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Heterogeneous Synthetic Vesicles toward Artificial Cells: Engineering Structure and Composition of Membranes for Multimodal Functionalities

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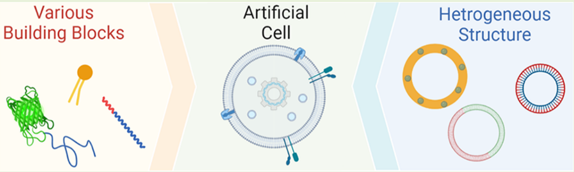

ABSTRACT: The desire to develop artificial cells to imitate living cells in synthetic vesicle platforms has continuously increased over the past few decades. In particular, heterogeneous synthetic vesicles made from two or more building blocks have attracted attention for artificial cell applications based on their multifunctional modules with asymmetric structures. In addition to the traditional liposomes or polymersomes, polypeptides and proteins have recently been highlighted as potential building blocks to construct artificial cells owing to their specific biological functionalities. Incorporating one or more functionally folded, globular protein into synthetic vesicles enables more cell-like functions mediated by proteins. This Review highlights the recent research about synthetic vesicles toward artificial cell models, from traditional synthetic vesicles to protein-assembled vesicles with asymmetric structures. We aim to provide fundamental and practical insights into applying knowledge on molecular self-assembly to the bottom-up construction of artificial cell platforms with heterogeneous building blocks.

1. INTRODUCTION

A vesicle is an essential cellular structure composed of a microcompartment enclosed by a lipid bilayer, which mediates the secretion, uptake, and transport of materials while protecting its components from being damaged by external shocks.[1] Natural biological vesicles usually contain heterogeneous components, such as a varying amounts of lipids and integral or peripheral proteins diffusing in the membrane.[2] For several decades, there has been growing interest in creating artificial cells to mimic the structure and functions of living cell membranes by engineering synthetic vesicles made of heterogeneous biological building blocks.[3]

Synthetic vesicles with a single lamellar phase bilayer are classified according to their size and complexity as small unilamellar vesicles (SUVs, <200 nm), large unilamellar vesicles (LUVs, 200 nm to 1 μm), and giant unilamellar vesicles (GUVs, >1 μm).[2] Vesicles can also have more than a single lamellar bilayer, and these vesicles are called multilamellar vesicles (MLVs) or multivesicular vesicles (MVVs, vesosomes).[4] In particular, GUVs are the most commonly used structure in studies of artificial cell development owing to their large size, which permits direct microscope observation and precise control of membrane properties and is similar to prokaryote cells.[5,6] The inner and outer leaflets of artificial vesicle bilayers can be made with either asymmetric or symmetric distribution. Also, heterogeneous components can render more complex functionalities of synthetic vesicles with different functional molecules.

Traditional homogeneous vesicles, such as lipid-based liposomes[7] and polymer-based polymersomes,[8] have provided engineered platforms that imitate the basic cellular membrane structure and allow for the transport of materials. In addition, the field is actively shifting from the design of these traditional platforms toward heterogeneous vesicles made of lipid/polymer hybrids,[9] nanoparticles (NPs),[10] or proteins to mimic complex cell structures and functions.[11] The direct incorporation of proteins into synthetic cell platforms is imperative for exhibiting protein-powered specific binding and biochemical cascade reactions in the cell.[12] The reconstitution of transmembrane proteins in liposomes has contributed to making protein-incorporated vesicle membranes. Other types of functionally folded, globular proteins have been incorporated into polymersome scaffolds (named proteinosomes) as hybrid forms of protein−polymer conjugates via chemical cross-linking in organic solvents.[11,13,14] Still, the membrane rupture or decrease in the protein activities during the incorporation process and the difficulties in controlling the protein orientation and proportion in synthetic vesicles remain significant challenges.[12,15,16]

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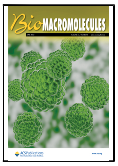

Herein we summarize recent research on synthetic vesicles toward artificial cell models to mimic the structure, composition, and essential biological functionalities of living cells. Notably, we discuss heterogeneous vesicles that exhibit advanced functionalities achieved by combining individual properties of different functional components, which are hard to realize via traditional homogeneous vesicles. The highly controllable compositions and structures of heterogeneous vesicles allow diverse applications for artificial cells, smart drug delivery systems, biosensing, *in vivo* imaging, and so on.[17,18] In this Review, we aim to provide a perspective of the state-of-the-art technology, materials, and science used in developing diverse types of synthetic vesicles with heterogeneous components and controlled structures for sophisticated functions toward artificial cells and their other potential uses (Figure 1).

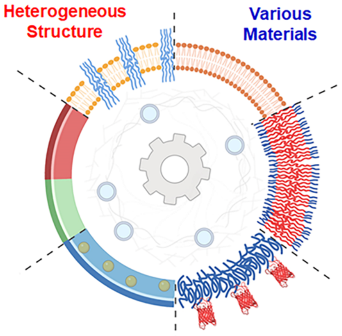

Figure 1. Schematic illustration of synthetic vesicles with a heterogeneous structure and compositions used for the construction of artificial cells.

2. TRADITIONAL HOMOGENEOUS SYNTHETIC VESICLES TO MIMIC CELL STRUCTURE AND FUNCTIONS

2.1. Liposomes toward Artificial Cell Platforms. Lipid-based materials, such as lipid NPs and liposomes, have been actively investigated for decades for various biomedical applications. In particular, lipid-based drug delivery carriers have recently been highlighted for Covid-19 mRNA vaccines.[19] Liposomes are spherical vesicles made from lipid bilayers.[20] Liposomes have been considered primary artificial cell platforms based on their nontoxicity, fluidity, biocompatibility, biodegradability, and nonimmunogenicity for *in vivo* administrations (Figure 2).[20] Liposomes can be made from differently charged building blocks using anionic, neutral, or cationic lipids.[21] Cationic liposomes are often used for gene therapy vehicles by forming ionic complexes with negatively charged nucleic acids (i.e., lipoplexes),[22] whereas neutral liposomes are employed to enhance drug delivery efficacy to solid tumors with preferential localization by decreasing electrostatic interactions with the anionic species in the circulation.[23] Anionic liposomes have shown higher adjuvant activities than cationic and neutral liposomes due to the capability of facilitating the uptake of protein antigens by antigen-presenting cells.[24] Micrometer-scale cell-sized GUVs play an important role in *in vitro* biological studies to investigate the cell membrane structure, morphology, and phase state due to their similarity with the cellular membrane in thickness and morphology.[25,26] They are capable of encapsulation, growth, and division to mimic the structural functions of a biological cell membrane,[27−30] as well as simple communication by exchanging chemical signals.[31]

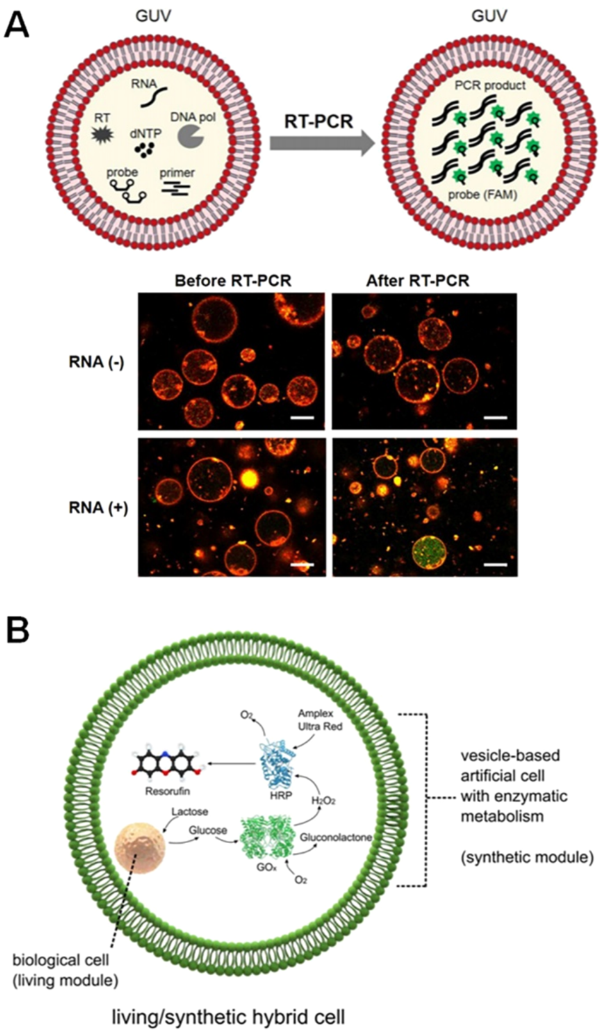

Figure 2. Traditional liposomes developed for artificial cell models. (A) Scheme (top) and confocal images (bottom, scale bar = 10 μm) that show the amplification of DNA from an RNA template driven by polymerases inside the giant liposomes.[33] Reproduced from ref 33, licensed under CC BY 4.0. (B) Scheme of the artificial cell model encapsulating the biological living cell to process chemical elements by an enzymatic cascade reaction.[38] Reproduced from ref 38, licensed under CC BY 4.0.

Recently, Lentini et al. demonstrated an artificial cell system enabling two-way communication with liposomes and bacteria.[32] They exploited cell extracts to generate liposomes capable of synthesizing molecules perceived by various species of bacteria, such as *E. coli*, toward biomimetic sensing applications.[32] In addition, a few attempts have been made to mimic cellular motility by housing artificial cytoskeleton networks[33−37] or living biological cells[38] into traditional liposomes. In addition, Tsugane and Suzuki encapsulated a reverse transcription−polymerase chain reaction (RT-PCR) mixture into giant liposomes to evaluate the applicability of RNA detection (Figure 2A).[33] Also, Elani et al. constructed liposomes including biological living cells as an organelle that showed an enzymatic reaction in the artificial cell platform (Figure 2B).[38]

Liposome-based artificial cell platforms can also be found from gene-expressing vesicles made via the self-assembly of phospholipids into vesicle membranes in an aqueous phase while encapsulating all of the materials needed for gene expression.[31] For example, Miller et al. suggested a concept of artificial cells in which different biochemical reactions can occur to replicate themselves and synthesize proteins by encapsulating cellular components step-by-step.[3] These gene-expressing liposomes can mimic one of the essential behaviors of living cells, such as protein synthesis from corresponding genes and chemical signal exchanges with biological cells.[31]

Whereas liposomes have made significant contributions for several decades toward creating an artificial cell platform via diverse approaches, significant drawbacks still exist with using liposomes to achieve the ultimate goal of life-like functioning.[12] Current procedures for making cell-like systems in liposomes are sensitive to the components incorporated and the experimental setup conditions.[12] Short half lives, instability under oxidation, hydrolysis, high concentrations of salts, or significant changes in pH, as well as low tunability of the materials' properties, all remain challenges with using liposomes.[8] Nonetheless, it is evident that liposomes serve as well-developed biological vesicles functionalized by surface modification or combination with different building blocks. Therefore, finding combinations of various types of phospholipids that can provide better stability to the *in vivo* environments of membranes can overcome these challenges. It warrants future work to mimic numerous cellular activities associated with metabolism and energy under dynamic environmental conditions.

2.2. Polymersomes toward Artificial Cell Platforms. In 1999, the term "polymersome" was coined,[39] meaning a class of artificial vesicles formed by amphiphilic synthetic block copolymers (BCPs). The membrane structure of polymersomes is a bilayer composed of a hydrophobic center covered with hydrophilic inner and outer surfaces organized from diblock or triblock copolymers.[40] The molecular weight of BCPs could reach up to the 100 kDa scale, hundreds of times higher than the liposomal counterpart, which manifests the building block of polymersomes to be tougher, less permeable, and less fluidic than liposomes (Figure 3).[41]

The high tunability and durability of polymersomes enable them to be rationally designed to imitate the structure and functions of living cells.[42] Peters et al. crafted a fully synthetic, multicompartmentalized system, polymersome-in-polymersome, which mimics a cellular hierarchical construct, such as organelles in a cell (Figure 4A).[45] Also, tunable membrane properties of polymersomes, switched on and off upon the external stimuli, enable the controlled enzymatic reactions to be demonstrated. For example, polymersomes of which membranes are composed of pH-responsive polymers can lead to expansion of the polymersomes upon pH trigger with the increase in membrane permeability.[46,47] van Hest et al. recently demonstrated enzymatic reactions using polymersomes encapsulating horseradish peroxidase (HRP) upon the addition of a substrate and acidic fuels, such as HCl and urea, in the polymersome solution.[48] The initial expansion of polymersomes made it possible for HRP to react with its substrate. Then, the reaction products of ammonia changed the pH conditions of the solutions, thereby decreasing the membrane permeability and switching off further enzymatic reactions (Figure 4B).[48] As the energy source for the biological process, ATP synthesis is one of the essential features of a living cell. Choi and Montemagno constructed a biomimetic polymersome capable of producing ATP by coupling the activity of the light-driven proton pump protein bacteriorhodopsin (BR) to ATP synthase. In this example, BR created a proton gradient across the polymersome membrane then drove ATP production by ATP synthase (Figure 4C).[49]

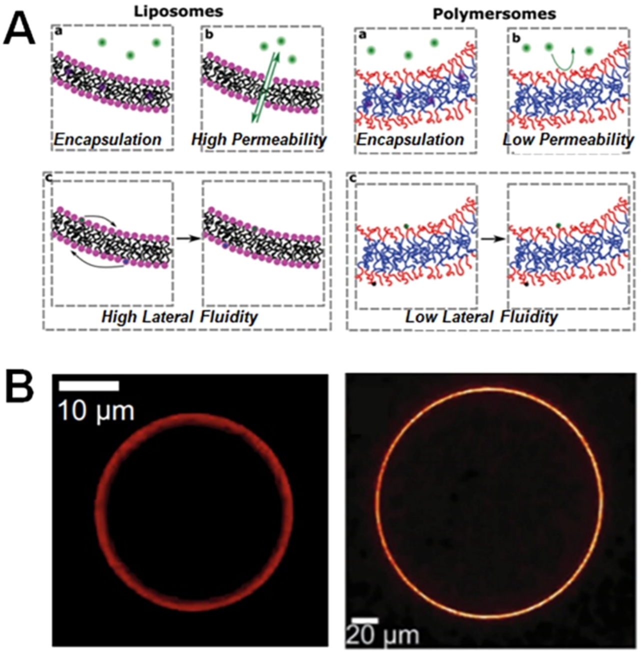

Figure 3. (A) Schematic illustrations of the traditional building blocks and membrane structures of liposomes and polymersomes.[42] Reproduced from ref 42, licensed under CC BY 3.0. (B) Microscopic images of the liposome (left)[43] and polymersome (right).[44] Left panel reproduced from ref 43, licensed under CC BY 4.0. Right panel reproduced from ref 44, licensed under CC BY 3.0.

Given the broad tunability of polymer-based-vesicles, they have been extensively used in recent decades for many applications such as smart drug delivery, *in vivo* imaging, and microreactors.[17] Nevertheless, the high stiffness and low fluidity of polymer membranes sometimes limit the application of homogeneous polymersomes for biological studies. To resolve these problems, researchers have tried to optimize the biocompatibility and permeability of polymersomes through hybrid fusion with lipids or chemical conjugation with other molecules.[50] For instance, Palivan et al. developed biocatalytic polymersomes with the encapsulation of phosphoglucomutase (PGM). These polymersomes produce glucose-6-phosphate (G6P) through the incorporation of α-hemolysin in the polymer membrane.[51] In Section 3, we further discuss such state-of-art approaches in synthetic vesicles mimicking one of the cellular functions, mainly made from heterogeneous components with asymmetric membrane structures.

2.3. Polypeptide-based Vesicles toward Artificial Cell Platforms. Several reports recently demonstrated the vesicle formation from rationally designed recombinant proteins, such as recombinant oleosin,[52] elastin-like polypeptide (ELP),[53,54] and collagen-like polypeptide (CLP).[55] Recombinant protein technology is a powerful tool for creating potent protein molecules in which the amino acid length and sequence are precisely controlled to confer specific activities.[56,57] In particular, recombinant ELP fusion proteins, derived from a structural protein (tropoelastin) responsible for providing

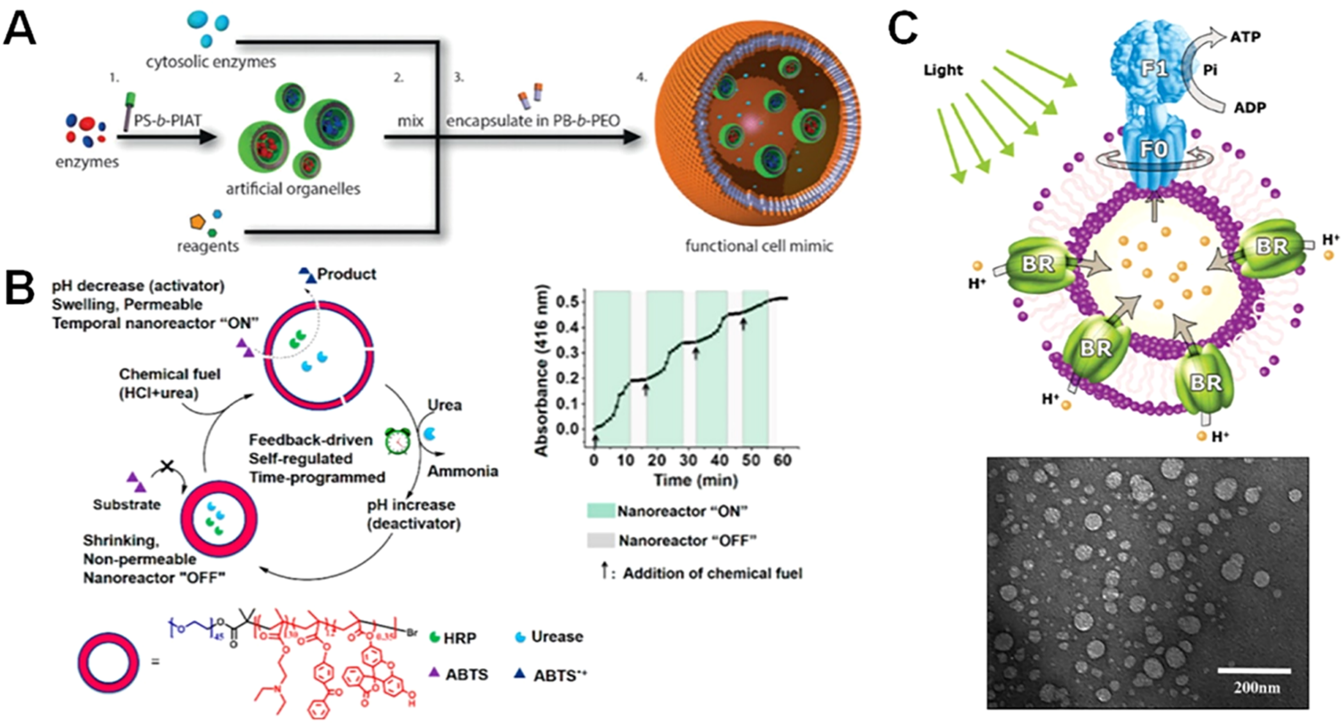

Figure 4. (A) Scheme illustration of the construction of the cell-mimicking multicompartment polymersome, which encapsulates enzyme-filled nanoreactors, free-enzymes, and substrates.[45] Reprinted with permission from ref 45. Copyright 2014 Wiley-VCH Verlag Gmbh & Co. KGaA, Weinheim. (B) Scheme and experimental data to show the stimuli-induced control of the membrane permeability of polymersomes with reversible on-and-off modulation.[48] Reprinted with permission from ref 48. Copyright 2018 American Chemical Society. (C) Schematic representation of a polymersome reconstituted with a proton pump and ATP synthase (top) and transmission electron microscopy (TEM) micrograph of the polymersomes (bottom).[49] Reprinted with permission from ref 49. Copyright 2005 American Chemical Society.

strength and elasticity to tissues in the human body, have been widely used to create temperature-responsive, self-assembled materials for various applications.[58−61] ELP, consisting of a penta-repeat $(\mathrm{VPGXG})_n$, where X can be any amino acid except proline (P),[62] undergoes a temperature-responsive inverse phase transition from soluble to insoluble due to lower critical solution temperature (LCST) behavior.[63] Vogele and coworkers developed "peptidosomes" from amphiphilic ELP fusion proteins consisting of hydrophilic glutamic acid-rich and hydrophobic phenylalanine-rich domains.[54] Similarly, Frank et al. showed the formation of polypeptide-based giant vesicles using recombinant amphiphilic ELP. These polypeptide-based vesicles are capable of housing cell-free gene expression reactions, demonstrated by the transcription of a fluorescent RNA aptamer and the production of a fluorescent protein.[64] Additionally, Hammer et al. showed that the membrane thickness of different types of peptidosomes, self-assembled from triblock copolymer-like recombinant oleosin proteins, was controlled by the ratio of the length of the hydrophilic arms to the length of the hydrophobic central block of the membrane.[52] These vesicle examples made from recombinant proteins shed light on the use of recombinant protein technology to design synthetic protocells.[54]

In addition to intrinsically disordered polypeptides like ELP, full-sized folded globular proteins play a critical role in performing a wide variety of specific biological functions, including catalysis,[65] sensings,[66] and therapeutics.[66] Hence, creating recombinant globular fusion proteins that serve as building blocks to construct vesicles would be promising for precise control over recognition, catalytic, or assembly functions as an advanced artificial cell. However, devising strategies to create highly tunable vesicles from globular proteins is still in the early stages because of the incredible complexity of protein−protein interactions. Schiller et al. have recently developed protein membrane-based compartments using an amphiphilic BCP ELP library directly fused with a few globular domains.[67] The authors demonstrated the vesicle assembly of recombinant fusion proteins by hybridization with lipids. Moreover, thermosensitive vesicles made of lipid-conjugated ELPs using click chemistry have been reported by Ibrahimova et al. as a new artificial model of hybrid lipoprotein vesicle membranes.[68] However, to our best knowledge to date, there are only a few reports of utilizing polypeptide- or protein-based vesicle platforms to mimic cell-like functions toward artificial cell development. The future direction in utilizing protein-incorporating, heterogeneous synthetic vesicles through hybridization for artificial cell applications will be discussed in more detail in Section 4.

3. HETEROGENEOUS SYNTHETIC VESICLES IN STRUCTURE AND COMPOSITION FOR MULTIMODAL FUNCTIONALITIES

Traditional methods for constructing a vesicle structure via the spontaneous assembly of amphiphilic lipids or a hydrophilic-*block*-hydrophobic (AB-type) diblock copolymer mainly result in a symmetric membrane structure. However, natural vesicle membranes are often dictated by an asymmetric distribution of a fluid mosaic of lipids and other molecules, creating a heterogeneous structure in both the radial and azimuthal directions.[69] To realize such heterogeneous membranes in synthetic vesicles, researchers have developed diverse strategies, from the rational design of building blocks to a controlled interfacial assembly based on emulsion transfer or with the aid of microfluidics.[70−73] Herein we discuss the methods for creating heterogeneous synthetic vesicles by controlling the membrane asymmetry in either the radial or the azimuthal direction (i.e., transverse or lateral asymmetry) and using different types of building blocks in hybrid conjugates or blending forms.

3.1. Heterogeneous Liposomes or Polymersomes with Transverse Membrane Asymmetry.

Natural vesicles often display transverse membrane asymmetry in the bilayer, implying that the compound on the inner leaflet differs from those on the outer leaflet. Such biomimetic synthetic transverse asymmetric vesicles can have benefit of transporting different ingredients efficiently, which improves their applications in artificial cells.[74,75] Heterogeneous liposomes are usually made with different compositions of inner and outer leaflets. Weitz et al. demonstrated a single-step microfluidic approach based on the use of water-in-oil-in-oil-in-water (W/O/O/W) triple emulsion templates, which lead to the formation of uniform asymmetric vesicles.[76] In addition, Malmstadt et al. reported a two-step route, microfluidic generation combined with emulsion transfer to form asymmetric vesicles (Figure 5A).[77] The first monolayer can be formed inside a microfluidic device (a,b) followed by the formation of the second monolayer by pouring these droplets into a solution containing a layer of oil over a layer of water (c,d). The composition of each leaflet of the resulting vesicle membrane can be tuned by dissolving different lipid compositions in each oil phase.

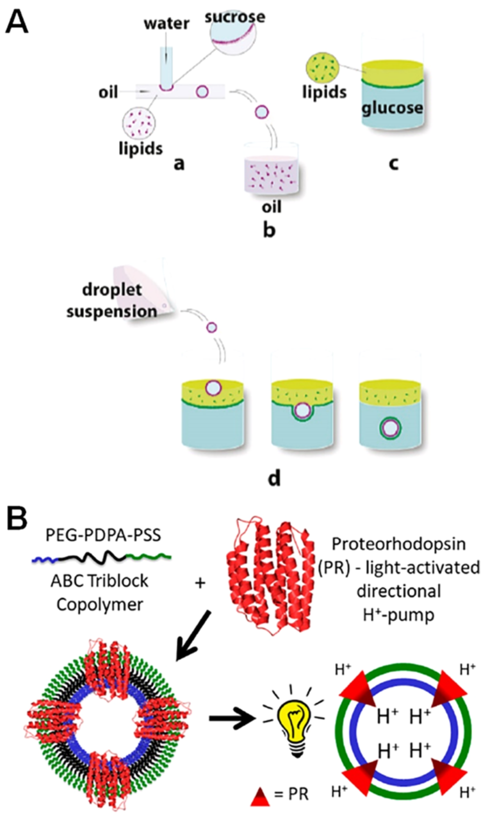

Figure 5. (A) Methods for constructing heterogeneous, asymmetric liposomes using microfluidic devices and emulsion transfer.[77] Reprinted with permission from ref 77. Copyright 2011 American Chemical Society. (B) Heterogeneous asymmetric polymersomes consisting of a triblock copolymer and a light-activated proton pump.[78] Reprinted with permission from ref 78. Copyright 2019 American Chemical Society.

In polymersomes, such transverse asymmetric membrane structures can be obtained by the self-assembly of ABC triblock copolymers with heterogeneous block patterns.[79] Triblock copolymers consisting of two different hydrophilic block ends (A and C) self-assemble into asymmetric membranes with the center B layer.[80] The ABC asymmetric triblock structure in vesicle membranes can expand the functionality and flexibility of the polymersomes for the development of an artificial cell model. For instance, Stoenescu et al. demonstrated that the asymmetry and orientation of ABC triblock copolymer-made-vesicle membranes play a critical role in controlling the incorporation of a membrane protein into the artificial cell membrane.[18] Kumari et al. incorporated His-tag-labeled Aquaporin 0, which constitutes ∼45% of the total membrane proteins while serving as a water channel and a structural cell-to-cell adhesion protein,[81] into symmetric polymersomes and liposomes and asymmetric polymersomes. The results indicated that Aquaporin 0 was inserted randomly into the symmetric membrane, whereas the insertion into the membrane of the asymmetric polymersome always favored one side distribution. This promising result has inspired researchers to design asymmetric membrane structures for synthetic vesicles to control the orientation of transmembrane proteins to mimic protein-mediated functions in artificial cell platforms.

Several reports demonstrated that the self-assembly of ABC triblock copolymers facilitates the bottom-up construction of asymmetric vesicle membrane structures. A novel ABC triblock copolymer (poly(ethylene oxide)−polycaprolactone−poly(2-methyl-2-oxazoline) (PEO-*b*-PCL-*b*-PMOXA)), consisting of biocompatible (PEO, PCL, PMOXA) and biodegradable (PCL) blocks, can self-assemble into asymmetric vesicles.[82] In this example, the PEO outer surface and the PMOXA inner surface possess the capability of conjugating different functional groups, providing new opportunities toward the specific targeting of the desired molecules of the asymmetric polymersomes. Furthermore, Gaitzsch and coworkers incorporated a functional transmembrane protein, the light-activated proton pump proteorhodopsin (PR), into asymmetric polymersomes using self-assembly with poly(ethylene glycol)-*block*-poly(diisopropyl aminoethyl methacrylate)-*block*-poly(styrenesulfonate) (PEG-*b*-PDPA-*b*-PSS).[78] These two hydrophilic chains of PEG and PSS also enabled control of the specific orientation of the proton pump PR, inducing a gradient of protons across the membrane (Figure 5B). Whereas the previous work to create asymmetric vesicles using lipids or ABC triblock copolymers successfully shed light on designing the multifunctional properties of vesicle membranes in radial directions, it is still challenging to mimic the heterogeneous vesicle structure of living cells in azimuthal asymmetry.

3.2. Heterogenous Janus Vesicles with Lateral Membrane Asymmetry.

The Janus structure exhibits two-faced lateral asymmetry in the membrane structure and composition, imparting different chemical or physical properties localized at distinct parts within the structure.[83] Janus vesicles (JVs) exhibit spherical hollow vesicle shapes but different components and properties in two-faced locations at the membrane, enabling distinct multimodal functionalities in one platform. Given the unique structural properties along with the broad usage of synthetic vesicles, JVs have gained interest in many attractive and feasible applications, such as emulsion stabilizers and facilitators for engineering liquid/liquid and liquid/air interfaces, optical nanoprobes, electronic inks, and self-propelling beads.[84] However, to date, there are very few successful examples that show Janus vesicles for artificial cell manipulations.

Wang et al. have developed Janus liposomes consisting of two different phospholipids and cholesterol based on gel-assisted thin-film hydration methods.[85] The authors created JVs having two halves composed of cholesterol with either 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) or 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), which exhibit immiscibility at the interface due to the hydrophobic mismatch to each other. They achieved control of the degree of heterogeneity by adjusting the proportion of three lipids (DPPC, DOPC, cholesterol) (Figure 6A). This asymmetric Janus liposome possessed heterogeneous surface chemistry by displaying different biotin−avidin binding affinities at two half sides. Furthermore, such Janus liposomes embedded with biotin−avidin binding affinity, can self-assemble into higher ordered domain-specific clusters/aggregations whose structures are dependent on the domain configuration of individual liposomal halves. Among the diverse types of heterogeneous vesicles with asymmetric membrane structures, the Janus structure makes vesicles exhibit disjunctive properties, showing more tendency for fission.[83] Thus the challenge remains in JV formation to overcome the membrane fission while stabilizing the interface between two distinct halves. It also motivates the development of JVs with heterogeneous components by using lipid/polymer hybrids or other potential building blocks.

Synthetic vesicles consisting of lipids, polymers, or lipid/polymer hybrids can be further functionalized by incorporating inorganic NPs.[86,87] There are several examples of the creation of heterogeneous JVs by tailoring the self-assembly of block polymers conjugated with functional NPs, such as polymer/NP hybrid JVs made from amphiphilic BCPs and BCPs conjugated with magnetic nanoparticles (MNPs) and gold nanoparticles (AuNPs) (Figure 6B).[88] Depending on the size and mass fraction of two NPs, the assembly process produces spherical or hemispherical JVs with two distinct halves or simple homogeneous vesicles with uniform distribution. The ordering and dense packing of the NP-hybridized BCP building blocks enhances the functionalities of heterogeneous JVs for many applications based on the responsiveness of MNPs and AuNPs to a magnetic field and near-IR, respectively.[88]

Heterogeneous vesicles hybridized with NPs can mimic the essential features of living cells, such as the autonomous motility, by leveraging the catalytic functions of NPs. Wang and coworkers developed JVs composed of inorganic NPs with BCPs, where the ratio of two half surfaces was controlled by the ratio of individual building blocks in the mixture (Figure 6C).[89] A poly(ethylene oxide)-*b*-poly(styrene) (PEO-*b*-PS) BCP was used as the main building block for vesicle formation. Then, several inorganic NPs (e.g., Au nanorods, AuNPs, PtNPs), whose surfaces were modified with PEO-*b*-PS, were incorporated into the half of the vesicle membrane by self-assembly with the aid of microfluidic generation. These NPs-incorporating JVs can lend multimodal functionalities to the vesicles. In particular, the PtNP/polymer hybrid JVs exhibited autonomous propulsion motility through the catalytic decomposition of hydrogen peroxide (H_2O_2) fuel upon the irradiation of near-IR light. Another method for creating a novel type of JVs would be to hybridize with NPs and other building blocks using specific molecular recognition. For instance, a molecular recognition group of either β-cyclodextrin (CD) or adamantane (Ada) can be incorporated into the surface of polymersomes with quantum dots (QDs).[90] By a CD−Ada host−guest interaction, the fusion of CD-functionalized vesicles and Ada-modified vesicles occurred, leading to the production of JVs with two hemispheres containing

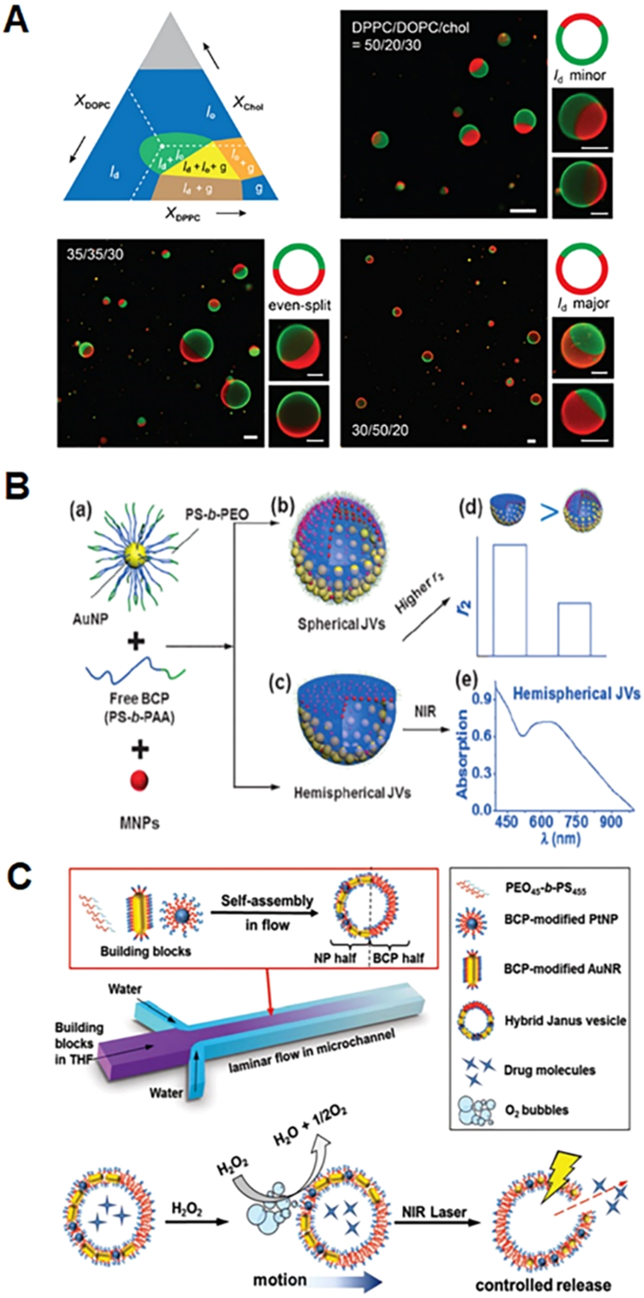

Figure 6. (A) Phase diagram and micrographic images of Janus liposomes with controlled domain configuration.[85] Reprinted with permission from ref 85. Copyright 2018 American Chemical Society. (B) JVs with the controlled distribution of AuNPs and MNPs with different morphologies.[88] Reprinted with permission from ref 88. Copyright 2016 Wiley-VCH Verlag Gmbh & Co. KGaA, Weinheim. (C) Hybrid JVs generated from a mixture of BCPs and inorganic NPs in a microfluidic device. Stimuli-triggered motility and controlled release of JVs in the presence of hydrogen peroxide upon the irradiation of near-IR.[89] Reprinted with permission from ref 89. Copyright 2015 Wiley-VCH Verlag Gmbh & Co. KGaA, Weinheim.

different QDs. The functionalities of JVs made with magnetic NPs (e.g., Fe_3O_4) were also demonstrated in a magnetically controlled printing application, inspiring the magnetic-field-driven motion toward artificial cells.[90]

3.3. Engineering Hybrid Vesicles with Heterogeneous Components and Structures. Despite liposomes exhibiting overall good fluidity, permeability, and biocompatibility, the nonadjustable thickness of lipids (~5 nm) with low molecular weights limits the stability and tunability of the vesicle membranes.[91] On the contrary, the tunable membrane thickness of polymersomes (~8 to 50 nm) renders them higher strength but results in lower permeability, fluidity, and flexibility.[92] Thus the field is shifting to create hybrid vesicles by integrating two or more complementary systems of liposomes and polymersomes into one versatile vesicle structure.

Many researchers have tried to hybridize polymers with lipids to make highly tunable and biologically active synthetic vesicles by importing the complementary properties of both polymersomes and liposomes. Miele et al. designed giant hybrid vesicles made from the mixtures of poly(methyl methacrylate)-based copolymers and lipids by means of a droplet transfer method based on a water-in-oil emulsion.[40] Another example of hybrid vesicles can be found from poly(ethylene oxide)-*block*-polycaprolactone-*block*-poly(2-methyl-2-oxazoline) (PMOXA-*b*-PCL-*b*-PMOXA) polymersomes hybridized with egg-phosphatidylethanolamine (PE), made by thin-film rehydration methods.[40] These hybrid vesicles presented intermediate properties of the copolymer and the lipid components, allowing for precise control over the overall membrane properties by varying their molar ratios. Also, the hybridization of polymers and lipids into heterogeneous vesicles can be achieved through layer-by-layer deposition, where the conformation and architecture of high-molecular-weight polymers play a critical role in determining the membrane properties. For example, Tan et al. created lipid/polymer heterogeneous vesicles using surface modification of pristine liposomes, which facilitated the layer-by-layer deposition of chitosan polymers on lipid bilayers, leading to an asymmetric membrane structure (Figure 7A).[93] The chitosan/lipid heterogeneous vesicles showed tailored properties of fluidity, morphology, and hydrophobicity by using the three different forms of chitosan, determined by its concentration. As a result, the deposition of crimped chitosan on liposomes led to less fluidity in the vesicle membrane surface with higher structural order than the case of using the extended chitosan chains at a lower concentration.

The structural asymmetry of lipid/polymer heterogeneous vesicles is strongly correlated to the phase separation of the lipid and the polymer. In the following study, we can find the detailed heterogeneous membrane structure and domain formation process of lipid/polymer hybrid vesicles. Dao et al. created asymmetric heterogeneous vesicles using copolymers of poly(dimethylsiloxane) (PDMS) and poly(ethylene oxide) (PEO) with different molecular weights and architectures (i.e., grafted vs block) when the copolymers were mixed with either 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, in fluid state) or dipalmitoylphosphatidylcholine (DPPC, in gel state).[94] The authors found that the copolymer architectures and molecular weights play significant roles in not only the successful formation of heterogeneous vesicles but also the domain size of the hybridized vesicles (Figure 7B).

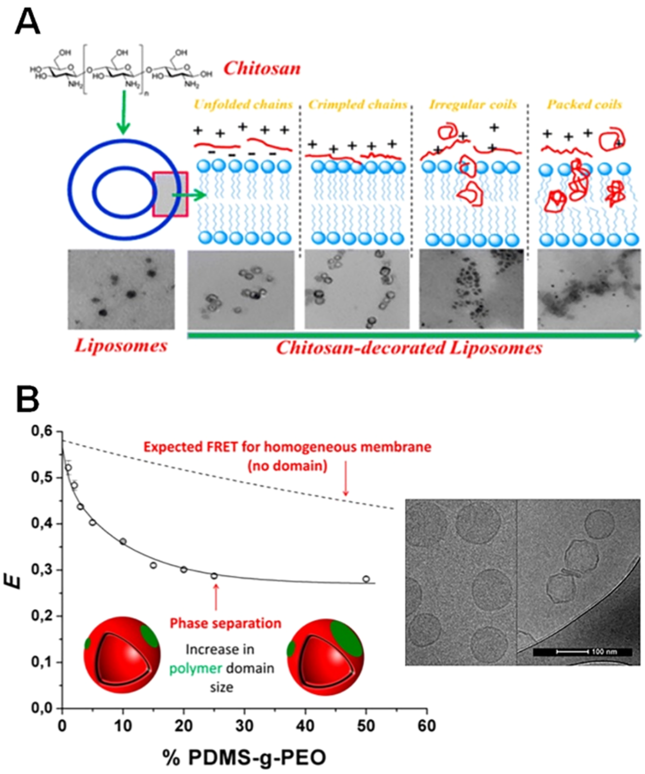

Figure 7. (A) Scheme and TEM micrograph of chitosan-decorated chitosan/lipid hybrid vesicles with different chitosan concentration.[93] Reprinted with permission from ref 93. Copyright 2015 American Chemical Society. (B) Fret efficiency graph as a function of the mole fraction of PDMS-g-$(PEO)_2$ and cryo-TEM micrograph of lipid/polymer hybrid vesicles.[94] Reprinted with permission from ref 94. Copyright 2017 American Chemical Society.

Indeed, great attention has been paid to making heterogeneous vesicles using a mixture of lipids and polymers along with an intensive effort to characterize the asymmetric membrane structure at the submicrometer scale. However, classical characterization methods based on scattering and microscopy techniques would not be sufficient to fully understand the dynamic properties of such heterogeneous vesicle membranes. Also, lipid/polymer heterogeneous vesicles have some practical challenges with phase separation due to thermodynamic instability, resulting in budding and fission into single homogeneous liposomes or polymersomes. The lateral phase separation is mainly induced by a temperature and thickness mismatch, which warrants future investigation for the engineering stability of heterogeneous vesicles.[91] Recent advances in coarse-grained molecular dynamics simulations have helped to elucidate the factors governing lipid−polymer interactions in heterogeneous vesicles.[95] Ultimately, a deep understanding of the thermodynamics and kinetics behind the self-assembly of heterogeneous components into vesicles will advance the development of artificial cells with more structural and functional complexity.

4. CAN GLOBULAR PROTEINS SERVE AS POTENTIAL BUILDING BLOCKS TO FACILITATE BIOLOGICAL FUNCTIONS OF HETEROGENEOUS SYNTHETIC VESICLES?

Proteins are one of the basic building blocks of life. Proteins also have potential for many applications, such as catalysis,[65] sensing,[66] and therapeutics,[96] based on their specific activities originating from their functionally folded three-dimensional structures. The unique functions of proteins are hard to mimic using other synthetic or short biological molecules.[56] Although proteins are generally considered as potential molecules, soluble-free proteins suffer from short circulation kinetics with a low localized concentration, a low recovery yield, and a short-term half life.[97] Therefore, a number of studies have been devoted to encapsulating functional globular proteins within the lumen of synthetic vesicles, offering promising potential in the fields of nanomedicine, biotechnology, and materials engineering as novel drug-delivery vehicles, bioreactors, and artificial cells.[97] Moreover, the vital functions of living cells, such as cellular communication, metabolic uptake, and product release, are all performed by transmembrane proteins that comprise membrane structures like cell surface receptors, ion channels, and molecular transporters.[3] The efforts to use folded proteins as critical building blocks in artificial cell construction include the examples that we discussed in Section 3, such as BR,[49] α-hemolysin,[51] or PR-loaded heterogeneous polymersomes.[78] Thus the incorporation of proteins into synthetic vesicle membranes is a critical step in the rational design and construction of artificial cells to achieve advanced cellular functions mediated by proteins.[98,99] Herein we highlight the diverse approaches and challenges for incorporating globular proteins into synthetic vesicle platforms. We also discuss one of the promising ways to utilize recombinant protein building blocks toward achieving more cellular functions mediated by folded proteins.

4.1. Encapsulation of Globular Proteins in Synthetic Vesicle Lumens. The encapsulation of globular proteins into polymersomes that are stable in biological environments allows for maintaining the protein structure and enables their controlled release through tunable polymer membranes.[97] The film rehydration method offers versatility for encapsulating proteins in the aqueous lumen of vesicles without the loss of their functional conformation, regardless of the types of therapeutic molecules, ranging from small drugs to proteins.[97] Bovine serum albumin (BSA) has also been encapsulated as a model protein drug within the aqueous lumen of synthetic vesicles composed of diverse BCPs by the film rehydration method (Figure 8A).[100]

Globular proteins empower synthetic vesicles to imitate the complex "life-like" functions of living cells and offer a cutting-edge approach that can overcome current limitations in medical treatments.[97,101] Seasonal blood shortages, the short storage shelf life of blood, and the high demand for blood during unfortunate scenarios have all led to multiple attempts to mimic the structure and functions of red blood cells (RBCs) by engineering synthetic vesicles. The first step for the construction of artificial RBCs would be to mimic the simple intracellular structures of RBCs containing nucleus and organelles.[97,101] During the last few decades, liposomes encapsulating the metalloprotein hemoglobin (Hb) have emerged as an encouraging candidate of RBC substitutes in transfusion medicine.[101] Diverse techniques, such as detergent dialysis, reverse-phase evaporation, and microfluidics, have been used to encapsulate Hb into liposomes. In particular, microfluidic generation enables the efficient scale-up production of liposome-encapsulated hemoglobin (LEH).[101] Nonetheless, the short circulation kinetics of LEHs and their tendency to aggregate and fuse together after several days of storage remain challenges.[101] Protein-loaded polymersomes can overcome these limitations of LEHs with an improved circulation half life and the *in vivo* stability of vesicle membranes.[102] Arifin et al. successfully demonstrated that Hb can be readily encapsulated within polymersomes, thus creating polymersome-encapsulated hemoglobin (PEH), which has oxygen affinities comparable to those of human erythrocytes.[102]

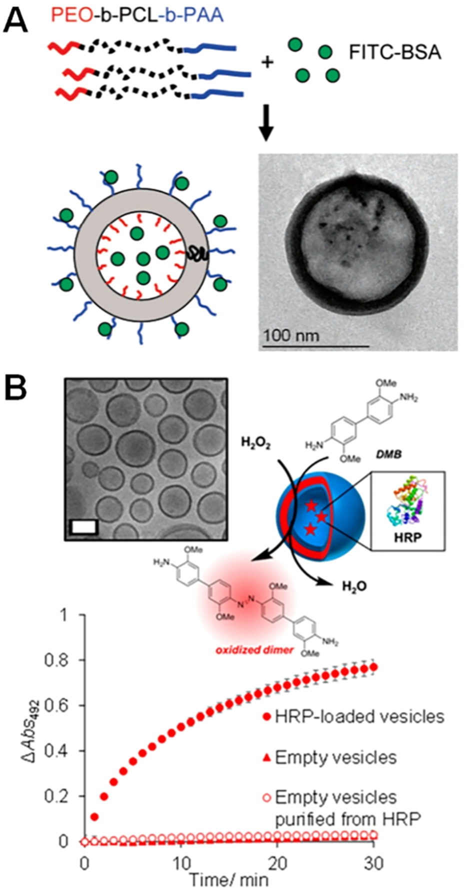

Figure 8. (A) Schemes and TEM micrograph of a triblock copolymer-made asymmetric vesicle encapsulating fluorescence-labeled bovine serum albumin (FITC-BSA).[100] Reprinted with permission from ref 100. Copyright 2007 American Chemical Society. (B) Schematic illustration of the HRP-catalyzed cascade reaction in polymersomes (inserted cryo-TEM image of HRP-loaded vesicles). Experimental data of the activity of HRP-loaded vesicles against empty vesicles (right bottom).[103] Reprinted with permission from ref 103. Copyright 2017 American Chemical Society.

Accordingly, such multicomponent heterogeneous vesicles encapsulating proteins can have an increasingly versatile and potent role in imitating the structure and essential function of cells. For example, polymersomes can encapsulate diverse types of enzymes while retaining their functions, which allows for the modeling of elaborate cellular metabolic functions in artificial cell platforms.[104,105] Blackman et al. demonstrated the feasibility of polymersomes for this application by encapsulating the HRP and glucose oxidase enzymes (GOx).[103] Because GOx and HRP interact with each other to induce the catalytic cascade reaction, GOx- and HRP-loaded vesicles were used to validate that the compartmentalized enzymes between separate vesicles were capable of mimicking enzyme cascade reactions in living cells (Figure 8B).[103] In addition to modeling the metabolic functions of enzymes in living cells by using engineered heterogeneous vesicles, researchers have further tried to develop approaches to recreate other protein functions in artificial cells. Incorporating the enzyme β-lactamase, which catalyzes the hydrolysis of ampicillin to form ampicillinoic acid in bacteria, into the vesicle lumen of polymersomes enables them to mimic the bacterial function and allows for nanoreactor applications.[97] These findings demonstrated the promising potential for the use of globular proteins in heterogeneous synthetic vesicles to simplify and model sophisticated cellular functions.

Globular proteins can also serve as bioactive building blocks in heterogeneous component vesicles capable of metabolism and sensing.[103] However, the process of encapsulating globular proteins remains a challenging procedure. During the process of encapsulating globular proteins, proteins can be exposed to hydrophobic interfaces in harsh organic solvents, which can denature proteins and lead to inactive folding structures.[106] Furthermore, the process can result in undesirable spatial organizations of proteins attached to the external interface of vesicles.[100] To overcome these drawbacks, multidisciplinary research efforts have recently been devoted to developing novel strategies to incorporate globular proteins into an essential cellular vesicle structure in diverse forms by rationally designing the protein building blocks via recombinant protein engineering and synthetic biology.

4.2. Incorporating Functionally Folded Proteins at Synthetic Vesicle Membranes. The inclusion of transmembrane proteins into liposomes has been achieved by various methods, such as partial drying and rehydration of protein-containing lipid bilayers,[107] mechanical insertion,[108] and detergent-mediated reconstitution.[109] Numerous studies have successfully utilized specific transmembrane proteins, such as mechanosensitive channels, Ca^{2+} ATPase, and potassium channels, at vesicle membranes to engineer synthetic vesicles for a multitude of precise protein functions.[3] Li et al. developed a protocell model to synthesize ATP from light by incorporating the motor protein BR into a protein−polymer hybrid membrane.[110] The researchers demonstrated a systematic bottom-up construction of the artificial cells by engineering intermolecular interactions between building blocks based on electrostatic layer-by-layer assembly and $\pi-\pi$ stacking to incorporate the motor protein and photoacid generators within the protocell membrane.[110] This work suggests the potential of protein-conjugated synthetic vesicles in remotely controlled ATP-consumed biosensors, biocatalysts, and biodevices.[110]

Incorporating light-sensitive proteins, such as rhodopsin, into synthetic vesicle membranes facilitates communication with neighboring vesicles through light stimuli while not affecting biological components.[8] Palivan and coworkers utilized polymersomes to build a biologically functional nanoreactor, an "artificial peroxisome", capable of clearing its surroundings of superoxides.[98] To achieve this, the researchers built an artificial cell from ABA triblock copolymers and incorporated the channel protein OmpF within its membrane. This artificial peroxisome marked a significant milestone in the development of applications of synthetic vesicles as nanoreactors and drug delivery systems,[98] which has been inspired by living cell mimetics.

Despite the encouraging use of transmembrane proteins in expanding the functionality of artificial cells, prominent challenges exist in incorporating transmembrane proteins in synthetic vesicle membranes. The reconstitution of transmembrane proteins in liposomes has contributed to making protein-incorporated vesicle membranes, but the membrane rupture and denaturation of the proteins incorporated and the difficulties in controlling the protein orientation and proportion remain significant challenges.[12,15,16,111] Correct protein orientation is of the utmost importance when fashioning synthetic vesicles to ensure proper control of inward and outward transport.[98] In this regard, engineering an asymmetric structure of heterogeneous vesicles, as discussed above, plays a significant role in enabling control over protein orientation.

The construction of an artificial cell containing a globular protein at the membrane, named a "proteinosome", was first suggested by Mann in 2013.[11] Protein−polymer conjugated building blocks were made through chemical conjugation of primary amine groups of bovine serum albumin (BSA-NH_2) to synthetic poly(*N*-isoprophylacrylamide) (PNIPAAm). The self-assembly of BSA-PNIPAAm conjugates results in oil-in-water Pickering emulsion droplets, and cross-linking of the membranes followed by oil removal produces BSA-incorporated proteinosomes.[11] Coupling mercaptohiazoline-activated PNIPAAm chains to primary amine groups of cationized BSA-NH_2 produces the protein−polymer building blocks BSA-NH_2/PNIPPAm. This heterogeneous protein−polymer building block has recently been proposed as a promising artificial protocell model in the form of coacervate droplets or emulsions for guest molecule encapsulation, selective membrane permeability, and programmable renewal.[13,112−114] Huang et al. rationally designed biomimetic artificial cells capable of guest molecule encapsulation, selective permeability, gene-directed protein synthesis, and membrane-gated enzyme catalysis.[11] The globular protein BSA was also coupled to the polymer poly(ε-caprolactone) using a maleimide−sulfhydryl coupling reaction to form another amphiphilic protein−polymer conjugate BSA-PCL.[115] An emulsion evaporation method was used to create BSA-PCL self-assembled into vesicles that can serve as novel drug delivery vehicles while also exhibiting a well-defined structure, excellent biocompatibility, and low cytotoxicity.[115]

Nonetheless, the chemical coupling BSA to different polymer units could hinder the biological functions of globular proteins at the vesicle membrane.[116] This is due in part to the use of organic solvents during the chemical coupling of BSA to polymer subunits, the high molecular weights preventing conformational arrangement, and the possible shielding.[116] Finally, the direct incorporation of globular proteins into self-assembling polymers has the drawbacks of intricate thermodynamics and kinetic profiles of the amphiphiles caused by the high molecular weight of the globular moiety and the associated steric hindrance.[117] Currently, substantial design obstacles exist in the development of a synthetic vesicle membrane that efficiently encapsulates internal components while allowing the selective ingress and egress of precursor and product molecules via transporter proteins and pores traversing the membrane.[3] Furthermore, the folding, function, and successful incorporation of proteins are heavily reliant on the membrane composition and the environment and require future investigation.[3]

4.3. Self-Assembly of Recombinant Globular Fusion Proteins into Heterogeneous Vesicles. Recombinant globular fusion proteins are attractive and promising building blocks in the design and creation of artificial cells.[118] Recent advances in recombinant protein technology have paved the way for the creation of novel protein molecules in which the amino acid length and sequence are strictly controlled to convene precise bifunctionalities for synthetic vesicles.[56] Furthermore, this approach enables detailed tuning of the physicochemical characteristics of the protein building blocks. For example, the amphiphilicity of recombinant protein building blocks for vesicle assembly can be manipulated by varying the total length and ratio between hydrophilic and hydrophobic protein domains.[118]

Recent research has demonstrated the feasibility and encouraging potential of vesicles self-assembled from rationally designed recombinant proteins. In Section 2.3, we introduced several examples of peptidosomes made from recombinant polypeptides.[52,54,55] To overcome some of the drawbacks described in the polymer−protein conjugates, Huber et al. utilized recombinant ELPs as part of a synthetic biological approach to control the formation of dynamic membrane-enclosed compartments.[118] The researchers suggested a unique approach to constructing organelles from nonlipid molecules. This synthetic vesicle platform advances the capabilities of recombinant protein vesicles for artificial cells by enabling metabolic regulation, recreating a cell's structural and functional network, and using them as nanobioreactors.[118] Although the prior work was limited to intrinsically disordered polypeptides, it motivates the broader application of recombinant protein technology to obtain vesicles containing functionally folded, full-sized proteins. The creation of recombinant globular fusion proteins that serve as building molecules to construct vesicles will allow for precise control over catalytic,[65] recognition,[66] or therapeutic[96] functions as an advanced artificial cell model. Nevertheless, devising strategies to create highly tunable vesicles from globular fusion proteins is still in the early stages because of the greater complexity of protein−protein interactions.

Park and Champion designed versatile recombinant protein building blocks to make globular protein vesicles (GPVs) solely via aqueous self-assembly based on the high binding affinity of leucine zipper pairs (Figure 9A).[119] The protein building blocks for vesicle assembly are composed of a globular domain fused with a glutamic-acid-rich leucine zipper (globule-Z_E) and an arginine-rich leucine zipper fused with an elastin-like polypeptide (Z_R-ELP). The use of leucine zipper motifs with high binding affinities to fuse either a globular protein or an ELP domain facilitates the versatile, modular design of protein amphiphiles. Jang et al. demonstrated the mechanism behind the vesicle assembly of globule-Z_E/Z_R-ELP fusion protein complexes using fluorescent globular domains.[56,120,121] The globular fusion proteins form an amphiphilic complex then self-assemble into GPVs upon warming due to the LCST behavior of the ELP Figure 9B. The authors elucidated a temperature-induced phase transition of the recombinant protein complexes from soluble proteins through metastable coacervates to vesicles by microscopy and scattering techniques.[120] The understanding of the self-assembled structure as a function of temperature provided the fundamental information to tune the size and membrane structure of GPVs by altering the molecular packing parameter.[120] The ability to incorporate diverse globular proteins into vesicle membranes via hierarchical self-assembly (molecular and supramolecular) under mild aqueous solutions makes GPVs attractive candidates to perform their functions as protein-powered artificial cells.

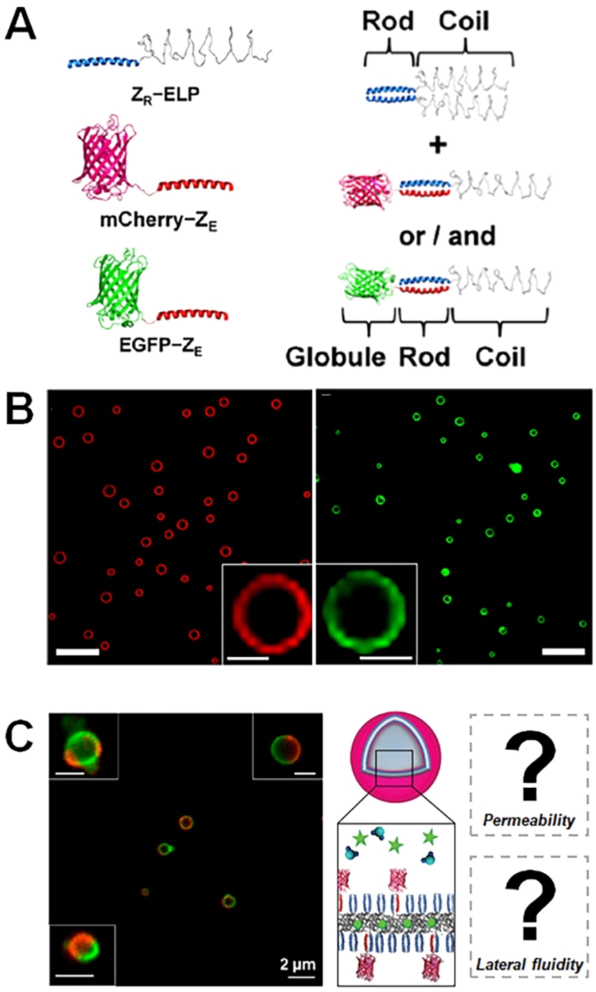

Figure 9. (A) Recombinant fusion protein building blocks. (Left) Z_R-ELP, mCherry-Z_E, EGFP-Z_E, and their self-assembled complexes. (Right) Rod−coil Z_R-ELP homodimer, globule−rod−coil mCherry-Z_E/Z_R-ELP, and EGFP-Z_E/Z_R-ELP heterodimers that form vesicles. (B) Confocal micrographs of globular protein vesicles (GPVs) made from (left) mCherry-Z_E/Z_R-ELP and (right) EGFP-Z_E/Z_R-ELP. Scale bars are 10 and 1 μm (inset).[119] Reprinted with permission from ref 119. Copyright 2014 American Chemical Society. (C) Confocal micrograph of the heterogeneous GPVs with asymmetric membrane distribution.[121] Reprinted with permission from ref 121. Copyright 2019 American Chemical Society.

The deep understanding of the coacervate-to-vesicle transition of the globule recombinant protein building blocks enabled the development of an engineering strategy to make heterogeneous GPVs displaying different globular proteins at different locations.[121] Among the few types of heterogeneous vesicles made of lipids, polymers, or lipid/polymer hybrids that exist,[9,85,122,123] this report suggested a versatile approach to the construction of synthetic vesicles made of multiple full-sized, globular functional proteins with asymmetric membrane distributions by utilizing the self-assembly of recombinant proteins. This work inspires future investigations to build up an artificial cell platform composed of fully folded, globular proteins in controlled proportion with controlled properties (e.g., permeability and lateral fluidity) and at the desired locations (Figure 9C).

5. CONCLUSION AND FUTURE PERSPECTIVES

Motivated by results arising in the past two decades, we highlighted and compared the research outcomes about the structure and composition of synthetic vesicle membranes and the future research directions of potentially feasible materials. Lipids and polymers have emerged as components for symmetric synthetic vesicles for several decades; consequently, a great number of mature outcomes have been made. However, the functionalities of synthetic vesicles can be further enhanced by the incorporation of more than one component. Thus we conclude the hybrids of lipids/polymers, NPs, or proteins can serve as next-generation building blocks to construct artificial cells that exhibit asymmetric structures and multimodal functionalities with more versatility. We also discussed the present developing stages of protein-incorporating vesicles, including transmembrane proteins through vesicle membranes, globular proteins as the encapsulant, and the self-assembly of globular proteins into GPVs. As the encapsulant, proteins carried in synthetic vesicles have been proved to imitate the basic structures and realize the versatile functions of living cells, like metabolic function or bacterial function. As a part of the vesicle membrane layer, protein conjugates exhibit high potential in selective transportation and allow for precise control by means of domain tuning. Recombinant protein engineering will make it promising to design potent building blocks for the bottom-up construction of heterogeneous artificial cells toward multiple protein functionalities.

Heterogeneous protein vesicles would be an innovative class of artificial cells to enable higher ordered protein assembly in a vesicle structure. Utilizing proteins as building blocks for artificial cells allows them to be engineered with specific properties at both the molecular and supramolecular levels, advancing the versatility of artificial cell applications. A heterogeneous protein vesicle platform paves the way for artificial cells to be used as "smart" functioning autonomous particulates in a wide range of future technologies from biomedical devices to protein drug delivery and self-regulating microbiofactories. With the vast potential to advance the human condition, it remains critical to expand our understanding of the structure−property correlations of heterogeneous protein vesicles and bring to fruition the myriad of promising and versatile applications of these artificial cells. Ultimately, the goal of developing synthetic vesicles is to create artificial cells capable of executing the necessary functions of living cells by utilizing nonliving materials. A summarization of this work would be of a great interest to researchers in the constituent tuning of heterogeneous vesicles toward artificial cells.

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Notes

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