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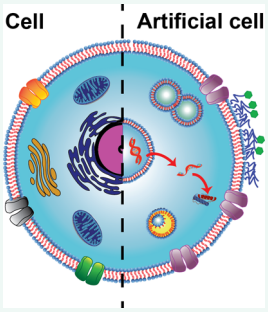
REVIEW

Engineering Compartmentalized Biomimetic Micro- and Nanocontainers

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ABSTRACT: **Compartmentalization of biological content and function is a key architectural feature in biology, where membrane bound micro- and nanocompartments are used for performing a host of highly specialized and tightly regulated biological functions. The benefit of compartmentalization as a design principle is behind its ubiquity in cells and has led to it being a central engineering theme in construction of artificial cell-like systems. In this review, we discuss the attractions of designing compartmentalized membrane-bound constructs and review a range of biomimetic membrane architectures that span length scales, focusing on lipid-based structures but also addressing polymer-based and hybrid approaches. These include nested vesicles, multicompartment vesicles, large-scale vesicle networks, as well as droplet interface bilayers, and double-emulsion multiphase systems (multisomes). We outline key examples of how such structures have been functionalized with biological and synthetic machinery, for example, to manufacture and deliver drugs and metabolic compounds, to replicate intracellular signaling cascades, and to demonstrate collective behaviors as minimal tissue constructs. Particular emphasis is placed on the applications of these architectures and the state-of-the-art microfluidic engineering required to fabricate, functionalize, and precisely assemble them. Finally, we outline the future directions of these technologies and highlight how they could be applied to engineer the next generation of cell models, therapeutic agents, and microreactors, together with the diverse applications in the emerging field of bottom-up synthetic biology.**



KEYWORDS: *compartmentalization, artificial cells, synthetic biology, lipid membrane, vesicles, polymersomes, double emulsions, droplet interface bilayers, microfluidics*

Compartmentalization is a universal organizational principal in biology, whereby all living cells are encapsulated by a biological membrane consisting of lipids arranged into a bilayer. This fluid membrane not only serves as a barrier between the external medium and the inner cytosolic compartment but also is a highly dynamic surface that mediates the exchange of molecular components and chemical signals *via* protein assemblies embedded into the lipid matrix.[1] The theme of compartmentalization, however, is seen in length-scales that are both smaller and larger than cellular ones. Eukaryotic cells contain membrane-bound subcompartments that are specialized to perform specific tasks (organelles), while the pervasiveness and importance of bacterial subcompartments are also increasingly apparent.[2] Chemical species can also be compartmentalized on surfaces, for example, on cytoskeletal filaments, inside invaginations/folds as found inside the inner membrane of mitochondria, or on membranes that exhibit lateral segregation of content in the form of rafts,[3] or colocalized in supramolecular complexes.[4] In the other direction, cells can be brought together to form higher-level structures, namely tissues and organs that exhibit collective properties.

The ubiquity of compartmentalization in biology is ultimately derived from the fact that it allows segregation of content and function, enabling multiple processes to occur simultaneously, which has a host of associated advantages; it allows distinct chemical environments (*e.g.*, redox states, pH, chemical potentials) to coexist, the buildup of chemical gradients, the maintenance of nonequilibrium states, and the isolation of components that would otherwise be incompatible with one another. It also enables the principle of division of labor to be employed, increasing process efficiency and productivity.

The rise of bottom-up synthetic biology and the potential of cell-like structures across a range of applications, from microreactors and therapeutic delivery vehicles capable of on-board chemical synthesis to artificial cells that perform computing operations and interact with neighboring cells, has led researchers to ask the following question: Can the principle of compartmentalization and its associated advantages be transferred to synthetic cell-like structures? (Figure 1) This review will outline recent progress related to this question, with a focus on the engineering strategies used to manufacture biomimetic micro- and nanocompartments.

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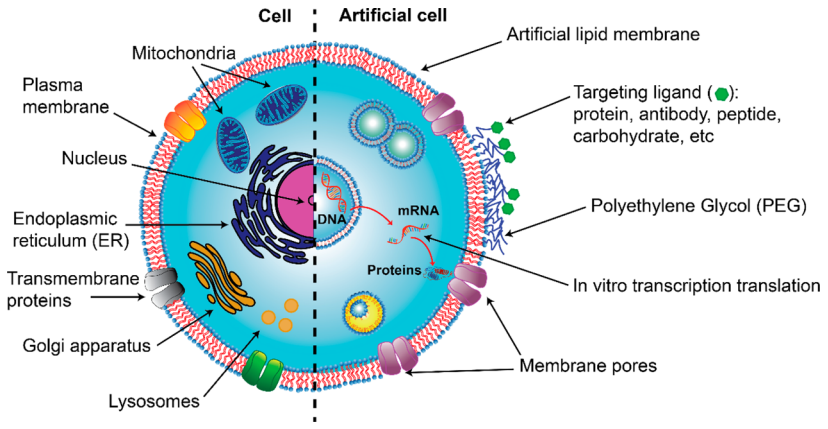


Figure 1. An analogy of a biological and a synthetic cell. The artificial membrane of the cells is engineered according to the end application. Poly(ethylene glycol) (PEG)-ylated lipids to suppress the cell uptake by the immune system, insertion of protein pores to act as gateways, and functionalization of membrane with targeting ligands for stimuli-responsive features. Membrane-bound subcompartments inside the artificial cell can segregate content and perform distinct functions, such as *in vitro* transcription−translation.

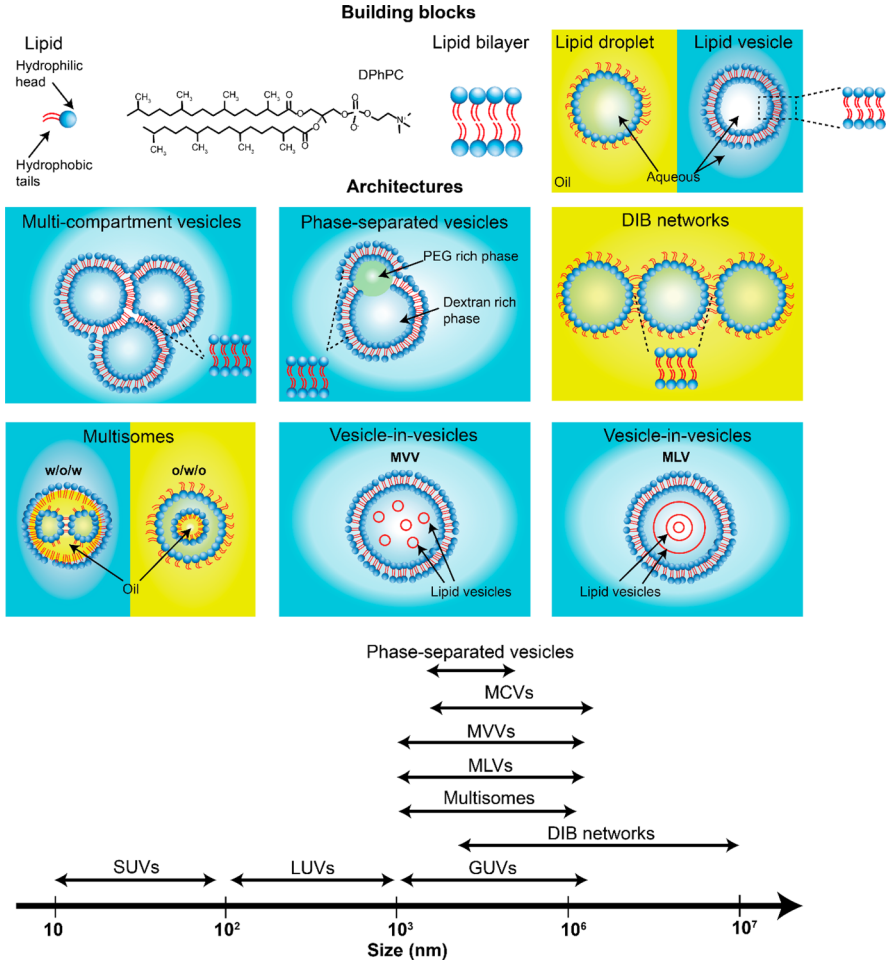


Figure 2. Compartmentalization architecture for lipid-based systems. The building blocks of these systems are amphiphilic lipids which arrange in a monolayer (lipid droplet) or bilayer (lipid vesicle) depending on the oil/water environment. Different compartmentalization architectures involve multicompartment vesicles, phase-separated vesicles, DIB networks, multisomes (or double emulsions), and vesicle-in-vesicle systems such as multivesicular vesicles (MVV) and multilamellar vesicles (MLV). The length-scale of these architectures is indicated at the bottom of the figure.

At the heart of all these strategies is the self-assembly of amphiphilic molecules, with hydrophilic head groups facing the aqueous environment and the hydrophobic tails facing an oil environment which can either be the membrane core or an external oil solution. This can be achieved using bulk approaches, where molecular design of constituent building blocks influences the final architecture of the supramolecular assembly.[5] This has been supplemented in recent years with

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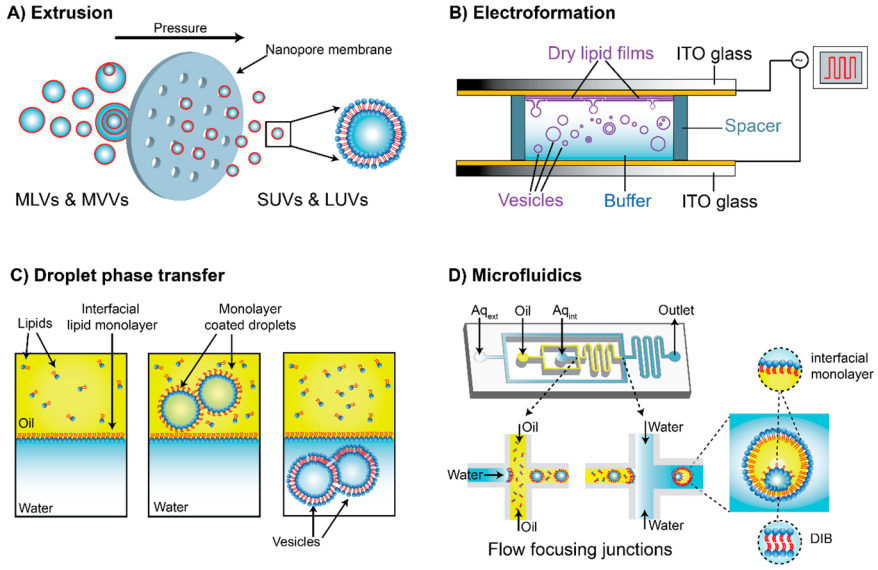


Figure 3. Engineering strategies for constructing lipid vesicles and multicompartment vesicles. (A) SUVs and LUVs are prepared through extrusion of a polydisperse vesicle population. (B) Electroformation of dry lipid films results in the formation of polydisperse GUVs, MLVs, and MVVs. Note that lipid films can be deposited on one or both ITO slides. (C) Vesicles and multicompartment vesicles are generated *via* droplet phase transfer through a lipid-stabilized oil−water interface. (D) Microfluidic generation of w/o/w double emulsions and subsequent oil extraction results in the formation of unilamellar vesicles.

microfluidic and nanofluidic methods,[6,7] where compartmentalized structures are assembled on-chip, one-by-one and often using droplets as precursors, through the handling of fluids in channels of micro- and nanoscale dimensions. This has, in part, been responsible for the upsurge in the number of investigations in this area, due to the high control over the size, content, molecular organization, degree of compartmentalization, and connectivity that it affords.[8]

In this review, we deal with structures that have lipid bilayers and related soft-matter assemblies as a fundamental structural motif delineating compartments. The size of the assemblies that we cover spans 6 orders of magnitude, from tens of nanometers to several millimeters, and include lipid vesicles, droplet interface bilayers (DIBs), polymer-based structures, and hybrid architectures. Within these, a range of compartmentalization principles exists, including nested vesicles-in-vesicles, multilayered assemblies, and higher-order networks of interconnected units. In addition, efforts to impart functionality to these systems through the introduction of biological machinery are discussed, as are the attractions and limitations associated with the various engineering strategies for their assembly. This review concludes with perspective applications of these structures as cell-mimics to investigate biological phenomena, in therapeutics, as microreactors and in bottom-up synthetic biology, and outlines a future roadmap for the field.

VESICLES AND COMPARTMENTALIZED VESICLE ARCHITECTURES

Vesicles (or liposomes) are fully enclosed shells of lipid bilayers that have an aqueous interior and exterior. They are the most common type of lipid architecture due to their stability at a range of different lipid compositions and their structural and functional compatibility with biological machinery such as membrane proteins and DNA. Vesicles are classified by their size and number of bilayers (or lamellarity),[9] where unilamellar vesicles are separated into small unilamellar vesicles (SUVs, <100 nm), large unilamellar vesicles (LUVs, 100−1000 nm), and giant unilamellar vesicles (GUVs, >1 μm), while vesicles consisting of multiple lipid bilayers are classed as multilamellar vesicles (MLVs).

The different size and lamellarity of vesicles largely underpins their application. The small aqueous volumes contained within SUVs make them particularly useful for performing leakage assays to study the activity of protein pores and channels, making SUVs the preferred platform for a wide range of applications including screening for drug/membrane and protein/membrane interactions,[10,11] assembling synthetic cells[12] and as vehicles for targeted drug administration.[13] Given their comparable size to intracellular compartments, LUVs are considered as biologically relevant drug carriers and also offer a higher aqueous space-to-lipid ratio. GUVs are usually employed to construct reduced cell analogs in the laboratory, referred to as "minimal cells",[12] as they are similar in size to a biological cell. MLVs are suitable for the encapsulation of a variety of substances and are the choice for multiple-stage drug delivery.

There have been several efforts to introduce compartmentalization into vesicles and segregate materials into distinct, spatially organized compartments. The present work reviews state-of-the-art architectures of multicompartment vesicles and phase-separated vesicles.

Multicompartment Vesicles (MCVs). MCVs are systems that consist of multiple hemifused vesicles which share a single lipid bilayer with each other, thus enabling the insertion of membrane proteins that facilitates the communication between two neighboring compartments (Figure 2). Individual steps are isolated in distinct compartments, and their products traverse into adjacent compartments with the aid of transmembrane protein pores, initiating subsequent steps. Therefore, an engineered multistep enzymatic pathway can be carried out.[14−16] For example, a two-compartment vesicle was employed to demonstrate communication between vesicle compartments and between each compartment with the external environment depending on the spatial location of the

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small, water-soluble, heptameric β-barrel pore-forming toxin alpha hemolysin (αHL).[14] In the first scenario, one compartment contained $CaCl_2$, while the other compartment contained Fluo-4, a Ca^{2+} sensitive fluorescent dye. Protein insertion in the internal partitioning bilayer resulted in Ca^{2+} ions flux between compartments, followed by gradual increase of fluorescent signal. In the second scenario, αHL was inserted at the external bilayers, and $CaCl_2$ was added to the external solution. Fluorescent intensity of both compartments was increased simultaneously, as Ca^{2+} diffused from the external environment into each vesicle compartment. The same group later demonstrated a three-step enzymatic cascade performed in a three-compartment vesicle.[15] In this application demonstrator, each compartment was loaded with lactase, glucose oxidase, and horseradish peroxidase (HRP) together with Amplex Red, respectively. Lactose was initially hydrolyzed to glucose by lactase, which then translocated through the bilayer pores to the second compartment. There glucose is oxidized using glucose oxidase, yielding H_2O_2. Finally, H_2O_2 diffused through the bilayer into the third compartment, where it oxidized Amplex Red in the presence of HRP, yielding a fluorescent molecule resorufin. In a more recent study, a two-compartment vesicle containing an *in vitro* transcription and translation system was employed to demonstrate spatial segregation of protein synthesis in an artificial cell construct.[16] In this system, one compartment contained a green fluorescent protein (GFP) plasmid, while the other compartment contained a red fluorescent protein (RFP).

Phase-Separated Vesicles. Phase-separated vesicles are composed of solutions that are capable of phase separating if they contain both poly(ethylene glycol) (PEG) and dextran (Figure 2).[17] Vesicles then "bud", resulting in distinct compartments. The main limitation of these systems is that its functional use is limited, since there is no bilayer to separate compartments. Moreover, these systems are restricted to solutions that can be phase separated.

TECHNOLOGIES FOR ENGINEERING VESICLES AND MULTICOMPARTMENT VESICLES

There is a wide selection of available methods to generate individual vesicles. These range from bulk techniques, such as electroformation, hydration, and droplet phase-transfer for GUVs, extrusion and sonication for SUVs and LUVs, to more automated and high-throughput technologies, such as droplet microfluidics (Figure 3).

Extrusion. Extrusion is the most common method to prepare SUVs (Figure 3A).[18] A lipid film is hydrated with an aqueous buffer to create a dispersion which is subjected to five freeze−thaw cycles to make the dispersion more homogeneous before it is passed at least 13 times through a polycarbonate filter, resulting in the formation of monodisperse unilamellar vesicles.

Electroformation. Electroformation is a particularly simple and widely accessible method for the generation of GUV and multivesicular vesicles (MVV).[19] The process involves the application of an alternating electric current across a hydrated film of lipid deposited between two indium tin oxide (ITO) plates (Figure 3B). The alternating electric field causes a periodic electroosmotic movement of water between the individual bilayer lamellae in the film. It has been suggested that these perpendicular to the electrode surface vibrations pull the bilayers off the surface to form a mushroom-like structure which continues to grow until the neck closes and the vesicle detaches from the surface.[20] The main limitations of this method is the lack of control over the vesicle size, asymmetry, and inner compartment content. In addition, this technique is limited to buffers with low ionic strength and is unsuitable to encapsulate large, charged molecules such as DNA and proteins, due to the extremely slow interleaflet movement of these molecules.

Phase Transfer. GUVs can also be formed by transferring a lipid monolayer-coated water in oil droplet to an aqueous phase through an interfacial monolayer (Figure 3C).[21,22] As the droplet is transferred, the interfacial monolayer envelopes the droplet, leading to the formation of a vesicle. Phase transfer is primarily driven by gravitational forces where droplets are loaded with a dense internal solution relative to the surrounding oil and aqueous phase, which allows them to sink through the column. This process can also be aided using centrifugal forces[23,24] or by physically driving droplets through the interface using a micromanipulator.[25] A key advantage of the phase transfer method compared to electroformation is that it allows for asymmetric GUVs to be engineered by incubating droplets in a lipid/oil solution containing one lipid type (inner leaflet) and then adding them to a water-oil column containing another lipid type (outer leaflet).[26] Other advantages of the phase-transfer methodology include control over size distribution and a 100% encapsulation efficiency, since they both depend on the size and number of initial droplets. This is particularly useful when encapsulating large, charged biological macromolecules, which is more difficult to achieve using alternative methods. One of the disadvantages of this technique is the lack of control over the number of compartments of resulted vesicles, which is a stochastic phenomenon dependent on the density of the droplets made during the emulsion formation stage.

Multicompartment vesicles were showcased by Elani *et al.*[14−16] using the phase transfer of two or three droplets across a water−oil interface; nanoliter volume aqueous droplets loaded with the appropriate reaction components were initially prepared manually by pipetting. Two or three droplets were subsequently pipetted in the same locality into an oil-water column separated by an interfacial lipid monolayer. This technique facilitates the production of both symmetric and asymmetric lipid bilayers, but the minimum compartment size is 500 μm, below which droplets would not penetrate the interfacial lipid monolayer.

Microfluidics. Monodisperse and asymmetric GUVs in the nanoliter and picoliter regime can be engineered either by using water/oil/water (w/o/w) double emulsions as templates with subsequent extraction of the middle oil phase (Figure 3D)[27,28] or by mediating phase transfer of lipid stabilized w/o droplets by inertial lift force[28−30] or microfabricated posts.[31−33] The efficiency of the latter, however, was extremely low with only ~1−5% of droplets surviving the phase transfer. Others used an approach where individual monolayers were deposited on droplet templates, enabling asymmetric vesicles of defined lamellarity to be generated depending on how many monolayers were sequentially deposited.[34] Additional efforts have focused on generating vesicles using planar membranes as starting points and then employing a fluidic pulse jet flow to pinch off vesicles.[35] This technique, however, lacks the control and versatility of water in oil methods, while also suffering from a low production rate.

In the femtoliter regime, microfluidic hydrodynamic focusing has been demonstrated as a simple technique for nanoscale

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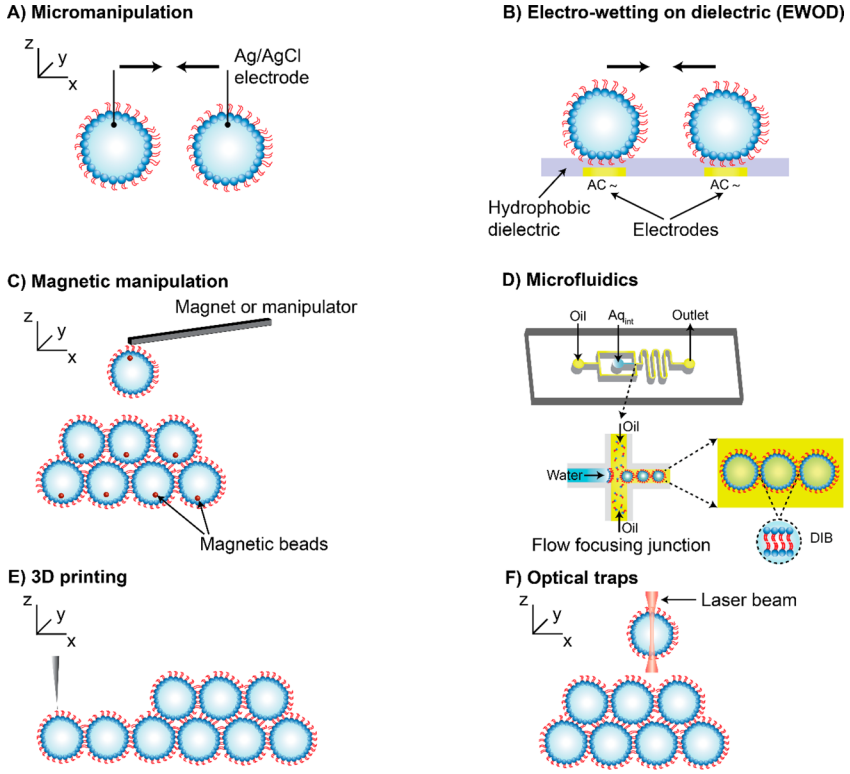


Figure 4. Strategies for assembling DIBs and DIB networks. (A) Micromanipulation of two lipid-coated water-in-oil droplets into contact using Ag/AgCl electrodes. (B) Assembly of DIBs using electric fields to drive droplet motion by electrowetting-on-dielectric (EWOD). (C) DIB network assembly using a magnet to assemble droplets containing magnetic beads. (D) Microfluidic generation of water-in-oil lipid-coated droplets and subsequent assembly of DIB networks. (E) High-throughput production of 3D DIB networks *via* 3D printing. (F) 3D DIB network construction using optical traps.

liposome formation.[36,37] The mechanism of formation relies on the diffusive mixing at two miscible liquid interfaces at a microfluidic flow focusing junction. The disperse phase contains an alcohol solution with diluted lipids, while the continuous phase is an aqueous solution. Diluting the alcohol concentration below the solubility limit of lipids initiates lipid self-assembly into small unilamellar vesicles.

DROPLET INTERFACE BILAYERS

DIBs are model membranes assembled between two lipid-coated water droplets positioned into contact inside a well of oil (Figure 2). The method, pioneered by both the Bayley[38] and the Takeuchi[39] groups, works in two different operating modes depending on how phospholipids are supplied to the system. In the lipid-out approach, the lipids are dissolved directly into the oil, whereas using the lipid-in mode, the lipids are supplied to the water droplets in the form of vesicles, usually prepared by extrusion or sonication. In both approaches, a lipid monolayer assembles at the water−oil interface, and a bilayer is formed when monolayer encased droplets are manipulated into contact.

As a lipid bilayer is only present at the interface of the droplets, DIBs typically have a much smaller bilayer area compared to GUVs made from equivalent sized droplets. On the other hand, DIBs are much simpler to assemble, can be connected together to form networks, and, from a compartmentalization perspective, offer the distinct advantage of having one micro compartment positioned either side of the membrane.

TECHNOLOGIES FOR ASSEMBLING DROPLET INTERFACE BILAYERS

Devices for assembling DIBs were initially reported by the Takeuchi group, who showed both the assembly of bilayers from two 15 μL droplets inside overlapping Parylene-coated poly(methyl methacrylate) (PMMA) wells filled with lipid-oil, and at the interface of two continuous aqueous flows inside a microfluidic chip with a lipid-oil cross-flow.[39] A similar microfluidic approach was also reported by the Schmidt group who assembled lipid bilayers inside 100 μm wide microchannels controlled by pneumatic valves, except in this instance the oil was removed *in situ* by exploiting the absorbent properties of polydimethylsiloxane (PDMS).[40] The material properties of PDMS were also utilized by the Leo group who showed that bilayer size could be controlled by compressing a PDMS device consisting of two overlapping wells. In this elegant approach, an initial compression separates a single 1.2 μL droplet in lipid-oil into two, and the interdroplet interface is controlled by the level of release.[41] Other approaches to assemble DIBs from microliter-scale droplets include the use of micromanipulators to position droplets *via* electrodes (Figure 4A)[38] or by a pin anchor[42] inserted into the droplets, which can also be used as a guide for falling droplets[43] and the use of electric fields to manipulate droplets either by droplet dielectrophoresis[44] or by electro-wetting on dielectric (EWOD) (Figure 4B),[45] which can also be used to control bilayer size.[46] The assembly of DIBs from nanoliter-scale droplets has also been shown using microfluidics (which also facilitated droplet exchange),[47] sliding wells,[48] and magnetic beads (Figure 4C)[49] and using fluid motion generated either by

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the thermocapillarity effect or by Marangoni convection.[50] Several of these techniques can also be used to assemble DIB networks, which was demonstrated by the Bayley group using 200 nL droplets dispensed inside Perspex wells[51] and was later shown inside an automated microfluidic platform capable of stacking droplets ranging from 5 nL to 100 nL in volume.[52] Microfluidic approaches have also been used to demonstrate the controlled assembly of alternating 40 pL droplets in branched channels in addition to more complex 3D structures (Figure 4D).[53] In other approaches, thousands of 65 pL droplets have also been assembled into 3D DIB networks using 3D printing (Figure 4E),[54] while the smallest stable 3D networks reported to-date were assembled from 15 pL droplets using optical tweezers (Figure 4F).[55]

FUNCTIONALIZATION OF DROPLET INTERFACE BILAYERS WITH MEMBRANE PROTEINS AND PORES

While droplets may be supplied with small molecules such as hydrogen peroxide[15] or resorufin[48] which can passively diffuse across DIBs, higher-order activity requires the membrane to be functionalized with membrane proteins or pores supplied to the droplets. This has been reported extensively in the literature using αHL pores, as demonstrated using fluorescence assays[56] and ion channel electrophysiology.[39] Other examples of DIB functionalization using water-soluble species include the linear peptide gramicidin,[44] which forms cation-selective channels,[57] Alamethicin,[41] a 20 amino acid peptide that produces a voltage-dependent conductance in lipid bilayers,[58] and OmpG,[38] which is known to demonstrate pH-dependent gating and voltage-dependent closure.

In contrast to most water-soluble peptides, which can be added directly to droplet compartments with minimal preparation, membrane proteins require reconstitution into proteoliposomes before supplying to droplets for membrane insertion. While this has been achieved in DIBs with potassium channels KcsA,[44] hERG,[59] and hBK,[60] the mechano-sensitive channel MscL,[61] the temperature and methanol activated channel TRPM8,[62] and the light-sensitive proton pump bacteriorhodopsin,[51] these proteins are not all commercially available and typically need to be prepared in advance by the investigator. This usually involves overexpression in cells, followed by protein purification and reconstitution which is a laborious, time-consuming, and low-yielding process.

In vitro transcription−translation (IVTT) has recently emerged as a "cell-free" alternative method for expressing membrane proteins inside a few hours with minimal molecular biology infrastructure. The method works by incubating a cell-lysate containing all of the necessary ribosomal machinery for assembling proteins with amino acids, nucleotides, a system for metabolic energy generation and an engineered DNA (or RNA) template.[63] While membrane proteins have been successfully expressed using IVTT and purified before supplying to DIBs,[38] electrical measurements of the potassium channels Kcv,[38] KvLm,[64] KcsA, and $hERG_{S5-S6}$,[65] in addition to optical measurements of the transporter lactose permease,[66] have also been reported directly from droplets of pre-incubated IVTT mixture, without any purification or reconstitution.

Although efforts to couple protein expression and characterization using IVTT in DIBs has been limited by bilayer instability,[65] recent reports have shown that this can be overcome using PEGylated lipids to stabilize the membrane, while fluorescent proteins or αHL are expressed using IVTT in the presence of a DIB or DIB network under an external light trigger.[67] The ability to control protein expression inside microdroplets using external triggers combined with the ability to engineer genetic circuits[68] could set a paradigm shift for functionalizing droplet compartments used to assemble DIBs.

The ability to assemble functionalized droplet compartments into bilayer networks with user-defined architectures has enabled microsystems to be engineered with collective properties. Examples of these were demonstrated by the Bayley group who showed the assembly of a light-sensing DIB network and a biobattery from 200 nL droplets functionalized with bacteriorhodopsin and αHL pores.[51] The same group later showed the construction of DIB networks that displayed electrical properties such as full- and half-wave rectification and a current limiting using 7R-αHL, a mutated form of the αHL pore engineered to behave like a diode.[69]

MULTISOMES

Multiple emulsions are polydispersed systems where both water-in-oil and oil-in-water emulsions exist simultaneously and are stabilized by hydrophilic and lipophilic surfactants, respectively. There exist two types of multiple emulsions: water-in-oil-in-water (w/o/w) and oil-in-water-in-oil (o/w/o) double emulsions. In the w/o/w, small water droplets are encased in bigger oil droplets, and these oil droplets are dispersed in a continuous aqueous phase. In the o/w/o, oil droplets are dispersed in larger aqueous droplets, and these droplets are again dispersed in a continuous oil phase. In synthetic biology, multiple emulsions have been stabilized by amphiphilic lipids which act as biological surfactants to prevent droplets of the same phase coalescing (Figure 2). These systems, termed multisomes, were introduced by the Bayley group in 2011[70] in order to overcome the main limitation of DIB architectures, being their inability to work in physiological environments since they are restricted to a bulk oil phase. In this work, water droplet networks were microinjected into a larger oil/water droplet, crucially forming w/o/w-type multisomes. Lipid bilayers formed between the inner water droplets but also connected the inner droplet networks to the external aqueous medium through embedded protein pores. O/w/o-type multisomes were recently demonstrated,[71] which can find applications as carriers for hydrophobic molecules that are insoluble in water, however, these systems natively do not form lipid bilayers and, therefore, communication between distinct compartments is solely based on diffusion through the lipid monolayers. Among w/o/w- and o/w/o-type multisomes, the former has wider areas of applications due to its ability to operate in physiological environments. W/o/w multisomes are also precursors for templating vesicles by removing the intermediate oil phase and zipping the two lipid monolayers together to form a lipid bilayer.

TECHNOLOGIES FOR ENGINEERING MULTISOMES

Bulk Emulsification Methods. Bulk emulsification methods have traditionally been used to generate double emulsions in the microliter regime and typically involve a two-step procedure.[72] A simple w/o or o/w emulsion is first prepared by adding the internal phase to the external phase and mixing through manual pipetting. The emulsion is then emulsified again with the external (bulk) phase. A pre-emulsification step can be added to this process to achieve proper emulsification.[73] Despite its simplicity this technique fails to precisely control the size distribution of the produced multiple emulsions. The

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membrane emulsification technique is an alternative method to produce monodispersed emulsions. In this process w/o or o/w emulsions are pressed through the pores of a microporous membrane into the continuous phase.[74]

Microfluidics. Recently, methods for the high-throughput generation of double emulsions and miniaturization to cell-sized volumes have been developed, using traditional microfluidic chips assembled *via* soft lithography as well as using capillary-based devices.[75] These developments are advantageous, as they allow the generation of picoliter-scale emulsions with greatly narrow size distribution profiles and at rates as high as thousands of emulsions per second. They also allow a greater degree of control over double emulsion architecture to be achieved. In addition, one of the most attractive features of microfluidic techniques is that they enable the fabrication of higher order (double, triple, and quadruple) emulsions to be generated.[76−80]

The production of double emulsions in microfluidic devices requires synchronization of the droplet formation frequencies and very specific channel wettability; oil droplet formation can only be realized at a hydrophilic microfluidic junction, whereas the aqueous droplets can only form at a hydrophobic junction. Modification strategies have been leveraged to precisely pattern the surface chemistry within a network of microchannels, enabling the production of oil and water droplets at different parts of the microfluidic chip. One such example is the selective deposition of hydrophilic molecules on plasma-activated PDMS microchannels that are inherently hydrophobic.[27,73,78,81] Nevertheless, while microfluidic generation of surfactant-stabilized systems is well established, lipid-stabilized systems are notoriously more cumbersome to produce, since they require specific surface chemistries and many surface modification techniques are incompatible with lipids. To date, there are few reports detailing the microfluidic generation of lipid-stabilized double emulsions[27,78] and inverted double emulsions.[71]

As a step toward applications in biological settings, there have also been efforts at stabilizing multisomes generated in glass capillary devices by encapsulating the internal nanoliter compartments in gelled alginate, providing them with the robustness needed for eventual academic, industrial, and clinical applications.[82] There have also been efforts to eliminate the intermediate oil phase in multisomes, which might pose problems with respect both to biocompatibility (*e.g.*, if the oil is toxic) and to the encapsulated chemical partitioning into the oil phase. Zhang *et al.*[83] leveraged a microfluidic device to encapsulate a lipid-stabilized aqueous core with a flexible poly(lactic-*co*-glycolic acid) shell that was further coated with another lipid monolayer to form "rigid nanovesicles" in the femtoliter regime, although no bilayer was actually present in this system.

APPLICATIONS OF MULTISOMES

Multisomes were initially envisaged to act as modular chemical microreactors operating in physiological environments, for applications including smart drug delivery and for *in vivo* biosensing. Villar *et al.* explored the potential of w/o/w multisomes for *in situ* release of chemicals and demonstrated temperature and pH-triggered release of internal droplet content by taking advantage of lipid phase behavior.[70] In this work, multisomes were generated with membranes that would destabilize at temperatures above their phase transition and at high pHs, leading to compartment rupture and exposure of chemical species previously isolated in the internal compartments to one another. The use of picoliter volumes of multisomes as *in situ* drug synthesis machines was showcased in a study where w/o/w multisomes consisting of two inner droplets were generated using a PDMS microfluidic device.[78] In one droplet, a membrane impermeable fluorogenic pyrylium compound was encapsulated, while the second droplet was loaded with a membrane permeable primary amine. Amine was gradually diffused through the DIB into the adjacent pyrylium-loaded droplet, yielding a fluorescent pyrylium salt.

W/o/w multisomes have also been employed as templates for the formation of lipid vesicles. Abraham Lee's group developed a technique for the fabrication of lipid vesicles based on multisomes consisting of oleic acid, a nontoxic derivative of olive oil that rapidly dissolves in ethanol.[27,84] Water droplets containing the target encapsulated species were first produced in oleic acid in high throughput using a PDMS microfluidic device. The emulsion was then injected into an ethanol/water mixture, where oleic acid was rapidly dissolved in ethanol, leading to removal of the excess solvent and eventually forming lipid vesicles with both ethanol soluble and insoluble phospholipids. The fabricated lipid vesicles were used to encapsulate fluorescent proteins and carcinoma cells. A few years later, this process was entirely transferred "on-chip" by the same group[27] which showcased on-board GFP synthesis. The fabricated multisomes were loaded with a reaction mix consisting of RNA and enzymes, which was mixed "on-chip" with a plasmid solution, and the expression of GFP in the resulted vesicles was monitored over time.

HIGHER-ORDER STRUCTURES

As demonstrated in the preceding sections, compartmentalizing chemical species in space has a host of advantages. These are, however, higher degrees of order which can exist, defined by the organization of a population of compartments in relation to one another. As technologies for compartmentalizing chemistry and biology have continued to progress, there has been an increasing interest in exploring this space for the engineering of materials with emergent and collective features. An analogy with biological systems is instructive: Collections of cells are networked with one another as a collective to yield tissues and organs which are capable of increasingly sophisticated functionalities.

Vesicles in Vesicles. An alternative compartmentalization strategy centers upon the construction of hierarchical structures where vesicles are encapsulated in larger vesicles, leading to structures known as vesosomes (Figure 2). These can be classed according to the size regimes of the internal and external compartments. SUVs-in-SUVs have been formed *via* a spontaneous encapsulation approach,[85,86] and GUVs-in-GUVs generated *via* osmotic-induced membrane invagination and budding.[87] LUVs can be encapsulated in GUVs by hydration of lipid films in the presence of LUVs,[88,89] although this results in low encapsulation efficiencies, as the mechanism of encapsulation largely prohibits the encapsulation of nanoparticles and colloids. Recent development of emulsion-based generation strategies has circumvented these issues.[90] These have also allowed three-tiered vesicle assemblies to be generated, where SUVs in GUVs were put through a third GUV encapsulation step.[91] Once more, precision engineering of structures with respect to the number of encapsulated vesicles, and lipid composition and encapsulated content of each of the vesicles, has only recently been achieved using microfluidic technologies using sequential vesicle generation modules using dewetting of

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double emulsion templates.[92] Deng *et al.* used this method to generate a eukaryotic mimic, where the inner vesicles acted as a "nucleus", in which *in vitro* transcription of DNA took place.[92]

Others have functionalized nested vesicle structures for responsive microreactors for multistep enzymatic reactions. Bolinger *et al.* encapsulated two populations of SUV in a larger vesicle, each one carrying a different cargo and each composed of distinct lipid compositions, thus possessing different phase transition temperatures.[88] Using phase-transition-mediated leakage, sequential release of reagents by an external stimulus was achieved, and multistep enzymatic reactions could be performed in the vesicle microreactor.

Multilamellar Vesicles. MLVs are related structures to vesicles-in-vesicles and are composed of several bilayers layered directly above one another in an onion-like arrangement. These structures are formed simply by hydration of lipid films where submicron particles self-assemble.[93] Multilamellar giant vesicles are generated during electroformation, however, because this process is stochastic, only a small percentage of vesicles possess more than one lamellae. These extra layers can be visualized under fluorescence microscopy[94] and result in distinct vesicle mechanical properties,[95] affecting both their surface tension[96] and bending rigidities.[97] There have been recent efforts in microfluidic technologies for controlled deposition of a number of defined bilayers around a droplet template using a layer-by-layer approach, leading to the demonstration of the controlled construction of multilamellar giant vesicles.[98]

Directed Assembly of Vesicle Aggregates. There have been efforts at designing vesicles capable of aggregation through both specific and global forces,[99] with aggregation being driven by electrostatic forces[85] as well as site-specific biotin−streptavidin interactions.[100,101] Others have demonstrated aggregation of vesicle-based cells into "colonies" through the addition of polypeptide-induced electrostatic interactions.[102] These aggregates were shown to have enhanced fusion rates and increased permeability to charged biologically relevant molecules including ADP and tRNA due to the stresses resulting from membrane deformation. Second generation technologies have centered on vesicles containing single-stranded DNA on the surface, which are embedded to the membrane through single or double cholesterol anchors[99,103] or using hydrocarbon anchors.[104] The use of DNA allows specific intervesicle interactions to be made through the formation of Watson−Crick base pairs. These interactions are asymmetric allowing controlled assembly of two different vesicle populations, each possessing complementary DNA strands, unlike biotin−streptavidin interactions where a single population of biotin-containing vesicles are bridged by streptavidin molecules. Such systems were used to construct ordered and amorphous colloidal particles that were coated with a membrane containing surface-mobile DNA linkers.[105] Others have used DNA-coated oil-in-water droplets for sequence-specific self-assembly[91] and to yield assemblies with distinct geometries based on steric considerations, including droplet pairs, chains, and multivalent close-packed structures.[106] There have also been several examples of forming heterogeneous GUV and LUV collections[107−109] and vesicles that clustered through site-specific adhesion between DNA functionalized "Janus vesicles" exhibiting phase separation, where aggregation was limited by steric effects.[110] DNA-tethered vesicles were also engineered to exhibit higher-level properties including negative thermal expansion and tunable porosity through the intervesicle space,[111] with potential sensing and responsive cargo-release applications. Finally, DNA linkers can also be designed to induce vesicle fusion and mixing of internal content through the anchoring geometry of the DNA, a process which has been exploited in a vesicle microreactor context.[104,112,113] Although selective communication between vesicles in such assemblies has not been demonstrated, prizing routes to achieve this include the use of protein engineering, reconstitution of cellular machinery, and the design of DNA-origami pores.[114,115]

The primary advantage of using bulk-aggregation methods is that it exploits the principles of self-assembly, meaning aggregates can be generated *en masse* without external input or human manipulation. As a result, however, the structures are largely amorphous aggregates whose geometries are uncontrolled. Alternative strategies involve constructing vesicle assemblies one-by-one. Orwar and others developed a platform of vesicle networks linked by open nanoethers that enabled flow of encapsulated material between compartments[111,116,117] and subsequent chemical reactions that could be controlled by network geometries[118] and shape and volume changes.[119]

Higher-Order Droplet Interface Bilayer Networks. There have been several recent examples of using microfluidic methods to tackle the issue of throughput[53] while still achieving a fine degree of control over network architecture. Villar *et al.* developed a 3D printer capable of depositing lipid-coated droplets of set compositions in defined locations, using this to construct a "tissue-like" material consisting of tens of thousands of interconnected bilayers.[120] This material exhibited collective properties such as osmosis-induced self-folding from a flat to a spherical architecture, and through selective insertion of protein pores, electrical signals along specific pathways were achieved. By further functionalizing this system with a cell-free expression system and a light-activated DNA promoter, a functional mimic of neuronal transmission that can be controlled in a precise, real-time way was assembled.[121]

POLYMER-BASED COMPARTMENTALIZATION ARCHITECTURES

There has been increasing interest in using polymers as alternatives to liposomes both in biotechnology (*e.g.*, in drug delivery) and as the construction of cell mimics. Although this review focuses on lipid membrane-based compartmentalized systems, polymer-based structures are briefly discussed here.

Polymersomes are hollow spherical structures with an aqueous core enclosed by a polymer-based membrane and can thus be considered as synthetic analogues to lipid membranes. Polymers may be composed of a mono- or bilayer of amphiphilic block copolymers, which self-assemble spontaneously in aqueous solution to minimize unfavorable interactions between the hydrophobic regions and the aqueous surroundings (Figure 5A). The self-assembly of monomers can result in distinct architectures, such as planar, spherical, or cylindrical shape, depending on the hydrophilic fraction of the block copolymer.[122] Polymer vesicles, therefore, are not composed of bilayers, but instead of a single polymer-thick membrane. Other types of polymers include diblock copolymers which do assemble as bilayers, comb copolymers, and dendrinized copolymers. Polymersomes are physically more robust than liposomes and are capable of withstanding larger stresses and deformations while still mainlining their physical integrity. They are also more resistant to chemical and biochemical degradation. Polymer monomers are easier to synthesize, meaning finely tuning their chemical structure to impart desired properties of the membrane (*e.g.*, thickness,

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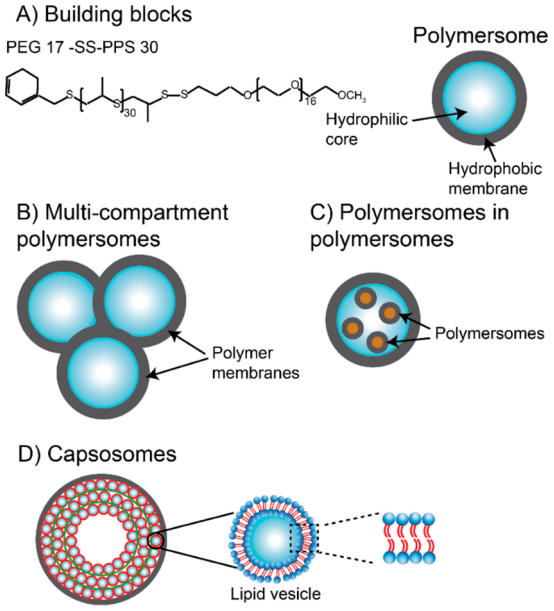


Figure 5. Compartmentalization architecture for polymer-based systems. (A) The building blocks of these systems are amphiphilic block copolymers which assemble to form a polymer membrane encasing an aqueous core. Compartmentalized polymer-based architectures include (B) multicompartment polymersomes and (C) polymersomes-in-polymersomes. (D) Capsosomes as hybrid compartmentalized systems consisting of a polymer membrane which encases small unilamellar liposomes.

permeability, release rates, stability, and responsiveness). Furthermore, on account of their chemical composition and increased thickness, they are less permeable than lipid membranes, which is advantageous for certain applications. However, polymers also possess several limitations compared to liposomes. They inherently lack the biological relevance of lipids and have a diminished ability for functionalization with biological components, such as transmembrane channels and membrane-associated proteins and antibodies, although there have been some examples of successful reconstitution of several membrane pores (synthosomes).[123] In addition, block copolymers do not possess the rich phase behaviors of lipid membranes, which can give rise to dynamic and responsive behaviors. Due to their diminished biomimetic potential, their use as models of cell membranes to study biological phenomena (*e.g.*, protein−membrane interactions) is limited. Polymersomes can be formed the same way as liposomes, depending on the solubility of the block copolymers; film rehydration, electroformation, extrusion, direct injection, dissolution, and double emulsion production using microfluidics.[124,125]

Multicompartment Polymersomes. Multicompartment polymersomes have been implemented from w/o/w emulsions with block copolymers as the stabilizing agent (Figure 5B).[126] A volatile intermediate oil phase consisting of chloroform and hexane was subsequently removed *via* evaporation. Multicompartment polymersomes, where essentially the polymersome is divided in two by a partitioning membrane, have been formed using a microfluidic device which generates w/o/w double emulsions containing a volatile oil as the intermediate phase, which is then removed, leaving behind polymer membranes.[126]

Polymersomes-in-Polymersomes. Other compartmentalized structures, referred as polymersomes-in-polymersomes (Figure 5C), are based on the generation of polymersomes and their subsequent encapsulation in larger polymersomes.[127−130] Polymersome-in-polymersomes have been generated both by bulk sequential emulsification strategies[127,129,130] and using controlled microfluidic methods.[128]

APPLICATIONS OF POLYMERSOMES AND COMPARTMENTALIZED POLYMERSOME SYSTEMS

Due to their enhanced stability by comparison to liposomes, polymersomes hold great promise as capsules for drugs, cosmetics, and nutrients, facilitating long-term storage and controlled payload release.[122] Polymersome-in-polymersomes systems have been leveraged for the encapsulation and controlled and sequential release of multiple distinct components.[128] In this work, programmable payload release of materials was achieved by selectively rupturing the different membranes (outer, middle, and inner) of multilayer polymersomes based on the presence or absence of (PEG)-*b*-poly(lactic acid) (PLA) diblock copolymers on the distinct membranes. For example, in a triple polymersome, the absence of PLA homopolymers in all membranes results in sequential rupturing of the membrane from the outermost to the middle and inner membranes, whereas addition of PLA homopolymers in the outer and middle membranes only results in spontaneous degradation of the PLA in water and rupturing of the innermost membrane first. Polymersomes-in-polymersomes have also been used as structural and functional eukaryotic cell mimicry, where smaller polymersomes were loaded with distinct enzymes inside larger polymersomes and served as artificial organelles, facilitating multistep reaction pathways.[127,129,130] Multicompartment polymersomes have been used in synthetic biology as cell-like nanocompartments with embedded transmembrane channels, allowing the influx of substrates which are enzymatically acted upon in the interior.[123]

HYBRID ARCHITECTURES

In addition to compartmentalized structures that are engineered from either lipids or polymers, there also exists another class of hybrid structure that benefits from the material properties inherited from both systems, capsosomes, together with an additional system based colloidal inclusions of lipid aggregates (coacervates).

Capsosomes. Capsosomes are multilayered polymer capsules that contain intact liposomes in engineered subcompartments (Figure 5D). Here, the polymer capsule provides structural stability, while the liposomes present biocompatibility. Capsosomes were initially reported by the Caruso group, who showed that 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes could be embedded in polyelectrolyte multilayers consisting of poly(styrenesulfonate) and poly(allylamine hydrocholoride). In their approach, 3 μm silica particles were used as scaffolds for depositing polyelectrolyte films *via* layer-by-layer assembly, and capsosome formation was completed by dissolving the silica core.[131] The Caruso group have also shown that liposome adsorption by the polymer film can be improved significantly using a cholesterol-modified poly(L-lysine) precursor layer and a poly(methacrylic acid)-*co*-(cholesteryl methacrylate) capping layer[132] and presented a detailed study outlining the structural integrity of the capsosomes, the cross-linking of the thiols in the film, the

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encapsulation efficiency under different conditions, and the long-term stability of the system.[133] A modified, oxidization-free method to stabilize the carrier capsules was also reported[134] in addition to a bespoke liposome-anchoring cholesterol-containing block copolymer that enables the "free-floating" of liposomal subcompartments.[135] To date, capsosomes have been successfully employed to encapsulate biological components such as β-lactamase and luciferase into two spatially separated compartments inside the same structure[133] and show great promise as compartmentalized bioreactors.

Coacervates. Coacervates are spherical aggregations of lipid molecules making up a colloidal inclusion which measures 1−100 μm across and is held together by hydrophobic forces. Coacervates possess boundaries that are not as clearly delineated as cellular membranes. Nevertheless, coacervates favor the formation of molecular concentration gradients in their "pseudo-compartments" which were proved to be able to perform catalysis.[136] Coacervates have been suggested by Alexander Oparin as possible precursors of biological cells.[137] Oparin hypothesized that coacervates formed in the prehistoric sea and underwent a selection process, during which they developed microcompartments with chemical reactions taking place in each compartment, which was eventually considered to be a protocell. Recent studies have highlighted the possibility of creating structured microscale entities resembling cells based on coacervates. Multicomponent protocell-like constructs were generated with fatty acid membrane assembly on coacervate microdroplets.[138] The same group later discretely leveraged microfluidic technologies to generate discrete membrane-free coacervate microdroplets[139] and demonstrated the potential of these systems to act as microreactors which are able to perform cell-free gene expression and folding of a fluorescent protein.[140]

NONLAMELLAR ARCHITECTURES

Recently, aqueous dispersions of reversed lyotropic nonlamellar liquid crystalline mesophases have also shown promise as therapeutic agents.[141] The most well studied of these lyotropic nanostructured containers are hexosomes and cubosomes, which consist of colloidal nanoparticles with confined internal structures of 2D columnar hexagonal phases[142] or well-ordered 3D bicontinuous cubic phases,[143] respectively. The structural characteristics of these well-defined compartments can potentially facilitate highly efficient encapsulation of medicinal cargo and, by coating the surface with targeting ligands, can be engineered to target specific cells, tissues, or organs.[144]

Another type of nonlamellar structures are solid lipid nanoparticles (SLN), which generally consist of a spherical solid lipid core in the nanometer range having a monolayer of phospholipid coating.[145] These dispersions have been proposed as an alternative type of colloidal drug carrier system with their solid core containing the drug dissolved or dispersed in the solid high melting fat matrix.[146] Although these systems are less compatible with biological machinery, they combine advantages of polymeric nanoparticles, fat emulsions, and liposomes, which make them attractive for drug delivery applications; they are biodegradable and stable against coalescence, and their solid core enables drug release over a prolonged period.

The second improved generation of SLN was introduced as nanostructured lipid carriers (NLCs).[147] NLCs are produced by using a mixture of solid and liquid lipids. Compared to SLN, NLCs offer the advantage of increased and firmer loading of actives inside the particle matrix due to the presence of many imperfections in the crystalline structure of the particle matrix.[148] The latter has significantly increased their shelf life and facilitated their use in cosmetic and pharmaceutical formulations.

DISCUSSION

Comparing and Contrasting Compartmentalized Architectures. The selection of the appropriate compartmentalized system from the variety of architectures described above mainly depends on the end application. An important consideration when using these systems as cell mimics for fundamental research or as functional microdevices is how closely they can reconstitute cellular machinery, namely how biomimetic they are. Truly biomimetic systems are also less likely to trigger immune responses. For other applications, the biomimetic feature may not be important. For instance, for drug delivery applications, polymer systems may be more versatile than their liposome counterparts, because they are mechanically more stable, more deformable, and more resistant to their biochemical environment.

In this context, vesicle-based systems are far more advantageous, since their basic unit—a unilamellar lipid vesicle—resembles the basic compartment structure of all biological cells, in the sense that the vesicle membrane is a mimic of the self-closed lipid matrix of the plasma membrane. This advantage enables them to incorporate cellular machinery, such as embedded proteins and DNA. This feature is ideal not only for synthetic cell fabrication but also for further studying drug/membrane and protein/membrane interactions. Many vesicle-based compartmentalized systems can be employed as biological systems analogs; for example, nested vesicle structures can be used to mimic a biological cell and its internal compartments/organelles, each of which can act as a responsive microreactor for multistep enzymatic reactions. Vesicle-in-vesicle systems are great candidates for targeted multistaged delivery, where each vesicle compartment is functionalized differently to initiate a specific payload release under distinct stimuli (multiresponsive vesicles). MCVs can be employed to build a cluster of cells that are networked with one another, yielding tissues and organs which are capable of increasingly sophisticated functionalities. The main limitation regarding MCVs arises from how they are engineered; the fabrication of these systems relies on the production of individual water droplets manually through pipetting or microfluidically and their subsequent transformation into lipid vesicles through phase transfer. Thus, compartment volumes are restricted to the nanoliter volume regime, since below this size, droplets are not heavy enough to overcome the water−oil interfacial tension and thus cannot penetrate the oil−water interface. In addition, MCVs lifetimes are limited to a maximum of 1 h due to the large osmotic differences that are required to drive phase transfer.

DIB networks and w/o/w multisomes can also be functionalized to produce biologically relevant systems. Both architectures are great candidates to act as modular chemical microreactors, although multisomes benefit from the extra advantage of being able to operate in physiological environments, making them suitable for applications such as smart delivery, microreactors for synthetic chemistry, and *in vivo* biosensing. In addition, the multiphase nature of these systems enables them to act as carriers of both hydrophilic and hydrophobic molecules. An additional advantage of DIB networks and multisomes is that they can be scaled down to

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the attoliter regime and be produced in high throughput with the aid of droplet microfluidics, something that currently comprises a major technical bottleneck for the manufacturing of vesicle-based compartmentalized systems.

Critical Discussion on Generation Strategies. Depending on the application, different engineering strategies or a combination of these are required to assemble multicompartment systems, although the focus nowadays is shifting toward leveraging techniques that facilitate self-assembly and intercompartment communication in an automatic and high-throughput fashion. Electroformation and film hydration are popular and simple methods particularly for engineering higher order structures, however, they lack control over the vesicle size, bilayer asymmetry, and intercompartment content. Phase transfer, on the other hand, addresses the aforementioned issues, however, since it is driven by gravitational forces, it cannot be employed for the production of femtoliter and attoliter systems. The use of optical traps to assemble preprepared droplets and vesicles into DIB networks and VIMs, respectively, can achieve high control over the system's architecture, however, it is a low-throughput method.

Microfluidic approaches allow the design of compartmentalized systems with narrow size distributions and excellent control over the number of compartments and asymmetry of the individual compartments. Recent advances in microfluidic device rapid prototyping, such as 3D printing and acrylic micromachining, have accelerated the use of these platforms in biomembrane engineering. However, with increasing demand not only for high-throughput production but also for minimum compartment size (scaled-out *versus* scaled-down systems), microfluidic technologies have reached a technical bottleneck. It is indisputably a manufacturing challenge. The state-of-the-art in lipid droplet and vesicle microfluidic generation is almost entirely based on nonscalable technologies such as soft lithography, which restricts applications to microsized carriers (>10 μm). Work on microfluidic nanodroplet and nanovesicle production has recently started to emerge,[36,149] however, we are still far from exploiting the full potential of microfluidics in assembling compartmentalized systems of these dimensions. Unlocking this technical challenge with the aid of emerging materials and nanofabrication technologies will lead to further applications in artificial organelle manufacturing and therapeutic delivery.

APPLICATIONS AND FUTURE DIRECTIONS

A multitude of discipline-spanning applications for compartmentalized systems have been proposed over the years. From modular computers and tissue scaffolds to biosensors and functional materials in soft robotics, and their potential to be used in academia, industry, and the clinic is wide-ranging. Four of the most prominent areas that have been most actively pursued are the use of such structures as cell models for the study of fundamental biology, as microreactors, as therapeutic agents, and as artificial cells in synthetic biology. These are outlined below in more detail.

Cell Models for the Study of Cell Biology. Compartmentalized soft-matter systems have the potential to be used as models of artificial cells with which to quantitatively investigate biological phenomena in a simplified environment, where variables can be precisely defined for systematic studies, without interference from a living and responsive cell.[150] Cell mimics have already been used to shed light on processes such as cytokinesis (cell division),[151,152] cell−cell adhesion,[153] and cell motility[154] and the biological effects of molecular crowding, confinement, and compartmentalization.[155,156] They have also been integral in studying the physical principles behind the membrane's role in modulating cellular processes, specifically regarding the effects of bilayer asymmetry,[157] curvature,[158] and mechanical properties.[159,160] Some facets of cell biology are easier to isolate and recapitulate in an artificial system, and it goes without saying that these are better suited for this reductionist approach. This strategy is expected to play an increasingly important role in the study of cell biology going forward. In a general sense, as artificial cells become ever more complex, they will more accurately mimic their biological counterparts, and the more effective this strategy will be.

Microreactors. Another prospective application is the use of compartmentalized structures as self-assembled reactors for chemical and biochemical synthesis.[15,16,161] Their use as reactors is attractive for several reasons: (i) They have ultralow reaction volumes (approaching the femtoliter scale), allowing low-quantity/scarce reagents to be used; (ii) they can be self-assembled, are free of defects, are often self-healing, and do not have to be constructed one-by-one *via* low-throughput nanofabrication techniques; (iii) they can be directly coupled with biological machineries to impart functionality (*e.g.*, selective diffusion of feedstocks/products through embedded membrane channels); and (iv) they can exist as free-standing units in an aqueous environment, enabling reactor applications in physiologically relevant settings.

Compartmentalized Reactors. These systems hold specific promise in biocatalysis, for the orchestration of spatially and temporally segregated enzyme cascades.[161] Segregating catalytic steps in separate compartments, where each compartment possesses a distinct chemical environment (*e.g.*, different pH, temperatures, redox states, presence of cofactors, *etc.*), will allow the coupling of enzymatic reaction steps that would otherwise be incompatible with another. Compartmentalization will also facilitate hybrid chemo-enzymatic hybrid cascades, where reaction incompatibilities are especially pronounced, by preventing poisoning of chemical catalysts by enzymatic cofactors and *vice versa*.

Future directions will likely involve the design of tunable reactors, where temporal control of reactions will be controlled by embedded actuators that can modulate activity in response to the external cues (*e.g.*, chemical environment, light, presence of biomolecules, *etc.*). In addition, a crucial aspect that needs to be addressed is the development of techniques to allow trans-compartment material transfer. When there is a single bilayer separating compartments (*e.g.*, in the case of DIBs), this can be easily achieved through simple protein pores.[38] However, when two bilayers separate compartments from another, this becomes more difficult. Trans-compartment communication will likely rely on DNA origami structures,[115] engineered protein pores, or reconstitution of complex cellular structures that can span double membranes (*e.g.*, connexions, nuclear pore complex and bacterial efflux proteins).

Therapeutic Applications. Liposomes have been used as drug delivery vehicles for several decades.[162] They can encapsulate hydrophobic and hydrophilic drugs in the aqueous core or in the membrane itself, can improve the therapeutic index of drugs by influencing drug absorption and metabolism, and can extent drug half-life as well as reduce toxicity. Drug distribution can be controlled by the physicochemical properties of the delivery vehicle itself, and not of the drug, providing crucial extra degrees of freedom in the design of therapeutics.

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There are several areas in therapeutic delivery where using biomimetic containers can prove advantageous. The first is to increase the robustness and circulation time of the delivery vehicles. This can be achieved by modulating the composition, size, and charge of the compartment,[163,164] by incorporating molecules such as glycolipids and PEG on the vesicle surface,[165] by mimicking the erythrocyte membranes,[166] and by using cell membrane mimetic polymers.[167]

The second concerns targeted delivery, enabling delivery of drugs to the organs, tissues, or cells affected by the disease. Related to this is the need to release the drug at defined rates, according to the diseased state. This can be achieved through a biomimetic approach of attaching moieties to recognize, bind, and induce internalization of the delivery vehicle. They can be decorated with targeting molecules (*e.g.*, antibodies, aptamers, receptor ligands, peptides, and growth factors)[168,169] and elements incorporated to evade the body's immune response.[165] There have also been several efforts at using biomimetic surface engineering to coat nanoparticles with material found on the outer surface of cell membranes to mask it from the biological environment and to allow specific targeting.[170] This strategy has been shown to lead to increased uptake by tumors.[171]

The third is in the design of liposomes that are smart or responsive, releasing drugs only when the target or diseased site is met. This can be achieved through the incorporation of lipid components that are sensitive to pH[172] or temperature,[173] leading to fusiogenic properties or increased permeability due to phase transitions. Use of stimuli-responsive structures that respond to exogenous triggers (*e.g.*, magnetic fields, light, electric pulses) is also an increasingly explored strategy.[174]

The fourth is cell encapsulation in microcapsules (*e.g.*, ones based on hydrogels or polymers). This has the potential for *in situ* delivery of secreted proteins and for cell-based therapeutics more generally, where living cells treat pathological conditions. Encapsulation in a material that allows outflow of therapeutic material (and waste) and inflow of molecules needed for cell metabolism, while shielding the cell from the larger molecules of the immune system, is a potentially powerful concept in therapeutic delivery.[175]

The use of more elaborate compartmentalized structures for such applications is highly attractive for the introduction of smart and responsive functionalities in next generation therapeutic delivery vehicles. These include multicompartment architectures where several drugs can be isolated from one another yet still delivered simultaneously to the target site, *in situ* synthesis of active therapeutics from isolated drug precursors that are brought together upon encountering a target site, multilayer delivery vehicles with tunable drug release kinetics and staged payload delivery, and implanted tissue-like therapeutic devices. Compartmentalization of content is also valuable in the context of multimodal constructs, such as theranostics, which combine diagnostic and therapeutic capabilities in a single agent.

The introduction of biological components to the delivery vehicle surface adds an extra layer of functionality.[15] Addition of responsive elements to the delivery chassis (*e.g.*, functional proteins channels, DNA origami modules, and light-sensitive lipids) raises the possibility for the construction of dormant systems with biovalves that can open/close in the presence/absence of diseased states, and the use of liposome systems as prophylactics that can be administered before a disease is even present.

One of the most pressing issues that needs to be addressed for such applications to be realized is the scaling down of the compartmentalized structures to the submicron regime for them to pass through narrow physiological constrictions and the scaling up of generation throughput. Advanced microfluidic and nanofluidic advances are expected to help in this regard.

Bottom-up Synthetic Biology. Biological systems are highly compartmentalized across several length scales. This includes biomolecule compartmentalization through attachment to membranes and cytoskeleton scaffolds, lateral organization on membrane rafts, compartmentalization in membrane-bound or protein-based organelles, as well as higher-order arrangements of cells in the form of tissues. Therefore, recapitulating such levels of compartmentalization is key in the quest to construct synthetic cell-like systems capable of performing precisely engineered bespoke tasks.

As we have seen, efforts at achieving this are accelerating, and synthetic analogues of increasingly intricate biological structures are being developed. The repertoire of cell-like features and components successfully mimicked is expected to expand in the coming years to include, for example, the construction of synthetic exosomes for the transport of cargo between cells, synthetic gap junctions and synapses, and highly specialized analogues of membranous organelles such as the endoplasmic reticulum and the Golgi apparatus through the incorporation on nonbilayer forming lipids. In addition, methods to exert fine control over synthetic cell architecture will lead to the production of diverse cell morphologies such as elongated neutral-like cells.

Related to this, the isolation and reconstitution of whole cellular machineries, including flagella, biomolecular motors, cytoskeletons, lipid synthesis machinery, and components needed for membrane fission, will lead to the engineering of emergent behaviors such as self-healing, dynamic morphology changes, directed propulsion, homeostasis, replication, and evolution. For these higher-order behaviors to be mimicked, however, it is also necessary to integrate components that have to date been developed in isolation. Finally, applications utilizing synthetic cells will also be aided by the development of hybrid systems, where synthetic cells are interfaced with biological ones either through the construction of synthetic communication pathways or through hijacking and re-engineering existing biological ones.[176−178]

CONCLUSIONS

There is little doubt that compartmentalizing chemistry and biology in soft-matter assemblies is an active and fast-growing area of research with both the number and scope of investigations increasing year after year. There are several trends which will play important roles in dictating the future direction of the field and help in achieving the diverse proposed applications.

The first is increasing synergies with other disciplines, specifically with the micro/nanofluidics community to develop devices needed for the controlled construction of functional structures, and with biochemists whose familiarity with cellular extraction and reconstitution protocols will prove invaluable. Collaboration with chemists to synthesize advanced building-blocks capable of self-assembly is key, as is the involvement of modeling and simulation researchers to better predict system behavior prior to fabrication. Finally, interfacing the soft-matter structures with the external world will require partnerships with engineers, both biological and electronic, and with medics,

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especially for clinical translation. These will be key in addressing one of the bottlenecks in this area, namely how processes contained inside synthetic cell-like structures can be continually powered by external energy sources, rather than coming to a halt when encapsulated chemical sources of energy are depleted.

The emergence of 3D printing and of publically accessible hackspaces in research will also have a part to play. It will serve to democratize the discipline and facilitate multidisciplinary collaborations and will allow biochemists and molecular biologists to exploit microfluidic technologies in their laboratories with limited infrastructure and training.

On the other side of the coin, the development of cheap and easy to use cell-free expression kits will enable physical scientists to directly exploit research areas that have previously been the preserve of trained biologists familiar with cell culture and genetic engineering techniques and will prove useful in rapid prototyping of biological parts and devices. This will be aided by the emergence of made-to-order gene synthesis and protein engineering providers, robotic cloud laboratories, and by ever decreasing costs of DNA sequencing (indeed the cost per megabase of DNA sequencing has been outpacing Moore's law since the mid-2000s).

In conclusion, the above trends, in combination with developments in supramolecular chemistry, will allow the fabrication of increasingly complex compartmentalized architectures with useful cell-like functionalities that can serve functional purposes across a range of applications. Although an effort at outlining a roadmap for the field had been made, there will surely be many unpredictable developments that will take this research area to unexpected directions; what these are remains to be seen.

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VOCABULARY

Compartmentalization, spatial segregation of content into distinct locations; **Biomimetic**, able to mimic processes, elements, and systems of nature; **Artificial cell**, an engineered machine mimicking biological cell; **Bottom-up synthetic biology**, construction of artificial cells and systems module by module; **Lipid droplet**, an aqueous sphere coated with a monolayer of amphiphilic lipids with their hydrophilic heads facing toward the aqueous core and their hydrophobic tails facing toward the external oil environment; **Lipid vesicle**, an aqueous core coated with a bilayer of amphiphilic lipids that arrange with their hydrophilic heads facing toward the internal and external aqueous environment and their hydrophobic tails facing each other within the membrane

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