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Advancing Artificial Cells with Functional Compartmentalized Polymeric Systems – in Honor of Wolfgang Meier

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KEYWORDS

Polymersomes, Compartmentalization, Self-Assembly, Synthetic Organelles, Artificial Cells, Coacervates

ABSTRACT

The fundamental building block of living organisms is the cell, the universal biological base of all living entities. This micrometric mass of cytoplasm and the membrane border have fascinated scientists due to the highly complex and multi-compartmentalized structure. This specific organization enables numerous metabolic reactions to occur simultaneously and in segregated spaces, without disturbing each other, but with a promotion of inter- and intra-cellular communication of biomolecules. At present, artificial nano- and micro-compartments, whether as single components or self-organized in multicompartment architectures, hold significant value in the study of Life development and advanced functional materials and in the fabrication of molecular devices for medical applications. These artificial compartments also possess the properties to encapsulate, protect, and control the release of bio(macro)molecules through selective transport processes, and they are capable of embedding or being connected with other types of compartments. The self-assembly mechanism of specific synthetic compartments and thus the fabrication of simulated organelle membrane is one of the major aspects to gain insight. Considerable efforts have now been devoted to design various nano- and micro-compartments and understand their functionality for precise control over properties. Of particular interest is the use of polymeric vesicles for communication in synthetic cells and colloidal systems, to reinitiate chemical and biological communication and thus close the gap towards biological functions. Multicompartment systems can now be effectively created with a high level of hierarchical control.

In this way, these structures can not only be explored to deepen our understanding of the functional organization of living cells but also pave the way for many more exciting developments in the biomedical field.

1. Introduction

Cells provide an extraordinary self-assembly material model for inspiring the development of novel systems designed to mimic precise properties or functionalities inherent in nature selectively. This basic unit of life, with an incredible compositional organization and complex metabolic paths, relies on a perfect synchronization of parallel or successive biological and chemical reactions. Regarding the internal organization of the cell, the compartmentalization process is one of the crucial features that characterize the highly functional internal organelles (nuclei, mitochondria, vacuoles, peroxisomes, etc.). This specific organization enables numerous metabolic reactions to occur simultaneously in segregated spaces (without disturbing each other) and produce compounds sequentially required by a different pathway. Therefore, the compartmentalization of biomolecules promotes inter- and intra-cellular communication. When it comes to mimicking cells, there are two strategies for creating functional compartments: i) the top-down strategy, based on extraction/isolation of minimal natural compartments with intrinsic or induced functionality, and ii) the bottom-up strategy, which combines building blocks of natural and synthetic origin to generate compartments with advanced properties and functionality. Given its capacity to simplify the complexity inherent in natural counterparts, the bottom-up strategy is particularly appealing for generating functional systems capable of performing selective chemical and biological reactions, with superior control over the components or the spatiotemporal functionality compared to natural organelles.[1–3] Therefore, the bottom-up strategy is not limited to natural components,

and the use of synthetic ones (ranging from catalysts to polymer assemblies) can bring new properties and even introduce new-to-nature orthogonal reactions if specific applications are intended to achieve multi-component, multi-scale, and multi-functional systems.

To leverage specific characteristics inherent in nature, these artificial compartments exhibit a diverse array of architectures, from single compartments (micelles,[4] vesicles,[5] or nanoparticles[6]) to multi-scale structures (compartments-in-compartments).[7] These compartments are based on various building blocks, including lipids, polymers, peptides, proteins, or a combination thereof, and have a size ranging from nano- up to micrometers. Compartments exhibiting vesicular architecture are particularly interesting, enclosing an inner confined space isolated from the external environment by a membrane. The specific architecture of vesicles offers: i) an aqueous inner cavity for encapsulation of hydrophilic entities, ii) a membrane that allows insertion of hydrophobic molecules, and iii) an external interface that can be functionalized for attachment of specific molecules favoring targeting approaches or immobilization on surfaces. This structure can also be designed to protect any biological compound present in the core of the vesicles from harmful environmental conditions or to allow molecules to diffuse through for desired *in situ* reactions or signaling.[8]

Considerable efforts have been currently devoted to the design of various block copolymers with diverse multiple compositions, thus enabling the formation of polymer-based vesicles with diameters ranging from a few tens of nanometers (small unilamellar vesicles, SUVs) to hundreds of micrometers (giant unilamellar vesicles, GUVs). Although there are various notations for designating polymer vesicles as membrane-bound compartments and to clarify the relation with the lipid-based vesicles, the term “polymersomes” for nanometer-sized vesicles (or SUVs) will be employed and the term “microcompartments” for micrometer-sized ones (or GUVs). Depending

on the chemical nature of the block copolymers, polymersomes and synthetic GUVs offer multiples benefits: i) an improvement of mechanical stability, ii) an increase of the membrane flexibility that enables the encapsulation of a large variety of compounds (drugs, contrast agents, proteins, nucleic acids) and protecting them from proteolytic attack, iii) and the structural modification in the presence of external or internal stimuli.[9–11] Polymersomes and GUVs are defined as functional when active compounds or assemblies are encapsulated/inserted and their *in situ* activities are properly measured.[10,12,13] Starting with single-enzyme reactions inside polymersomes,[14] the composition and functionality of compartments have evolved first to replicate reactions occurring inside natural organelles.[15]

To address increasingly complex scenarios of reactions and signaling events, a significant advancement was achieved by creating multicompartments (**Figure 1**).[16] This includes the encapsulation of nano-sized compartments inside micrometer-sized ones (compartments-in-compartments),[7,17–19] as well as integrating different compartments (clusters of compartments), where at least one component is composed of a polymer block.[20] Over the last decade, the production of hierarchical and sub-compartmentalized polymer systems was attempted with the goal to mimic the most complex structures and biological functions found in cells, including the division of labor between subcompartments. At present, nano- and micro-compartments, whether as single components or self-organized in multicompartment architectures, hold significant value in studying the origin of life, developing advanced functional materials, and fabricating molecular devices for medical applications.

In this Perspective Article, we will highlight the latest innovations in the design of polymer-based vesicles incorporating active modules (molecules, assemblies, combinations thereof). These advanced structures aim to deepen our fundamental understanding of the biological functions of

natural organelles and cells while also facilitating applications where precise control over properties and multifunctionality is indispensable. We will indicate the mechanisms employed in multicompartmentalization that enables active molecules within different compartments to participate in cascade reactions.[21,22] The interest in using multicompartmentalization to support theranostic approaches,[20] mimic signaling pathways, dynamic assembling, or intra- and intercellular communication will be also discussed.[18,23] Ultimately, the life-like behavior of multi-compartmentalized hierarchical systems will be addressed, and a summary and outlook regarding the future directions will be presented at the end.

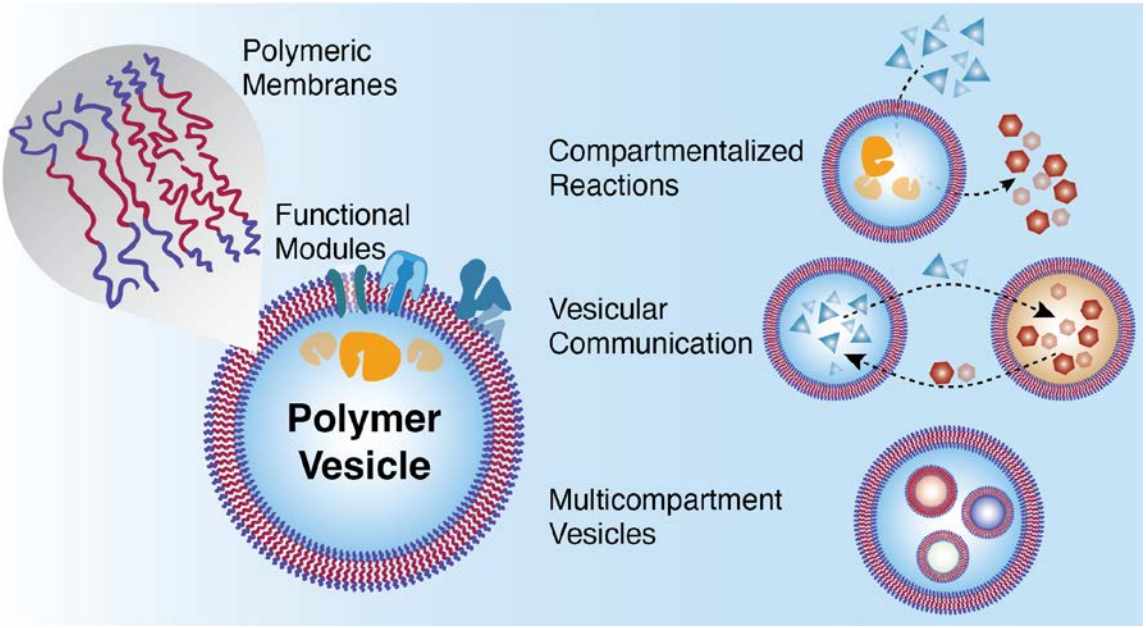

Figure 1. *Overview of polymeric vesicle systems and selected applications. Polymer vesicles are composed of a polymeric membrane made from di- or triblock copolymers and gain functionality by adding functional modules such as membrane pores and proteins or enzymes encapsulated within. Polymer vesicles can be used as compartments for catalysts for biochemical reactions, compartments for communication between segregated catalysts or to build multicompartment systems based on the encapsulation of smaller vesicles in a bigger vesicle.*

2. Vesicles as Compartments

As one of the most fascinating artificial mimics of cell membranes, compartmentalized polymeric systems, have drawn extensive attention due to their excellent physical stability and chemical designability compared to phospholipid bilayers.[24–31] The membrane-based compartment, which can be divided into two types (nano compartments or small unilamellar vesicles (SUVs) and micro-compartments or giant unilamellar vesicles (GUVs)), means that the membrane compartmentalization is caused by phase separation between the film-forming components during and after self-assembly. Though the overall structure of small vesicles and giant vesicles is very similar, still many differences remain. For instance, the ratios of the surface area to the luminal volume and membrane thickness to the luminal volume differ. While the surface area increases with the diameter of the vesicles, the ratio of the surface area to luminal volume decreases. Furthermore, the membrane thickness of giant vesicles is not proportional to the size of the vesicles and thus, the ratio of membrane thickness to vesicle size decreases significantly with increasing size. In specific conditions, the membrane induces segregation into compartments inside one compartment. Different compartments can be created by adjusting the properties (block length, nature of the blocks, *f* ratio, etc.) and proportions of the different components. By selecting stimuli responsive building blocks and changing the external environment, such as solvent and temperature, architectures with desired properties such as permeability can be achieved. In contrast to conventional polymersomes, subcompartmentalized polymeric structure can contain incompatible cargoes in the cavity and release them in a programmed manner. Various mechanisms have been used to induce membrane compartmentalization or change the membrane properties, including achieving desired permeability or specific composition: polymer phase separation,[32] polymer/lipid phase separation,[33,34] photo-cross-linking/photoreaction, insertion of

proteins into membranes (including Outer membrane protein F (OmpF) and Concanavalin A),[35] or embedding of nanoparticles into the membrane.[36] Therefore, such systems show great potential in the encapsulation, delivery, and controlled release of drugs, biological macromolecules, and nanoparticles.

Nanocompartments (Polymersomes)

The fundamental design principle underlying the creation of polymer-based vesicles involves using multiple chemically compatible membrane-forming components that coalesce to form a robust and stable membrane.[37] A critical aspect of this formation process is the strategic introduction of controlled incompatibility among these components, leading to the spontaneous emergence of distinct domains through phase separation.[38] Different types of polymersome architectures can be meticulously fabricated using various self-assembly techniques, including solvent exchange, film rehydration, co-assembly with heterogeneous components, and polymerization-induced self-assembly (PISA).[39] In addition, copolymeric vesicles can display various properties intrinsically related to the chemical nature of the components, such as varying size, shape, pH response, amphiphilicity, or modular assembly.[40,41] Besides, the membrane compartmentalization produced by polymeric phase separation is rooted in the thermodynamic behavior of polymers.[38] When a controlled degree of incompatibility among membrane-forming components is present, it elevates the Flory-Huggins interaction parameters, consequently increasing the system's free energy.[37] Therefore, the membrane undergoes phase separation to reach an equilibrium state that minimizes overall free energy.[32] Amphiphilic polymers with a hydrophilic block and one or more incompatible hydrophobic blocks can self-assemble into polymersomes, where the fine-tuning of the membrane structure can be achieved by adjusting the molar ratios between the components forming the membrane. The architecture and properties of

synthetic polymer vesicles can also be tuned with the hydrophilic and hydrophobic ratio of the block copolymer that dictates assembly into micellar, rod-like, or lamellar structures.[42–45,100] For instance, polymersomes with distinct surface domains were successfully designed by mixing various block copolymers at different proportions.[32] Patchy polymersomes were fabricated through self-assembly using variable molar ratios of poly(ethylene oxide)-*block*-poly(butylene oxide) (PEO-*b*-PBO) and poly((2-methacryloyloxy)ethyl phosphorylcholine)-*block*-poly(2-(diisopropylamino)ethyl methacrylate) (PMPC-*b*-PDPA), resulting in polymeric phase separation within both the hydrophilic coronas and hydrophobic membrane. PISA presents an appealing approach for fabricating polymersomes as it combines polymer synthesis and self-assembly processes concurrently at high polymer concentrations (10-50 wt%).[46–48] External stimuli can trigger transition from a homogeneous to a phase-separated membrane. For example, a strategy to induce membrane phase separation in nano-polymersomes was devised *via* photo-cross-linking.[49] Poly(ethylene oxide)-*block*-poly(2-(diethylamino)ethyl methacrylate-*stat*-7-(2-methacryloyloxyethoxy)-4-methylcoumarin) (PEO-*b*-P(DEA-*stat*-CMA)) contained hydrophilic PEO and hydrophobic P(DEA-*stat*-CMA) blocks, which could self-assemble into nuclear envelope-like polymersomes (NEVs) due to π-π stacking of coumarin groups (**Figure 2**). The NEVs were designed to phase separate into coumarin-rich and coumarin-poor region, the latter forming polymer pore complexes, similar to the nuclear pore complex found in cells, based on nanophase segregation. Upon lowering the pH below 6.0, the polymer pore complex can be opened, enabling the transport of biomacromolecules across the membrane. Subsequently, this strategy was employed to design dually gated polymersomes for delivering biomacromolecules[50] and sugar-regulating polymersomes.[51]

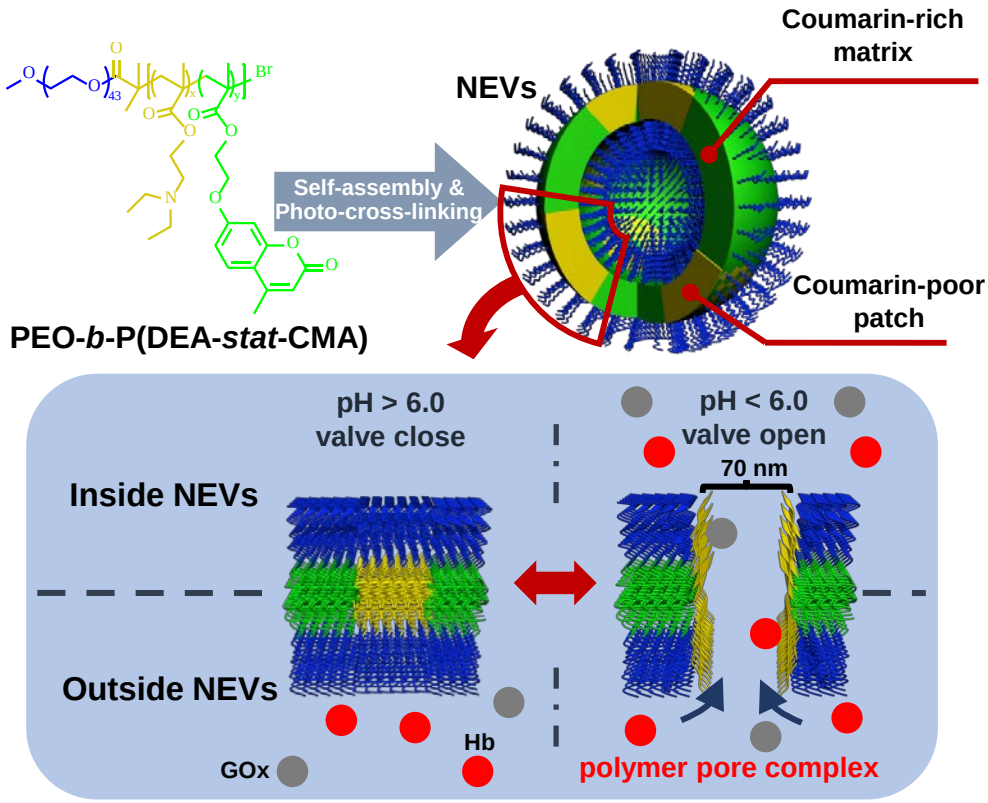

Figure 2. *Diblock copolymer self-assembly into nuclear envelope-like polymersomes (NEVs) with distinct coumarin-rich (coumarins + DEAs) and coumarin-poor (predominantly DEAs) domains. The former domains readily undergo cross-linking under UV exposure. Owing to phase separation, the latter domains exhibit multifunctionality as large valves, proton sponges and polyelectrolytic filaments. The large valves undergo reversible opening and closing upon pH variation. Adapted with permission from ref* [49]. *Copyright 2014, American Chemical Society.*

Furthermore, framboidal polymersomes were synthesized *via* seeded RAFT emulsion polymerization using poly(glyceryl monomethacrylate-*b*-2-hydroxypropyl methacrylate) (PGMA-*b*-PHPMA) polymersomes as seeds.[52] The surface topology of polymersomes was controlled by adding a third monomer (benzyl methacrylate (BzMA)) to the membrane of the smooth precursor polymersomes. The demixing enthalpy between the PHPMA and PBzMA blocks is responsible

for the occurrence of phase separation. In another example, polymersomes possessing hydrophilic and hydrophobic sub-domains in the membrane were constructed by self-assembly of the 21-arm star polymer poly(ethylene glycol) [poly(acrylic acid)-*block*-polystyrene]$_{20}$ (PEG(PAA-*b*-PS)$_{20}$).[53] The 21-arm star polymer's topology is crucial for forming hydrophilic and hydrophobic subdomains in the polymersome membrane because the water-soluble PAA blocks are confined within the membrane, creating to hydrophilic subdomains. Recently, phase separated vesicles self-assembled from the fluorine-containing triblock copolymer PEG-*block*-polystyrene-*block*-poly(4-vinylbenzyl pentafluorophenyl ether) (PEG-*b*-PS-*b*-PVBFP) were reported.[54] Due to the large volume of the polymersome and the thin polymersome membrane (28 nm), the fluorinated PVBFP block formed 9 nm columnar microdomains in the membrane that were shielded by the PS phase.

Polymersomes can be constructed with complex surface topologies to facilitate membrane-confined self-assembly. This is achieved by introducing an interfacial energy through the interaction of hydrophilic blocks, e.g. AB, with CB and a stabilizing triblock copolymer, which acts as a surfactant and thus contains elements compatible with both phases, e.g. ABC.[55] Such copolymer mixture self-assembles into domains resembling 2D versions of micelles. Like polymeric phase separation, the induction of membrane phase separation between polymers and lipids is also an essential method for constructing polymersomes. This approach synergistically combines the biocompatibility and flexibility of lipid membranes, and the stability and versatility of polymeric membranes within one global system. During the synthesis of polymer/lipid hybrid polymersomes, forming a composite membrane is facilitated by the moderate yet distinct solubility parameter mismatch between the polymer and lipid components, which permits a degree of compatibility while maintaining sufficient thermodynamic drive for membrane phase separation.[33] Moreover, the disparity in size between polymer chains and lipid molecules gives rise to

substantial hydrophobic interface stress at the polymer-lipid interface. The polymer and lipid will coalesce into polymer- or lipid-rich domains to minimize the interfacial energy.[56] A pivotal control parameter of polymer/lipid phase separation is the adjustment of the polymer-to-lipid molar ratio. Inserting proteins into the membrane is another strategy employed to design polymersomes, combining the specificity and efficiency of proteins with the robustness and versatility of polymersomes. Hydrophobic interaction is the primary driving force behind fabricating polymer/protein composite polymersomes, while charged membranes can influence the orientation of protein insertion.[57] Furthermore, the polymersome membrane thickness and the polymer chains' flexibility are crucial factors in determining successful protein insertion.[58] Due to the thicker membrane of polymersomes (5-50 nm)[59], a substantial mismatch exists between membrane thickness and protein size, affecting protein insertion and function.[60] Therefore, the polymer chains must be flexible enough to ensure that the conformational rearrangement of membrane-forming hydrophobic blocks matches these proteins. Embedding nanoparticles into the membrane can also modify polymersomes, thereby combining the attributes of both nanoparticles and polymers. There are two principal approaches to creating such composite polymer/nanoparticle polymersomes: i) anchoring polymers to nanoparticles and then self-assembling, or ii) co-assembling polymers with nanoparticles. For the former method, end-functionalized polymers can be “grafted to” nanoparticles *via* chemical bonding or “grafted from” initiator-modified nanoparticles.[36] For the latter method, nanoparticles can be encapsulated within the polymersome membrane through precipitation and non-covalent interactions such as electrostatic and hydrophobic interactions.[61]

Active polymersomes

One of evolution's game-changers was when cells learned to move purposefully toward or away from stimuli.[62] This marked the shift from aimless drifting to directed movement. The most ancient and widespread stimulus is the chemical signal, sparking movement through a process known as chemotaxis. This is seen from the tiniest bacteria to our immune cells.[62] Inspired by this natural behavior, scientists have created synthetic micro- and nanoparticles that mimic chemotaxis.[63] Polymersomes stand out among these. They are biocompatible and can be tailored for targeted delivery, adapting flexibly to their surroundings. A crucial factor for autonomous motion in these systems is asymmetry. This was demonstrated using Janus-like polymersomes, obtained from shape transformation of polymersomes,[64] forming dumb-bell shapes known as stomatocytes.[65] These stomatocytes, loaded with metal catalysts or enzymes, showed enhanced movement in hydrogen peroxide.[65] They also encapsulated enzymes like catalase and glucose oxidase, improving efficiency and motion even at low fuel levels.[66] Typically, the loading efficiency is closely related to their preparation techniques, especially for large sized biomacromolecules. For instance, the loading efficiency is usually quite low for polymersomes prepared by film rehydration or solvent exchange. In contrast, a loading efficiency of nearly 100% has been achieved for micrometer-sized vesicles by microfluidics. Nevertheless, the introduction of specific interactions between the vesicles and the cargoes such as hydrogen bonding, π-π interaction and other noncovalent interactions can improve the loading efficiency of cargoes, though the number of enzymes and transmembrane proteins encapsulated per polymersome is rarely reported. Up to now, it is still challenging to confirm the number and distribution of encapsulated biomacromolecules per polymersome. In addition, it was reported that polymersomes can evolve between shapes, such as elongated nanotubes and peanut shapes, depending on the polymer used (**Figure 3**A-D),[65,67] or thermo-responsive stomatocytes controlled their propulsion by adjusting

their openings based on temperature.[68] Stomatocytes mimicking glycolysis showed sustained movement regulated by glucose and adenosine triphosphate (ATP), reflecting how biological systems adapt to environmental changes through enzymatic reactions and feedback loops.[69]

Another approach to induce asymmetry was reported by combining two copolymers, creating patchy polymersomes with varying surface permeabilities.[70] These encapsulated enzymes demonstrated chemotaxis toward glucose (**Figure 3**E-G**)**. Moreover, polymersomes functionalized with low-density lipoprotein receptor-related protein 1 (LRP-1) significantly enhanced blood-brain barrier crossing, showing a four-fold increase compared to their symmetric counterparts. These advancements highlight how synthetic systems can emulate natural processes and offer new ways to tackle complex biomedical challenges.

While most polymersome systems are designed for *in vivo* use, alternative applications such as (blood) diagnostics, e.g. for the detection of viral proteins[71,72] or small molecules,[73,74] increase the translation potential as no *in vivo* safety studies are needed.

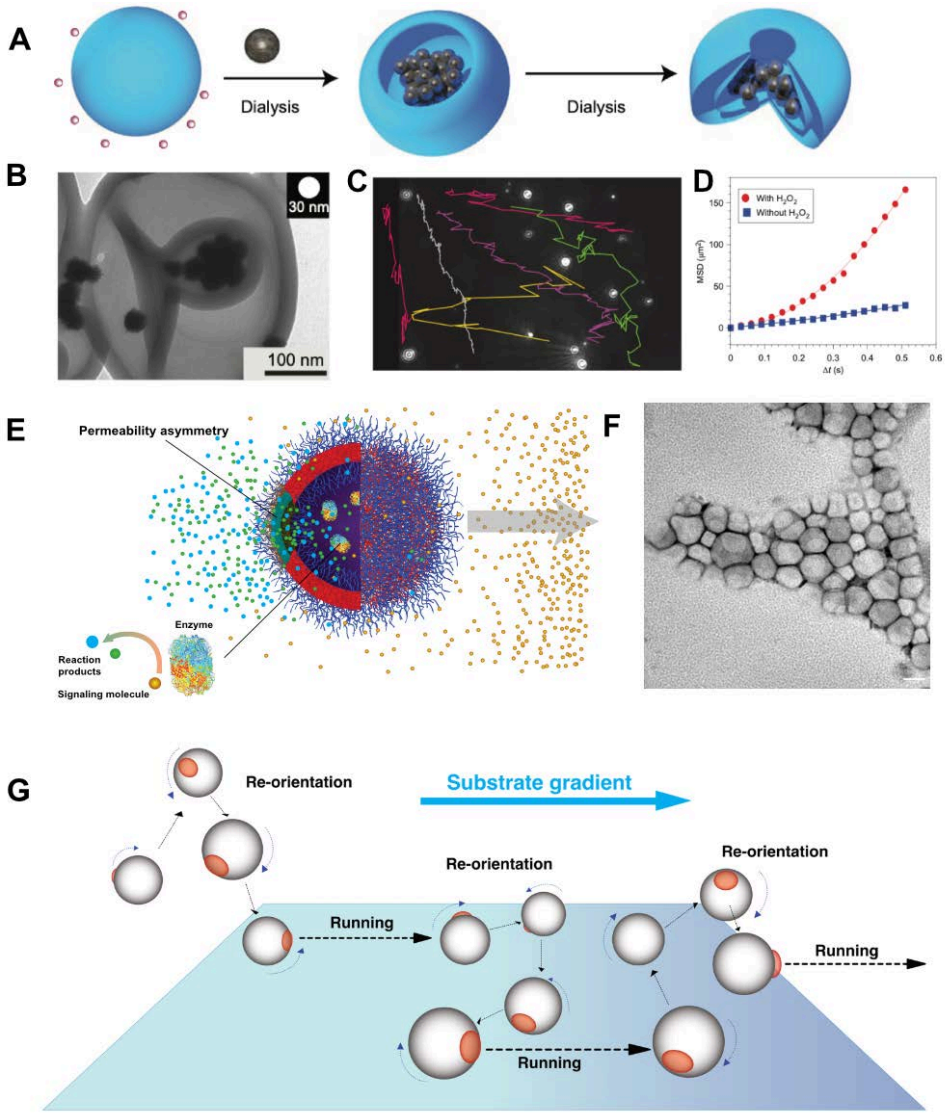

Figure 3. *(A) Graphical representation of stomatocytes with entrapped platinum nanoparticles. (B) TEM image of stomatocytes containing 80 nm PVP-capped platinum nanoparticles. (C) Dark-field microscopy imaging of stomatocytes and their tracking. (D) Average mean-square displacements (MSD) of stomatocytes with entrapped Pt-nanoparticles, comparing conditions without and with H_2O. Reprinted with permission from ref [65]. Copyright 2012, Nature Chemistry. (E) Graphical representation of asymmetric polymersomes. (F) TEM image of PMPC-PDPA/PEO-PBO polymersomes. (G) Illustration of the proposed chemotaxis mechanism for*

asymmetric polymersomes. Reproduced under terms of the CC-BY license.[70] *Copyright 2017, Science Advances.*

Microcompartments

Polymeric microcompartments can be used for the development of artificial cells, offering precise control over spatial organization and reaction conditions. These systems enhance the functionality of synthetic cells by enabling compartmentalized biochemical processes, crucial for applications in synthetic biology and fundamental studies of cellular processes through mimicry. However, compared to the complex structure and abundant functionalities of natural cells, the development of polymeric microcompartments as cell mimics is still in their infancy, both in structure and functionality. The combination of spatial compartmentalization (e.g. vesicle-in-vesicle) and functional membranes might be an alternative strategy, however the challenge in their preparation techniques remain to date. The phase separation during the self-assembly of amphiphilic polymers is also an important way to construct micrometer-sized membrane-based compartments. For instance, the two block copolymers of poly(acrylic acid)-*block*-poly(butadiene) (PAA-*b*-PBD) and PEO-*b*-PBD were co-assembled into vesicles with uniform membranes.[75] However, with the addition of multivalent ligands (such as calcium and copper ions), the microphase separation of the microcompartments was observed, leading to the formation of spotted GUVs. As illustrated in **Figure 4** after the addition of calcium ions, microdomains were formed on the surface of the microcompartments at highly restricted pH and salt concentrations (**Figure 4**A-D). The highly anionic and fluorescent-labeled lipid phosphatidylinositol-4,5-diphosphate (PIP_2) was added to the polymer membrane and found to be enriched in the PAA-*b*-PBD domain of the vesicles (**Figure 4**E). In this process, the addition of calcium ions was very

important, as this divalent cation acted as a crosslinker between polyanionic PAA chains to produce locally rich PAA fragments on the membrane. In addition, copper ions had a similar effect to induce the microphase separation of microcompartments, leading to the formation of patched systems (**Figure 4**F) and asymmetric Janus microcompartments (**Figure 4**G).

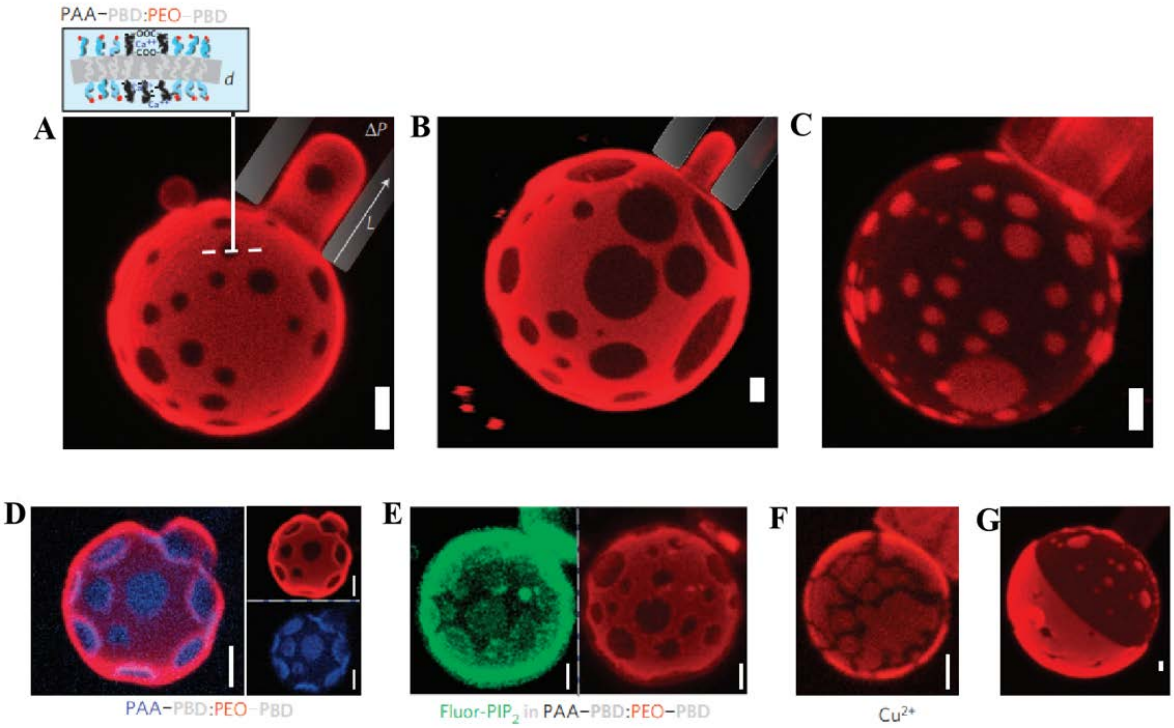

Figure 4. *Spotted microcompartments.*[75] *(A-C) Cation-induced, lateral phase segregation of the formed* two *microcompartments* at pH 4, *0.1 mM calcium at (A) 25%, (B) 50% and (C) 75% of PAA-b-PBD. (D) Merged images of a phase-separated microcompartment (34% of PAA-b-PBD). (E) Two-color images of phase-separated microcompartment (50% of PAA-b-PBD) with 2% PIP_2–BodipyFL (green). (F) Phase separation of PAA-b-PBD (50%) induced by copper(II) at pH 3.5, 0.075 mM copper(II). (G) Janus microcompartment resulting from domain coarsening in a 50% PAA-b-PBD. Scale bars: 2 µm. Reprinted with permission from ref* [75]*. Copyright 2009, Springer Nature.*

Inspired by the structure of cell membrane, patchy polymersomes based on the co-assembly of two BCPs, PEO-*block*-poly(butylene oxide) (PEO_{16}-*b*-PBO_{22}) and poly((2-methacryloyloxy)ethylphosphorylcholine)-*block*-poly(2-(diisopropylamino)ethyl methacrylate) ($PMPC_{25}$-*b*-$PDPA_{70}$) were produced.[32] The two polymers co-assembled into hybrid polymersomes with different mole percentages of $PMPC_{25}$-*b*-$PDPA_{70}$. For mole percentages of 10% and 25%, different domains appeared on the vesicle surface. As the mole percentage was increased to 50% and 90%, a tendency to form stripes was observed. In another example, the aqueous self-assembly of binary mixtures of PEO-*block*-poly(ε-caprolactone)-*block*-PMOXA (PEO-*b*-PCL-*b*-PMOXA) triblock copolymers was reported to form polymersomes-in-polymersome by polymersome uptake with average sizes up to several micrometers.[76] The packing geometry of the $A_{45}B_{135}C_{10}$ and $A_{45}B_{135}C_{25}$ triblock copolymers leads to the formation of the polymersomes with an external A block and an external C block, respectively.

The commonly used preparation methods of microcompartments also include microfluidics, especially double emulsion methods. Microfluidic technology provides a general method for preparing monodisperse vesicles using single-emulsion (water/oil) or double-emulsion (water/oil/water) droplets as templates. Compartmentalized polymeric systems can be obtained by designing microchannels used to form emulsion droplets, which are usually composed of buffer droplet polymers within a polymer organic phase that can be further functionalized using proteins. For instance, an elegant strategy was proposed to study such a behavior by controlling the spatial separation of enzymes within different synthetic cell-sized GUVs (**Figure 5**).[77] GUVs were designed by incorporating pore-forming peptides (gramicidin) and membrane proteins (OmpF) into the poly(2-methyl-2-oxazoline)-*block*-poly(dimethylsiloxane) (PMOXA-*b*-PDMS) membrane to favor *in situ* reactions. Enzymatic contents in GUVs could be tightly controlled,

reaching nearly 100% encapsulation efficiency. GUVs containing pores or membrane proteins and encapsulating one or multiple enzymes were assembled in diverse configurations, facilitating investigating three-step reactions. Owing to the provision of spatiotemporal control, optimal conditions for reducing inhibitor accumulation were revealed, and total cascade efficacy was maximized, benefiting from reactive intermediates.

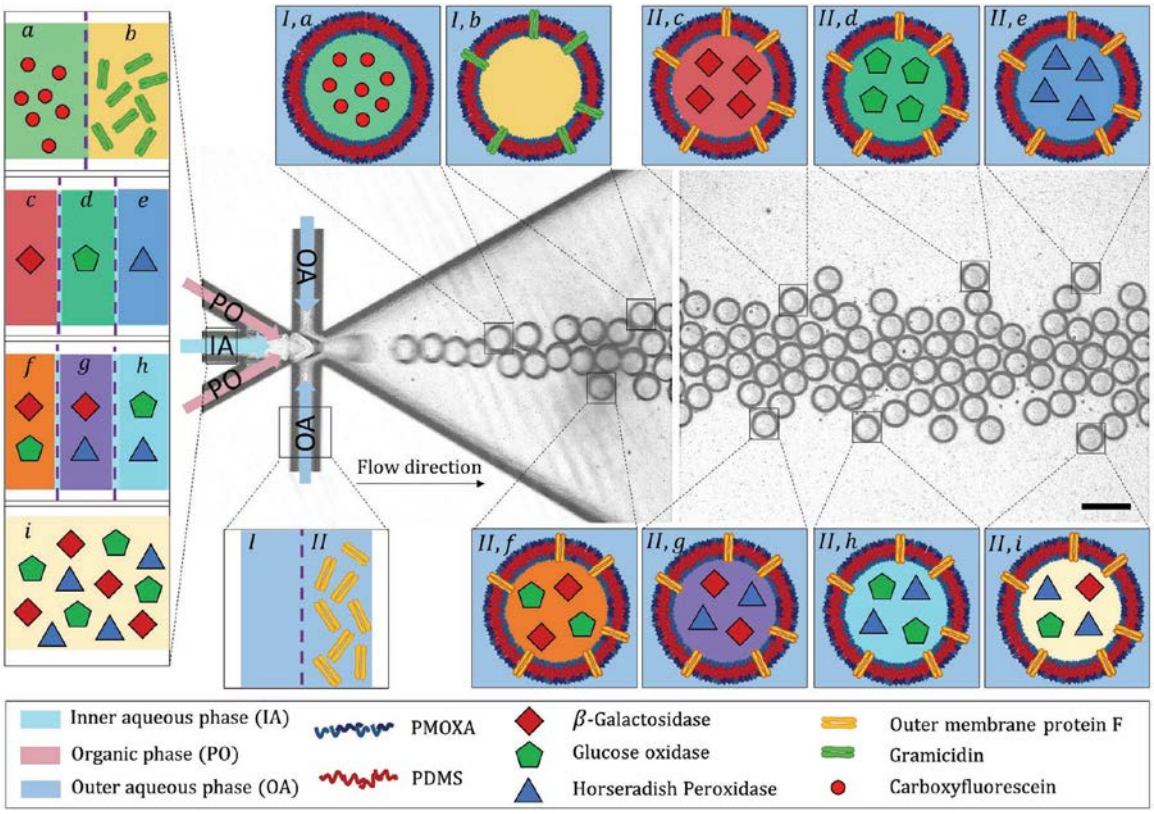

Figure 5. *Schematic illustration of high-throughput double emulsion generation process for producing GUVs containing precise amounts of biomolecules in various possible combinations in the inner aqueous lumen and inside the polymeric membrane. Reproduced under terms of the CC-BY license.*[77] *Copyright 2020, Wiley-VCH GmbH.*

Membrane-based systems that simulate both nano- and microscale cellular structures will continue to revolutionize nanotechnology, biomedicine, and synthetic biology. At the nanoscale, compartmentalization is poised to advance through complex polymer designs that simulate

organelle membrane behavior using phase separation mechanisms.[78] This includes the creation of robust and multifunctional polymersomes, where precise control over membrane composition and internal domains can be achieved through careful tuning of component ratios and external stimuli-responsive behaviors. The strategic combination of polymers and lipids or insertion of proteins and nanoparticles into these membranes will further refine their ability to encapsulate and deliver various cargoes with enhanced specificity and programmability. At the micro level, self-assembled amphiphilic polymers form microcompartments that mimic cell compartments, allowing for the exploration of intricate phase separation phenomena leading to microcompartments with spatially organized contents. Techniques like microfluidics enable the reproducible fabrication of monodisperse micrometer-sized vesicles with tailored internal environments, capable of accommodating complex biochemical cascades within well-defined boundaries and undergo complex dynamic behavior.[79,80] Adding multivalent ligands or specific molecular interactions facilitates controlled microphase separation, enabling the formation of compartmentalized structures with intricate patterns akin to cell membranes. The inherent incompatibility among distinct membrane constituents may potentially hinder their functionalities.[81] Hence, it is crucial to delve into the harmonious interplay between these various membrane components and optimize their compatibility to ensure seamless operation within the compartmentalized system.

3. Polymeric vesicles for communication in synthetic cells and in (re)clustered colloidal systems

One key argument for the future development of synthetic vesicles is their contribution to the development of synthetic cells,[82,83] therapeutic organelles,[11,84–86] and (proto)tissue engineering.[83,87–89] Their impact will rely on their ability to mimic : i) communication processes

of intra- und intercellular cell compartments,[7,88–91] ii) (regenerative) biological pathways,[69,88,92] and iii) the crowdedness of cellular processes by vesicle clustering in the micro-scale (**Figure 6**A,B).[22,89,93–97]

The tremendous success of nano- and micrometer-sized synthetic vesicles is thoroughly connected to their ability to show versatile and controllable membrane permeability. Communication between vesicular compartments relies on exchanging nutrients, metabolites, and larger bio(macro)molecules through various membranes.[77,84,87,98–100] Membrane permeability of polymersomes and proteins, or polymer-protein conjugates (proteinosomes), is intrinsic to the membrane components' chemical composition and molecular weight. They provide a given membrane porosity (**Figure 6**C**:** i), which also contributes to membrane stability and specific membrane interactions[84,87,98,99,101,102]. Moreover, the integration of transmembrane proteins allows for a defined uptake and release of nutrients and very small metabolites (**Figure 6**C**:** ii).[77,103,104] Thus, intrinsic and size-controlled molecular communication (**Figure 6**A) between various compartments, including different artificial organelles and synthetic cells, becomes possible and allows the exchange of chemical and biological information (**Figure 6**C: i + ii).[77,103,104]

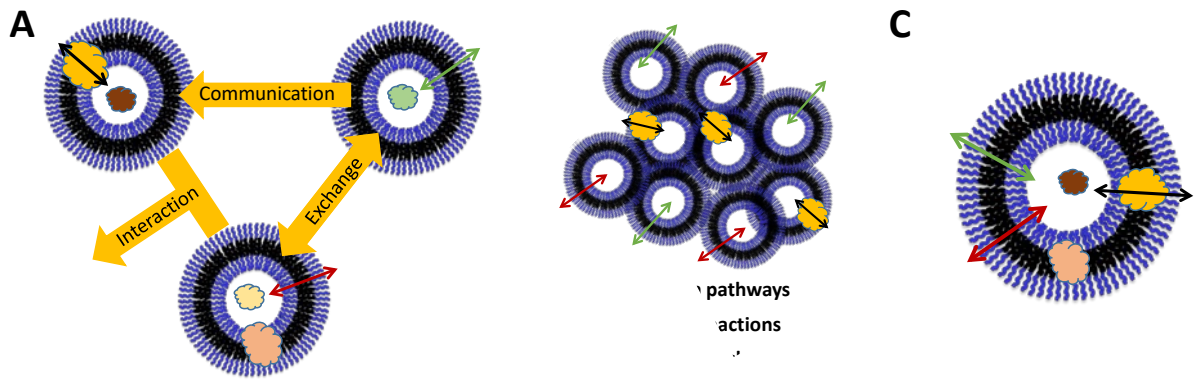

Figure 6*. (A) Communication between vesicles promotes unidirectional transfer for the transport of chemical and/or biological information from a source to a compartment, whereby the latter acts as a receiver. In response to this information, the receiver is able to transmit signals, biologicals*

and/or chemicals to the environment. This can result in the exchange of information between vesicles, which is necessary for mimicking biological processes and for the vesicle´s adaptation to changing environmental conditions. The interactions of vesicles are attributed to the preferential non-covalent assembly of several vesicles. (B) Intense communication between vesicles is enabled in vesicle clusters preferentially driven by non-covalent interactions, which enable short diffusion pathways for rapid cascade reactions and allow for simple mixing of several types of loaded and unloaded vesicles. Only unloaded vesicles, addressed with unique membrane permeability (Details in Figure 6C), are shown in Figure 6B. (C) Communication requires transmembrane traffic realized by: i) intrinsic diffusion with different molecular weight cut off (MWCO) (< 1kDa, 10kDa, ≤ 40 kDa or higher); ii) transmembrane proteins with uni- and multidirectional influx and/or efflux (MWCO ≤ 2 kDa); iii) switchable responsive membranes for, e.g. oscillating acornnd feed-controlled diffusion processes; iv) membrane-integrated enzymes for multidirectional exchange; and v) lumen-integrated enzymes producing chemical and optical information for switchable processes and unidirectional exchange. There are various examples of molecular communication and exchange of information, including vesicles´ adaptation to their environment,[19,83,86,103,105–108] *while other examples explore the vesicles´ interactions.*[20,85,90,92]

In living cells, a cyclic and repeatable on-demand uptake and release of metabolites controls the biological processes. The fabrication of switchable biomimetic synthetic vesicles (**Figure 6**C: iii), responsive to external stimuli (pH, light, temperature etc.),[11,19,20,84,105–107,109–112] allows researchers to establish sophisticated molecular communication between different (living) compartments and their environment with regulated transmembrane diffusion. To achieve this, switchable dye-molecules, or pH-, temperature, redox- and other responsive building blocks must be integrated in the membrane of synthetic vesicles, while retaining their structural integrity under the influence of

applied stimuli and changing environmental conditions. Therefore, the integration of enzymes into the synthetic vesicles, that can specifically produce triggering chemical information (**Figure 6**C: i+v), is of importance. This enables the conduct of oscillating, feedback controlled, cyclic, or self-adaptive processes in and between biomimetic/living cell compartments (**Figure 6**A).[105,107–110,112–115]

The interdisciplinary field of synthetic vesicles merges biology and polymer science, including biological components like cell extracts, genetically expressed proteins or (bacteria) cells. Especially the latter are especially essential in the construction of biomimetic compartments and show high potential to mimic or manipulate specific biological pathways. Nanometer and/or micrometer-sized synthetic vesicles with integrated bio(macro)molecules allow to generate of generic temporal, local, dynamic, out-of-equilibrium, and spatially separated aspects for intra- and intercellular communications. They inspire researchers to establish such cell mimics in synthetic biology. Integrating different transmembrane proteins into synthetic vesicles enables the screening of enzymatic cascade reactions via the controlled influx and efflux of metabolites within biomimetic cell compartments (**Figure 6**A,C: ii).[77] The known principle of membrane-integrated enzymes from cells can be used in the intracellular cascade communication of proteinosomes with membrane-integrated enzymes (**Figure 6**C: iv).[116] Controlled enzymatic reactions are used to fabricate tubular proto-tissues for processing bioactive nitric oxide to avoid any platelet activation and blood clot formation.[88] Here, a logic gate processing is applied to validate the correct sequential assembly of enzyme-loaded protocells, including horseradish peroxidase (HRP), glucose oxidase (GOx), and catalase (CAT), within an artificial vessel to have an on/off production of nitric acid over the space of tubular photo tissue. Finally, cell-integrated bioluminescent

synthetic vesicles (**Figure 6**C: i + v) are able to activate the influx of membrane proteins in opto-genetically-modified cells (cardiomocytes).[117]

Overall, the already obtained progress in the molecular communication between synthetic vesicles paves the way to provide an even higher level of complexity in the design and fabrication of synthetic cells. The field now even allows for dynamic and out-of-equilibrium states in (parallelized) cascade reactions.[90,91,112] Moreover, recent advances in genetic methods also offer the potential in the synergistic combination with organelle-mimicking vesicles to fabricate biohybrid cellular systems with tailored transport characteristics.[117]

Mimicking fast intra- and intercellular molecular communications, meaning between cells and between cell organelles as mitochondria and endoplasmic reticulum, also requires short distances, thus the control of the distance and crowding of vesicular compartments is also of interest (**Figure 6**B).[87,91,118,119] Di-/Trimerized and, especially, clustered synthetic GUVs (from 0.3 up to 50 µm and larger), fabricated by covalent and non-covalent crosslinking processes, exemplify enhanced short-distance communication (**Figure 6**B).[22,89,93–97] These approaches allow the design and fabrication of even more sophisticated biomimetic cell structures. To break down this complexity, each synthetic compartment (**Figure 6**C: i, ii or iii combined with iv and/or v) within these clusters or colonies can carry out an own step within a series of reactions.[87,91] Unique detections, transductions, signal amplifications, logic circuits and/or feedback loops are still available, but also combinations of them are within reach. With an additional twist, clusters of synthetic vesicles can be declustered by reversing the cluster-forming bonds.[95–97] If fully reversible bonds are used, this even allows for dynamic crowding processes between different biomimetic compartments.[95–97] As recently discussed, singular or clustered synthetic and biohybrid compartments are of high

interest in tissue engineering and organoid research with the potential to reinitiate the chemical and biological communication and thus, to close the gap towards biological functions.[83]

4. Multicompartmentalized systems

Organelles are crucial for the organization of many different processes within eukaryotic cells; the multicompartment cellular architecture enables the segregation of biochemical processes to be executed with unsurpassed efficiency. Cellular compartments play a key role in cellular recognition, immune responses, neuromuscular transmission, and cellular organization in tissues and organs. Synthetic multi-compartmentalized structures have thus been created,[3,16] using artificial vesicle systems, such as polymersomes, liposomes and coacervates.[120]

Compartment-in-compartment structures were developed via the encapsulation of nanoassemblies (polymersomes, liposomes, nanoparticles and combinations thereof) in microcompartments to mimic the hierarchical organization of living cells. Such hierarchical assemblies could communicate together or hold diverse functionalities under the roof of one system. In one example, semipermeable enzyme-loaded polymersome-based organelles were encapsulated in GUVs, mimicking the cellular compartment.[7] These organelles were able to catalyze cascade processes, in which the encapsulation of the enzymes also led to their physical separation, which prevented incompatibility issues. Multicompartment systems have also been employed for signaling and communication. In another example, redox-sensitive sub-compartments were encapsulated in a GUV. Upon providing a chemical signal, dithiothreitol (DTT), in the environment of the GUVs, it crossed the GUV membrane and induced the disassembling of the sub-compartments, resulting in the release of their cargo.[19] Depending on the chemical nature of the cargo (substrates, biopores, monomers), a specific reaction took place inside

GUVs (e.g. polymerization of actin) or a second chemical compound was generated and released from these simple artificial cells.[18,19]

Induction of hybrid structures between polymers and lipids is also essential for constructing compartment-in-compartment architectures. Several studies about liposome-polymersome hybrid structures have been published, showcasing the ability to incorporate different types of compartments within a single entity whilst resulting in assemblies that hold the unique properties of both polymersomes and liposomes. This concept was demonstrated by confining liposomes in PEO-*b*-PBO GUVs.[121] Interestingly, the authors showed temperature-controlled release of cargo from the liposomes inside the GUVs lumen, which could be utilized to initiate on-demand cascade reactions within such a confined space.

Using coacervates adds to the level of cellular mimicry, as the density of a coacervate phase is more similar to that of the cell's cytoplasm than the dilute aqueous phase normally observed in polymersomes and liposomes. Rather than confining an aqueous core with a membrane, coacervates are formed through liquid-liquid phase separation. These membrane-less compartments are often based on polycations and polyanions and can mimic biomolecular condensates by creating a crowded microenvironment.[122] This feature has been explored recently via the construction of coacervate mimics that were loaded with different types of semipermeable polymersomes (**Figure 7**).[100] The coacervates were furthermore stabilized by a permeable and dynamic polymer membrane. With this system, it was possible to add substrate to the medium, which could enter the artificial cell and be converted in a cascade reaction by the encapsulated polymer organelles. These compartment-in-compartments were generated via a simple co-assembly/formulation approach, which provides a highly versatile route by which functional (macromolecular) components can be introduced within the coacervate. Interestingly, the robust

nature of the polymersome-in-coacervate system was highlighted in a co-culture experiment in the same medium as living cells.

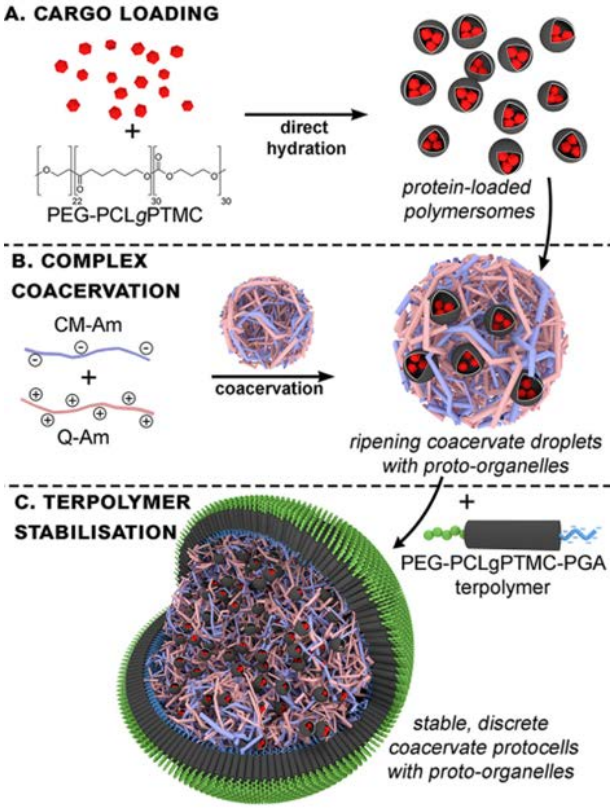

Figure 7*. Formation of Hierarchical Polymersomes-Coacervate Protocell. Reproduced under terms of the CC-BY license.*[100] *Copyright 2019, American Chemical Society.*

The previous examples demonstrate that multicompartment systems can be effectively created with a high level of control of hierarchical control. Many organelle features have already been conceptually demonstrated in these artificial cells. The important next step to take is to functionally integrate them with living systems, for example as programmable units for the spatiotemporal production and release of therapeutic components. In this way, these structures cannot only be explored to deepen our understanding of the functional organization of living cells but also pave the way for many more exciting developments in the biomedical field.

5. Conclusions and Future Perspectives

In this Perspective Article, we highlight recent innovations in the design of polymersome-based artificial cells that incorporate active modules such as molecules and assemblies. These advanced structures aim to enhance our understanding of natural organelles and cells while enabling precise control and multifunctionality. Despite notable progress, challenges still persist that are shaping the future directions of research. Addressing these challenges is pivotal for realizing the full potential of polymersomes, microcompartments and compartment-in-compartments for diverse applications.

Artificial cells created using polymer vesicles have shown remarkable potential in mimicking specific cellular functions. However, these artificial cells currently replicate only isolated functions or structures, lacking the complexity and multifunctionality of natural cells. Future research should focus on enhancing this complexity by integrating multi-step biochemical pathways and additional functionalities. This will be essential for creating more sophisticated artificial cells that better replicate the dynamic and multifaceted nature of living cells.

A significant challenge further lies in enabling artificial cells to respond to multiple and repeated stimuli, adapting to changing environments similar to natural cellular responses. Addressing this will involve developing advanced mechanisms for environmental sensing and adaptive responses. Additionally, most studies have so far mostly observed artificial cells in isolation. Future research must explore their behavior in more complex biological systems, such as cultures, communities, or prototissues, to understand their interactions and functionalities in a more realistic context.

Many current artificial cells exhibit irreversible reactions to environmental changes. Developing reversible response mechanisms will be crucial for enhancing their adaptability and functionality. Moreover, cellular replication remains a largely unexplored aspect of artificial cell development.

Achieving replication in artificial cells, akin to natural cellular division, poses a substantial challenge but is a critical area for future investigation.

Integrating polymer vesicles with biochemical signaling pathways presents an exciting opportunity to facilitate sophisticated interactions similar to cellular communication. However, designing multicompartmentalized systems with controlled architectures remains a significant challenge. Future research should aim to achieve precise control over each compartment's properties while maintaining the overall functionality of the artificial cell. The hierarchical self-assembly of these structures into higher programmed aggregates may also provide deeper insights into organ formation from individual cells.

In conclusion, the future direction of artificial cells lies in enhancing their complexity, functionality, and adaptability. Interdisciplinary collaboration across materials science, biology, engineering, and clinical research will be essential to overcome these challenges. By integrating advanced biochemical pathways, developing adaptive response mechanisms, and exploring artificial cells in complex biological systems, we can unlock their full potential and drive significant advancements in biomedical and technological applications.

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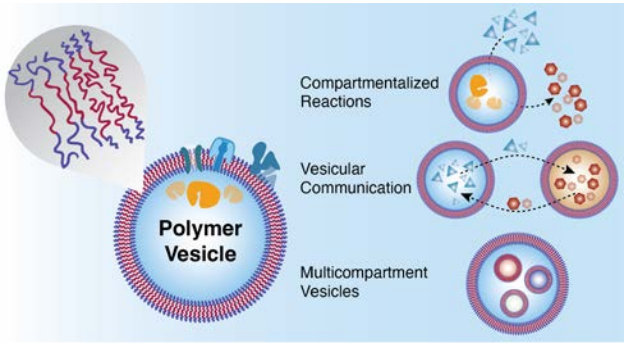
TOC FIGURE



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