TOPICAL REVIEW

Shaping up synthetic cells

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Physical Biology

Shaping up synthetic cells

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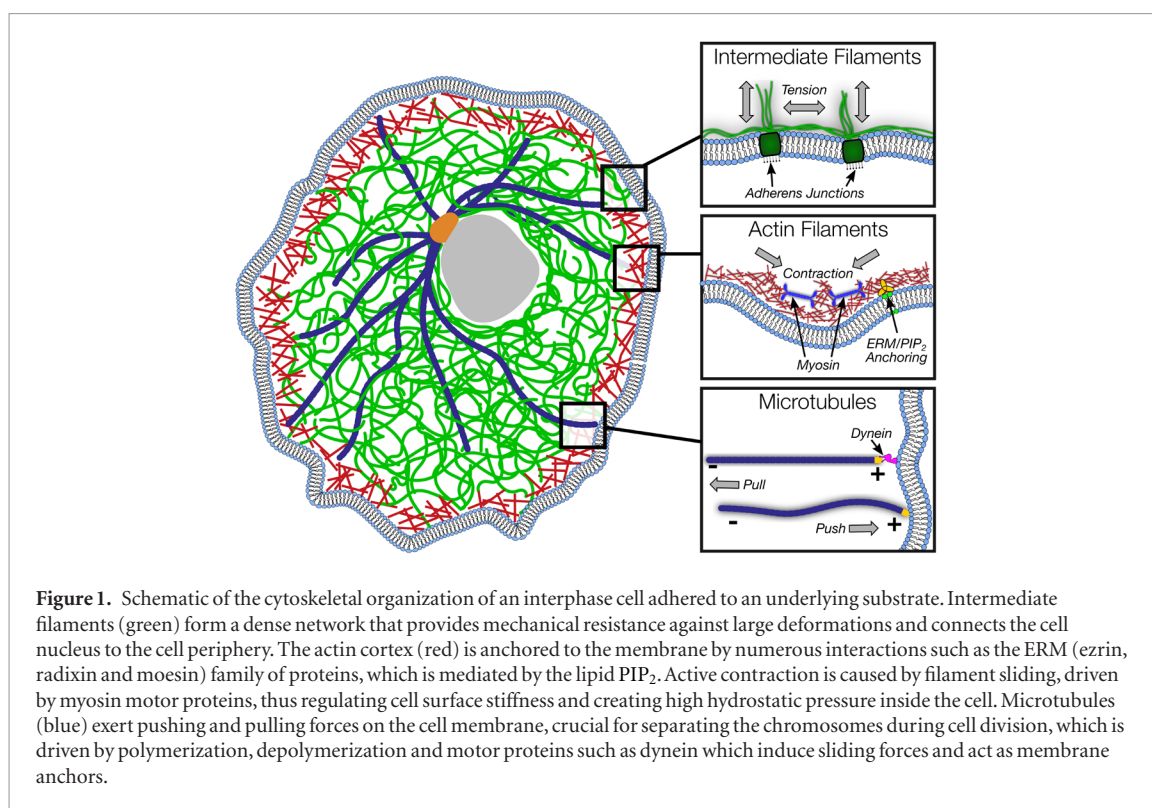E-mail: g.koenderink@amolf.nl

Abstract

How do the cells in our body reconfigure their shape to achieve complex tasks like migration and mitosis, yet maintain their shape in response to forces exerted by, for instance, blood flow and muscle action? Cell shape control is defined by a delicate mechanical balance between active force generation and passive material properties of the plasma membrane and the cytoskeleton. The cytoskeleton forms a space-spanning fibrous network comprising three subsystems: actin, microtubules and intermediate filaments. Bottom-up reconstitution of minimal synthetic cells where these cytoskeletal subsystems are encapsulated inside a lipid vesicle provides a powerful avenue to dissect the force balance that governs cell shape control. Although encapsulation is technically demanding, a steady stream of advances in this technique has made the reconstitution of shape-changing minimal cells increasingly feasible. In this topical review we provide a route-map of the recent advances in cytoskeletal encapsulation techniques and outline recent reports that demonstrate shape change phenomena in simple biomimetic vesicle systems. We end with an outlook toward the next steps required to achieve more complex shape changes with the ultimate aim of building a fully functional synthetic cell with the capability to autonomously grow, divide and move.

Introduction

Cells are soft biological machines with a perplexing degree of complexity. Their physical properties and functional behavior are the result of a complex collection of molecular processes that are highly regulated, dynamic and self-organizing. These processes allow complex functions such as information processing, metabolism and multicellular tissue formation. An overarching aspect to all of these processes is the cell's ability to reconfigure its shape to match its functional state, which can be condensed down to two fundamental features: the cell's ability to actively generate mechanical forces and its mechanical response to self-generated as well as external forces.

In animal cells these two features arise from interactions between the cell's plasma membrane and the cytoskeleton, a composite dynamic biopolymer network comprising three interconnected subsystems: filamentous actin (F-actin), microtubules, and intermediate filaments (figure 1) [49]. These three subsystems each have their own characteristic mechanical properties and are able to interact with one another and with the plasma membrane to define the cell's

mechanical properties and shape. Actin forms a filamentous cortex close to the plasma membrane that is able to exert contractile forces on the membrane through the action of myosin motor proteins that pull on the actin filaments [79]. Actin filaments are also able to treadmill through nucleotide-driven asymmetric polymerization, to facilitate pushing and pulling forces that cause the formation of membrane protrusions or invaginations [18]. Microtubules are highly rigid tubelike fibers that likewise exert forces through asymmetric (de)polymerization as well as motor-driven sliding [59]. These forces are the main driver for establishing cell polarity and separating the chromosomes in dividing cells. Intermediate filaments are more flexible than actin filaments and microtubules and form dense networks between the cell nucleus and the membrane with much slower turnover rates [25]. They serve a vital scaffolding role by providing resilience to large deformations, a phenomenon facilitated by their ability to withstand high tensile strains [61]. Finally the plasma membrane itself also plays a role in mechanics and shape control. Aside from having the flexibility required to accommodate forces generated by the cytoskeleton, the plasma membrane is shaped

Figure 1. Schematic of the cytoskeletal organization of an interphase cell adhered to an underlying substrate. Intermediate filaments (green) form a dense network that provides mechanical resistance against large deformations and connects the cell nucleus to the cell periphery. The actin cortex (red) is anchored to the membrane by numerous interactions such as the ERM (ezrin, radixin and moesin) family of proteins, which is mediated by the lipid PIP_2. Active contraction is caused by filament sliding, driven by myosin motor proteins, thus regulating cell surface stiffness and creating high hydrostatic pressure inside the cell. Microtubules (blue) exert pushing and pulling forces on the cell membrane, crucial for separating the chromosomes during cell division, which is driven by polymerization, depolymerization and motor proteins such as dynein which induce sliding forces and act as membrane anchors.

by changes in lipid composition [16] and interactions with curvature-inducing proteins [77]. Furthermore, the permeation of water and ions via aquaporins and ion channels regulates cell volume and membrane tension [53].

Importantly, the functions of the three cytoskeletal subsystems and the plasma membrane are interdependent. All filamentous systems are able to tether to the membrane [9] and interact with one another both through physical interactions mediated by crosslinkers and motors and through joint biochemical regulation [49]. This complexity makes it very difficult to disentangle the contributions of each subsystem to cell shape control. In recent years researchers have begun to utilize a so-called 'bottom-up' approach to elucidate the complex processes that drive shape change. In this approach cells are 'taken apart' with their individual working components reconstituted in minimal model systems. Such approaches have been used to synthetically recreate protein synthesis [97], vesicular transport [103], self-reproduction [80] and compartmentalized reactions [109] within giant unilamellar lipid vesicles (GUVs), large unilamellar lipid vesicles with a diameter larger than 1 μm. The ultimate goal of this work is a fully functional synthetic cell in which motility, metabolism and replication are combined together within a single membrane container [52].

Shape change is a cornerstone of this grand goal and has been a topic of interest since the first encapsulation of cytoskeletal proteins inside GUVs in 1989 [27]. Advances in encapsulation with improved yields and better reproducibility now make it possible to construct synthetic cells whose shape is controlled by just

a single cytoskeletal subsystem. In this review we outline the state of the art in the encapsulation of cytoskeletal components inside cell-sized GUVs, how these approaches have been used to elucidate the mechanistic basis of cell shape control, and our view on what the probable and necessary developments in this field will be. We focus our attention particularly on advances in the last five years, as summarized in table S2 (stacks. iop.org/PhysBio/15/041001/mmedia). For information on earlier reports we direct the interested reader to previous review articles [96, 114].

Cytoskeletal encapsulation

By far the most common encapsulation system for cytoskeletal proteins is GUVs because they share both the size (of the order of 10–50 μm) and unilamellarity of eukaryotic cells. Encapsulating cytoskeletal filaments within them leads to relatively simple cell models, but their preparation is a highly non-trivial task. Ideally, the encapsulation process should involve a simple procedure that does not harm the proteins, has a high throughput, and leads to GUVs that are uniform in size, defect-free, and unilamellar and have a 100% encapsulation efficiency, independently of the protein, lipid and buffer composition. Perhaps unsurprisingly, such an ideal method does not yet exist. However, extensive research has yielded many different methods [34, 48, 88, 99, 108], each performing well in at least some of these aspects (see table S1). Here we provide an overview of these different methods and direct the reader to a recent review which provides more details [110].

Swelling-based approaches

The simplest and most long-standing approach to preparing GUVs is the swelling of lipid films [121]. The method simply involves suspending lipids in an organic solvent, drying them on a glass substrate and then resuspending them in an aqueous buffer that may contain the proteins of interest to trigger self-assembly into GUVs. Although the procedure is straightforward, it is lengthy (usually of several hours) and tends to give a rather low yield of GUVs. Moreover, the yield and quality of GUVs strongly depend on the lipid and buffer composition [4], making it an inefficient approach for encapsulation. Fortunately there are two swelling-based approaches that boost GUV formation and increase encapsulation efficiencies over wider lipid and buffer composition ranges. These are electroformation and gel-assisted swelling [48] (see figure 2(A)).

With electroformation [34] the swelling process is accelerated by depositing the lipids on a conductive surface and applying an alternating electrical field via electrodes. The application of an electric field results in a much higher GUV yield as it continuously exerts forces on the (zwitter-)ionic lipids and thereby accelerates the separation of the membrane from the surface [5]. However, this method can only be performed in non-physiologically low salt concentrations [5]—which limits its effectiveness for biological applications. In recent years, this limitation has been overcome to some extent by using high-frequency oscillations of the AC field [66], although the reported GUV yield is significantly lower than that in conventional electroformation. The need for an electric field can be eliminated entirely by using gel-assisted swelling [48]. Here, an agarose hydrogel is spin coated on a glass slide before the application of the lipid film and the hybrid film is hydrated in a buffer. Unlike electroformation [5], this method has a good GUV yield in physiological buffers, and for a broad range of lipid types that include zwitterionic, cationic and anionic lipids as well as lipids with one or more headgroups [122]. Most importantly, proteins can be incorporated into the GUV by adding them to the swelling buffer. The encapsulation efficiency varies among the GUVs, but crucially the proteins have been found to still be functional after incorporation [115]. A significant downside to the use of agarose as a hydrogel is that it can become incorporated into the lipid bilayer [48], thereby changing the mechanics of the resulting GUV [69]. Recent work has circumvented this problem by replacing agarose with polyvinyl alcohol [122] or dextran(ethylene glycol) [72]. Furthermore, the size of the GUVs can be tuned by changing the pore size of the hydrogel [72], and the effect of buffer conditions on the GUV synthesis rate and yield has been mapped in detail [89].

Emulsion-based approaches

An elegant but fundamentally different approach to swelling-based methods is to template GUVs from oil/water interfaces stabilized by lipids (see figure 2(B)). This approach is known as the inverse emulsion method [88], and involves forming the inner and outer leaflets of the GUV in two sequential steps. First, an emulsion of water droplets is created by mixing a small volume of water with a large volume of oil containing dissolved lipids. The lipids form a monolayer on the oil–water interface that will ultimately form the inner leaflet of the GUV. Separately the outer leaflet is formed at a bulk interface between a water layer overlaid with an oil layer. The emulsion droplets are then added to the oil phase and forced through the interface by centrifugation, thus forming a bilayer around the droplet and creating a GUV. There are multiple advantages of inverse emulsion over conventional swelling approaches, including the possibility of creating asymmetric GUVs [87] and close control over the encapsulation [88]. The main disadvantages of emulsion-based approaches compared to swelling-based approaches are their lower yield and the presence of traces of oil in the GUV, which alter its mechanics in a way that is not easily quantifiable [17]. To upscale production, the method has been translated to microfluidic devices which allow better control over the steps of droplet creation and bilayer formation. Early attempts at this approach used a two-step process in which the initial emulsion droplet was created on a microfluidic chip and the rest of the inverse emulsion protocol remained unchanged. The two steps were later integrated into a single device specially tailored for GUV production [2], a technique known as continuous droplet interface crossing encapsulation (cDICE; see figure 2(C)). This method is effective for a wide range of buffer conditions and lipid mixtures [11], with the notable drawback that cholesterol incorporates poorly into the GUVs [11]. Compared to conventional inverse emulsion methods, cDICE allows a much higher yield and better control over GUV size [2]. However, cDICE is more technically complex and its use in the formation of asymmetric GUVs has not yet been demonstrated [88].

Other advanced microfluidic techniques in which all inverse emulsion steps are performed on-chip have also been reported [75, 86, 99]. Much like cDICE, these methods have reported higher yields than conventional inverse emulsion approaches, but they are technically more complex.

Other approaches

Swelling [45], gel-assisted swelling [20, 113], electroformation (EF) [102], cDICE [55, 71] and especially conventional inverse emulsion [17, 44, 73, 100] approaches have all been used to encapsulate cytoskeletal components. Since none of them are ideal, there have recently been many efforts to develop alternative approaches for GUV production, mostly based on microfluidic technologies [117]. To our knowledge, none of these newer techniques have been widely adopted beyond reports of proofs of principle,
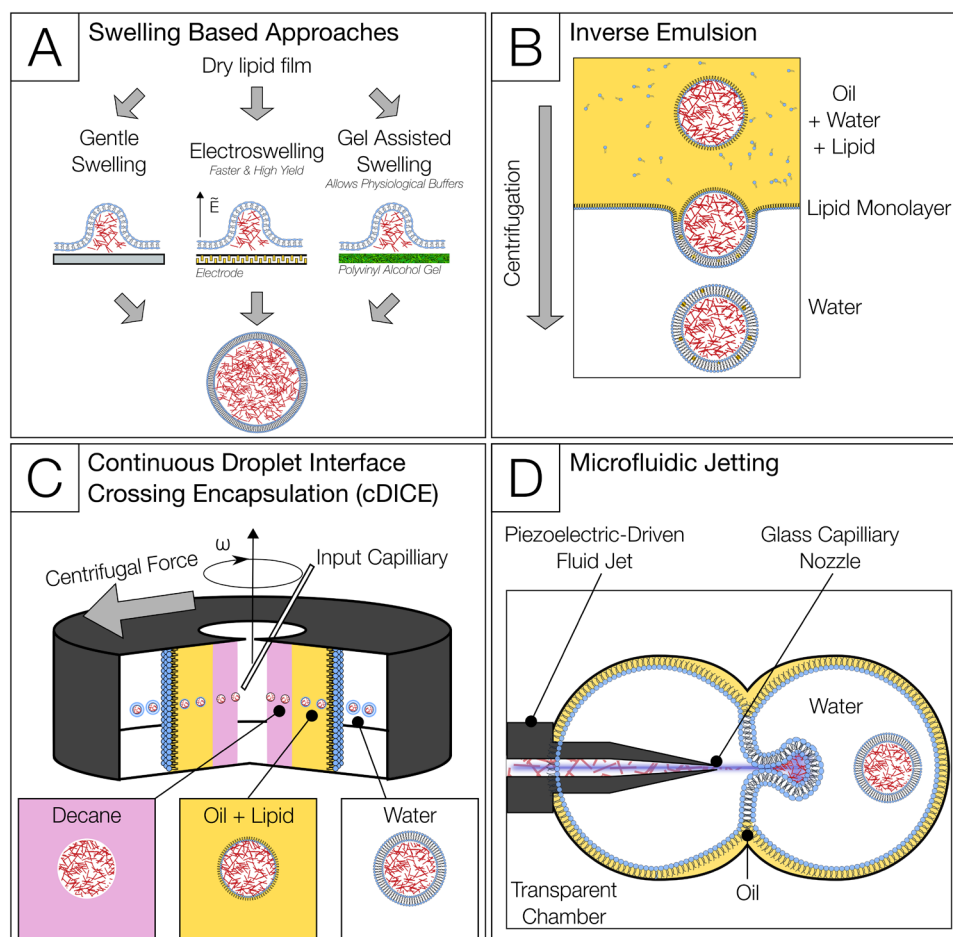
Figure 2. Overview of different methods used to encapsulate proteins inside GUVs (see main text for details): (A) Swelling-based methods [6, 88, 108] involve spontaneous GUV formation upon the swelling of a lipid film deposited on a surface by the addition of an aqueous solution. (B) With the inverse emulsion method [88], water-in-oil droplets are created by emulsification of a mixture of a water phase with an oil solution containing lipids. The lipids form a monolayer on the water droplets that will constitute the inner leaflet of the bilayer of the GUV. Next, the water-in-oil emulsion is centrifuged through a water–oil interface with another lipid film. As a result, both lipid films zipper and form a GUV. (C) Continuous droplet interface crossing (cDICE [2]) is based on the same principle as inverse emulsion. However, as the droplet formation process is well-controlled, the GUV yield is typically much higher and more monodisperse. (D) A thick planar lipid bilayer can be created using two water droplets submerged in a solution of lipids in oil. Afterward, GUVs can be formed by allowing an inkjet to blow on the planar lipid bilayer [108].

likely due to their technical complexity. Below we discuss the two most well-developed techniques, which are based on double emulsions [6] or jetting [108], as well as a recent method where GUVs are stabilized inside polymersomes during fabrication [123].

Microfluidic jetting
The basis of microfluidic jetting [108] is conceptually analogous to blowing a bubble: a microfluidic capillary applies a focused jet of fluid against a planar lipid bilayer, causing a protruded lipid microtube to form on the surface that eventually pinches off to form a GUV (see figure 2(D)). A drawback of this approach has been the inability to prevent oil inclusions in the resulting GUVs [57], but recent reports have circumvented this by setting the jetting parameters such that the lipid microtube disintegrates non-uniformly, creating two populations of GUVs with distinct sizes [54]. Raman spectroscopy showed that oil was present only in the population of larger GUVs —none was found in the smaller GUVs [54]. However,

this inhomogeneous break-up has the tendency to lead to a larger size distribution of the resultant GUVs compared to homogeneous jetting [108].

Double-emulsion approach
Double-emulsion droplets (DEDs) are water droplets encapsulated by a thin layer of oil, surrounded again by water [6]. DEDs can be used to form GUVs by first including lipids in the oil phase, which form monolayers on both interfaces. The oil phase can then be removed via ethanol-mediated evaporation [111]. Similar approaches have been reported for the creation of multilamellar liposomes [30] or assemblies of several GUVs [29]. A recent development of this technique involved replacing the oil phase with octanol, which spontaneously splits off from the droplet as it forms a GUV [31]. A downside of this method is that remnant octanol forms droplets inside the microfluidic chip, which adversely affect GUV stability. However, follow-up work showed that these octanol droplets can be separated from the GUVs

based on their density difference [31]. While this technique appears very promising, the quality of the membrane has so far only been assessed in terms of its ability to incorporate the membrane pore protein complex alpha hemolysin [31]. To truly assess whether this technique creates GUVs comparable to those of other approaches, more detailed characterization of the membrane by Raman spectroscopy [57] or mechanical measurements [17] is required.

Droplet-stabilized GUVs

Just this year, a novel microfluidic technique was reported that makes use of polymersomes as a precursor to GUVs [123]. Polymersomes are vesicles made from diblock copolymers [35]. They are significantly more stable against mechanical perturbations than lipid-based vesicles, and allow the pico-injection of controlled volumes of reagents using electro-microfluidics [1]. It was shown that the inner leaflet of a polymersome can serve as a template for a supported lipid bilayer, which is formed by bursting encapsulated LUVs. The polymersome can then be filled with proteins of interest using pico-injection and the polymeric template can be removed, releasing a GUV containing pico-injected proteins. Both actin and microtubules could be successfully encapsulated in this way, and transmembrane proteins like integrins have also been incorporated into the membrane [123]. Raman spectroscopy and mechanical experiments have indicated that there is no residual oil [123]. Quantitative control over the encapsulant has yet to be demonstrated, but earlier work on pico-injection suggests this should be possible [1].

Triggering cytoskeletal polymerization *in situ*

For encapsulation methods that depend on an intermediate droplet phase, droplet creation necessarily applies shear stresses on the cytosol [6, 88, 108]. Encapsulation methods depending on swelling require proteins to pass through membrane defects in order to be encapsulated [34, 121]. In both cases, this process can be problematic when the cytoskeletal proteins are in filamentous form. Therefore, it is preferable to encapsulate proteins in their monomeric form, and trigger the polymerization of filaments *in situ*. The trigger for polymerization differs per cytoskeletal component. For example, actin polymerization can be triggered using magnesium ions [68], whereas microtubule polymerization is often controlled by the addition of GTP [114]. One popular approach is to ensure that the encapsulation process proceeds more quickly than the polymerization process by performing the encapsulation process at a low temperature [115]. Alternatively, one can change the buffer conditions in the GUV after encapsulation. The membrane itself prevents ion exchange between the cytosol and the outer solution, but the inside buffer

conditions can nevertheless be changed by inserting pore-forming peptides or proteins into the membrane [92], or by adding ionophores to the outer solution, which transfer ions across the membrane [68].

Anchoring cytoskeletal systems to the membrane

Shape control of cells crucially depends on interactions between the cytoskeleton and the surrounding plasma membrane. This interplay is therefore a central consideration for synthetic cells. All three cytoskeletal subsystems are engaged in interactions with the membrane. Microtubules interact with the membrane via a host of different linkers and membrane proteins [14, 33, 82, 106] while in many cell types intermediate filaments interact with the plasma membrane at protein complexes such as desmosomes, which provide adhesive interactions with neighboring cells and the extracellular matrix [94]. Of the three subsystems, however, actin plays the central role because it forms a thin (150 nm) cortical layer on the inner face of the plasma membrane [23]. The actin cortex contributes significantly to cell surface mechanics by providing cortical stiffness and tension, and it also drives shape changes during cell migration and division [24].

One of the most straightforward approaches to mimicking actin–membrane coupling in synthetic cells involves the receptor–ligand pair streptavidin and biotin [120]. Streptavidin is a protein with four binding sites to biotin [90], and the resulting bond is among the strongest noncovalent interactions found in nature. Actin can be linked to the inner surface of GUVs by streptavidin when biotinylated actin monomers are incorporated into the actin filaments and biotinylated lipids are included in the membrane mixture [113]. A more transient interaction that is more reminiscent of actin–membrane linkage *in vivo* can be obtained by using 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)—a synthetic lipid that is not found in nature [51]. Actin directly interacts with this strongly positively charged lipid [46]. One of the most 'biomimetic' approaches that have been developed to anchor the actin cytoskeleton to the membrane of synthetic cells is the artificial linking of purified actin-binding proteins to the membrane [60, 71]. This approach utilizes polyhistidine motifs, which are frequently used as tags in the purification of actin-binding proteins and cleaved off from the proteins at the end of purification. When retained, the polyhistidine can be used to bind the proteins to synthetic lipids containing a nickel-NTA group. His-tagged actin-binding proteins can thus serve as a link between the membrane of a GUV and the encapsulated actin filaments. Actin-binding proteins that have been used in this manner include physiologically relevant actin–membrane anchors such as anillin [71] or one of the ERM (ezrin, radixin, moesin) proteins [60]. The ERM proteins are among the most important anchoring proteins in cells

[36]. To fully reconstitute their *in vivo* functionality, it is likely important to recruit ERM proteins to the membrane via phosphatidylinositol,4,5-bisphosphate (PIP$_2$), a negatively charged phospholipid involved in many signaling pathways [76]. It was recently shown by fluorescence recovery after photobleaching experiments that ezrin-mediated membrane–cortex tethering in cells involves long-lived interactions with the membrane via ezrin's FERM domain coupled with shorter-lived interactions with the actin cortex [39]. It was proposed that ezrin acts as a link that leads to low friction between the membrane and the cortex, which may be important for tuning cortical organization and contractility. It is not straightforward to reconstitute ERM/PIP$_2$ interactions due to experimental challenges in controlling the phosphorylation of ERM, which regulates its actin-binding activity [36], and in working with PIP$_2$, which is prone to degradation and is readily solubilized from the membrane into the aqueous solution [19]. Actin–membrane anchoring via the PIP$_2$-dependent membrane binding of ERM has been synthesized on supported lipid bilayers, but not yet in GUVs [12].

Another 'biomimetic' approach to anchoring the actin cytoskeleton to the membrane of synthetic cells is the nucleation of actin at the membrane using nucleation-promoting proteins such as the Arp2/3 activator VCA bound to the membrane [92]. In this case, actin filaments are only attached to the membrane at one end. This method has been demonstrated to result in GUVs with a thin actin cortex localized underneath the membrane [92].

Mechanics

Many different approaches exist for studying the mechanics of GUVs having an internal cytoskeleton. Like studies of shape change, the majority of studies, both recent and earlier, have focused on actin. Membrane-anchored actin cortices have been shown to contribute to the elastic response of GUVs [74, 101, 102], being significantly harder to compress by AFM than empty GUVs [101, 102] (see figure 3(A)). With micropipette aspiration, cortical stiffness was reported to increase when the actin was crosslinked by dynacortin [74] (see figure 3(B)). Microrheology measurements on GUVs obtained by observing the thermal fluctuations of beads attached to the outer surface of the GUVs or by actively exerting a force on these beads [68] revealed the bending and shear moduli of GUVs with an actin cortex. The resulting moduli as well as the time-dependent mechanics were found to be similar to those of bulk actin networks [43], confirming the importance of the actin cortex to cell surface mechanics.

An alternative approach to quantifying mechanics is to combine micropipette aspiration with optical tweezers [17] (see figure 3(C)). Here, the GUV is held in place with a micropipette and the optical tweezers are used to pull a membrane nanotube from the GUV via a bead attached to the membrane. To determine the membrane bending modulus, the force on the bead in the optical trap can be measured as a function of the pressure applied via the micropipette. Whereas steady-state values of the membrane bending modulus were not affected, the membrane dynamics were slowed down by the presence of an actin shell [17]. This result suggests that the actin cortex increases membrane friction [17]. This effect is attributed to actin decreasing the diffusion of lipids within the membrane, consistent with other studies [41, 47]. Membrane nanotubes can also be extruded via hydrodynamic drag at constant force [44]. Extruded nanotubes were observed to be shorter when the actin cortex was present, again pointing to the role of actin in reducing lipid mobility.

Generating shape change

A key functionality of the cytoskeleton in general and of the actomyosin network in particular is to dynamically control cell shape [79]. Recent work has demonstrated that some features of cytoskeletal shape control can be minimally reconstituted, either by encapsulating individual filamentous systems inside GUVs [62] or alternatively by encapsulating entire cellular extracts [3].

Reconstituted cytoskeletal systems

It is well established that due to their intrinsic high stiffness, microtubules polymerizing inside a GUV can cause the formation of membrane protrusions [62] that bear some resemblance to microtubule-based protrusions in cells [15]. It has been shown that microtubule protrusion can be be reversed in reconstituted systems by applying hydrostatic pressure to promote microtubule depolymerization [45] (see figure 4(A)). By cycling the applied pressure, the GUV shape could be repeatedly switched between more and less protruded. In the presence of GTP, microtubules exhibit a characteristic phenomenon known as dynamic instability, whereby the ends stochastically switch between growing and shrinking states. To our knowledge, there are no reports yet of the time dependence of protrusion formation by dynamic microtubules.

In contrast to microtubules, actin filaments are not stiff enough to independently deform GUVs [22]. However, work from our laboratory has shown that bundled actin can control the shape of a lipid bilayer [113] (see figure 4(B)). This was achieved by comparing uncrosslinked F-actin with actin bundled by the bundling protein fascin within GUVs. Whereas uncrosslinked actin GUVs were spherical, actin bundling caused a wide variety of shapes, including some with protrusions. The competition between membrane resistance and actin bundle stiffness was addressed by varying the fascin-to-actin ratio, where increased stiff-
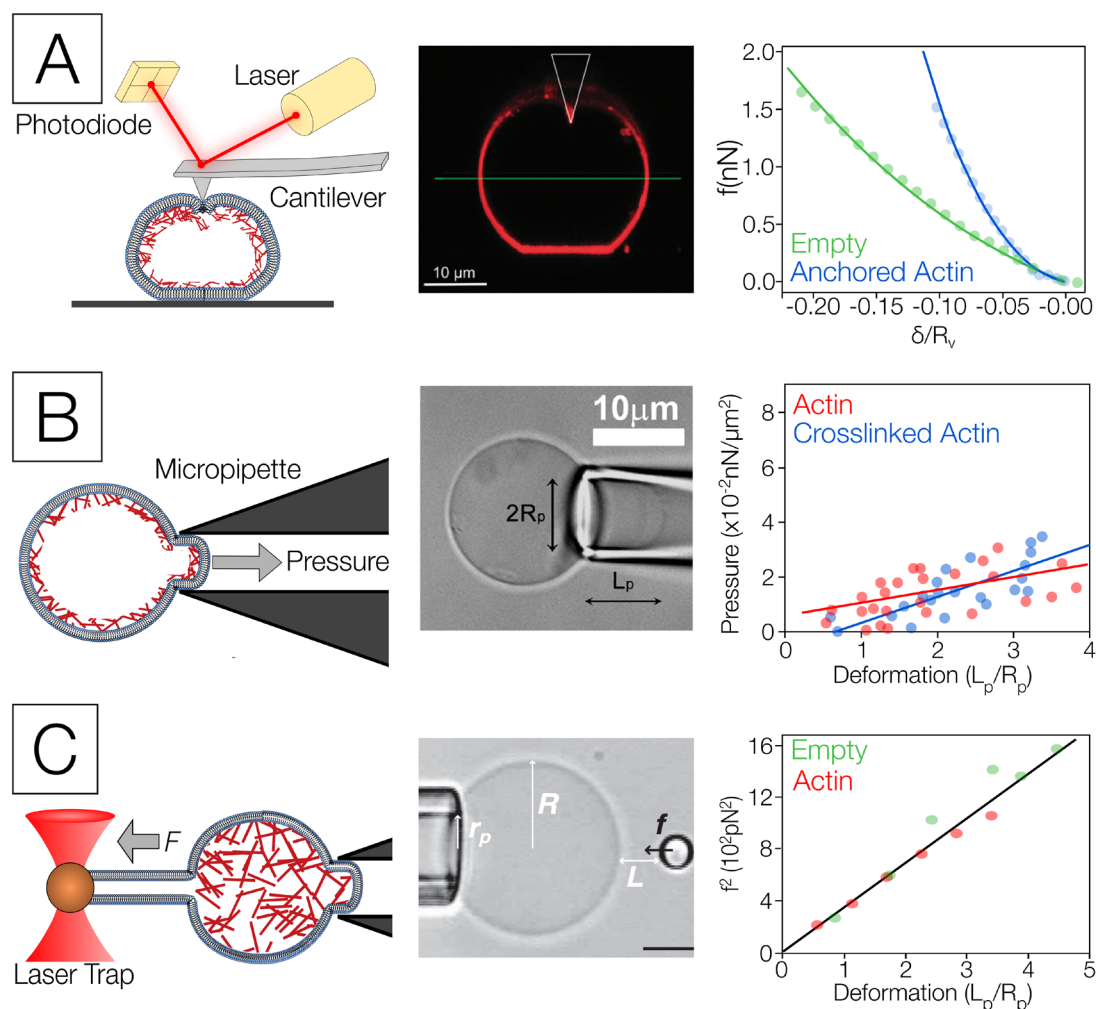
Figure 3. Examples of mechanical characterization of synthetic cells (see main text for details). (A) Nanoindentation of GUVs with (blue curve) or without (green) an actin cortex showed that cortices made the GUVs significantly harder to compress (reproduced from [102] CC BY 3.0). (B) Micropipette aspiration of synthetic cells containing actin with or without dynacortin showed hints of an influence of actin crosslinking (Reprinted from [74], with the permission of AIP Publishing). (C) Optical tweezers were used in combination with micropipette aspiration to pull membrane tethers from either empty GUVs (green) or GUVs containing actin in the cytosol (red) (reprinted from [17], Copyright 2013, with permission from Elsevier). The force required to pull a tether was insensitive to the presence of actin in the cytosol, consistent with the absence of membrane–actin anchoring (the scale bar is 5 μm).

ness caused the actin bundles to become increasingly straight, deforming the GUVs more severely.

In addition to the active (dis)assembly dynamics of actin and microtubules, cells also use the activity of molecular motors to achieve shape control. Motor-based shape control in synthetic cells has been demonstrated by co-encapsulating actin filaments and myosin II motors within a GUV [20, 115] (see figure 4(C)). Here, actin was anchored to the membrane via biotin–streptavidin linkages. Myosin activity was shown to be sufficient to cause the actomyosin network to contract and even to detach from the membrane in case of weak anchoring [20]. By contrast, when such an actomyosin network was attached to the outside of a GUV, actomyosin contractility caused GUV crushing. It was recently shown how cortical tension can also cause more subtle membrane remodeling in GUVs, using anillin as a membrane anchor and adjusting membrane tension by adding glucose to the outside solution [71]. Depending on the myosin and anillin concentrations, a wide variety of GUV shapes could be generated

such as faceted surfaces and extrusion of tether-like shapes. Moreover, GUVs with membrane blebs have been observed (see figure 4(D)). Blebs are spherical membrane protrusions that are thought to drive some forms of cell migration and to regulate cortical tension in dividing cells [8, 104]. Theoretical studies and observations of cells have suggested that bleb expansion is driven by the contractile actomyosin cortex, which generates hydrostatic pressure in the cytoplasm [112]. This hypothesis has been elegantly validated by the *in vitro* reconstitution of blebs in GUVs containing a minimal actomyosin system.

Reconstituted whole cellular extracts
Cytoplasmic extracts provide an interesting alternative approach to reconstituting a cytoskeletal system capable of mimicking complex aspects of cell shape control [7, 70, 118]. Extracts have a significantly more complex composition than bottom-up reconstituted mixtures, which makes a detailed mechanistic interpretation of experimental observations more
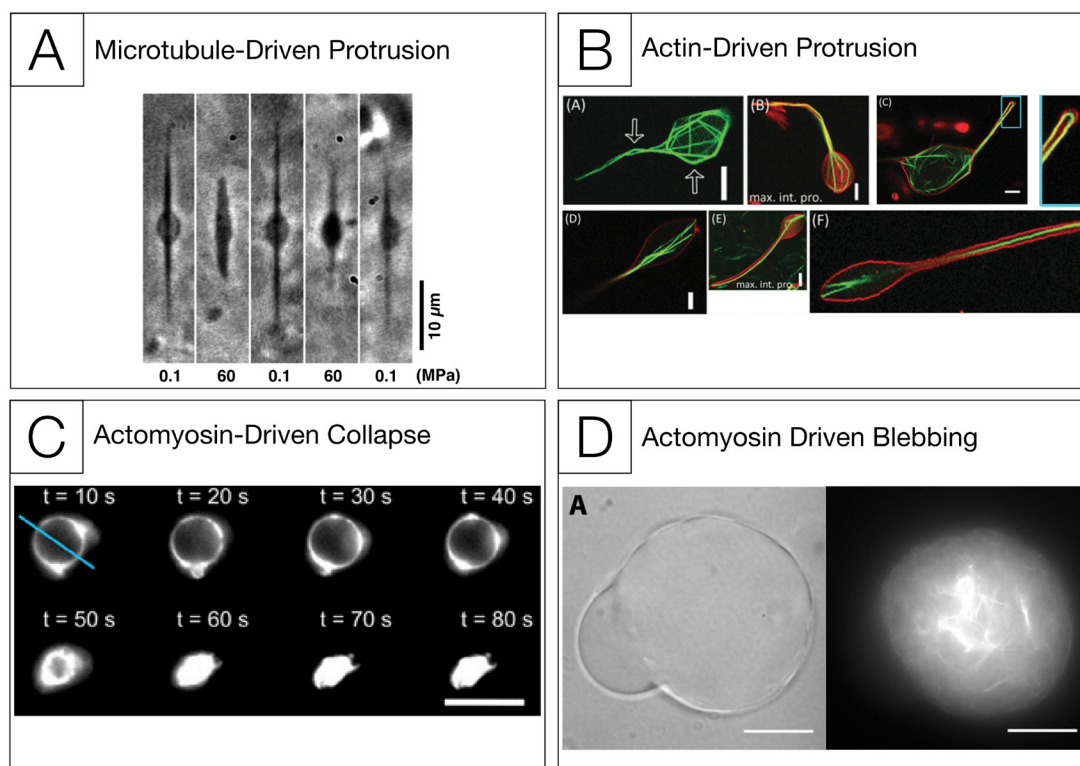
Figure 4. Several examples are shown of GUV shape changes achieved by encapsulation of cytoskeletal components. (A) Microtubules encapsulated in GUVs form membrane protrusions. These shape changes can be reversed by increasing the osmotic pressure to depolymerize the microtubules (reprinted with permission from [45]. Copyright 2016 American Chemical Society). (B) Actin bundles formed by co-encapsulation with the bundling protein fascin cause membrane protrusions, with geometries depending on the ratio of actin to fascin (the scale bars are 5 μm) (reproduced from [113] with permission of The Royal Society of Chemistry). (C) When an actomyosin cortex is attached to the outer surface of a GUV, the GUV is crushed due to myosin-driven network contraction (the scale bar is 10 μm) (reproduced with permission from [20]). (D) When an actomyosin cortex is attached onto the inner surface of a GUV, the balance between hydrostatic pressure and actomyosin contractility causes bleb-like shapes (the scale bar is 20 μm) (reprinted/adapted from [71]. © The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC).

difficult yet simplifies the reconstitution of complex processes that require a large set of proteins [10, 18]. Cytoplasmic extracts contain over 11 000 proteins [125], including a multitude of actin-binding proteins like cofilin, profilin, Arp2/3 and formin which together cause the continuous polymerization and depolymerization of actin filaments [3] and signaling complexes such as the chromosomal passenger complex and centralspindlin which allow communication between different cytoskeletal components [38]. Cytoplasmic extracts from amoeba have been encapsulated inside emulsion droplets and anchored to the interface via DOTAP [50, 81]. The actomyosin cortex caused an observable wrinkling of the lipid interface, which was modeled theoretically by considering the balance between cortex elasticity and contractility [50]. Extracts from oocytes reconstituted in droplets with actin nucleators localized at the surface have been shown to drive flows of cortical actin, reminiscent of the polarizing cortical flows observed in oocytes and early embryonic cells [3, 91]. The flows could be tuned by adding crosslinkers of actin and by tuning the spatial localization of F-actin nucleators and actin turnover. Recently, interactions of the actin and microtubule cytoskeleton were likewise reconstituted inside emulsion droplets. It was shown

that F-actin contractility in combination with actin-bound signaling proteins could drive microtubule nucleation [26].

Bio-inspired novel functionality

All of the papers that we have discussed used biological components with the goal of understanding aspects of cellular behavior. The same biological components can also be used to create entirely novel functionality. This philosophy has been applied to systems comprising microtubules and kinesin motor proteins encapsulated inside GUVs [55]. A dense layer of microtubules was found to form a two-dimensional liquid crystal phase. Kinesins caused active sliding between microtubules, creating a so-called active nematic [98]. This active nematic was confined to the surface of the GUVs and was shown to cause the GUVs to undergo constant shape changing [56]. Although this nematic microtubule phase is not directly biomimetic [124], the resultant shapes were surprisingly reminiscent of filopodium-like protrusions and even migration of droplet-confined active nematics was observed [98] (figure 5(A)).

In another approach toward bio-inspired functionality with encapsulation, a GUV referred to as a 'molecular robot' was created by encapsulating kinesins,
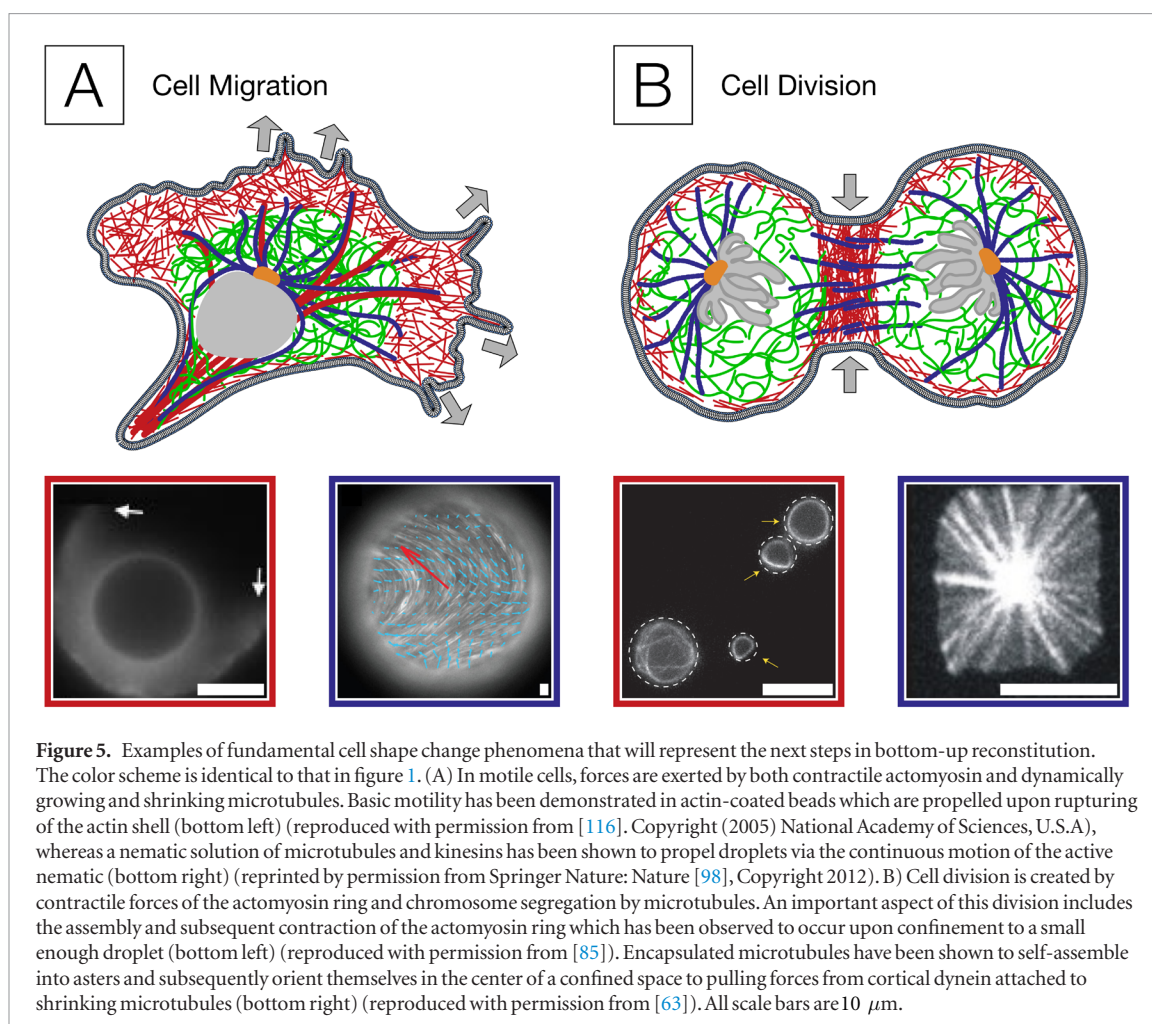
Figure 5. Examples of fundamental cell shape change phenomena that will represent the next steps in bottom-up reconstitution. The color scheme is identical to that in figure 1. (A) In motile cells, forces are exerted by both contractile actomyosin and dynamically growing and shrinking microtubules. Basic motility has been demonstrated in actin-coated beads which are propelled upon rupturing of the actin shell (bottom left) (reproduced with permission from [116]. Copyright (2005) National Academy of Sciences, U.S.A), whereas a nematic solution of microtubules and kinesins has been shown to propel droplets via the continuous motion of the active nematic (bottom right) (reprinted by permission from Springer Nature: Nature [98], Copyright 2012). B) Cell division is created by contractile forces of the actomyosin ring and chromosome segregation by microtubules. An important aspect of this division includes the assembly and subsequent contraction of the actomyosin ring which has been observed to occur upon confinement to a small enough droplet (bottom left) (reproduced with permission from [85]). Encapsulated microtubules have been shown to self-assemble into asters and subsequently orient themselves in the center of a confined space to pulling forces from cortical dynein attached to shrinking microtubules (bottom right) (reproduced with permission from [63]). All scale bars are 10 μm.

microtubules and photo-activatable DNA connectors within a GUV [100]. Three types of single-stranded DNA were used: one was attached to the membrane, another was attached to the kinesins and upon light activation a third DNA strand could connect the two other strands. As a result, light activation triggered the recruitment of kinesins to the membrane via DNA hybridization. Subsequently, kinesin-bound microtubules were recruited to the surface, causing the GUV to change shape. Due to the active sliding between the kinesins and microtubules, continuous shape changes were observed as long as the 'molecular robot' was exposed to light [100]. This study is an example of how different techniques derived from biology, herein DNA nanotechnology and protein reconstitution, can provide significantly enhanced function.

The shape of things to come

It is becoming increasingly clear that encapsulated systems are an essential tool to understanding the fundamentals of cell shape change and mechanics. Lately there has been a convergence of numerous technical advances that make reliable and biomimetic encapsulated systems a possibility. At the forefront of these are advances in encapsulation, with automated techniques such as cDICE now facilitating high yields and accessibility to a broad range of lipid compositions

and buffer conditions to suit the assembly and/or dynamics of the desired encapsulated cytoskeletal protein [2]. This improved versatility is likely to facilitate better control of encapsulated systems as a more comprehensive understanding of the influence of plasma membrane composition on encapsulated protein remodeling is reached. This in itself is a separate and vibrant field of study [9]. In addition, creative solutions to long-standing concerns such as the incomplete removal of residual oil from emulsion encapsulation are also being created through novel approaches [123]. The true test of the utility of these new approaches will be whether they become widely adopted outside of the laboratory of origin. The high technical barriers to realizing this may be a limiting factor.

Alongside advances in encapsulation, there now exists a broad library of different cytoskeleton–membrane anchoring approaches such that researchers may select anchoring that is weak, strong, fast or slow. Investigating the influence of membrane anchor dynamics and affinity in this way represents, in our view, a significant untapped source of research potential. While studies have shown, by proof of principle, that the actin cortex influences GUV mechanics [74, 102], complementary rheological data on bulk actin networks show a richness of mechanical response [58, 67, 126] that is almost entirely unstudied in encapsulated systems.

Even more pressing is that mechanical data on reconstituted active actin cortices are entirely nonexistent. For example, in bulk actin networks it has been shown that myosin activity dramatically stiffens actin networks by pulling out thermal fluctuations [58], but mechanical studies on cells have shown that myosin II activity can stiffen adherent cells while softening cells in suspension [21, 37]. To understand this varying behavior, it will be essential to measure the mechanical properties of reconstituted active cortices inside GUVs.

Considering the diversity of cytoskeletal components that have been successfully encapsulated, intermediate filaments are glaringly absent. This may be because their indirect contribution to shape change makes them a less obvious candidate for study. In contrast to actin filaments and microtubules, they have a slow turnover rate (of the order of 1 h) and this turnover is more difficult to reconstitute as it is governed by phosphorylation/dephosphorylation rather than by nucleotides and is dependent on interactions with the microtubule and actin cytoskeleton [83]. While this makes their influence on cell shape change difficult to study by reconstitution approaches, their influence on mechanics should be more easily measurable. For example, their reported high extensibility could have an influence on the GUV's nonlinear mechanics in response to nanotube extrusion as previously quantified for actin [17]. In this context, it would also be interesting to reconstitute membrane anchoring, which appears to play an important role in the mechanical scaffolding function of intermediate filaments in cells [94].

A major milestone in synthetic cells that has now been reached is the possibility of not only observing shape change in synthetic cells but also actively controlling it, either by composition [71] or by external triggers such as hydrostatic pressure [45] or light [100]. This paves the way for the reconstitution of dynamic cellular processes such as cell motility. A central requirement of motility is symmetry breaking, a phenomenon reported in cellular extracts [3] and also in purified actin systems polymerized on the outer surface of polystyrene beads and GUVs [116] (figure 5(A)). Studies of migration in cells have revealed that an important requirement for establishing and maintaining cell polarity is close coupling between all three cytoskeletal subsystems [49]. For example it has been shown that intermediate filaments in cells are reorganized by microtubule-induced cell polarization [65]; conversely, their organized structures serve as a template for further microtubule growth [42]. Similarly, traction forces are generated by the actin cytoskeleton but intermediate filaments play a role in their directional orientation [28]. Lastly, the co-organization of microtubules and actin plays an important role in controlling the front–back polarity of cells [13]. This crosstalk was recently reconstituted *in vitro* but was not yet encapsulated in GUVs [93]. Similarly, cell division requires close crosstalk between the actin and microtubule cytoskeleton: the

actin cortex facilitates the formation, positioning and orientation of the mitotic spindle, while the spindle directs the formation and activity of the actomyosin ring at the cell's equator [64]. Spindle-like microtubule structures have recently been reconstituted inside emulsion droplets by co-encapsulating centrosomes with tubulin, motor proteins, crosslinkers, and cortical dynein anchors [63, 119] (figure 5(B)). Likewise, actomyosin ring constriction has been successfully reconstituted inside emulsion droplets, where crowding agents caused actin to bundle and to form a ring at the droplet equator that contracted in the presence of myosin [78]. It will be exciting to combine these microtubule- and actin-based structures to achieve spatial control over cell division in synthetic cells. Until now, efforts to reconstitute cell division in synthetic cells have been based mainly on the prokaryotic FtsZ system, a tubulin homolog in *E. coli* that forms a so-called z-ring, which contributes to cell constriction in concert with the cell-wall building machinery [84]. This approach is significantly more mature than efforts based on eukaryotic systems. Not only anchoring to the plasma membrane via the membrane proteins FtsA and ZipA but also, under certain conditions, division of the entire GUV has been achieved [85] (figure 5(B)). Furthermore, spatial regulation of FtsZ assembly has been achieved by adding proteins of the Min family, which form spatial patterns by a reaction–diffusion mechanism [107].

In the long run, reconstitution of eukaryotic life by synthetic cell approaches has to consider the interactions between cells and their surrounding tissue matrix that drive multicellularity. Proof-of-principle evidence shows that GUVs can be made selectively adhesive to the extracellular matrix by incorporating integrin receptors into the membrane [40, 123]. Also, cadherin-based adhesion between cells has already been mimicked in biomimetic systems [105]. The ultimate challenge will be to combine all these individual aspects of cell shape control within a single system to achieve synthetic cells with truly life-like functionality.

Acknowledgments

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