Recent developments in microfluidic synthesis of artificial cell-like polymersomes and liposomes for functional bioreactors

Hanjin Seo and Hyomin Lee

AFFILIATIONS
Department of Chemical Engineering, Pohang University of Science and Technology (POSTECH), 77 Cheongam-Ro, Nam-Gu, Pohang, Gyeongbuk 37673, South Korea

ABSTRACT
Recent advances in droplet microfluidics have led to the fabrication of versatile vesicles with a structure that mimics the cellular membrane. These artificial cell-like vesicles including polymersomes and liposomes effectively enclose an aqueous core with well-defined size and composition from the surrounding environment to implement various biological reactions, serving as a diverse functional reactor. The advantage of realizing various biological phenomena within a compartment separated by a membrane that resembles a natural cell membrane is actively explored in the fields of synthetic biology as well as biomedical applications including drug delivery, biosensors, and bioreactors, to name a few. In this Perspective, we first summarize various methods utilized in producing these polymersomes and liposomes. Moreover, we will highlight some of the recent advances in the design of these artificial cell-like vesicles for functional bioreactors and discuss the current issues and future perspectives.

I. INTRODUCTION
Cells, the basic building blocks of life, are highly complex systems in which various biological reactions take place. The interior of these micro-scale bioreactors is compartmentalized by a membrane that separates the interior from the surrounding environment. The cellular membrane is semi-permeable and stimuli-responsive and thus adapts to changes in their surroundings and allows communicating with other adjacent cells. In addition, the membrane serves as a barrier protecting the cells from toxins that interfere with the cells’ vitality and also respond to specific biomolecules as well as ionic strength of the media. To exploit these cells’ unique structure and functionality, artificial cells that mimic the membrane structure as well as the biological functions are actively explored in various fields, including synthetic biology, molecular biology, drug delivery, biosensors, and bioreactors. For instance, vesicles that resemble the cells’ membrane structure have been presented for efficient delivery of cosmetic actives and therapeutics. The two most widely studied molecules that comprise these artificial cell-like vesicles are synthetic polymers with excellent chemical versatility and phospholipids that constitute an actual cell. The self-assembly of these molecules into a bilayer structure results in polymersomes for amphiphilic block copolymers, and liposomes for natural phospholipids as schematically illustrated in Fig. 1.

Among these two artificial cell-like vesicles, polymersomes comprising an amphiphilic block copolymer with a hydrophobic block and a hydrophilic block can be tailored to a desired molecular weight and composition to yield vesicles with osmotic-pressure responsive properties or complex structure with multi-compartments. Due to these advantages, polymersomes encapsulating specific substances in the interior can be selectively released in response to external stimuli. However, the versatility of these polymersomes is often offset by the low biocompatibility, thick membrane thickness, and incompatibility with membrane-bound proteins, which all lead to deviation from actual cell structure and function. This has been considered as one of the main obstacles of utilizing polymersomes as the artificial cell mimics.

On the other hand, liposomes composed of phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) have been actively studied as an
alternative artificial cell model. The excellent biocompatibility offered by liposomes has been exploited in drug delivery systems as well as for mimicking and understanding the complex metabolism occurring inside cells. In particular, liposomes provide a more simplified and controllable model than an actual cell with a complex internal structure, and a series of biochemical reactions occurring in organelles have been implemented in these artificial cells. As liposomes are comprised of natural lipids that constitute the actual cell membrane, they can more readily mimic the cell membrane and avoid any compatibility issues with various substances involved in these biochemical reactions. However, liposomes still have several challenges to overcome. The variability in the composition and chemical tunability is limited compared to polymersomes, and most importantly, liposomes are less stable than polymersomes and actual cells due to their low molecular weight as well as the simplicity of the cell membrane model which neglects any membrane-bound substances that are prevalent in actual cells.

Even with such shortcomings, various studies have been reported which have presented the potential of these artificial cell-like vesicles in regulating biological functions. Various biochemical reactions including in vitro protein expression, enzyme synthesis, and assay have been realized in artificial cell-like vesicles. To effectively carry out these biochemical reactions, subcellular structures including membranous organelles and non-membrane organelles have been actively investigated. Specifically, membraneless organelles (MOs) have been devised using liquid–liquid phase separation (LLPS) or coacervates to create a dynamic internal compartment within the vesicle; these MOs are known to involve in a variety of biological responses including regulation of gene expression. Moreover, membrane proteins have been incorporated in the vesicle membrane to tune the membrane permeability and regulate the biological function by controlling the mass transport across the artificial cell membrane.

In this Perspective, we will first summarize the methods used to prepare artificial cell-like vesicles. While conventional methods such as thin film hydration and the electroformation method will be presented, we will mainly discuss the up-to-date methods with specific emphasis on the droplet microfluidics approach with high uniformity, high encapsulation efficiency, and continuous production capability. After the detailed discussion on the methods utilized in the preparation of artificial cell-like vesicles, we will then focus on the physicochemical properties of these vesicles, methods to realize sub-compartmental structures in these vesicles, and their relation to the realization of various biochemical reactions in these vesicles. This will be followed by the recent research outcomes and current issues, as well as future perspectives.

II. METHODS FOR VESICLE FORMATION

Vesicles have been most commonly produced by the film hydration method in which a dried thin film comprising of amphiphilic molecules residing at the bottom of a solid support such as a glass flask is simply hydrated by exposure to an aqueous solution. Upon hydration and mechanical agitation of the swollen film, the ends of the film are spontaneously enclosed to form a vesicle that separates the inner core from the outer aqueous solution. Starting from this basic principle, similar concepts capable of fine-tuning the physical properties of the resulting vesicles have been recently reported. In this section, we will briefly review the principles, merits, and limitations of the conventional methods used in preparing vesicles and discuss the recent methods that notably improve the shortcomings of the previous methods.

A. Solvent-free methods

Before we discuss the methods used to prepare artificial cell-like vesicles, it is noteworthy to briefly re-visit the two representative artificial cell models, polymersomes and liposomes. Polymersomes consist of an aqueous core separated from the continuous aqueous media by a molecular bilayer membrane comprising of amphiphilic block copolymers as shown in the schematics of Fig. 1(a). Various types of amphiphilic block copolymers with a different combination
of hydrophobic and hydrophilic blocks have been used to prepare polymersomes (Table I). This clearly reveals the excellent chemical versatility and tunable functionality of the resulting polymersomes. While the complete structure–property relation of these block copolymers in polymersome membrane has not yet been fully revealed, the effect of molecular weight and the composition on the membrane thickness, modulus, and permeability are currently being investigated as we speak.

In comparison, liposomes consist of an aqueous core surrounded by a bilayer membrane comprising of natural amphiphilic molecules, phospholipids, as shown in the schematics of Fig. 1(b). Liposomes are often classified into small unilamellar vesicles (SUVs, <200 nm), large unilamellar vesicles (LUVs, <500 nm), and giant unilamellar vesicles (GUVs, >5 μm) depending on their overall size.27 We note that liposomes with diameters ranging from 10 to 100 μm have gained significant interest recently due to their sizes that are similar to an actual cell.28–30 Similar to polymersomes, phospholipids in liposomes are also amphiphilic and have a hydrophobic phosphatidyl tail group and a hydrophilic head group. Various phospholipids exist that have a variety of head groups and different numbers of carbon atoms and double bonds present in the tail groups. In the end, the appropriate selection of these basic building units, either a block copolymer or a phospholipid, leads to polymersome or liposome with desired characteristics and functionality.

While polymersomes and liposomes differ by the basic building units of the membrane, the methodology used for forming these vesicles is quite similar. These vesicles have been commonly prepared either by the thin film hydration method31,32 or the electroformation method.33,34 As briefly described above, the thin film hydration method relies on the formation of a vesicle by spontaneous budding of a dried film consisting of amphiphilic molecules. The injection of an aqueous solution onto the dried film deposited on a rotary flask, Teflon, or a metal substrate, followed by mechanical agitation via sonication or stirring results in budding of the film and formation of vesicles. However, this method does not allow fine control of the resulting vesicle membrane structure as well as the size. The vesicles tend to be polydisperse, and more importantly, the encapsulation efficiency is low as vesicle formation and encapsulant loading occur simultaneously.35 To further complement this method, the electroformation method has been proposed. In electroformation, a high alternating current is additionally applied during the budding process to yield vesicles with less dispersity as well as size that are similar to an actual cell.36 We note that while the electroformation method is known to generate high yields of unilamellar vesicles, multi-lamellar vesicle (MLV) structure is also observed37 and the size variation of the resulting vesicles still remains to be resolved.38 In this sense, the synthesis of vesicles through these two methods needs improvement in the ability to control the membrane structure, obtain narrow size distribution, and enhance the efficacy of the encapsulation process.

### B. Solvent-assisted methods

To resolve these issues and reliably induce self-assembly of amphiphilic molecules to form a single bilayer membrane in high yield, various solvent-assisted methods have been proposed. The inverted emulsion method allows the production of unilamellar GUVs with high uniformity and better encapsulation efficiency compared to the conventional thin film hydration and the electroformation method.35,37,38 [Figs. 2(a) and 2(b)]. Specifically, an organic solvent (oil) containing phospholipids is first placed on top of an aqueous solution. Then, a separate aqueous droplet that ultimately constitutes the inner core of the GUV is dispensed in the organic solvent phase using a micropipette. By injecting the inner aqueous droplet in the lipid-containing oil phase, a water-in-oil (W/O) single emulsion is first created at the pipette tip, thereby constructing a monolayer of phospholipids at the periphery of the water droplet. Due to the higher density of the aqueous droplet compared to the oil phase as well as another phospholipid monolayer formation at the bulk water/oil interface, the sedimentation of the water droplet into the underlying aqueous phase leads to the formation of a lipid bilayer and spontaneous transfer of the resulting GUV into the aqueous phase as shown in Fig. 2(a). While this method reduces the size dispersity of the vesicles and enables the production of GUVs with sizes in the range of few hundreds of micrometers, the production yield is very low and does not allow the construction of vesicles with more complex structures.

Another solvent-assisted method has been proposed to directly prepare asymmetric unilamellar liposomes (GUVs) by applying a pulsed jet into a planar lipid layer using glass capillaries [Fig. 2(c)]. In this microfluidic jetting method, two organic solvents containing different lipids are separately introduced and come into contact to form a single bilayer of phospholipids. An aqueous solution is directly pulsed and sprayed from a micronezzle to generate GUVs as it passes through this phospholipid bilayer formed by the two organic solvents containing different lipids. Since GUVs are formed by applying a constant pulse, the production yield is higher than the previous methods and the resulting GUVs are less disperse in size. Moreover, this method allows direct encapsulation of the desired internal aqueous solution in GUVs with high encapsulation efficiency. However, the produced GUVs contain large amounts of organic solvents (e.g., decane) in the membrane and thus are unstable.

The other approach, known as the droplet-shooting and size-filtration (DSSF) approach,39 was developed, which resolves the
issue of liposome size controllability in the jet nozzle jetting method. In the jet nozzle jetting method, the difficulty in tuning the jet dispensing time often leads to the formation of miniscule satellite vesicles. On the contrary, the DSSF approach is not only capable of forming cell-sized liposomes with high size controllability in a simple equipment setup using the size-filtration effect but also allows the formation of liposomes with a minimal amount of lipids in the solvent [Fig. 2(d)]. However, the size-filtration effect also implies that monodisperse GUVs are not produced directly but instead initially non-uniform vesicles are subsequently filtered to the desired size. While the production yield is also not sufficient, the strategy is quite unique in that it has demonstrated that filtration can be employed to separate out GUVs with the desired size and showed that GUVs can be prepared with a relatively small quantity of lipids, which are often very expensive.41

C. Droplet-based microfluidic method

While conventional solvent-free and solvent-assisted methods allow facile production of artificial cell-like vesicles, there still remain challenges in achieving sufficient encapsulation efficiency, production rate, as well as controllability in the membrane structure. Among these, the unilamellar structure in the vesicle membrane is essential as it enables the incorporation of membrane proteins and molecular pores/channels to mimic actual cell functions by controlling the membrane permeability as well as the
stimuli sensitivity.\textsuperscript{42} As a result, there has been an increasing demand for approaches that offer the formation of artificial cell-like vesicles with a unilamellar structure.

A promising solution to this challenge is the droplet microfluidics technology, which enables precise flow control of multi-phase fluids to synthesize various vesicles in a high-throughput manner.\textsuperscript{11} By tuning the fluid composition as well as the flow rate, emulsions with complex structures including the core-shell structure can be prepared with high encapsulation efficiency as well as monodispersity in size.\textsuperscript{43}

These multi-phase emulsion droplets produced via droplet microfluidics are of particular interest due to the compartments in these emulsion droplets that can be enlisting to impart functionality. By incorporating any desired substance to be encapsulated in the core and the basic building units of the membrane in the shell phase, monodispersive functional vesicles with a small volume ($10^{-12}$–$10^{-11}$ m$^3$) can be produced at a high speed in the order of kHz. This includes the polymersomes and liposomes described previously with a unilamellar structure, excellent encapsulation efficiency, and high monodispersity in size, which are comparable to actual cells, as shown in Figs. 3(a) and 3(b).\textsuperscript{28,48,49}

In the synthesis of polymersomes and liposomes using droplet microfluidics, water-in-oil-in-water (W$_1$/O/W$_2$) double-emulsion droplets are used as templates. The innermost aqueous phase (W$_1$) contains hydrophilic molecules including reagents or active ingredients while amphiphilic molecules comprising the membrane are dissolved in the oil phase (O). The continuous aqueous phase (W$_2$) containing suitable surfactants emulsifies the flowing biphasic stream into monodisperse double-emulsion droplets within the microfluidic device. Depending on the combination of organic solvent and amphiphilic molecules used in the oil phase (O), the emulsion droplets become either polymersomes or liposomes. For instance, Weitz and co-workers have recently shown that highly monodisperse GUVs can be robustly generated by controlling the spreading coefficient via the addition of Pluronic F68 surfactant in the continuous aqueous phase (W$_2$), as shown in Figs. 3(a) and 3(d).\textsuperscript{50} In the previous GUVs formation studies, chloroform/hexane cosolvent\textsuperscript{50} containing lipids were used as the oil phase and an aqueous solution without the Pluronic F68 surfactant was used as the continuous phase. The lipid bilayer formation was induced through either oil evaporation or spontaneous extraction of solvent for chloroform/hexane cosolvent and oleic acid, respectively. However, unwanted oil pockets reside in the resulting liposomes, and in the case of oleic acid, the bilayer formation involves a time-consuming oil extraction step. Huck and co-workers improved this drawback through systematic studies on the effect of Pluronic surfactant type and concentration on vesicle formation and found that the addition of F68 surfactant in the aqueous continuous phase (W$_2$) results in the complete dewetting process to occur within 10 min. In addition, the formation of unilamellar vesicles without any oil pockets was further confirmed via the incorporation of toxin pores such as α-hemolysin in the lipid bilayer. Moreover, a coacervate was induced through a temperature-responsive
FIG. 3. (a) Microfluidic preparation of liposomes by using water-in-oil-in-water (W/O/W) double emulsions as templates in a glass capillary-based microfluidic chip. Series of optical images showing the dewetting of the residual oil droplet to yield liposomes. Reproduced with permission from Deng et al., J. Am. Chem. Soc. 138(24), 7584–7591 (2016). Copyright 2016 ACS Publications. (b) Schematics illustrating the production of liposomes using the octanol-assisted liposome assembly (OLA) method. Reproduced with permission from Deshpande et al., Nat. Commun. 7, 10447 (2016). Copyright 2016 Author(s), licensed under a Creative Commons Attribution (CC BY) license. (c) Microfluidic preparation of polymersomes by using W/O/W double emulsions as templates in a glass capillary-based microfluidic chip. Reproduced with permission from Lorenceau et al., Langmuir 21(20), 9183–9186 (2005). Copyright 2005 ACS Publications. (d) Schematics and images showing the in situ detection of in vitro transcription and translation (IVTT) inside the liposomes, working principle of the RNA aptamer Spinach2, synthesis of RNA in the liposomes. Reproduced with permission from Deng et al., J. Am. Chem. Soc. 139(2), 587–590 (2016). Copyright 2016 ACS Publications. (e) Microfluidic production of liposomes by using W/O/W double emulsions as templates in a polydimethylsiloxane (PDMS)-based microfluidic device. Reproduced with permission from Petit et al., Eur. Phys. J. E 39, 59 (2016). Copyright 2016 Author(s), licensed under a Creative Commons Attribution (CC BY) license.
mechanism to mimic membraneless compartment within these GUVs [Fig. 3(d)] and in vitro transcription and translation (IVTT) was also successfully implemented. As this approach allows fine control of the internal structure as well as the membrane composition, versatile GUVs with tunable membrane property were realized by modulating the fluid composition as well as the value of the spreading coefficients. In a separate work, Weitz and co-workers have successfully prepared GUVs with a bilayer comprising of two different types of lipids, which are also known as asymmetric liposomes, using a glass capillary microfluidic device.

To fully exploit the advantages of PDMS-based microfluidic devices, which includes rapid prototyping, accurately controllable size of the microchannels, and the ability to prepare nearly identical devices via replica molding, several methods have been proposed. These include the usage of oleic acids as the oil phase followed by oil extraction into the continuous aqueous phase or the modification of the PDMS device channels with a glass coating to use chloroform as the oil phase in the microfluidic device. While these studies have their own merits, they still lack the ability to either effectively induce a single bilayer membrane structure or require additional device modification or solvent-extraction processes, which can be tedious and troublesome.

To avoid such issues, various studies on replacing the previously used solvents with more benign ones that are also compatible with a PDMS microfluidic device have been investigated [Fig. 3(e)]. For instance, Dekker and co-workers reported the production of GUVs in a PDMS microfluidic device by using 1-octanol as the oil phase or the modification of the PDMS device channels with a glass coating to use chloroform as the oil phase in the microfluidic device. While these studies have their own merits, they still lack the ability to either effectively induce a single bilayer membrane structure or require additional device modification or solvent-extraction processes, which can be tedious and troublesome.

Another factor that causes this difference in physical properties lies in the composition of their membranes. In the case of polymersomes, the amphiphilic block copolymer consists of a hydrophilic block and a hydrophobic block each with different numbers of repeat units. Thus, the physicochemical properties of the polymersome membrane are determined by the combination of these blocks as well as their volume ratio. This does not necessarily mean that any combination of blocks and volume ratios can be assembled into vesicles. For amphiphilic molecules to self-assemble, the packing parameter and the hydrophilic-to-hydrophobic ratio need to be considered. We note that other than these two factors, the Flory–Huggins interaction parameter as well as the degree of polymerization also needs to be considered for polymersomes.

Another factor that causes this difference in physical properties lies in the composition of their membranes. In the case of polymersomes, the amphiphilic block copolymer consists of a hydrophilic block and a hydrophobic block each with different numbers of repeat units. Thus, the physicochemical properties of the polymersome membrane are determined by the combination of these blocks as well as their volume ratio. This does not necessarily mean that any combination of blocks and volume ratios can be assembled into vesicles. For amphiphilic molecules to self-assemble, the packing parameter and the hydrophilic-to-hydrophobic ratio need to be considered. We note that other than these two factors, the Flory–Huggins interaction parameter as well as the degree of polymerization also needs to be considered for polymersomes.

In general, the packing parameter (P) should be between 1/2 < P < 1 to form a spherical shape, where χ is the volume of the hydrophobic block, a is the head area of the hydrophobic block, and I is the length of the hydrophobic polymer segment. However, DOPC with P > 1, which forms a planar structure in nanoscale, can form a GUV in the microscale (10−9−10−8 m). On the other hand, the volume ratio of the hydrophilic-to-hydrophobic block, f, provides a reference to whether the polymer could form a vesicle structure. While spherical vesicles can be usually formed in the range of 0.2 < f < 0.4, Spatz and co-workers predicted that polymers with a relatively large f value [e.g., poly(lactic acid)-b-poly(ethylene glycol), PLA10k-b-PEG5k with a large f value of 0.53] would result in polymersomes with a thick and densely packed bilayer...
membrane.\textsuperscript{64} As the authors also referred, while the packing parameter and $f$ value are both important factors with regard to the mechanism of self-assembly at the nanoscale, there are other factors that need to be additionally considered for microscale vesicles. It was suggested that for microscale vesicles, $f$ value must be regarded as one of many factors that influence the assembly. For example, Weitz and co-workers showed that Pluronic L121 triblock copolymer with a calculated $f$ value of 0.12 can form stable polymersomes with a size larger than 100 nm.\textsuperscript{65}

The physical property of the vesicles that depend on the molecular weight and composition of the basic building units also includes mechanical property and permeability. Polymersomes typically have a much higher bending modulus, surface shear viscosity, and lyso stress while exhibiting relatively low permeability and membrane fluidity compared to liposomes.\textsuperscript{25} This is due to the weaker van der Waals attractive force between the hydrocarbons comprising the lipids compared to block copolymers. This results in high lateral diffusion of lipids across the bilayer and thus the membrane to be fragile and exhibit fluid-like properties.\textsuperscript{26} On the contrary, block copolymers in polymersome membranes have a higher molecular weight and the hydrophobic blocks are in an entangled state in the bilayer membrane. As a result, the lateral fluidity as well as the permeability is relatively low. We also note that the lipid bilayer in liposomes is more sensitive to the temperature set by the glass-transition temperature ($T_g$), whereas polymersomes are less influenced by the values of $T_g$ and $T_m$.\textsuperscript{66} Although no tangible reasons have clearly revealed this fact, effects due to the complex behavior of the polymeric structure might suggest that it can be made more stable to the surrounding conditions than liposomes. Moreover, since amphiphilic block copolymers used for polymersomes can be prepared synthetically, block copolymers with various compositions can be designed to tune the physical properties of the polymersomes including membrane permeability and fluidity. However, the higher molecular weight of polymers compared to lipids often result in a thicker membrane, which limits the incorporation of membrane proteins or molecular pores/channels that can only be inserted into a membrane that has a thickness comparable to a phospholipid bilayer with a unilamellar structure.

On the contrary, phospholipids in liposomes are natural amphiphilic molecules with a low molecular weight. As lipids are the basic unit constituting an actual cell, proteins, receptors, and molecular pores/channels can be readily incorporated into the liposome membrane.\textsuperscript{1,25,46} However, this is also accompanied by some intrinsic drawbacks. Lipids are chemically unstable compared to synthetic polymers and are susceptible to oxidation and hydrolysis under trivial conditions. While several attempts have been made to post-functionalize the head group of phospholipids to increase their stability, the shelf-time of the resulting liposomes are still significantly shorter (~3 days)\textsuperscript{28} than that of polymersomes (>3 months).\textsuperscript{71}

\section*{IV. SUB-COMPARTMENTAL STRUCTURES IN ARTIFICIAL CELL-LIKE VESICLES}

Over the past decade, polymersomes and liposomes with sizes comparable or larger than an actual cell (>10 μm) have been exploited as an ideal cell model for the bottom-up approach in mimicking the cellular function. Specifically, controllable cell-free protein synthesis and in vitro transcription and translation (IVTT) have been realized within these artificial cell-like vesicles to implement the complex biochemical reaction pathways of an actual cell in a more simple and efficient manner. For example, Weitz and co-workers presented cell-free expression based on E. coli ribosomal extract and MreB DNA plasmid in polymersomes, whose membrane consists of PEG-b-PLA amphiphilic polymer and PLA homopolymer [Fig. 4(a)].\textsuperscript{60} In addition, Huck and co-workers demonstrated IVTT in liposomes composed of 1-α-phosphatidylcholine (egg PC) and even highly ordered cascade reactions in a liposome-in-liposome structure [Fig. 4(b)].\textsuperscript{66}

While the membrane in these artificial cell-like vesicles provides a separate compartment for these biochemical reactions to occur apart from the continuous media, an additional compartment within the vesicle, inspired from membraneless organelles (MOs) in an actual cell, has been recently gaining more interest as a tool for de novo synthesis, or to orchestrate a series of cascade biochemical reactions within these artificial cell-like vesicles. Inside an actual cell, there are membraneous organelles (e.g., mitochondria, lysosome, vacuole, etc.) as well as MOs composed of proteins and nucleic acids.\textsuperscript{6,7} These MOs include P bodies,\textsuperscript{19} Cajal bodies,\textsuperscript{60} nucleoli, regulate ribonucleoprotein,\textsuperscript{1,25} histone locus bodies,\textsuperscript{75} and speckles,\textsuperscript{7,8} which are essentially based on liquid-liquid phase separations (LLPS). Along with the membraneous organelles, LLPS including bodies, granules, and organelles all serve as cellular compartments for the various biochemical reactions to occur within the cells. However, while membraneous organelles with a lipid bilayer typically consume energy for the molecular transport of substances across the membrane, MOs do not require additional energy due to their membraneless nature. This characteristic aspect of MOs has motivated the usage of artificial MOs or LLPS for controlling the dynamic reactions within an artificial cell-like vesicles.\textsuperscript{71}

The two most widely studied artificial MOs or LLPS are complex coacervates and the aqueous two-phase system (ATPS), respectively. Complex coacervates most commonly involve polyelectrolyte complexation between two oppositely charged polymers (polycation and polyanion), while an aqueous two-phase system (ATPS) comprises two immiscible phases, in which both phases are aqueous. Complex coacervates are typically induced by external stimuli such as pH, light, temperature, and osmotic pressure. Among these, pH change is the most widely studied external cue for coacervate formation whose constituent is composed of polyelectrolytes. Polycations exhibit net positive charge at pH condition below the pKw, while polyanions become net negatively charged above pKa. Therefore, controlling the external pH condition for an aqueous solution containing two complementary polyelectrolytes with different pK values (Table II) allows the formation of complex coacervates. On the other hand, other stimuli can also induce complex coacervates, which includes temperature-responsive coacervates based on poly-U/ spermine,\textsuperscript{30} and azobenzene cation/DNA, which are light-responsive.\textsuperscript{76} Huck and co-workers demonstrated in vitro transcription and translation (IVTT) in GUVs consisting of egg PC with temperature-responsive and reversible coacervate inside these GUVs [Fig. 4(c)].\textsuperscript{69} IVTT was spatially coordinated by inducing coacervation at which the generated RNA can be detected by using aptamer Spniach2 and DFHBI dye. Dekker and co-workers have also shown...
the spatiotemporal coacervate formation inside GUVs using the OLA method in a PDMS-based microfluidic chip. The incorporation of toxin pores, α-hemolysin, in the GUV membrane allowed the passage of substances with molecular weights of less than 2 kDa, and thus directly injecting ATP (507.18 Da) solution around GUVs led to coacervation with the poly-l-lysine inside the GUVs. This concept of inducing coacervation inside GUVs by external stimuli or the influx of substrates via pores can be further extended to...
vesicles without the toxin pores. For instance, Dekker and co-workers induced coacervates inside the GUVs without using toxin pores but instead by external pH control as the liposome membrane exhibit proton permeability [Fig. 4(d)].78 In a separate work, GUVs containing membrane-bound coacervates were prepared by utilizing the hydrophobic (pLL/spermine coacervate + 5′ cholesterol-poly-U) and electrostatic interaction [ATP/pLL coacervate + phosphatidylinositol (3,4,5)-trisphosphate (PIP3)] between the inner coacervate and the lipid membrane. Here, Tang and co-workers have shown the reversible and temporally controllable coacervates for dormant enzyme reactions in GUVs containing poly- l-lysine and ATP by adjusting the external pH condition [Fig. 4(e)].79 To control the enzymatic activity in situ, the authors inserted enzymes (formate dehydrogenase), substrate, and cofactor (formate, and β-NAD+) in low concentrations inside the GUVs. While low concentrations of formate dehydrogenase are insufficient for enzymatic reactions, the change in pH triggers the local concentration within the coacervate droplets to increase, followed by the activation of the dormant enzyme reactions. This shows that complex coacervation can be controlled dynamically in GUVs without additional usage of toxin pores and is quite useful since toxin pores such as α-hemolysin are expensive, require an additional purification process prior to usage, and do not offer tunable permeability.

ATPS is the other representative artificial MO system that consists of two aqueous solutions that demix above a certain concentration and temperature. Several combinations of polymers [e.g., dextran (DEX), poly(ethylene glycol) (PEG) and salts (e.g., sodium carbonate, phosphates, and citrates) have been reported to separate into two phases above a designated concentration. As these two phases are mostly composed of water without any volatile organic compounds, ATPS provides a benign and non-denaturing environment for biomolecules. As a result, ATPS has been used for many years in biotechnological applications for the separation and purification of proteins, viruses, enzymes, exosomes, and nucleic acids. For example, Lee and co-workers presented ATPS-based polymersomes for particle separation and isolation, whose ATPS system based on DEX and PEG is enclosed within a membrane consisting of an amphiphilic block copolymer, poly(butadiene)-b-poly(ethylene oxide) (PB-PEO) [Fig. 4(f)].80 The authors showed that the model particles, nanoliposomes, can be effectively separated from impurities by utilizing the selective partitioning tendency of model particles in the DEX phase in ATPS-based polymersomes. While a similar structure incorporating ATPS inside liposomes has been reported,81,82 the advantage of utilizing ATPS in these vesicles has not been thoroughly explored, offering opportunities in the applications involving these functional vesicles.

Up to now, we have shown how complex coacervates and ATPS have been implemented in artificial cell-like vesicles for mimicking cellular processes. Such LLPS enables the formation of sub-compartmental structures in artificial cell-like vesicles while serving as a tool for effectively modulating the composition as well as the reactions occurring in each compartment. Although MOs found inside actual cells are quite different in composition compared to LLPS discussed so far, the insight provided by this series of research suggests that dynamically induced LLPS in artificial cell-like vesicles may lead to effectively mimicking the diverse biochemical processes occurring in actual cells. In fact, various types of actual organelle-like coacervate droplets have been reported recently.82,83 Specifically, Boekhoven and co-workers demonstrated that RNA-containing active coacervate droplets consisting of poly-U RNA and peptide successfully mimics the MOs through a fuel-driven reaction cycle.84 While many previous studies have focused on phase separation in the equilibrium state, the authors showed the dynamic mechanical behavior of spontaneous coacervate assembly and disassembly by fuel-driven reactions. Using Broccoli aptamer and DFHB1T ligand, ribosome activity was restrained by functional RNA, which remained in the folded active state inside the coacervate droplet. By showing the spontaneous emergence and decay of coacervates with transient partitioning of functional RNA in the folded state, the possible route of asymmetry division in protocells was suggested. Overall, the above-mentioned studies provide a guideline in tuning the kinetics of coacervation, the interaction between the vesicle membrane and LLPS, and the physicochemical property of the LLPS itself. We anticipate that further advancement in understanding and controlling various types of sub-compartmental structures such as coacervate droplets85 will lead to realizing various biochemical reactions occurring inside actual cells in artificial cell-like vesicles for functional bioreactors.

### Table II. List of polyelectrolytes with different pKa values.

| Polymer                        | Molecular weight (Da) | Concentrations (M) | pKa value | Reference |
|--------------------------------|-----------------------|--------------------|-----------|-----------|
| Poly(acrylic acid)             | ~6000                 | 0.01               | 6.21      | 109       |
| Poly(allylamine hydrochloride) | ~17 500               | 0.01               | 8.92      |           |
| Poly(methacrylic acid)         | ~9500                 | 0.01               | 7.03      |           |
| Poly(ethyleneimine)            | ~750 000              | 0.01               | 7.46      |           |
| Poly(sodium 4-styrenesulfonate)| ~75 000               | 0.01               | 1.22      |           |
| Poly(-l-lysine)                | ~4000–15 000          | NA                 | ~10       | 110       |
| Spermine                       | 202.34                |                    | ~8.4–10.9 | 82        |
| Spermidine                     | 145.25                |                    | ~8.4–10.9 |           |
| Poly-U RNA                     | ~600 000–1 000 000    |                    |           | Anionic   |
| ATP                            | 507.18                |                    | ~4.0–6.5  | 111       |
| Poly(diallyldimethyl-ammonium chloride) | ~50 000               |                    |           | Cationic  |

---

**Biomicrofluidics** 15, 021301 (2021); doi: 10.1063/5.0048441

Published under license by AIP Publishing.
V. OUTLOOKS

Artificial cell-like vesicles including polymersomes and liposomes effectively enclose an aqueous core from the surrounding environment to implement various biochemical reactions, serving as a diverse functional reactor. The inclusion of sub-compartmental structures such as complex coacervates and ATPS within these vesicles has been shown to provide an additional route to control series of cascade biochemical reactions within these artificial cell-like vesicles.

Among these artificial cell-like vesicles, polymersomes, whose membranes are comprised of self-assembled amphiphilic block copolymers, typically are more rigid compared to liposomes and exhibit less permeability and low lateral fluidity. Moreover, the chemical versatility offered by the block copolymers allows precisely controllable membrane thickness, elasticity, permeability, and most importantly, high mechanical stability compared to liposomes.\(^\text{11,26}\) On the basis of this chemical versatility and durability, unique systems capable of responding to various stimuli have been presented. For instance, Meier and co-workers reported multi-step enzymatic reactions in artificial cell-like polymersomes.\(^\text{85}\) Polymersomes based on poly(dimethylsiloxane)-b-poly(2-methyl-2-oxazoline) (PDMS\(_{26-}\)-b-PMOX\(_{86}\)) allow us to precisely control the interconnectivity of the combinational enzymatic cascade pathways and provide conditions for optimal \textit{in situ} reactions by incorporating membrane pores such as outer membrane protein F (OmpF) and gramicidin. Since these polymersomes with high encapsulation efficiency and enhanced stability can be produced in high-throughput using droplet microfluidics technology, these polymersomes may resolve some of the challenges associated with artificial cell-like vesicles. Polymersomes are somewhat inferior compared to liposomes in terms of biocompatibility, but their excellent stability provides a firm basis for inducing a variety of stimuli-responsive properties to realize the complex metabolism in actual cells. Moreover, the strategy of incorporating multi-compartmental structure inside polymersomes can also function as an important tool for designing versatile bioreactor platforms. However, the biocompatibility of the polymersome membrane as well as the methods to precisely tune the membrane permeability such as insertion of membrane proteins or molecular channels in the membrane needs to be devised for broader applicability.

Despite the numerous advantages of artificial cell-like vesicles in cell-free protein synthesis and mimicking various biochemical pathways in an actual cell, the current microfluidic methods to produce such vesicles have several limitations. During vesicle formation, the inner aqueous core containing enzymes, proteins, derived cells, and nucleic acids may come into contact with the organic solvent phase at the water/oil interface, decreasing their bioactivity.\(^\text{16,47}\) While conventional solvent-free methods (e.g., thin film hydration, electroformation) allow the preparation of artificial cell-like vesicles with minimal deterioration in the enzymatic activity, this method typically results in polydisperse vesicles with low encapsulation efficiency.\(^\text{85,86-90}\) Moreover, these methods generate vesicles with a rather simple and uncontrollable structure, which are inadequate for realizing complex biochemical processes including IVTT. Moreover, oil pockets may reside in the vesicles even after the dewetting of the oil droplet in double-emulsion templates, which can compromise the bioactivity of inner biomaterials and also restrict the incorporation of pores in the membrane. Thus, a new method capable of constructing a unilamellar vesicle membrane without any excess organic solvent residing in the vesicles is required. While the most well-known approach to generate unilamellar structures is based on the concept of spreading coefficients, other possible factors may contribute in the dewetting process for generating vesicles with the unilamellar membrane.\(^\text{91-95}\) Therefore, replacing the toxic organic solvent used in solvent-assisted and microfluidic methods with benign ones or unveiling the detailed mechanism of the OLA-based method may resolve such challenges. We also note that high-yielding methods of GUV generation have been proposed recently by using either coacervates or aqueous emulsions as a core template.\(^\text{96-98}\) By exploiting coacervates and aqueous emulsions as the inner compartment, recombinant proteins and combinatorial enzyme reactions can be easily employed for an integrative approach to synthetic protocells, \textit{in vitro} biological engineering, and elegant artificial bioreactors with cellular hallmarks of monodispersity, unilamellarity, and high encapsulation efficiency.

Indubitably, both polymersomes and liposomes possess excellent potential as artificial cell-like vesicles for functional bioreactors. By using droplet microfluidics, vesicles with remarkably high encapsulation efficiency, monodispersity, and cell-sized compartments can be produced in high-throughput. Moreover, vesicles with diverse functional membranes as well as sub-compartmental structures based on LLPS can be prepared with high precision. As these artificial MOs exhibit rapid mass transfer rate with the surroundings due to their membraneless nature and can be induced on-demand by external stimuli, various dynamic and multi-step biochemical reactions can be implemented in these vesicles. On the other hand, a different concept of artificial MOs based on proteins has been proposed recently to precisely mimic the cell interior by enhancing the biocompatibility as well as the stability. In particular, protein-induced MOs enable compartmentalization of intracellular spaces without a distinct structure by utilizing the intrinsically disordered region (IDR) of proteins.\(^\text{99-100}\) Intrinsically disordered proteins (IDPs), the major components of the intracellular signaling system, participate in the assembly of signaling substances as well as the MOs such as nucleus and cytoplasm organelles.\(^\text{96}\) While this field is currently under active investigation, these new types of MOs may provide insights for reproducing the complex intracellular phenomenon \textit{in vitro}. In fact, various methods to spatiotemporally control the dynamics of proteins have been studied along this line.\(^\text{97-99}\) For instance, Champion and co-workers showed self-assembled coacervation with functional recombinant proteins, leucine zipper motifs, which improved the controllability in self-assembly and biocompatibility, beyond the complex coacervation based on polyelectrolytes and ATPS.\(^\text{97}\) As these relatively new biocompatible coacervate systems are showing great potential as functional protein-based materials for drug delivery, and tissue engineering, or biocatalyst,\(^\text{100}\) we envision that these newly emerging MOs motifs will eventually evolve into powerful tools to control the intracellular biochemical pathways and pave the way in the design of advanced artificial cell-like vesicles for various applications related to synthetic biology, drug delivery, biocatalyst, and functional bioreactors.
ACKNOWLEDGMENTS

This work was supported by the National Research Foundation (NRF) of Korea grant funded by the Korean government (MSIT) (Grant No. 2020R1C1C1004642) and the grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (Grant No. HP20C0006).

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1C. Xu, S. Hu, and X. Chen, Mater. Today 19(9), 516–532 (2016).
2B. C. Buddingh’ and J. C. M. van Hest, Acc. Chem. Res. 50(4), 769–777 (2017).
3P. Oberholzer, K. H. Nierhaus, and P. L. Luisi, Biochim. Biophys. Res. Commun. 261(2), 238–241 (1999).
4K. Kurihara, M. Tamura, K.-I. Shohda, T. Toyota, K. Suzuki, and T. Sugawara, Nat. Chem. 3(10), 775–781 (2011).
5S. Wang, M. Yoshimoto, K. Fukunaga, and K. Nakao, Biotechnol. Bioeng. 83(4), 444–453 (2003).
6T. Osaki and S. Takeuchi, Anal. Chem. 89(1), 216–231 (2017).
7N. Dimov, E. Kastner, M. Hussain, H. Perne, and N. Sitta, Sci. Rep. 7(1), 12045 (2017).
8M. S. Kapoor, A. D’Souza, N. Aibani, S. S. Nair, P. Sandhorr, D. Kumari, and R. Banerjee, Sci. Rep. 8, 16122 (2018).
9S.-H. Kim, J. Nam, J. W. Kim, D.-H. Kim, S.-H. Han, and D. A. Weitz, Lab Chip 13, 1351–1356 (2013).
10A. Perro, C. Nicolet, J. Angly, S. Lecommandoux, J.-F. Le Meins, and A. Colin, Langmuir 27(14), 9034–9042 (2011).
11W. Li, L. Zhang, X. Ge, B. Xu, W. Zhang, L. Qu, C.-H. Choi, J. Xu, A. Zhang, H. Lee, and D. A. Weitz, Chem. Soc. Rev. 47(15), 5646–5683 (2018).
12N. Aibani, T. N. Khan, and B. Callan, Int. J. Pharm. 522, 100040 (2019).
13D. A. Hammer and N. P. Kumat, FEBS Lett. 586(18), 2882–2890 (2012).
14K.-I. Shohda and T. Sugawara, Soft Matter 2, 402–408 (2006).
15A. Pohorille and D. Deamer, Trends Biotechnol. 20(3), 123–128 (2002).
16S. Ugwu, A. Zhang, M. Parmar, B. Miller, T. Sardone, Y. Peikov, and I. Ahmad, Drug. Dev. Ind. Pharm. 31(2), 229–228 (2005).
17D. E. Discher and F. Ahmed, Annu. Rev. Biomed. Eng. 8, 323–341 (2006).
18B. T. Kelly, J.-C. Baret, V. Talv, and A. D. Griffiths, Chem. Commun. 18, 1773–1778 (2007).
19N. Wu, F. Courtioux, R. Surjadi, J. Oakeshott, T. S. Peat, C. J. Eastson, C. Abell, B. T. Kelly, J.-C. Baret, V. Taly, and A. D. Griffiths, Chem. Commun. 9(3), 439–446 (2008).
20I. T. Maseda, T. Nakada, J. Shin, K. Uryu, V. Noireaux, and A. Libchaber, ACS Synth. Biol. 2(2), 53–59 (2012).
21M. Hondele, S. Heinrich, P. De Los Rios, and K. Weis, Emerging Top. Life Sci. 4(3), 343–354 (2020).
22Y. Elani, R. V. Law, and O. Ces, Nat. Commun. 5, 5304 (2014).
23M. Chanasakulniyom, C. Martino, D. Paterson, L. Horsfall, S. Rossier, and J. M. Cooper, Analyst 137(13), 2939–2943 (2012).
24V. Noireaux and A. Libchaber, Proc. Natl. Acad. Sci. U.S.A. 101(51), 17649–17654 (2004).
25R. Gómez, R. Dimova, P. Schvile, F. R. Wurm, and K. Landfester, Chem. Soc. Rev. 47(23), 8572–8610 (2018).
26A. Akbarzadeh, R. Rezaei-Sadabady, S. Davaran, S. W. Joo, N. Zarghami, Y. Hanifehpour, M. Samiei, M. Kouihi, and K. Nejati-Koshki, Nanoscale Res. Lett. 8(1), 102 (2013).
27Y. Sato and M. Takinoue, Micromachines 10(4), 216 (2019).
28T. J. McIntosh and S. A. Simon, Annu. Rev. Biophys. Biomol. Struct. 35, 177–198 (2006).
29A. S. Utada, A. Fernandez-Nieves, H. A. Stone, and D. A. Weitz, Phys. Rev. Lett. 99(9), 094502 (2007).
30E. Lorenceanu, A. S. Utada, D. R. Link, G. Cristobal, M. Joanicot, and D. A. Weitz, Langmuir 21(20), 9183–9186 (2005).
31H. C. Shum, J.-W. Kim, and D. A. Weitz, J. Am. Chem. Soc. 130(29), 9543–9549 (2008).
32H. C. Shum, E. Santanach-Carreras, J.-W. Kim, A. Ehrlicher, J. Bibeau, and D. A. Weitz, J. Am. Chem. Soc. 133(12), 4420–4426 (2011).
33R. C. Hayward, A. S. Utada, N. Dan, and D. A. Weitz, Langmuir 22(10), 4457–4461 (2006).
34N.-N. Deng, M. Yelleswarapu, L. Zheng, and W. T. S. Huck, J. Am. Chem. Soc. 139(2), 587–590 (2017).
35N.-N. Deng, M. A. Vihbrute, L. Zheng, H. Zhao, M. Yelleswarapu, and W. T. S. Huck, J. Am. Chem. Soc. 140(24), 7399–7402 (2018).
36L. R. Arriaga, S. S. Datta, S.-H. Kim, E. Amstad, T. E. Kodger, F. Monroy, and D. A. Weitz, Small 10(5), 950–956 (2014).
37J. Peit, I. Polenz, J.-C. Baret, S. Herrnghaus, and O. Baumchen, Eur. Phys. J. 39, 39 (2016).
38L. R. Arriaga, Y. Huang, S.-H. Kim, J. L. Aragones, R. Ziblat, S. A. Koehler, and D. A. Weitz, Lab Chip 19(5), 749–756 (2019).
39J. N. Lee, C. Park, and G. M. Whitesides, Anal. Chem. 75(23), 6544–6554 (2003).
40S.-Y. Teh, R. Khnouf, H. Fan, and A. P. Lee, Biomicrofluidics 5(4), 044113 (2011).
41D. Krafft, S. L. Castellanos, R. B. Lina, R. Dimova, I. Ivanov, and K. Sundmacher, Chem. Bio. Chem. 20(20), 2604–2608 (2019).
42J. Thiele, A. R. Abate, H. C. Shum, S. Bachtler, S. Förster, and D. A. Weitz, Small 6(16), 1723–1727 (2010).
43G. Despande and C. Dekker, Nat. Protoc. 13(5), 856–874 (2018).
44V. Srinivas, D. E. Discher, and M. L. Klein, Nat. Mater. 9(9), 638–644 (2004).
45S. Jain and F. S. Bates, Macromolecules 37(4), 1511–1523 (2004).
46D. E. Discher and A. Eisenberg, Science 297(5583), 967–973 (2002).
47I. Israelachvili, Interfacial & Surface Forces, 2nd ed. (Academic Press, London, 1991).
