Synthetic cellularity based on non-lipid micro-compartmentalization and protocell models

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This review discusses recent advances in the design and construction of protocell models based on the self-assembly or microphase separation of non-lipid building blocks. We focus on strategies involving partially hydrophobic inorganic nanoparticles (colloidosomes), protein–polymer globular nanconjunctures (proteinosomes), amphiphilic block copolymers (polymerosomes), and stoichiometric mixtures of oppositely charged biomolecules and polyelectrolytes (coacervates). Developments in the engineering of membrane functionality to produce synthetic protocells with gated responses and control over multi-step reactions are described. New routes to protocells comprising molecularly crowded, cytoskeletal-like hydrogel interiors, as well as to the construction of hybrid protocell models are also highlighted. Together, these strategies enable a wide range of biomolecular and synthetic components to be encapsulated, regulated and processed within the micro-compartmentalized volume, and suggest that the development of non-lipid micro-ensembles offers an approach that is complementary to protocell models based on phospholipid or fatty acid vesicles.

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Introduction
Compartmentalization of primitive biochemical reactions within microscale water-filled environments is a promising route towards synthetic cellular systems, and an essential step in elucidating the origin of life [1,2]. Conventional studies on synthetic protocell models have focused on minimal systems that confine and support biological reactions in localized volumes of aqueous space, which are delineated by organic boundaries usually in the form of membrane-bounded lipid or fatty acid vesicles [3–5]. To date, such an approach appears to offer the most plausible scenario for the origin of life, as enzyme catalysis [6], enzymatic-mediated RNA synthesis [7] and replication [8], polymerase chain reaction (PCR)-induced DNA amplification [9], and gene expression of single components [10] or cascading networks [11,12] have been successfully demonstrated in lipid-based or surfactant-based micro-compartmentalized systems. However, such processes are often limited by the high impermeability of the lipid membrane, which prevents continuous activity within the vesicles due to mass transfer and osmotic pressure restrictions. Moreover, the absence of internal structuration within the vesicle micro-compartmentalizes restricts the ability of these synthetic models to mimic cell-like behaviours associated with molecular crowding or cytoskeletal assembly and disassembly. And in terms of general utility, the widespread instability of fatty acid vesicles to pH, ionic strength, multi-valent cations and temperature implies that these chemical micro-ensembles have limited technological application particularly in adverse environments. As a consequence, alternative types of synthetic cell-like entities are being developed based on the self-assembly of inorganic (colloidosomes), polymeric (polymerosomes) or protein (proteinosomes) membranes, or phase separation of liquid micro-droplets (membrane-free coacervates). Integrated micro-systems based on these new types of protocells should benefit from greater chemical stability and mechanical robustness, enhanced biocompatibility, and increased scope for designing membrane functionality. Moreover, a membrane-free protocell model based on coacervate micro-droplets could contribute to alternative scenarios for the origin of cellular life on the early Earth. Here, we discuss these new approaches to synthetic cellularity, focusing particularly on recent work published within the last two or three years.

Protocell models based on inorganic nanoparticle self-assembly
Colloidosomes (also known as Pickering emulsions) are microscale capsules that are produced from the spontaneous self-assembly of inorganic or organic colloidal particles in water/oil biphasic systems. Assembly is driven by a decrease in total free energy associated with placement of the particles specifically at the liquid/liquid interface [13], and produces stabilized water or oil droplets that are dispersed in an oil or water continuous phase, respectively. Since the seminal paper by Weitz et al. in 2002 [14*], a wide range of colloidosomes have been prepared using inorganic particles, such as silica [15**,16], clays [17], CaCO3 [18], graphene oxide [19],...
TiO₂ [20], Fe₃O₄ [21], cerium oxide [22], gold particles [23], metal organic frameworks [24] and polyoxometallate-organic hybrid particles [25]. In most cases, the colloidosomes are several tens of micrometres in diameter, and consist of a monolayer of closely packed particles in the form of a continuous, enclosed membrane. Significantly, the interstices between the particles form an array of pores that exhibit high levels of selective permeability based on solute size-exclusion. As a consequence, colloidosomes offer great technological potential in macromolecular storage and for controlling matter transport between internal and external microscale reaction environments.

Recently, a series of experiments have been initiated in which semipermeable inorganic colloidosomes were utilized as micro-compartments for housing biological or biomimetic functions [15**]. In principle, this approach should provide new routes to the engineering of artificial cellular systems and alternative membrane-bounded protocells based on inorganic nanoparticle self-assembly, which could have applications in synthetic biology, bionanotechnology and origins of life research. Water-filled colloidosomes, tens of micrometres in diameter, were prepared in oil by using partially hydrophobic silica nanoparticles, only 20–30 nm in size (Figure 1a). Many types of biomolecules could be entrapped without denaturation within the inorganic compartments; for example a large ensemble of enzymes, amino acids, nucleic acids, ribosomes, plasmids, among others, were successfully encapsulated and used as a cell-free gene expression system for spatially confined in vitro protein synthesis [15**] (Figure 1b). Three examples of sustained enzyme catalysis using entrapped proteins and oil-soluble membrane-permeable substrates were also demonstrated.

The permeability of the colloidosome shell has been tailored by engineering the nature of the membrane components. For example, control over the pore size and release rate of encapsulated dye molecules can be achieved by using organic colloids in the form of temperature-dependent polymer microgel particles that exhibit swelling/deswelling behaviour upon changing temperature [26]. Alternatively, sintering the temperature responsive polymer particles [27], coupling a pH-responsive crosslinker to the membrane components [28], or varying the size and shape of the nanoparticles [29] can be used to tune the permeability of the colloidosomes. However, these methods are only successful in suppressing the transport of molecules larger than a crucial value; in contrast, the diffusion of small molecules through the colloidosome monolayer is difficult to regulate over a broad range of membrane particle diameters [30]. This is a major challenge for the use of colloidosomes as water-dispersible, semipermeable inorganic protocells, as the rapid discharge of small molecule substrates from the micro-compartment would severely restrict their potential as primitive artificial cells.

As a step towards overcoming this problem, water-filled protocell constructs with self-activated, electrostatically mediated permeability have been recently prepared and investigated in continuous aqueous media [31**]. Before transfer from dodecane into water, silica nanoparticle-based colloidosomes were crosslinked with tetramethoxysilane (TMOS) and then functionalized by grafting a pH-responsive copolymer onto the inorganic membrane (Figure 1c). Once in water, these modifications produced inorganic protocells with an outer polymer-based coronal layer that was capable of electrostatically mediating the diffusion of small molecules through the interstitial nanospaces of the silica nanoparticle shell. Changes in the zeta potential of the polymer (+20 and −30 mV at pH 2 and pH 10, respectively; pH₅₀, 5.76) were used to prepare colloidosomes with net positive or negative surface charges at low or high pH, respectively, and provide the basis of an electrostatically gated inorganic protocell. For example, at low pH, small molecule cationic solutes (acriflavine hydrochloride, rhodamine 6G) were rejected from entry or egress by electrostatic repulsions arising from positive charges on the colloidosome membrane, whereas at high pH small molecule anionic solutes (calcine) were unable to diffuse across the membrane. Significantly, this gating mechanism was exploited for the self-regulation of an enzyme reaction within the inorganic protocells [31**,32]. The small molecule anionic substrate 4-nitrophenyl phosphate (4NPP) was added to the continuous aqueous phase of a dispersion of polymer-grafted, crosslinked colloidosomes containing encapsulated alkaline phosphatase, and the reaction switched on or off by pH-mediated electrostatically induced gating of 4NPP into the micro-compartment interior [31**]. A related strategy has been reported using a thermally gated process to control the diffusion of small molecules across a semipermeable colloidosome membrane [33*]. For this, a thermo-sensitive triblock copolymer was encapsulated into the interior of organic nanoparticle-based colloidosomes and used to inhibit the diffusion of dye molecules through the membrane pores by temperature-dependent adsorption or desorption of the polymer to the inner surface of the semi-permeable shell.

In addition to tuning membrane permeability, other recent studies have explored the possibility of preparing colloidosome-based protocells with molecularly crowded interiors that mimic the structural reversibility and chemical milieu of the cytoskeletal matrix. This was achieved by encapsulation of alkaline phosphatase and a derivatized amino acid, fluorenlymethylcarbonyl-tyrosine-(O)-phosphate (FMOG-TyrP) into silica nanoparticle-based colloidosomes, followed by self-assembly of a supramolecular hydrogel within the micro-compartments
Figure 1

Protocell models based on inorganic nanoparticle self-assembly. (a) Optical micrograph of silica nanoparticle-stabilized water micro-droplets (colloidosomes) dispersed in oil; scale bar = 200 µm. (b) Fluorescence microscopy image of a single colloidosome undergoing cell-free gene expression. Plasmid pEXP5-NT/eGFP and components of a cell-free gene expression system were encapsulated within the inorganic protocells and left for 24 hours at 37 °C, and the increase in green fluorescence due to in vitro gene-directed synthesis of the protein eGFP was monitored with time [15**]; scale bar = 100 µm. (c) Construction of water-dispersible aqueous inorganic micro-compartment with self-activated gated membrane permeability. The scheme illustrates the design and construction of TMOS-crosslinked, polymer-grafted colloidosomes. The branched copolymer corona is linked to the semipermeable outer surface of a closely packed shell of silica nanoparticles. pH-induced changes in the net charge associated with cationic dimethylamino (blue) and anionic carboxylate (red) groups of the copolymer layer generate a gated response to the transfer of charged small molecules across the inorganic membrane. The structure of the branched copolymer is shown (right). (d) Fluorescence microscopy image recorded after 1 day from a single colloidosome containing encapsulated alkaline phosphatase and FMOC-TyrP. The intense blue fluorescence originates from binding of Hoechst 33258 dye to amino acid nanofilaments that are self-assembled into a hydrogel matrix inside the micro-compartment [34**]; scale bar = 20 µm. (e) Enzyme kinetic plots for alkaline phosphatase-mediated dephosphorylation of substrate 4NPP showing changes in initial rates with substrate concentration for reactions undertaken in colloidosomes containing a hydrogel core (30 °C, blue circles) or a disassembled FMOC-Tyr hydrogel interior (45 °C, red triangles). Corresponding values for the maximum velocity (Vmax) and Michaelis constant (Km) are shown [34**]. (f) Optical microscopy images showing time course of growth and division of inorganic protocells. A single liquid bud occurs at a discrete rupture point after approximately 10 minutes, and continues to develop into a 2G micro-compartment to produce a pair of conjoined inorganic protocells [35]; scale bar = 100 µm.
due to enzyme-mediated FMOG-TyrP dephosphorylation (Figure 1d). [34**]. Significantly, the kinetics of hydrogel growth were strongly influenced by confinement within the colloidosome micro-environment. Moreover, increasing the temperature above the gel–sol transition point was used to reversibly disassemble the amino acid hydrogel nanofilaments, such that protocols with modulated internal viscosities were produced. As a consequence, auxiliary enzyme reactions inside the protocells could be effectively switched on or off in the absence or presence of the cytoskeletal-like internalized matrix (Figure 1e).

Taken together, the above reports demonstrate the potential of nanoparticle-based inorganic colloidosomes as novel microscale bio-reactors, and suggest that studies on bio-inorganic protocells could provide an alternative paradigm of primitive cell formation that is not based on lipid compartmentalization [15**]. Although the design and construction of inorganic-based micro-compartmentals with biomimetic cell-like properties is clearly feasible, it remains difficult to envisage how these non-lipid protocells could undergo processes of growth and division analogous to the behaviour of organic vesicles. In this regard, a recent study described a remarkable process of primitive growth and division involving buffer-filled inorganic colloidosomes dispersed in oil [35]. Growth of the colloidosomes was induced by organosilane-mediated methanol formation inside the micro-compartmentals, and resulted in a localized rupture of the inorganic membrane followed by outgrowth and separation of a second-generation protocell that was stabilized by surface adsorption of silica nanoparticles present in the oil phase (Figure 1f). Addition of the organosilane simultaneously generated a slow increase in internal pressure by methanol production, as well as an increase in the membrane rigidity via silica crosslinking of the colloidosome membrane. Under optimum conditions, the balance between volume expansion and increased surface elasticity associated with methanol production and silica crosslinking of the membrane, respectively, gave rise to stable growth conditions and the inception of a single rupture point to produce conjoined protocells that readily separated. Multiple divisions were also achieved by changing the amount of organosilane added to the colloidosome dispersion [35].

**Proteinosome-based protocells**

Very recently, a new type of membrane-bounded protocell model based on the spontaneous interfacial assembly of amphiphilic protein–polymer nano-conjugates has been reported [36**]. The protein–polymer nano-conjugates, which consisted of three or so polymer molecules per protein, assembled at water droplet/oil interfaces into micrometer-scale water-filled capsules termed proteinosomes. The proteinosomes were delineated by a closely packed monolayer of conjugated protein–polymer building blocks that could be crosslinked such that the micro-compartmentals were transferred into water without fragmentation (Figure 2a). As a consequence, a wide range of guest molecules from small molecule fluorescence dyes to colloidal particles could be encapsulated at high efficiency inside the micro-compartmentals. Significantly, it was possible to entrap a hundred or so components of a cell-free gene expression system within the proteinosome internal volume (ca. 1 pL), and perform *in situ* protein synthesis (Figure 2b). Moreover, the protein–polymer membrane was sufficiently elastic and robust to withstand partial dehydration and rehydration, and remained structurally intact when held at a temperature of 70 °C for 90 min, suggesting that the proteinosomes could provide a useful protocell model system for studying the microscale confinement of thermophilic enzymes, or for developing artificial cells capable of undertaking temperature cycling procedures, such as PCR-mediated amplification of entrapped genetic polymers.

A key advantage of the proteinosome system lies with the ability to rationally design the structure and function of the proteinosome membrane by controlling the chemistry of the protein–polymer nanoscale building blocks. This makes the system an attractive candidate for the development of a range of functional protocells. For example, recent studies indicated that the proteinosome membrane was semipermeable, stimulus-responsive, enzymatically active, and mechanically elastic [36**]. It also exhibited size-selective permeability, only allowing molecules less than 40 kDa in molecular mass to enter or leave the proteinosome interior. Moreover, conjugation of the temperature sensitive polymer, poly(N-isopropylacrylamide) (PNIPAAm) to the protein molecules generated a thermally gated system that operated via the swelling or deswelling (at $T > 33 ^{\circ}C$) of the polymer chains present on the outer surface of the proteinosome membrane. As a consequence, enzyme-mediated peroxidation within the proteinosomes of a small molecule initially present in the external solution was switched on or off by thermally gating the diffusion of the substrate through the polymer shell barrier layer [36**] (Figure 2c,d).

As a development of these ideas, a recent study has exploited the fact that the proteinosome system provides a means of transforming water-soluble proteins and enzymes into membrane-bounded ensembles. Thus, provided that insertion and crosslinking into the membrane does not seriously perturb biomolecule function, a wide range of multi-functional, biocompatible protocellular constructs can be envisaged. For example, glucose amylase (GA), glucose oxidase (GO) and horseradish peroxidase (HRP) have been used to prepare protein–PNIPAAm amphiphilic nano-conjugates to construct proteinosomes comprising an enzyme triad capable of undertaking a multi-step membrane-mediated cascade reaction using starch as the starting substrate [37]. The
Proteinosome-based protocells. (a) AFM image of a dried proteinosome after transfer to water showing intact, collapsed micro-compartment enclosed by a continuous and flexible protein-polymer crosslinked membrane; scale bar = 5 μm [38*]. (b) Gene-directed synthesis of eGFP in proteinosomes. The fluorescence microscopy image shows a population of proteinosomes with encapsulated plasmid pEXP5-NT/eGFP and cell-free gene expression components recorded after 2 hours of incubation at 37 °C. Green fluorescence associated with in vitro expression of eGFP is observed in the proteinosome micro-compartment; scale bar = 100 μm [36*]. (c) Scheme showing polymer chain gating of enzyme reactions inside proteinosomes. Access to an entrapped enzyme depends on the hydrophobicity and porosity of the protein–polymer membrane, which can be influenced by the swelling/deswelling behaviour of the PNIPAAm chains. As a consequence, access to the internal compartment is curtailed for certain small molecule substrates above 33 °C due to polymer chain collapse on the outer surface of the membrane. (d) Temperature-dependent catalytic reaction rates of free myoglobin (black lines) and proteinosome-encapsulated myoglobin (red lines) in the presence of 2-methoxyphenol (5 mM) and H₂O₂ (20 mM), showing membrane-gated enzyme catalysis in proteinosomes dispersed in bulk water [36**]. (e) Three-step cascade reaction in multi-enzyme proteinosomes. Summary chart of the catalytic activity of enzyme–polymer proteinosomes prepared with different membrane and internal spatial organizations. Values are initial reaction rates of product formation (Vₒ) at 25 °C. Grey circles represent the proteinosome membrane, and locations of GA, GO and HRP are indicated. Starch is added to the external aqueous phase to initiate the cascade. The Vₒ values for the cascade reaction were essentially unchanged when native GO or HRP was encapsulated in the interior of the proteinosomes. By contrast, Vₒ was reduced by 90% when native GA was encapsulated within the aqueous interior and the GO-PNIPAAm and HRP-PNIPAAm conjugates crosslinked into the membrane [37]. (f) Mechanical properties of hydrogel-containing proteinosomes. Optical microscopy images showing time-dependent self-healing behaviour of a single hydrogel proteinosome subjected to deformation using a soft plastic fiber [38]; scale bar = 10 μm.

tandem reaction was regulated by the positional assembly and spatial separation of the three enzymes within the membrane of the protocell (Figure 2e), as well as by temperature-dependent changes in the conformation of the covalently attached polymer chains located on the external surface.

In recent work, proteinosome-based protocells with new types of higher-order functionality based on controlled membrane disassembly and permeability, internalized assembly of a cytoskeletal-like matrix, and self-production of a membrane-enclosing outer wall have been described [38]. Genetic polymers were encapsulated in the proteinosomes and their release triggered by protease-mediated hydrolysis or chemical cleavage of the membrane building blocks. In addition, proteinosome-encapsulated, enzyme-mediated amino acid dephosphorylation was used to generate a supramolecular amino acid hydrogel within the interior of the protein–polymer micro-compartment. As a consequence, the mechanical properties of the protocells were considerably enhanced. For example, the spherical morphology of the hydrogel-containing proteinosomes was quickly re-established after protocells were mechanically deformed or deflated
due to dehydration (Figure 2f). Interestingly, prolonged growth of the encapsulated hydrogel resulted in penetration of the amino acid nanofilaments through the porous shell of the proteinosome to produce a continuous, protease-resistant outer wall that totally enclosed the protein–polymer membrane [38], analogous in some respects to the cell wall/cell membrane layered structure of bacterial cells.

**Polysome-based protocells**

The above sections highlight the fact that synthetic polymers play important structural and functional roles in the design of artificial cell-like micro-systems based on hybrid building blocks. In particular, covalent grafting of polymer chains to inorganic nanoparticles (colloidosomes) or globular proteins (proteinosomes) is an effective means to generate microscale water-filled compartments enclosed by semipermeable monolayer membranes of functional nanoscale components. These membranes are therefore very different from the molecule-based bilayer or multi-lamellar structures that are characteristic of fatty acid and phospholipid vesicles. By contrast, the self-assembly of amphiphilic block copolymers into vesicle-like micro-architectures (polyosomes [39]) provides a more closely related synthetic analogy with liposome-based protocell models [40,41]. Polyosomes are generally more stable than liposomes, can be prepared with a wide range of membrane chemistries, and engineered towards semipermeability by introducing porosity into the block copolymer bilayers [42–46]. For example, channel-forming proteins can be reconstituted without loss of function within the polymer membrane to produce polyosomes with size-selective or substrate-specific pores [43]. Alternatively, stimuli-responsive block copolymers can be used as pore-generating components to control membrane-permeability, induce protocell disassembly, or regulate the activity of encapsulated enzymes in response to changes in the external environment [44]. In an attempt to mimic the morphological diversity of living cells, approaches have been developed to induce controllable shape transformations in polyosomes. In particular, kinetic manipulation of the phase behaviour of the glassy hydrophobic segment of an amphiphilic polymer [47**,48], and crosslinked induced changes in the composition and structure of the polyosomes membrane [49], have been successfully developed. For example, well-defined bowl-shaped polyosomes (polymer stomatocytes) were constructed using a poly(ethylene glycol)-polystyrene block copolymer [47**]. The hydrophobic polystyrene domain displays a phase transition from a solvent-swollen rubbery state to a rigid glassy state, and kinetic manipulation of this phase behaviour was exploited to capture transient morphologies in the shape of the polyosomes. Moreover, by selectively entrapping catalytically active platinum nanoparticles within the nanocavities the polymer stomatocytes were capable of moving autonomously in the presence of aqueous hydrogen peroxide solutions [47**].

It is well known that multi-compartmentalization is a key characteristic of eukaryotic cells, and it is therefore an interesting challenge to design models of primitive compartmentalization with integrated synthetic organelles. Although this strategy has been developed extensively in liposomes-in-liposome systems, it has only recently been advanced in non-lipid models involving polymesomes [50–53]. Polymesomes-in-polysome microarchitectures have been prepared by an emulsion/centrifugation method, and used to develop a three-enzyme cascade reaction across multiple compartments using either compatible or incompatible enzymes [54**]. In other studies, a multi-compartment system (capsosome) was prepared by incorporating liposomes inside the shell of a polymeric capsule using a layer-by-layer technique [55,56]. Co-encapsulation of glutathione reductase and disulfide-linked polymer-oligopeptide conjugates was then used to trigger the enzyme-mediated reduction of glutathione specifically within the multicompartmentalized protocells.

**Membrane-free protocell models**

As discussed above and elsewhere in this edition, the ubiquity of the cell membrane in living organisms has stimulated the development of a range of models of protocellular micro-compartmentalization based on the self-assembly of amphiphilic building blocks such as phospholipids, fatty acids, functionalized inorganic nanoparticles, block copolymers and protein–polymer nanoconstructs. Although it seems obvious that the modelling of primitive cells would be based primarily on membrane assembly, it is less evident that these systems have much relevance to questions concerning the origin of life. Indeed, except for fatty acid self-assembly, most of the other models are implausible in this regard, although they have considerable technological merit as platforms for synthetic cellularity. Unfortunately, origin of life theories of fatty acid vesicle self-assembly are also deficient in several respects, notably in terms of low encapsulation efficiencies, sensitivity to changes in pH and ionic strength, and the absence of internal structuration. In principle, these limitations can be overcome by invoking an intriguing alternative model of prebiotic organization based on the spontaneous formation of membrane-free, chemically enriched liquid droplets via aqueous phase separation or complex coacervation.

Aqueous phase separation in multicomponent solutions containing two types of polymers or macromolecules is driven by a change in entropy [57], which results in two distinct thermodynamic phases comprising different polymer compositions [58]. Polymer phase separation has been demonstrated in aqueous systems comprising neutral polyethylene glycol/dextran solutions [59]. Formation of
the two liquid phases, which was dependent on the temperature, concentration and ionic strength, gave rise to separated liquids with different polymer compositions that could be tuned by changing the molecular weight or stoichiometric ratio of the components [60**]. Significantly, liquid–liquid demixing can give rise to membrane-free fluid-like organelles in living cells that are molecularly distinct from the surrounding cytoplasm [61**]. Interest in these systems continues to grow [62,63]. Complex coacervation also involves aqueous two-phase separation but is typically driven by electrostatic and entropic interactions between charged polymers or macromolecules, and results in the formation of component-enriched microdroplets suspended in a chemically deficient aqueous continuous phase [64,65**]. The spontaneous formation of micro-droplets in these equilibrium systems provides a facile mechanism for membrane-free compartmentalization, and as noted by Oparin in the 1930s, could be highly relevant to the evolution of the cell on early Earth [66].

Hundreds of complex coacervate systems have been characterised and prepared from a range of components such as biological macromolecules, multivalent proteins and synthetic and natural polymers [67]. However, there are few reports of coacervate droplets produced from low molecular weight components. In this regard, a recent study described the formation of coacervate micro-droplets prepared by electrostatic complexation of cationic oligolysine and anionic nucleotides such as ATP [68**]. The droplets were tens or hundreds of micrometres in size, highly enriched in peptides and nucleotides, stable across a large pH and temperature range, and capable of sequestering a wide range of low and high molecular weight solutes including enzymes, substrates, nanoparticles and photoactive molecules (Figure 3a,b). The ease of preparation, molecular simplicity of the components, fundamental biological relevance of peptides and nucleotides, as well as the ability of the droplets to undergo growth and disassembly, suggested that a model of prebiotic organization based on coacervation should be seriously considered. Moreover, partitioning of molecules into the coacervate phase was based on differences in dielectric constant between the external aqueous solution and the molecularly crowded interior of the droplets [68**]. As a consequence, compared with bulk aqueous solution, the sequestration of biomolecular reactants into polylsine/ATP coacervate micro-droplets resulted in a significantly increased reaction rate for a glucose phosphorylation/dehydrogenation two-cascade enzyme reaction (Figure 3c). Similarly, elevated concentrations of a minimal multi-enzyme complex actinorhodin polyketide synthase were obtained by partitioning within the droplets, and used to increase the synthesis yield of two complex natural products [69].

Considerable enhancements in reaction rates have also been demonstrated in the molecularly crowded environments of aqueous two-phase systems. For example, compared to a polymer free solution, RNA catalysis by the hammerhead ribozyme was increased approximately 70-fold when partitioned preferentially into the dextran-rich phase of a polyethylene glycol/dextran phase-separated system [60**]. Similarly, urease activity was significantly increased by preferential sequestration into the dextran phase, and could be further regulated by tuning the relative volumes of the polyethylene glycol/dextran phases [70]. In other studies, enhanced levels of gene expression were reported in a cell lysate that was subjected to phase separation in polyethylene glycol-rich droplets by in situ dehydration of the aqueous phase within a microfluidic device [71**].

**Hybrid protocell models**

Although coacervate micro-droplets can be stabilized by excess surface charge [72], under many conditions they exhibit a propensity to coalesce into larger droplets or undergo macroscopic phase separation. As a consequence, their use as a protocell model can be compromised by their liquid-like behaviour and low surface tension. To circumvent this problem, and to generate a new type of hybrid protocell, Mann and colleagues have recently developed several methods to produce membrane-bound coacervate micro-droplets via spontaneous assembly or partitioning of auxiliary components on the surface of the liquid micro-compartments [73,74**,75]. For example, partitioning of high concentrations of the organic molecule, 8-anilinonaphthalene sulphonate (ANS) into micro-droplets prepared from mixtures of oligolysine and ATP resulted in the formation of a distinct ANS-rich membrane on the surface of the droplet [73]. In other work, a continuous inorganic membrane was assembled onto the surface of polyelectrolyte/ATP coacervate micro-droplets via electrostatic interactions between the polycationic polymer and inorganic polyanions (phosphotungstate) that were added to the continuous phase of the dispersion (Figure 3d) [75]. Interestingly, formation of the inorganic membrane was associated with sub-division of the droplet interior to produce a novel three-tiered vesicle architecture comprising a semipermeable negatively charged phosphotungstate/polyelectrolyte membrane, a submembrane coacervate shell enriched in polyelectrolyte and ATP, and an internal water-filled lumen (Figure 3e). As a consequence, proteins encapsulated in the coacervate vesicles were inaccessible to proteases, and could be exploited for the spatial localization and coupling of two-enzyme cascade reactions.

Very recently, a fatty acid multi-lamellar membrane has been spontaneously assembled on the surface of coacervate micro-droplets containing RNA and oligopeptides [74**]. Membrane assembly occurred under conditions not conducive to vesicle formation in free solution to produce well-defined fatty acid-coated droplets
Membrane-free and hybrid protocell models. (a) Optical bright field image of coacervate micro-droplets prepared in water by mixing cationic poly(diallyldimethyl ammonium chloride) (PDDA) with anionic ATP. The micro-droplets also contain a sequestered low molecular weight dipeptide, N-fluorenylmethoxy carbonyl dialanine; scale bar = 50 μm. (b) Confocal fluorescence microscopy image showing localized blue fluorescence associated with sequestration of 8-anilinonaphthalene sulphonate (ANS) into oligolysine/ATP coacervate micro-droplets [73]; scale bar = 1 μm. (c) Catalytic reactions in peptide/nucleotide coacervate droplets. Time profile of increase in β-NADPH concentration associated with a two-enzyme cascade reaction in polylysine/ATP coacervates containing the ATP-dependent enzyme hexokinase and glucose-6-phosphate dehydrogenase (filled dark red circles). The reaction rate is approximately doubled compared with the corresponding reaction in bulk aqueous solution (open blue circles) [88**]. (d) Optical microscopy image showing spherical inorganic membrane-bounded coacervate vesicles prepared by addition of aqueous sodium phosphotungstate (PTA) to a vigorously stirred dispersion of PDDA/ATP coacervate droplets [75]; scale bar = 50 μm. Inset shows single coacervate vesicle viewed between cross-polarizers showing birefringence due to ordering in the PTA/PDDA membrane. (e) Optical microscopy image showing initial stage of development of a three-tiered micro-architecture in PTA/PDDA/ATP coacervate vesicles prepared as in (d); locations of the PDDA membrane (M), PDDA/ATP coacervate sub-membrane layer (C), and water-filled lumen (W) are shown [75]. Image was taken 30 s after addition of PTA to a coacervate dispersion; scale bar = 20 μm. (f) Graphic showing hybrid protocell model based on the self-assembly of a fatty acid multi-layered membrane on the surface of coacervate micro-droplets containing ATP, RNA and cationic oligopeptides or polyelectrolytes. (g) Fluorescence microscopy image of an oleate-coated PDDA/ATP coacervate droplet stained with lipid-soluble BODIPY FL C16 dye, and showing green fluorescent ring associated with membrane formation [74**]; scale bar = 1 μm. (h) Small angle X-ray scattering profiles of oleate/PDDA/ATP micro-droplets prepared at different oleate/PDDA/ATP molar ratios (PDDA:ATP molar ratio = 1:0.25 = P in all cases). Peaks at 5.46 (peak (i)) and 4.77 nm (peak (ii)) are observed; the latter is highly dependent on increasing oleate concentration (increasing x: P) and was associated with assembly of a multi-lamellar fatty acid membrane on the surface of the coacervate droplets [74**].

(Figure 3f,g). The membrane-coated droplets were stabilized with regard to coalescence and exhibited selective uptake or exclusion of small and large molecules. Moreover, changes in the ionic strength were employed to disassemble the coacervate interior without loss of membrane integrity. Together, the results indicated that simple physical and chemical processes could give rise to hybrid protocols based on membrane-compartmentalization, chemical enrichment and internalized structuration (molecular crowding), and that key aspects of vesicle-based and coacervate-based models of prebiotic compartmentalization could be successfully integrated.

Finally, we note that aqueous two-phase polymer systems have been encapsulated in phospholipid vesicles to generate internalized sub-compartmentalization features [76]. As a consequence, lipid-based protocells exhibiting protein localization and phase transfer [77] were produced, and...
vesicle budding and division into compositionally different daughter vesicles demonstrated [78,79].

Conclusions
This review has highlighted recent advances in the design and construction of protocell models based on the self-assembly or microphase separation of non-lipid building blocks. We have focused particularly on strategies involving partially hydrophobic inorganic nanoparticles (colloidosomes), protein–polymer globular nano-conjugates (proteinosomes), amphiphilic block copolymers (polymersomes), and mixtures of oppositely charged biomolecules and polyelectrolytes (coacervates). Significant advances have been made in the design and construction of these micro-compartmentalized models, notably in the engineering of membrane functionality for gated responses and control over multi-step reactions, as well as in the structuring of the interior space for molecular crowding and cytoskeletal-like reversible hydrogel assembly. As a result, a wide range of biomolecular and synthetic components can be encapsulated, regulated and processed within the micro-compartmentalized space, suggesting that these non-lipid micro-ensembles offer attractive alternatives to phospholipid or fatty acid protocell models. Taken together, the studies reviewed herein exemplify a modern approach to synthetic cellularity that advances the physical and chemical basis of cell structure and function, proposes steps towards understanding the emergence of primitive cells on the early Earth, and facilitates the development of smart autonomously functioning chemical micro-compartmentalized models. These attributes should provide novel opportunities in bioinspired microstorage and delivery, micro-reactor technologies, cytomimetic engineering, and the development of integrated constructs for diverse procedures in synthetic biology.

References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


An interesting example of using a thermally gating strategy to control the diffusion of small molecules across a semipermeable colloidosome membrane.


A new protocell model based on the self-assembly of protein-polymer conjugates at water droplet/oil interfaces to produce membrane-bounded micro-compartment systems (proteinosomes) that exhibit biomimetic properties such as guest molecule encapsulation, selective permeability, gene-directed protein synthesis and membrane-gated internalized enzyme catalysis.


47. Wilson DA, Nolte RJ, van Hest JC: Autonomous movement of platinum-loaded stomatocytes. Nat Chem 2012, 4:268-274. A bowl-shaped polymersome was constructed by the controlled deformation of polymer vesicles. Loading platinum nanoparticles into the cavity turned this stomatocyte morphology into a nanorobotic and made the model move autonomously in a hydrogen peroxide solution.


56. Strulson CA, Moldent RC, Keating CD, Bevilacqua PC: RNA catalysis through compartmentalization. Nat Chem 2012, 4:941-946. RNA was partitioned into a dextran/polyethylene glycol aqueous two phase system. Molecular crowding of the RNA in the dextran-rich phase resulted in a 70-fold increase in ribozyme cleavage.


Theory of coacervation described by a two-step thermodynamic mechanism based on Debye Hückel equations for electrical interaction, and Flory-Huggins theory for entropy and experimental ITC and rheological data.


Low molecular weight mononucleotides and simple cationic peptides were shown to form micro-droplets in water. The stability of the membrane-free protocells with respect to temperature, pH and ionic strength was investigated, and sequestration of enzymes and nanoparticles into the molecularly crowded interior demonstrated.


A cell lysate was used as a cell free expression kit and dehydrated to form an artificial cell-like compartment via coacervation. The transcription rate was increased by five to six times.


First example of a hybrid protocell model that combines fatty acid membrane assembly (vesicle formation) and micro-droplet phase separation (coacervation).


