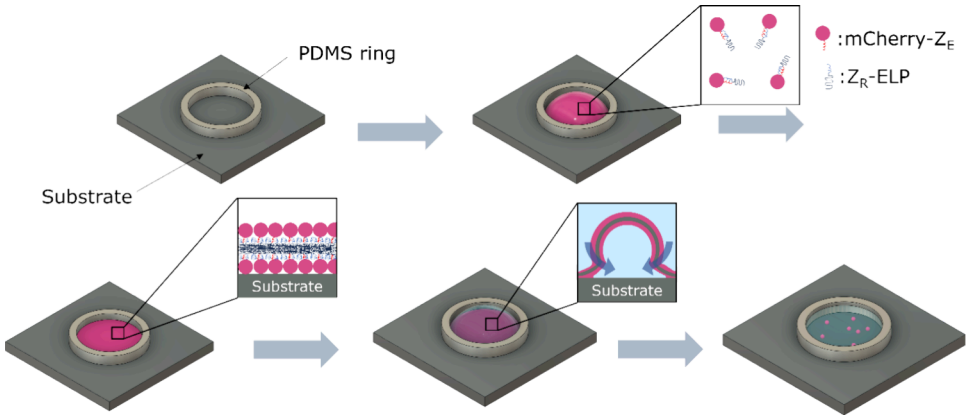
Scheme 1. Schematic Illustration of the Thin-Film Rehydration Process to Produce Giant GPVs



elastomer and curing agent (Dow Chemical) were mixed at a 10:1 weight ratio. The mold for the PDMS ring was fabricated through a 3D printer (Prusa3D). The mixed PDMS solution was poured into the mold to make a ring (diameter: 10 mm, height 3 mm). The formed ring and a glass substrate (Fisher, 25 mm × 50 mm) bonded to each other after oxygen plasma treatment. A well with the PDMS ring and glass substrate was stabilized in an oven (65 °C) for 1 h.

Formation of Giant GPVs. Z_R-ELP (2 mM) and mCherry-Z_E (0.1 mM) were mixed in a 1.5 mL Eppendorf tube (total volume: 200 μL). The tube was incubated at 4 °C for 15 min for stabilizing leucin zipper bonding. After incubation at 4 °C, protein mixed solutions were deposited in the well. Solvents were evaporated at 37 °C to make protein thin films. Hydration solution (2.2× PBS, 200 μL) was poured into the well, and giant GPVs were formed after 3 h.

Formation of Giant Polymer-Hybrid GPVs. PGM-PPO-PGM water-soluble block copolymers (50 μM) were added into the protein mixture. The protein−polymer mixture was incubated at 4 °C for 15 min. Protein−polymer mixtures were deposited in the well. Solvents were evaporated at 37 °C to make protein−polymer hybrid thin films. Hydration solution (2.2× PBS, 200 μL) was poured into the well, and giant polymer-hybrid GPVs were formed after 3 h.

Encapsulation of Soluble Macromolecules. FITC-PEG and FITC-dextran were dissolved into hydration solution to be encapsulated into giant GPVs through a swelling process. The hydration solution contained 1 wt % of FITC-PEG or FITC-dextran. Giant GPVs containing macromolecules were formed after 3 h hydration.

Formation of Multicompartment Giant GPVs. eGFP coacervates and eGFP vesicles were prepared through mixing Z_R-ELP and eGFP-Z_E in a molar ratio of 0.05 (eGFP-Z_E to Z_R-ELP) at a concentration of 120 μM Z_R-ELP. The preparation of eGFP vesicles and eGFP coacervates is described in a previous work.[25] Salt concentrations were 0.3 and 0.91 M for coacervates and vesicles, respectively. Then, 200 μL of eGFP coacervate solution or eGFP vesicle solutions was poured to the protein thin films. Multicompartment giant GPVs were formed after 3 h hydration.

RESULTS AND DISCUSSION

Formation of Giant GPVs through Straightforward Thin-Film Rehydration Methods. We employed the thin-film rehydration method to generate giant GPVs composed of two recombinant fusion proteins, mCherry-Z_E and Z_R-ELP (Scheme 1). The thin-film rehydration method stands out for its low cost, simplicity, and high versatility, making it a widely used approach for synthesizing giant vesicles.[29] The interaction between the complementary leucine zipper pairs (Z_E/Z_R) results in the spontaneous formation of protein complexes in an aqueous solution. The solution of fusion proteins is prepared by mixing two stock solutions of the fusion proteins (2 mM Z_R-ELP and 0.1 mM mCherry-Z_E, total 200 μL). Below the critical temperature (4 °C), all of the fusion proteins dissolve in water. The protein solution is placed in a poly(dimethylsiloxane) (PDMS) well, with a diameter of 10 mm, affixed to a glass substrate by ozone plasma treatment. The solvent in the PDMS well is evaporated at 37 °C, above the transition temperature, to form protein bilayer thin films. Subsequently, the protein thin films are immersed in the hydration solution (2.2× PBS) for 3 h, allowing them to self-assemble into giant vesicles via a swelling process, as shown in Figure 1A. Salt in the hydration solution is required for vesicle formation since it influences the conformation of ELP molecules. Higher salt concentrations help ELP to collapse and become more hydrophobic, leading to variations in the packing parameters of protein building blocks. Therefore,

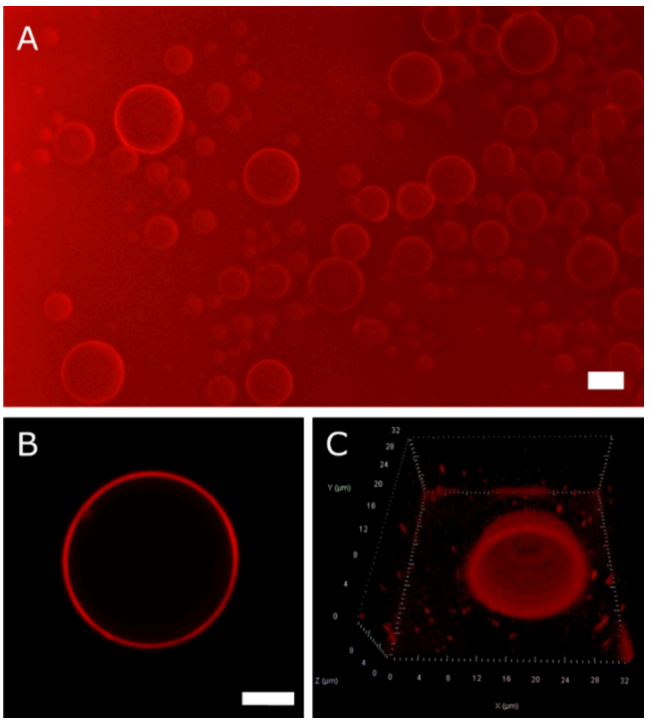


Figure 1. mCherry-fused giant GPVs. (A) Fluorescence microscopy image of mCherry-fused giant GPVs in hydration solution. (B) Confocal microscopy image of mCherry-fused giant GPVs formed by thin-film rehydration. (C) 3D confocal microscopy image of swelling thin films. Scale bars are 10 μm.

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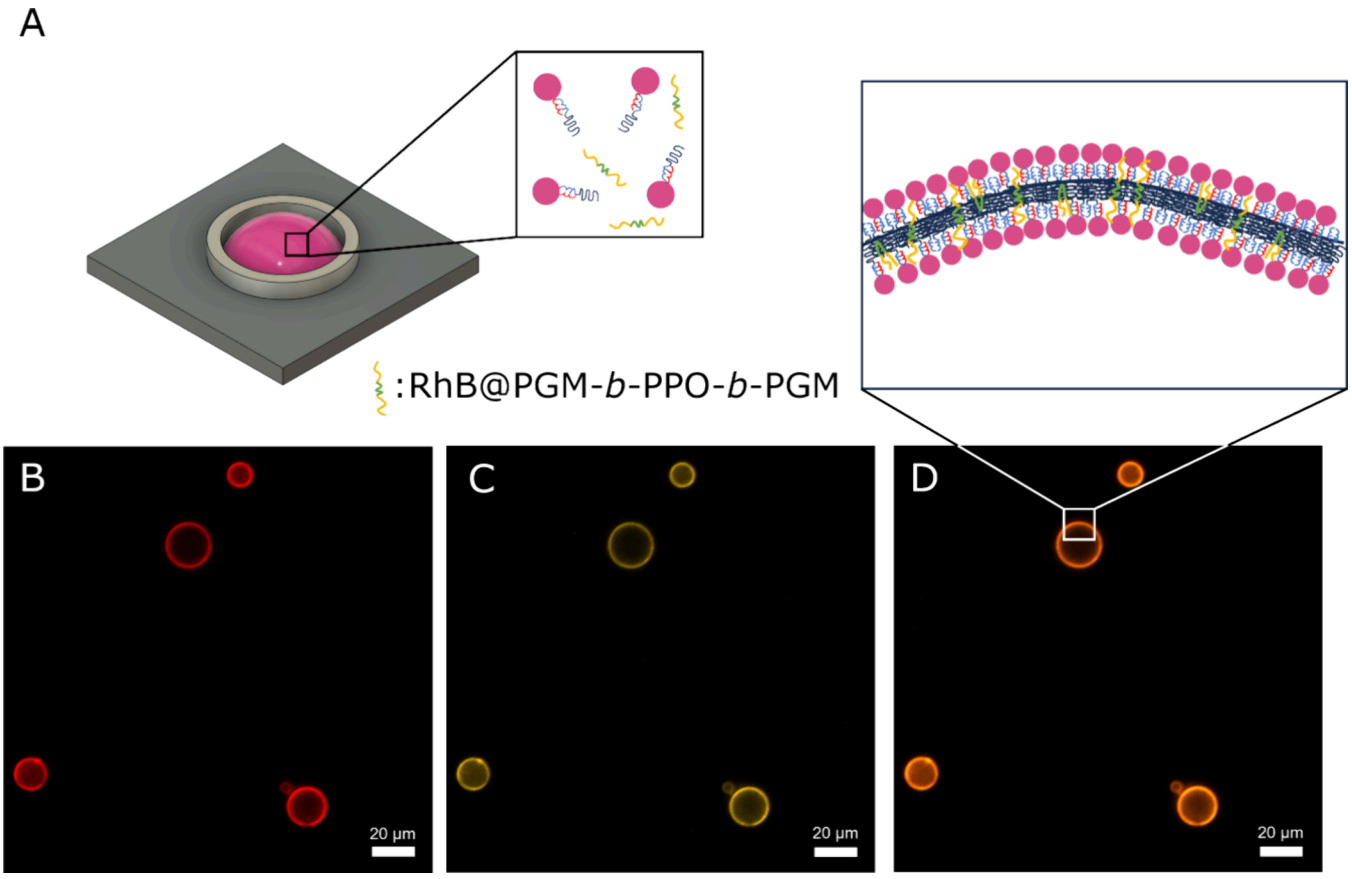

Figure 2. Incorporation of water-soluble fluorescent triblock copolymer RhB@PGM_{15}-*b*-PPO_{34}-*b*-PGM_{15} into giant GPVs. (A) Co-dissolution of polymers with fusion proteins at 4 °C. Confocal microscopy images of the polymer incorporated into giant GPVs with different fluorescence channels: (B) mCherry, (C) Rhodamine B, and (D) overlay.

vesicle formation is only observed when the salt concentration is higher than the critical point, which is 0.3 M (i.e., 2.2× PBS) for mCherry GPVs.[23] As the salt concentration increases in the solution, the average GPV size decreases due to the reduced packing parameter.[30] The hollow vesicle structure of generated giant GPVs is confirmed by confocal microscopy (Figure 1B). The swelling process of the protein thin film during hydration is observed through confocal microscopy (Figure 1C). The LCST behavior of ELP domain drives the formation of the bilayer protein thin film, and hydrophilic mCherry proteins allow swelling through hydration. Furthermore, the thin-film rehydration method also produces multilamellar protein vesicles, a common outcome resulting from the swelling of thin films, as shown in Figure S1 (Supporting Information). In addition, mCherry globular proteins can be replaced with other globular proteins, indicating that this method has wide applicability for self-assembling fusion proteins. Enhanced green fluorescent protein (eGFP) thin films are successfully swelled to form giant eGFP vesicles (Figure S2).

Facile Surface Modification Strategy Based on Self-Assembly of Fusion Proteins and Water-Soluble Block Copolymers. One of the most significant advantages of employing the thin-film rehydration method lies in its capacity to construct complex giant GPV structures and compositions through modifying the synthesis process. The incorporation of proteins into the polymer vesicles has been investigated for extending synthetic vesicle applications by leveraging and tuning essential properties of the polymer backbones, such as stability, functionality, or responsiveness to external stimuli.[31−33] Despite substantial investigations into these hybrid vesicles, the creation and precise control of GPVs, whose surface can be endowed by protein specific activities at high valency, through a blending approach remain relatively underexplored. To fill this gap, our study showcases the viability of blending techniques for manipulating the characteristics of protein-rich vesicles via their aqueous self-assembly with other amphiphilic macromolecules, thereby expanding the toolbox for engineering advanced vesicular systems.

A rhodamine-labeled water-soluble fluorescent triblock copolymer, poly(glycerol monomethacrylate)-*b*-poly(propylene oxide)-*b*-poly(glycerol monomethacrylate) (PGM_{15}-*b*-PPO_{34}-*b*-PGM_{15}), is utilized as a model polymer to show the possibility of incorporation of a polymer into giant GPVs to form polymer−protein hybrid vesicles. Triblock copolymers are synthesized through atom-transfer radical polymerization (ATRP) (Scheme S1) and comprehensively characterized by ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, and size exclusion chromatography (SEC) (Figures S3−S8 in the Supporting Information). PGM block is a well-known hydrophilic polymer and has been developed for applications where water adsorption or interactions are required, such as drug delivery or hydrogels.[34] Additionally, a fluorescent dye, rhodamine B (RhB), was conjugated with alcohol terminated hydrophilic PGM blocks using the Steglich esterification method to visualize the polymer under a fluorescence microscope (Scheme S1). For hydrophobic building blocks, PPO exhibits LCST behavior, similar to ELP, and the critical temperature of PPO_{34} is around 10 °C.[19] The RhB@PGM_{15}-*b*-

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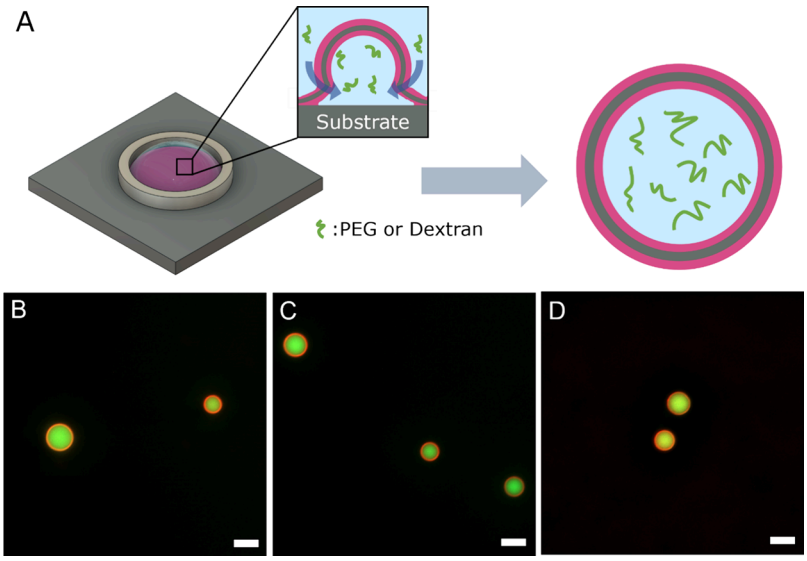

Figure 3. Encapsulation crowding agents into the giant GPVs. (A) Schematic illustrations about the swelling process with crowding agents suspended in 2.2× PBS. Fluorescence images of encapsulation of (B) FITC-PEG (8 kDa), (C) FITC-dextran (10 kDa), and (D) FITC-dextran (250 kDa). All scale bars are 20 μm.

PPO_{34}-*b*-PGM_{15} triblock copolymer is straightforwardly blended with fusion proteins in an aqueous solution and formed into polymer−protein hybrid thin films by evaporation above the transition temperature (Figure 2A). After the hydration step, the fluorescent triblock copolymers are incorporated into the giant GPV membrane, as shown in Figure 2B,C.

The main driving force of incorporation of the synthesized triblock copolymer is the LCST behavior of the PPO_{34} middle blocks. This LCST behavior makes the PGM_{15}-*b*-PPO_{34}-*b*-PGM_{15} triblock copolymer water-soluble block copolymers at 4 °C and facilitates their incorporation into the protein thin film due to the hydrophobic interaction between PPO blocks and ELP domains. These hybrid protein−polymer giant vesicles maintain their structure for a week, which indicates that coexistence of protein and the polymer in the membrane is stable (Figure S9).

Moreover, the substitution of hydrophilic blocks with other functional or stimuli-responsive domains holds the potential to confer additional functionalities to giant GPVs, thereby enhancing their potential for advanced artificial cells. For instance, the hydrophilic PGM blocks can be functionalized with biotin through esterification, providing sites for avidin binding. The interaction between biotin and avidin represents one of the most robust noncovalent bonds in nature, widely utilized in the field of biomedicine.[35]

Encapsulation of Crowding Agents and Microarchitectures in Giant GPVs toward Multicompartment Cell Structure Mimetics. During the hydration process, thin films repel the surface, enabling the aqueous solution to induce swelling and the formation of giant GPVs. By leveraging this process, various biomacromolecules and compartments are encapsulated into giant GPVs, simulating the structural and compositional aspects of cells. Within the biological cell, the intracellular space is filled with various macromolecules such as proteins, nucleic acids, and polysaccharides. These substances are densely packed within the confined space and affect several biochemical and biophysical reactions, such as diffusion, protein folding, or enzyme activity. To mimic the crowded environment of a cytosol, we employed poly(ethylene glycol) (PEG) and dextran as crowding agents, which have been widely used due to their simple structure and relative inertness.[36] The successful encapsulation of green fluorescent FITC-tagged PEG (8 kDa) and FITC-dextrans at different molecular weights (i.e., 10 and 250 kDa) in the thin-film rehydration process was observed through fluorescence microscopy (Figure 3).

These results suggest that the construction of giant GPVs emulating the composition of the cytosol is possible by loading different types of macromolecules across a wide range of molecular weights. The construction of cytosol-mimetic artificial cells is crucial for investigating protein regulation behavior as it enables researchers to recapitulate more complex intracellular environments, facilitating the study of protein−protein interactions,[37] enzymatic reactions,[38] and signaling pathways[39] within confined geometry.

Eukayotic cells are characterized by their complex internal structures, which include various membrane-based organelles and membraneless organelles. These structures play essential roles in cellular processes, allowing cells to perform specialized functions efficiently. Introduction of such internal biomolecular-assembled compartment structures into the hydration solution enables the construction of multicompartment giant GPVs. A mixture of eGFP-Z_E and Z_R-ELP is self-assembled into coacervates, or vesicles based on the salt concentration at the same temperature. Below the critical salt concentration (i.e., 0.3 M NaCl in the hydration solution) for vesicle formation, as reported in the previous work,[25] eGFP-Z_E and Z_R-ELP fusion protein complexes self-assemble into coacervates through liquid−liquid phase separation driven by the hydrophobic interaction between ELP domains (Figure 4A). At a high salt concentration (0.91M), eGFP-Z_E and Z_R-ELP fusion protein complexes self-assemble into small-sized GPVs with an average diameter of less than 2 μm by forming a molecular packing parameter suitable for vesicle assembly with the increased ELP hydrophobic interaction, resulting in tight packing (Figure 4B). After hydrating mCherry-Z_E/Z_R-ELP thin films with solutions containing eGFP-Z_E/Z_R-ELP coacervates

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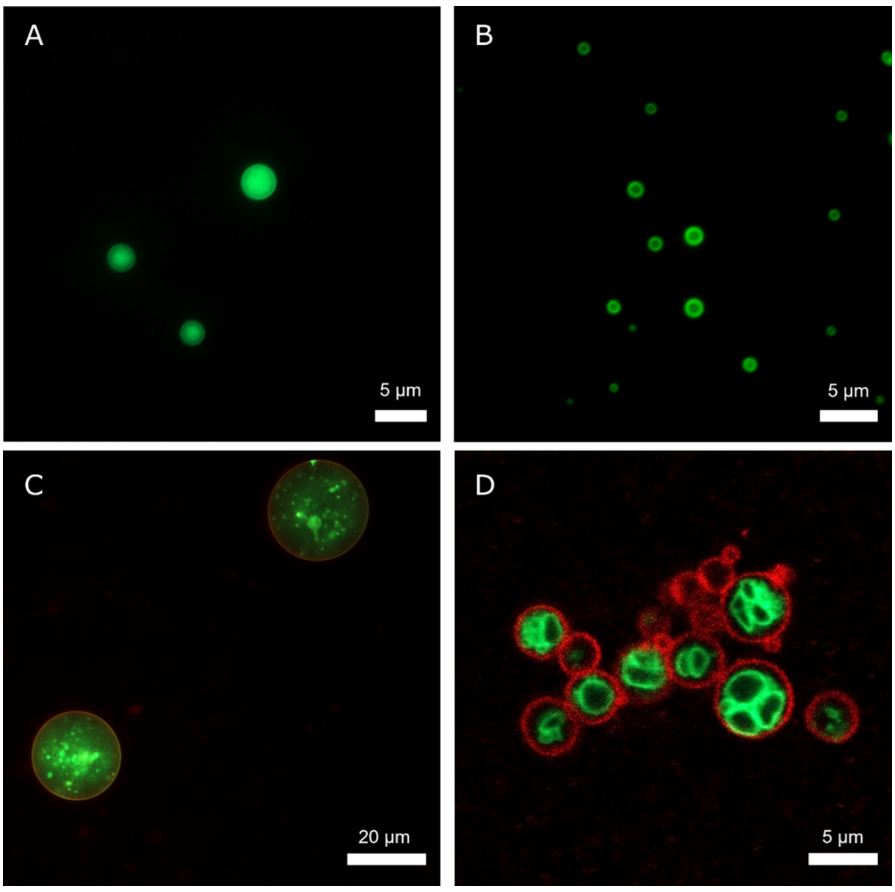

Figure 4. Construction of multicompartment giant GPVs. Fluorescent micrographs of microcompartment encapsulants, showing the formation of (A) self-assembled, small-sized eGFP-Z_E/Z_R-ELP coacervates and (B) self-assembled, small-sized eGFP-Z_E/Z_R-ELP vesicles. Confocal micrographs of the multicompartment giant mCherry-GPVs encapsulating (C) small-sized eGFP- coacervates and (D) eGFP-vesicles.

for 3 h, we observed the formation of green eGFP-coacervate-encapsulated red mCherry giant GPVs (Figure 4C). In addition to protein coacervates, we successfully encapsulated small-sized, self-assembled eGFP-GPVs, achieving multicompartmentalization, as confirmed by confocal microscopy (Figure 4D). This was also accomplished by a simple hydrating process of mCherry-fusion-protein-based thin films with solutions containing eGFP-GPVs. We observed that the multicompartmentalized giant mCherry-GPVs become smaller in size compared to as-prepared giant GPVs. This size reduction is attributed to the difference in salt concentrations used for making the outer and inner protein vesicles. The high salt concentrations in the inner-compartment eGFP-GPVs solution decrease the size of the outer-compartment giant mCherry-GPVs due to the salt-induced further hydrophobic collapse of ELP domains, resulting in an increased curvature of the vesicle membrane.

CONCLUSIONS

We demonstrated the application of the organic solvent-free thin-film rehydration method as an effective means for architecting giant GPVs with cell-like structures and compositions, contributing to the advancement of artificial cell development. We anticipate that giant protein vesicles could have diverse potential applications. They can encapsulate therapeutic proteins for targeted drug delivery, model cellular processes in biomimetic research, and serve as biosensors for detecting biological molecules. These vesicles also hold promise for studying biophysics and cellular mechanisms regulated by proteins in the realm of artificial cell development. This is the first report, to the best of our knowledge, that demonstrates the construction of multicompartmentalized cell-like structures through the hierarchical self-assembly of recombinant fusion proteins. The multicompartment GPV platform facilitates the localization of full-sized globular proteins on the vesicle membrane and in and at microcompartments within the lumen, thus preserving protein function without compromising protein activity. By leveraging the proteins' functions and biocompatibility, multicompartment giant GPVs can serve as advanced artificial cell models. These models encapsulate enzyme-localized organelles within membranes to achieve a highly efficient enzymatic activity. Consequently, multicompartment giant GPVs hold promise for diverse applications in biotechnology including as platforms for advanced artificial cells, bioreactors, and drug delivery systems. Furthermore, stable protein−polymer hybrid vesicles were constructed by decorating the surfaces of GPVs with fluorescent-labeled functional triblock copolymers. Therefore, we believe that this work could provide versatile engineering strategies that involve the incorporation of functional enzymes or receptor ligands into the vesicle membranes, enabling these structures to serve as artificial organelles or bioreactors within a cell-like environment for practical applications.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.4c00807.

Additional experimental details, materials, and methods: fluorescent microscope images and ^{1}H NMR spectrum, ^{13}C NMR spectrum, and size exclusion chromatography (SEC) traces for chemicals used in the synthesis of water-soluble block copolymer (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Salehi-Reyhani, A.; Ces, O.; Elani, Y. Artificial cell mimics as simplified models for the study of cell biology. *Exp. Biol. Med.* **2017**, *242* (13), 1309−1317.

(2) Xu, C.; Hu, S.; Chen, X. Artificial cells: from basic science to applications. *Mater. Today* **2016**, *19* (9), 516−532.

(3) Cook, A. B.; Novosedlik, S.; van Hest, J. C. Complex Coacervate Materials as Artificial Cells. *Acc. Mater. Res.* **2023**, *4* (3), 287−298.

(4) Torre, P.; Keating, C. D.; Mansy, S. S. Multiphase water-in-oil emulsion droplets for cell-free transcription−translation. *Langmuir* **2014**, *30* (20), 5695−5699.

(5) Hilburger, C. E.; Jacobs, M. L.; Lewis, K. R.; Peruzzi, J. A.; Kamat, N. P. Controlling secretion in artificial cells with a membrane and gate. *ACS Synth. Biol.* **2019**, *8* (6), 1224−1230.

(6) Martino, C.; Kim, S. H.; Horsfall, L.; Abbaspourrad, A.; Rosser, S. J.; Cooper, J.; Weitz, D. A. Protein expression, aggregation, and triggered release from polymersomes as artificial cell-like structures. *Angew. Chem.* **2012**, *124* (26), 6522−6526.

(7) Maffeis, V.; Heuberger, L.; Nikoletić, A.; Schoenenberger, C.; Palivan, C. G. Synthetic Cells Revisited: Artificial Cells Construction Using Polymeric Building Blocks. *Adv. Sci.* **2024**, *11* (8), No. 2305837.

(8) Komiya, M.; Kato, M.; Tadaki, D.; Ma, T.; Yamamoto, H.; Tero, R.; Tozawa, Y.; Niwano, M.; Hirano-Iwata, A. Advances in artificial cell membrane systems as a platform for reconstituting ion channels. *Chem. Rec.* **2020**, *20* (7), 730−742.

(9) Vance, J. A.; Devaraj, N. K. Membrane mimetic chemistry in artificial cells. *J. Am. Chem. Soc.* **2021**, *143* (22), 8223−8231.

(10) Nakano, S.-i.; Miyoshi, D.; Sugimoto, N. Effects of molecular crowding on the structures, interactions, and functions of nucleic acids. *Chem. Rev.* **2014**, *114* (5), 2733−2758.

(11) Lee, K. Y.; Park, S.-J.; Lee, K. A.; Kim, S.-H.; Kim, H.; Meroz, Y.; Mahadevan, L.; Jung, K.-H.; Ahn, T. K.; Parker, K. K.; Shin, K. Photosynthetic artificial organelles sustain and control ATP-dependent reactions in a protocellular system. *Nat. Biotechnol.* **2018**, *36* (6), 530−535.

(12) Robinson, T. Microfluidic handling and analysis of giant vesicles for use as artificial cells: a review. *Adv. Biosyst.* **2019**, *3* (6), No. 1800318.

(13) Van de Cauter, L.; van Buren, L.; Koenderink, G. H.; Ganzinger, K. A. Exploring Giant Unilamellar Vesicle Production for Artificial Cells−Current Challenges and Future Directions. *Small Methods* **2023**, *7*, No. 2300416.

(14) Wang, X.; Qiao, X.; Chen, H.; Wang, L.; Liu, X.; Huang, X. Synthetic-Cell-Based Multi-Compartmentalized Hierarchical Systems. *Small Methods* **2023**, *7*, No. 2201712.

(15) Schmitt, C.; Lippert, A. H.; Bonakdar, N.; Sandoghdar, V.; Voll, L. M. Compartmentalization and transport in synthetic vesicles. *Front. Bioeng. Biotechnol.* **2016**, *4*, 19.

(16) Deng, N.-N.; Yelleswarapu, M.; Zheng, L.; Huck, W. T. Microfluidic assembly of monodisperse vesosomes as artificial cell models. *J. Am. Chem. Soc.* **2017**, *139* (2), 587−590.

(17) Peyret, A.; Ibarboure, E.; Le Meins, J.; Lecommandoux, S. Asymmetric hybrid polymer−lipid giant vesicles as cell membrane mimics. *Adv. Sci.* **2018**, *5* (1), No. 1700453.

(18) Magsumov, T.; Ziying, L.; Sedov, I. Comparative study of the protein denaturing ability of different organic cosolvents. *Int. J. Biol. Macromol.* **2020**, *160*, 880−888.

(19) Amado, E.; Schöps, R.; Brandt, W.; Kressler, J. r. Spontaneous formation of giant bioactive protein-block copolymer vesicles in water. *ACS Macro Lett.* **2012**, *1* (8), 1016−1019.

(20) Behnke, J.-S.; Urner, L. H. Emergence of mass spectrometry detergents for membrane proteomics. *Anal. Bioanal. Chem.* **2023**, *415* (18), 3897−3909.

(21) Wang, M.; Altinoglu, S.; Takeda, Y. S.; Xu, Q. Integrating protein engineering and bioorthogonal click conjugation for extracellular vesicle modulation and intracellular delivery. *PLoS One* **2015**, *10* (11), No. e0141860.

(22) Göpfrich, K.; Haller, B.; Staufer, O.; Dreher, Y.; Mersdorf, U.; Platzman, I.; Spatz, J. P. One-pot assembly of complex giant unilamellar vesicle-based synthetic cells. *ACS Synth. Biol.* **2019**, *8* (5), 937−947.

(23) Park, W. M.; Champion, J. A. Thermally triggered self-assembly of folded proteins into vesicles. *J. Am. Chem. Soc.* **2014**, *136* (52), 17906−17909.

(24) Jang, Y.; Choi, W. T.; Heller, W. T.; Ke, Z.; Wright, E. R.; Champion, J. A. Engineering globular protein vesicles through tunable self-assembly of recombinant fusion proteins. *Small* **2017**, *13* (36), No. 1700399.

(25) Jang, Y.; Hsieh, M.-C.; Dautel, D.; Guo, S.; Grover, M. A.; Champion, J. A. Understanding the coacervate-to-vesicle transition of globular fusion proteins to engineer protein vesicle size and membrane heterogeneity. *Biomacromolecules* **2019**, *20* (9), 3494−3503.

(26) Moll, J. R.; Ruvinov, S. B.; Pastan, I.; Vinson, C. Designed heterodimerizing leucine zippers with a ranger of pIs and stabilities up to 10− 15 M. *Protein Sci.* **2001**, *10* (3), 649−655.
(27) Morgenstern, J.; Gil Alvaradejo, G.; Bluthardt, N.; Beloqui, A.; Delaittre, G.; Hubbuch, J. r. Impact of polymer bioconjugation on protein stability and activity investigated with discrete conjugates: alternatives to PEGylation. *Biomacromolecules* **2018**, *19* (11), 4250−4262.
(28) Rosano, G. L.; Ceccarelli, E. A. Recombinant protein expression in Escherichia coli: advances and challenges. *Front. Microbiol.* **2014**, *5*, 172.
(29) Rideau, E.; Wurm, F. R.; Landfester, K. Self-Assembly of Giant Unilamellar Vesicles by Film Hydration Methodologies. *Adv. Biosyst.* **2019**, *3* (6), No. 1800324.
(30) Li, Y.; Champion, J. A. Photocrosslinked, tunable protein vesicles for drug delivery applications. *Adv. Healthcare Mater.* **2021**, *10* (15), No. 2001810.
(31) Liu, X.; Gao, W. In situ growth of self-assembled protein−polymer nanovesicles for enhanced intracellular protein delivery. *ACS Appl. Mater. Interfaces* **2017**, *9* (3), 2023−2028.
(32) Gaitzsch, J.; Hirschi, S.; Freimann, S.; Fotiadis, D.; Meier, W. Directed insertion of light-activated proteorhodopsin into asymmetric polymersomes from an ABC block copolymer. *Nano Lett.* **2019**, *19* (4), 2503−2508.
(33) Liu, D.; Sun, H.; Xiao, Y.; Chen, S.; Cornel, E. J.; Zhu, Y.; Du, J. Design principles, synthesis and biomedical applications of polymer vesicles with inhomogeneous membranes. *J. Controlled Release* **2020**, *326*, 365−386.
(34) Zhang, H.; Grinstaff, M. W. Recent advances in glycerol polymers: chemistry and biomedical applications. *Macromol. Rapid Commun.* **2014**, *35* (22), 1906−1924.
(35) Jain, A.; Cheng, K. The principles and applications of avidin-based nanoparticles in drug delivery and diagnosis. *J. Controlled Release* **2017**, *245*, 27−40.
(36) Ou, X.; Tang, Z.; Ye, Y.; Chen, X.; Huang, Y. Macromolecular Crowding Effect on Chitosan-Hyaluronic Acid Complexation and the Activity of Encapsulated Catalase. *Biomacromolecules* **2024**, *25* (6), 3840−3849.
(37) Bhattacharya, A.; Kim, Y. C.; Mittal, J. Protein−protein interactions in a crowded environment. *Biophys. Rev.* **2013**, *5*, 99−108.
(38) Pastor, I.; Pitulice, L.; Balcells, C.; Vilaseca, E.; Madurga, S.; Isvoran, A.; Cascante, M.; Mas, F. Effect of crowding by Dextrans in enzymatic reactions. *Biophys. Chem.* **2014**, *185*, 8−13.
(39) Junker, N. O.; Vaghefikia, F.; Albarghash, A.; Höfig, H.; Kempe, D.; Walter, J.; Otten, J.; Pohl, M.; Katranidis, A.; Wiegand, S.; Fitter, J. Impact of molecular crowding on translational mobility and conformational properties of biological macromolecules. *J. Phys. Chem. B* **2019**, *123* (21), 4477−4486.

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