EurJOC
European Journal of Organic Chemistry

Review
doi.org/10.1002/ejoc.202200968

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Colloidosomes as a Protocell Model: Engineering Life-Like Behaviour through Organic Chemistry

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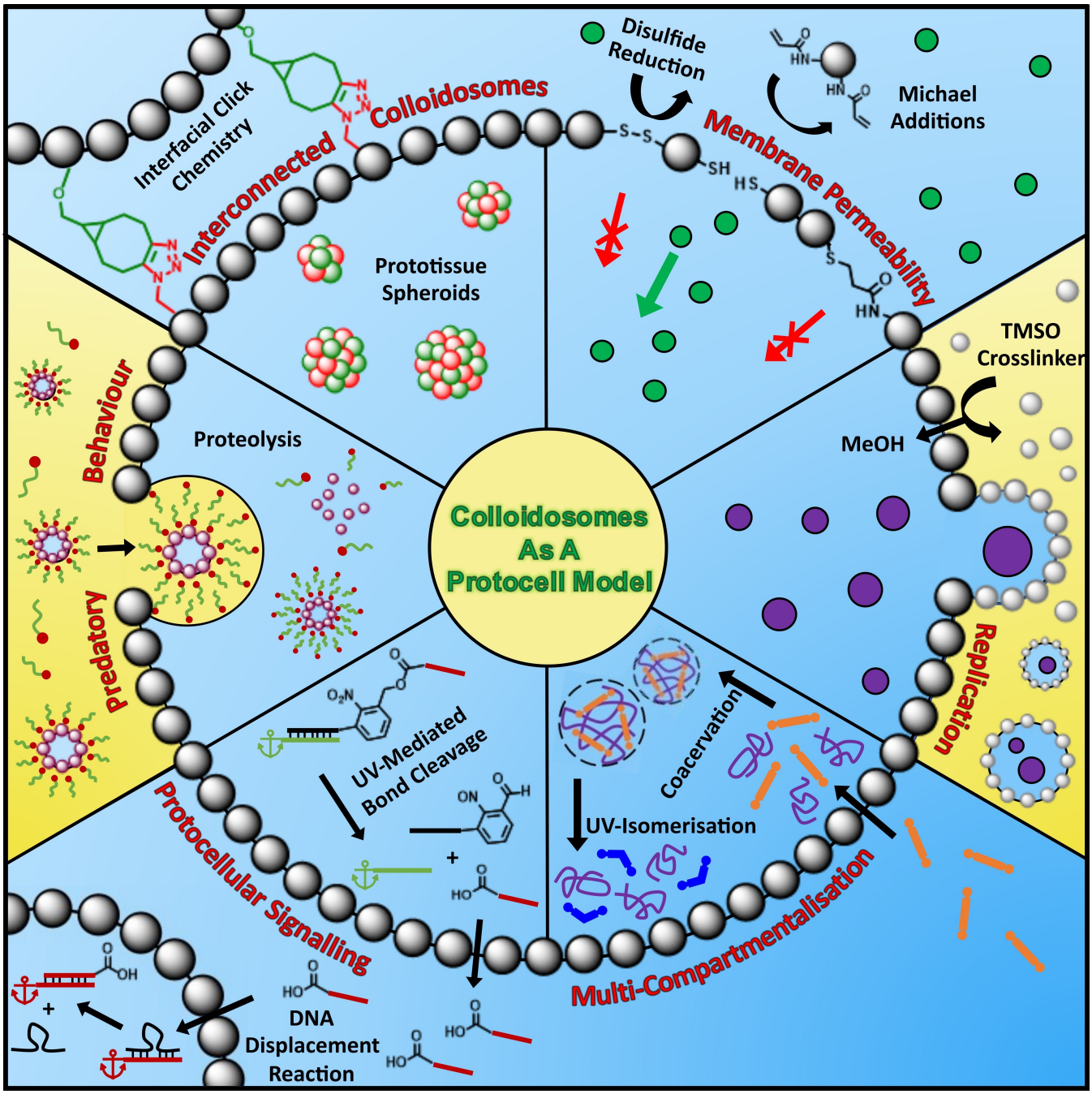

The bottom-up synthesis of self-assembled micro-compartmentalised systems that mimic basic characteristics of living cells is rapidly evolving. These types of systems are termed "protocells" and can be chemically programmed to grow and divide, to send and receive chemical signals, to transcript and translate chemical information, to adhere to surfaces or to other protocells, and to perform rudimental enzyme-mediated metabolic processes. An emerging protocell model that is attracting great attention is the colloidosome. Colloidosomes are microcapsules with a chemically crosslinked, semipermeable membrane composed of amphiphilic nanoparticles. Colloidosomes display important advantages over other protocell models (e.g., vesicles and coacervate micro-droplets) due to their physical-chemical properties that can be easily tuned through the careful engineering of their synthetic building blocks. In this review, we deliver an overview of the different types of colloidosomes that have been developed thus far and discuss how organic chemistry contributes to the design and bottom-up synthesis of novel types of colloidosomes endowed with advanced chemically programmed bio-inspired functions.

1. Introduction

Bottom-up synthetic biology is an emerging research field that aims to bridge the gap between physical and biological sciences. This field seeks to construct synthetic micro-encapsulated systems from scratch, that are capable of mimicking basic functions of biological cells.[1] These synthetic micro-encapsulated systems are called protocells.

The generation of protocells has attracted a great amount of attention and has been the subject of extensive investigation for several years. This has resulted in a large number of unique protocell models, but also a wide range of definitions of what exactly constitutes a protocell. Some propose that a protocell must be a full biological cell replica, capable of information processing, growth and evolution, whereas others suggest that a protocell must mimic at least one biological function.[2] It is therefore sensible to categorise different protocell models, separating them on the basis of their complexity. Xu *et al.* separated protocells into 'typical' and 'non-typical' models. Typical protocells are full biological replicas with regards to structure and function, whereas non-typical models are a range of synthetic structures which mimic at least one biological function.[3]

Protocells have the potential for applications in a wide range of fields including biotechnology, medicine, industry, environmental science, as well as in the study of the origin of life. For example, in biotechnology, protocells are used to construct (bio)reactors to replace engineered organisms in fields such as fuel, pharmaceuticals, tissue engineering, and wastewater treatment.[4] Protocells also have great potential in the medical industry, particularly as stimuli-responsive drug delivery vesicles.[5] While functional microcapsules have previously been applied in this field, protocells provide further advancements as autonomous drug delivery systems that utilise biomimetic processes and more biocompatible vessels. Additionally, the construction of protocells with sufficient similarity to biological cells would provide a non-invasive route to explore and further understand cellular life.[3] Although typical protocells are closely related to their biological templates and are useful for studying the formation and evolution of primitive cells in a controlled environment, it would be advantageous not to be limited to a specific range of materials or structures. This would be of great utility for technological applications that require high stability under a broader range of physical-chemical conditions compared to biological systems, including pH, ionic strength, temperature, and non-aqueous environments. In fact, non-typical protocells constructed from synthetic materials are generally robust and through organic synthesis can be chemically engineered to achieve much more than their living counterparts. Non-typical protocells will be the focus of this review due to their many potential applications.[6]

So far, the various non-typical protocells that have been synthesised using bottom-up methods can be divided into three main categories (Figure 1):

1. Vesicles
2. Membrane-free compartments
3. Colloidosomes

1.1. Vesicle Models

These include liposomes, polymersomes, and hybrid lipo-polymersomes. These models have bilayer membranes formed from lipids, polymers, and a combination of lipids and polymers respectively.[7] Various methods are used to form vesicles, including solvent free or solvent displacement methods. Solvent free methods principally consist of film rehydration processes and solvent displacement methods include solvent injection and emulsion phase transfer using microfluidic devices.[8] These protocell models have structures that most closely resemble their biological archetypes because their membranes consist of

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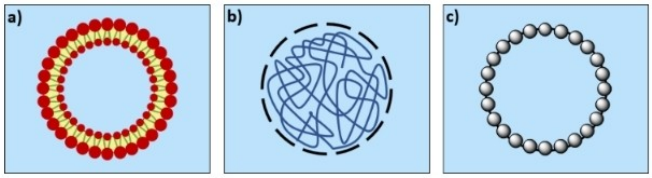
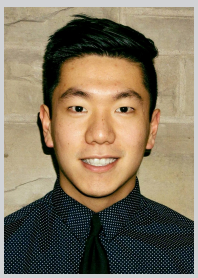


Figure 1. Illustration of the three main categories of protocell models currently available. a) Vesicle models include liposomes, polymersomes and lipo-polymersomes, which have membranes consisting of a bilayer structure similar to biological cells. b) Membrane-free models include microgels and coacervates; the former consisting of a micrometre-sized network of cross-linked polymers dispersed in an aqueous phase, and the latter consisting of aqueous microdroplets containing high concentrations of at least two polyions of opposite charge that undergo liquid-liquid phase separations. c) Colloidosomes are semipermeable microcapsules characterised by a membrane formed from a range of self-assembled colloidal particles.

dynamic bilayer structures. Therefore, they have been one of the most attractive solutions to build protocell models, particularly due to their high biocompatibility. These structures have been extensively studied as protocell models and a number of review articles highlighting vesicle protocells have been published in recent years.[3,6a,9]

1.2. Membrane-Free Models

These include coacervates and microgels; coacervates are macromolecule-rich liquid droplets that are formed through liquid-liquid phase separation[10] and microgels consist of polymer networks that are swollen with water and have sizes in the range of tens of micrometres.[11] Membrane-free protocell models have been employed less frequently compared to membrane-bound models.[12] Although using membrane-free protocell models may seem counterintuitive because cell membranes are a common feature of all biological cells, there are significant advantages in using these structures as protocells. For example, their ability to sequester biomolecules produces chemically enriched microdroplets which consequently display increased (bio)chemical reaction rates. More specifically, microgels are highly tuneable in several properties such as mechanical strength, elasticity, and biocompatibility, whereas coacervates represent valid pre-biotic protocell models which allows for the testing of important hypotheses on the origin of life.[13]



Jun Hyeong Park received his MSc in 2020 at the University of Western Ontario (Canada) under the supervision of Prof. M. S. Workentin. In January 2021, he joined the research groups of Dr. P. Gobbo and Dr. S. Rochat at the University of Bristol (UK) for a dual doctorate program joint with the Workentin group at the University of Western Ontario. The aim of his PhD project is to create gold nanoparticle-based protocells and inorganic tissue-like materials.

Agostino Galanti obtained his PhD at the Institut de Science et d'Ingénierie Supramoléculaires of the University of Strasbourg (France) in 2018, under the supervision of Prof. P. Samorì. He joined the group of Dr. P. Gobbo in 2020 at the University of Bristol (UK), where he started working on the synthesis of bioinspired materials using protocells as building blocks. The group then moved to the University of Trieste (Italy), where he is currently working.

India Ayling graduated from the University of Bristol in 2021, where she received a first class honours in a Master's of Chemistry. In her final year, her research focussed on the synthesis and application of colloidosomes as protocell models – in particular how polymerisation induced self-assembly could be used to synthesise this protocell model.

Sebastien Rochat obtained a PhD in supramolecular chemistry from the Swiss Institute of Technology in Lausanne, Switzerland (EPFL) in 2010. After postdoctoral stays at the Massachusetts Institute of Technology (USA) and at the University of Bath (UK), and a short role in industrial R&D, he started his independent research career at the University of Bristol in 2019. His research interests cover a broad range of functional organic materials, from bioinspired synthetic microcapsules to advanced materials for energy harvesting, conversion and storage.

Mark S. Workentin received his PhD in 1992 at McMaster University, was a Research Associate at the National Research Council of Canada, and has been a Professor of Chemistry at The University of Western Ontario (Canada) since 1995. His group's research has focused recently on the physical organic chemistry of materials, by studying interfacial reactions on surfaces, noble metal nanoparticles and nanoclusters.

Pierangelo Gobbo received his PhD in 2016 at the University of Western Ontario (Canada). In 2016 he joined the research group of Prof. Stephen Mann FRS at the University of Bristol (UK) as an NSERC of Canada Postdoctoral Fellow first, and then as an EU Marie Curie Postdoctoral Fellow. In 2019 he established his independent research group at the University of Bristol and started to develop protocellular materials. In 2021 he moved his research group to the Univeristy of Trieste (Italy), where he is currently working.

1.3. Colloidosome Models

The colloidosome is an emerging non-typical protocell model that is becoming increasingly popular, primarily due to its unusual properties compared to other membrane-bound protocell designs. Vesicle models are formed by self-assembled bilayer membranes which typically have low permeability and are stable only within a narrow range of external conditions (*e.g.*, pH, ionic strength, temperature).[8,9b,14] On the other hand, colloidosomes are characterised by a robust membrane with intrinsic porosity due to the imperfect packing of the amphiphilic colloidal particles that constitute the protocell membrane (*vide infra*). Moreover, the colloidal particles are generally covalently crosslinked, therefore yielding a membrane that is robust and stable in a wider range of physical-chemical conditions compared to vesicles. Taking advantage of this design, several examples of colloidosomes capable of mimicking a myriad of cellular processes have been synthesised.[15]

Several review articles that cover the synthesis methods and the applications of colloidosomes as (bio)reactors are available in the literature,[16] however this review is aimed at providing an overview on the use of colloidosomes as a protocell model. More specifically, our objective is to highlight the role of organic chemistry in the design and construction of unprecedented "life-like" micro-compartmentalised systems based on the colloidosome design.

2. Colloidosomes

The term colloidosome was first coined by Dinsmore *et al.* and describes a microcapsule with a shell formed from colloidal particles.[17] Colloidosomes can be formed using a range of techniques including thermal annealing,[18] polyelectrolyte complexation,[17] gel trapping,[19] and Pickering emulsion (PE) polymerisation[20] or PE crosslinking.[21] Since no heating step or oxygen removal is required, the latter method is preferred as it is compatible with applications involving temperature-sensitive biomolecules, such as enzymes, lipids, and nucleic acids. For these reasons, we will focus on colloidosomes formed using PEs, followed by chemical crosslinking techniques.[16a,22]

The PE technique involves the formation of an emulsion that is stabilised by the adsorption of amphiphilic solid species at the interface between two immiscible liquids (Figure 2). The solids act as stabilisers and are typically nano/microparticles, but other structures such as nanosheets,[23] worms,[24] or vesicles[25] have also been used. In general, two types of PEs can be formed: water-in-oil (*w/o*) or oil-in-water (*o/w*) emulsions, where different nonpolar solvents can be used, such as dodecane, *n*-octanol, 2-ethyl-1-hexanol, toluene, or hexane. The type of emulsion formed will be dependent on the hydrophobicity of the stabiliser and the contact angle (θ) it forms at the interface between the two immiscible fluids. In a PE the contact angle is measured between the stabiliser surface and the aqueous phase.[23] Hydrophilic species (such as unmodified silica, metal oxides and clays) are dispersed primarily in the water phase, with $\theta < 90°$, whereas hydrophobic species (such as modified hydrophobic silica) are dispersed in the oil phase (with $\theta > 90°$). Upon formation of a PE, a monolayer of the stabilising species is generated at the interface between the two immiscible liquids and will curve to maximise the contact with the preferred phase. For example, when hydrophobic stabilisers are used (*i.e.*, $\theta > 90°$), contact with the oil phase will be maximised so that *w/o* emulsions will be formed.[23,26]

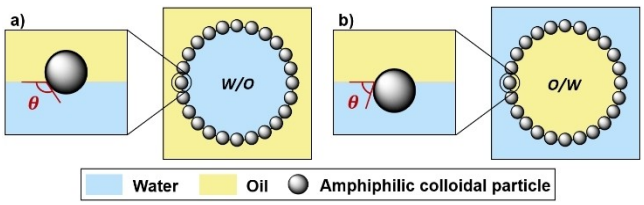


Figure 2. Diagram showing formation of water-in-oil (*w/o*) or oil-in-water (*o/w*) Pickering emulsions, depending on the hydrophilicity of the colloidal particle used as the stabiliser. a) To form a *w/o* emulsion, hydrophobic particles are used which have a high contact angle (*i.e.*, $\theta > 90°$) with the aqueous phase. b) For an *o/w* emulsion, hydrophilic particles are used which have a low contact angle (*i.e.*, $\theta < 90°$) with water and primarily sit in the aqueous phase.

From a general perspective, we can therefore say that the *w/o* or *o/w* microdroplets of a PE act as templates to form the colloidosomes. Once they are formed, it is then necessary to fasten the colloidal particles together to form robust membranes able to resist de-emulsification once the bulk external phase (oil or water) is removed. This can be achieved through two main methods: sintering or chemical cross-linking. Sintering joins the loose particles into a coherent mass through heating, whereas chemical crosslinking is based on the covalent binding of the particles to form a semipermeable membrane.[27] The latter is the most commonly-used method to fabricate colloidosomes, especially for applications involving biomolecules, because no heating step is required.[16a] Chemical cross-linking has been achieved *via* substitution reactions,[15a] amide couplings,[28] Schiff base chemistry,[29] disulfide formation,[30] ionic interactions,[31] Michael additions,[32] and using click chemistry.[21] Once crosslinked, the fortified colloidosomes can then be transferred to a continuous water phase for applications in aqueous environments. This is normally achieved using dialysis in a solvent system compatible with both the oil and water phases (typically ethanol or ethanol/water) and progressively swapping to only water. The resulting solution of colloidosomes can then be used for applications in aqueous biological systems.

The intrinsic permeability of the colloidosome's membrane is due to the interstices displayed by the colloidal particles when closely packed together as a monolayer, which is characterised by a molecular weight cut-off (MWCO).[16a] This means that high molecular weight molecules can be selectively retained inside or outside the colloidosomes, while molecules smaller than the pores' MWCO can freely diffuse in and out of the membrane. Cargo can therefore be encapsulated in the inner phase of the PE based on its partition coefficient between the two phases used to make the emulsion (Figure 3). For instance, a hydrophilic cargo such as an enzyme can be loaded

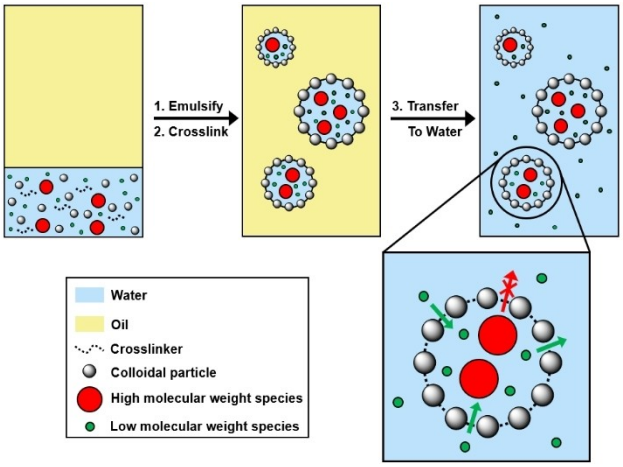


Figure 3. Scheme depicting a w/o PE of colloidal particles that encapsulate small and large molecular weight species in the aqueous phase of the emulsion. The resulting colloidosomes are crosslinked and then transferred into water. The permeability of the colloidosomes is determined by their MWCO. High molecular weight species (red circles) are retained in the colloidosomes and cannot diffuse out, while low molecular weight species (green circles) are able to passively diffuse in and out of the interstices of the membrane.

in the interior phase of a *w/o* PE by dissolving it in the aqueous phase prior to emulsification. On the contrary, a hydrophobic cargo can be encapsulated in a *o/*w emulsion by dissolving it in the oil phase before generating the PE.

The scope of applications of colloidosomes is broad, as this model has been used in various fields, including medical and analytical chemistry.[33] Primarily, colloidosomes are used for controlled drug delivery, as they can successfully encapsulate a range of different drugs, as well as releasing the cargo in response to external stimuli.[16b,34] A recent example of drug encapsulation and controlled delivery include the synthesis of silica colloidosomes that were shown to encapsulate a hydrophilic anti-cancer drug and release it upon changes in pH or ionic strength and on exposure to an enzyme.[35] Similarly, colloidosomes can also be used to encapsulate and deliver other molecules, for example insect repellents.[36] In analytical chemistry, colloidosomes have been used as microfluidic substrates for protein analysis[37] and as surface-enhanced Raman spectroscopy substrates.[38] Further uses of colloidosomes include biocatalysis,[39] photothermal anti-cancer therapies,[40] water treatment[4b,41] and anti-corrosion coatings.[42]

3. Colloidosomes as a Protocell Model

Protocell models based on colloidosomes are typically formed using the PE crosslinking technique described above from *w/o* emulsion droplets, chemically crosslinked and then transferred into water media *via* dialysis in ethanol/water. The colloidal stabilisers that are adsorbed at the interface between the oil and the water phase constitute the protocell membrane. As discussed above, although these solid species do not form a bilayer structure as found in biological cells, they serve as a boundary allowing the interior of the structure to be separated from the external environment. Protocell models based on colloidosomes have been fabricated from a wide range of bespoke colloidal nanoparticles synthesised or interfacially modified through a range of synthetic chemistry techniques. In general, all these protocell models can be broadly categorised in two main groups: inorganic colloidosomes and proteinosomes.

3.0.1. Inorganic Colloidosomes

Protocells based on inorganic colloidosomes have been fabricated from a wide range of colloidal materials such as silica nanoparticles (SiNPs),[15a] Au–Ag nanorods (AuAgNRs),[31] clay,[43] and magnetic iron nanoparticles.[15b] These inorganic stabilising species must be amphiphilic, or modified to be so in order to stabilise emulsions, which may then have to be chemically crosslinked to produce stable colloidosomes. The most extensively studied inorganic colloidosomes are composed of SiNPs due to their ease of functionalisation.[44] Their hydrophobicity can be readily tuned by reacting the hydroxyl groups of the surface silanols with varying amounts of dichlorodimethylsilane in substitution reactions. As a result, the silica colloidosomes with more dimethylsilane surface groups become more hydrophobic.[15a] These amphiphilic SiNPs can then be used to form PEs and be crosslinked using tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS) to further stabilise the colloidosomes and allow them to be transferred to a single continuous phase. Colloidosomes composed of AuAgNRs can be produced by performing PEs with nanorods coated with amphiphilic polymers, such as poly(*N*-isopropylacrylamide) (PNIPAAm) and poly(diallyldimethylammonium chloride) (PDDA). These emulsions can be stabilised through covalent crosslinking of the polymers, van der Waals forces, and ionic interactions.[31] Clay-based colloidosomes have been generated with Fe(III)-rich montmorillonite (FeM-clay).[43] The FeM-clay microparticles were made partially hydrophobic by covalently coupling methyltriethoxysilane to the surface hydroxyl groups of the FeM-clay precursors. Colloidosomes can be formed using these amphiphilic microparticles in *w/o* PEs and then crosslinked with TMOS *via* silylation of the individual FeM-clay microparticles. Magnetic iron oxide (magnetite) nanoparticles made hydrophobic using fatty acids (*e.g.*, oleic acid) can also be used to create *w/o* magnetic PEs.[15b]

3.0.2. Proteinosomes

In contrast to inorganic colloidosomes, proteinosomes are fully organic protocell models. Proteinosomes are prepared employing the PE technique using a protein–polymer nanoconjugate as the amphiphilic colloidal nanoparticle. To synthesise protein–polymer nanoconjugates, typically mercaptothiazoline-activated PNIPAAm chains are conjugated to cationised proteins, such as bovine serum albumin (BSA), *via* the reaction with the protein's interfacial primary amines.[45] For example,

BSA is cationised by coupling its accessible aspartic and glutamic acid residues with 1,6-hexanediamine, using *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) as the coupling agent (Figure 4a). This increases the number of interfacial primary amines on the surface of the protein with a degree of cationisation of typically 30% (meaning that 30% of all the protein's aspartic and glutamic acids have been functionalised). The interfacial primary amines of the cationised BSA then react with the mercaptothiazoline-activated headgroup of the amphiphilic PNIPAAm polymer. The resulting BSA/PNIPAAm nanoconjugates can form a *w/o* PE and can then be subsequently crosslinked by reacting the remaining primary amines on BSA with polyethylene glycol (PEG)-bis(*N*-succinimidyl succinate). This chemical crosslinking of the membrane allows for the transfer of the proteinosomes into a continuous water phase *via* dialysis in ethanol/water.

The incorporation of thermoresponsive PNIPAAm polymers on the proteinosomes allows for their permeability to be thermally regulated due to the lower critical solution temperature (LCST) of PNIPAAm. Close to physiological temperature, PNIPAAm undergoes a transition from an extended to a globule conformation.[46] This causes the diameter of the proteinosomes' membranes to shrink, lowering their MWCOs and leading to a decrease of the proteinosomes' permeability (Figure 4b).[47]

Proteinosomes present important advantages with respect to inorganic colloidosomes, as they possess numerous valuable properties such as high biocompatibility, enzymatic activity, and membrane elasticity.[45] In fact, using protein molecules to

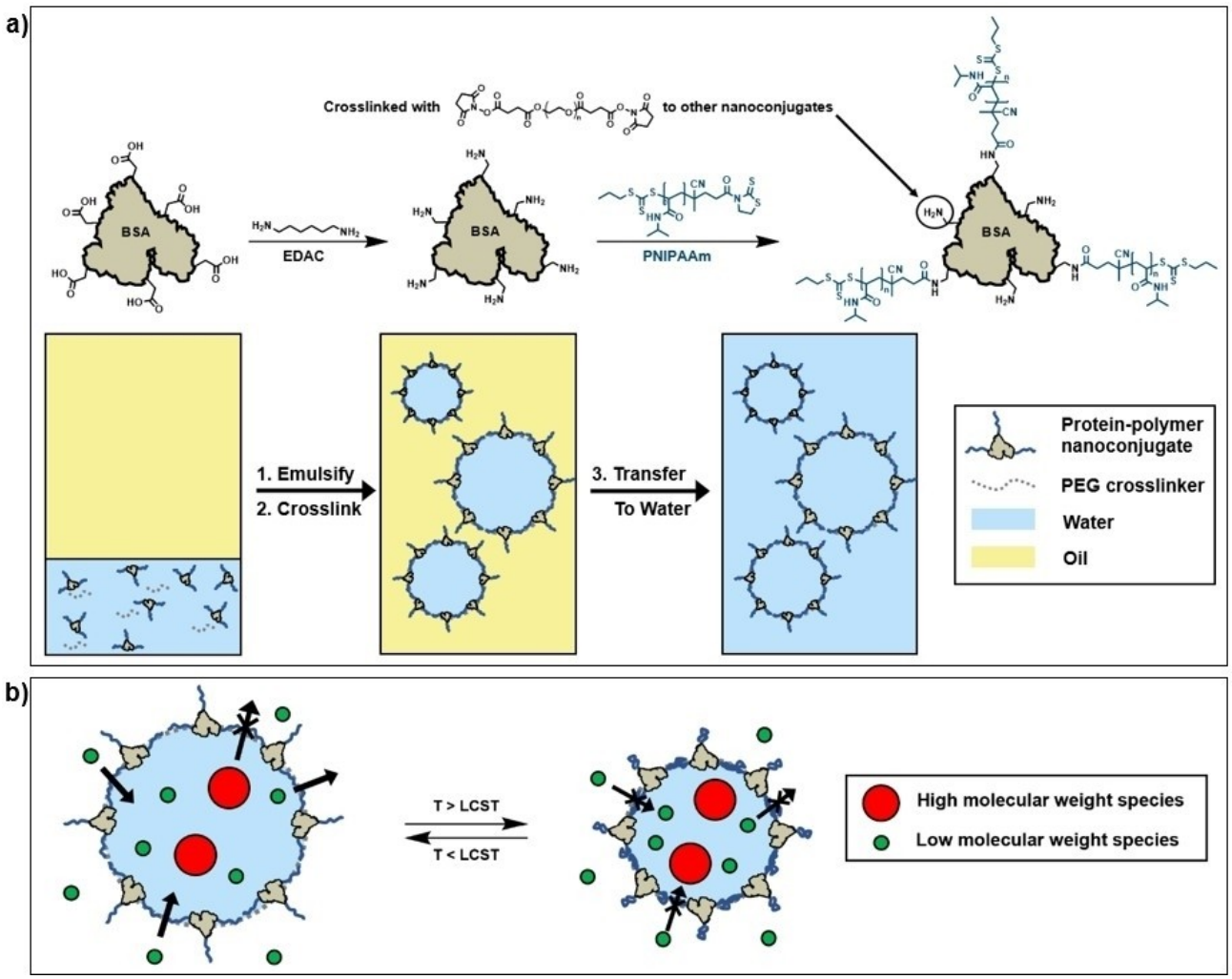


Figure 4. Fabrication and properties of proteinosomes. a) *Top:* Schematic depicting the synthesis of protein–polymer nanoconjugates from BSA and mercaptothiazoline-activated PNIPAAm. First, the aspartic and glutamic acid residues of BSA are cationised with 1,6-hexanediamine to increase the number of surface primary amines. A portion of the BSA amines are then coupled with approximately 3 mercaptothiazoline-activated PNIPAAm chains to produce amphiphilic BSA/PNIPAAm nanoconjugates. Remaining primary amines on the nanoconjugate surface can react with (PEG)-bis(*N*-succinimidyl succinate) molecules to crosslink the proteinosome together after PEs are formed. *Bottom:* Scheme depicting *w/o* PEs of protein–polymer nanoconjugates. After the emulsions are formed, PEG-based molecules in the water droplets are used to crosslink them to produce proteinosomes, that can then be transferred into water. b) Scheme demonstrating the change in permeability of proteinosomes as the temperature is modulated about the LCST. Below the LCST, low molecular weight species (green circles) can passively diffuse in and out of the interstices of the membrane, while high molecular weight species (red circles) are retained. As the temperature surpasses the LCST, the proteinosomes shrink due to the entropic coil-to-globule transition of the PNIPAAm chains. This consequently contracts the proteinosome, lowering its MWCO and inhibiting the diffusion of species in and out of the interstices of the membrane.

form protocell membranes also increases the biocompatibility, biodegradability and biofunctionality of the membrane. In some cases, these colloidosomes were more suitable for medical and *in vivo* applications compared to their vesicle counterparts.[9a] They can be engineered to have inherent enzymatic activity by using enzymes [*i.e.*, glucose amylase (GA), glucose oxidase (GOx), horseradish peroxidase (HRP)] in the synthesis of the nanoconjugates instead of BSA.[48] The literature on proteinosomes is extensive and only the most relevant works utilising organic chemistry to introduce biomimetic functionalities will be discussed in this review – for more detailed information see the various reviews published.[6a,9b,49]

In the following sections we describe and discuss a wide range of colloidosome-based protocells categorising them by their biomimetic function, with an emphasis on the use of synthetic organic chemistry to achieve such functions. These functionalities can be broadly separated into two main categories: *colloidosomes as chemical micro-(bio)reactors* and *networks of interacting colloidosomes*.

On one hand, colloidosomes can be engineered to mimic the living cells capability to function as chemical micro-(bio)reactors. In fact, from a broad perspective, a living cell can be seen as a micro-compartmentalised system capable of taking in input reagents found in the external environment and transforming them into useful products.[6b,50] The achievement of this function relies on two main properties of the overall micro-compartmentalised system: *membrane permeability* and *multi-compartmentalisation*. Membrane permeability allows the diffusion of reagents inside and of the products outside the micro-compartment, while simultaneously maintaining the chemical machinery required to produce the transformation from reagents to products caged inside the membrane.[45,51] Multi-compartmentalisation instead allows for the spatial sub-segregation of specific reactions to avoid chemical interferences, and for the local concentration of the reagents to increase the efficiency of chemical processes.

On the other hand, networks of interacting colloidosomes work between multiple dynamic populations of protocells and include advanced biomimetic functions such as *replication*, *protocellular signalling*, and *predatory behaviour*. The fabrication of *networks of interconnected colloidosomes* will also be discussed as the next frontier in the development of novel bioinspired materials, called "protocellular materials" (PCMs), from the controlled assembly of specialised protocell units.

3.1. Colloidosomes as Chemical Micro-(Bio)reactors

The simplest use of colloidosomes is as chemical micro-(bio)reactors. Here this concept is intended as the capability of the colloidosomes to receive a chemical species as input and to convert it into a desired product that is diffused outside the colloidosome and collected. A common practice to achieve this is by encapsulating enzymes in the protocell interior, followed by selective substrate diffusion through the membrane pores. The diffused substrates are then processed inside the colloidosomes, and the products of the catalytic reaction are allowed to diffuse outside the membrane. Some of the enzymes applied in protocells include lipases,[52] proteases,[53] chymotrypsin,[15a] alkaline phosphatase (ALP),[15b,54] glucose oxidase (GOx),[28,55] glucose amylase (GA),[48] horseradish peroxidase (HRP),[56] and benzaldehyde lyase (BAL).[33e]

In addition to encapsulating enzymes inside the protocell lumen to produce (bio)reactors, various functional biomacromolecules can be incorporated into the colloidosomes' membranes to advance the biochemical functionality and complexity of these protocell models. Huang *et al.* successfully incorporated three different enzymes (GA, GOx, and HRP) into a single, mixed proteinosome membrane, simply by conjugating them to PNIPAAm in a similar fashion to what was previously done with BSA.[48] This resulted in catalytically active and semipermeable membranes, which combined with the thermoresponsive nature of PNIPAAm, were shown to modulate the activity of enzyme cascade systems with external temperature triggers. Similarly, Sun *et al.* expanded upon this work by fabricating GOx/PNIPAAm and BAL/PNIPAAm based proteinosomes using an ATRP (atom-transfer radical polymerisation) "grafting-from" method. The authors utilised these proteinosomes to demonstrate enzyme cascades with chloroperoxidase and Candida antarctica Lipase B in the external aqueous phases.[33e] These systems advanced the concept of "carrier-free Pickering interfacial biocatalysis", where hollow protocells contain functional biocatalysts in the individual building blocks of the membrane. These thermoresponsive proteinosomes could then be loaded with desired cargo to work cooperatively with the membrane enzymes for complex, higher-order enzymatic processing.[33e] The establishment of these catalytically active membranes allows for intricate enzyme cascade reactions between the colloidosomes' external environment, surface, and internal lumen.

3.1.1. Membrane Permeability

Colloidosomes have a peculiar characteristic where they have porous, semipermeable membranes. The strategy that has been most extensively employed to increase the complexity of the colloidosomes' biomimetic functionalities is to enable the control of their membrane permeability. External stimuli applied to colloidosomes to control their permeability include temperature modulation,[45,48] electrostatic interactions,[57] light irradiation,[52a,56a] pH changes,[28–29] protein unfolding,[30] reducing agents,[30,58] proteases,[58] and the addition of small molecules able to self-assemble into protective coatings around the membranes.[32,59] The selective permeability of the membrane is key to the use of colloidosomes as a protocell model, as it allows various metabolic processes to occur within the micro-compartment, by granting the diffusion of the reactants and products across the membrane.

The earliest example of externally controllable permeability was briefly discussed above. This was obtained on proteinosomes where the nanoconjugate was made using a thermoresponsive polymer, PNIPAAm, thanks to which the membrane contraction and hydrophobicity were controlled by the external

temperature.[45] The incorporation of PNIPAAm allowed the permeability of the membrane to be controlled by thermal cycling around the polymer's LCST (*ca.* 32 °C). The proteinosome membranes exhibited a reversible decrease in diameter (~4%) above the LCST (40 °C), due to entropic coil-to-globule transitions of the PNIPAAm chains. Above the LCST, intramolecular hydrogen bonding between the membrane polymers is dominant over hydrogen bonding with the aqueous media. Consequently, the proteinosomes contract, lowering their MWCOs and hindering the diffusion of species in and out of the interstices of the membranes. This functionality in the colloidosome architecture was further advanced by achieving additional selectivity in the membrane permeability. This was accomplished with silica stabilised colloidosomes by functionalising the surface of the nanoparticles with a pH-responsive polymer.[57] The novel copolymer was composed of a branched architecture of 3-(trimethoxysilyl)propyl methacrylate (TMSPMA), 2-(dimethylamino)ethyl methacrylate (DMAEMA), methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA) and 1-dodecanethiol (DDT) with the approximate composition $TMSPMA_{23}/DMAEMA_{40}/MAA_{37}$–$EGDMA_8$–$DDT_{10}$. The EGDMA moieties were used to crosslink the polymer network and the TMSPMA moieties crosslinked the individual SiNPs together. The copolymer also had charged side groups, such as cationic DMAEMA and anionic MAA groups. By increasing the pH, the cationic DMAEMA groups were deprotonated, shifting the net charge of the polymeric membrane coating towards a negative value. This permitted small, positively charged molecules to be selectively transported across the membrane, therefore achieving electrostatically gated membrane permeability. This permitted the regulation of the enzymatic dephosphorylation of 4-nitrophenyl phosphate (PNPP) to 4-nitrophenolate (4NP) by controlling the electrostatic diffusion of the substrate into ALP-loaded colloidosomes. Overall, Li *et al.* were able to demonstrate that these silica colloidosomes could maintain a non-equilibrium compartmentalised process and electrostatically regulate the permeability of the protocells, which could be key in the development of electrostatically controlled delivery systems.

As well as utilising electrostatic interactions, it is also possible to use other stimuli, such as light, to control the proteinosomes' membrane permeability. Zhou *et al.* functionalised BSA/PNIPAAm proteinosomes with photoswitchable spiropyran moieties.[52b] Upon exposure to ultraviolet light, this class of photoswitches can isomerise between the closed-ring spiropyran and the open-ring merocyanine isomers. The process is thermally reversible and can also be reversed upon exposure to visible light.[60] The switch between these two forms was used to mediate interface reactions between various enzymes and their substrates, as the closed-ring form is hydrophobic, whereas the open-ring form is hydrophilic. As a result of the switch in membrane hydrophilicity, the substrates for the enzymatic reactions could be selectively released, and the activity of the enzymes encapsulated within the proteinosome could be modulated (Figure 5a). The incorporation of photoresponsive moieties in the colloidosome membrane is important for the synthesis of protocells with light-regulated permeability, increasingly advanced functionalities, and manipulable hydrophobicity to coordinate complex responses to external stimuli.

More recently, Su *et al.* engineered photoresponsive protocells by covalently grafting gold nanorods onto the surface of proteinosomes made from BSA/PNIPAAm nanoconjugates.[56a] Upon exposure to near-infrared light these nanostructures could contract, therefore regulating their membrane permeability. This reversible contraction behaviour was due to the surface plasmon resonance and the photothermal effect of the gold nano-objects. The gold nanorod-coated proteinosomes were irradiated with light to promote localised heating, which triggered the phase transition of PNIPAAm. The contraction of the proteinosomes decreased the MWCO of the membrane pores, allowing encapsulated enzyme cascade reactions (GOx, HRP, and dextran hydrolase) to be externally controlled. This was made possible by modulating of the diffusion of the substrate for the enzymatic reaction into the protocell. Using light as a stimulus to control the membrane permeability has several advantages over other types of stimuli, as it has high selectivity, spatiotemporal resolution, it allows for remote control and it can also be used to trigger photosynthesis-like reactions.[49] The fabrication of these photoresponsive colloidosomes is also an important step towards implementing novel signal transduction mechanisms, such as a photo-mechano-chemical transduction in protocell populations.

Changes in pH have also been used as an external stimulus to control the permeability of proteinosomes. Recently, Myrgorodska *et al.* designed acid-degradable BSA/PNIPAAm proteinosomes that were loaded with carboxymethyl chitosan (CM–chitosan) hydrogels.[28] Both the proteinosome membrane and the internal hydrogel were crosslinked with acid-degradable crosslinkers. The PEG-based crosslinker contained an acid-degradable ketal group in the centre and electrophilic, *N*-hydroxysuccinimide (NHS)-activated ester moieties on either end of the molecule. The NHS-activated esters were reacted with the interfacial primary amines of the BSA/PNIPAAm nanoconjugates and the primary amines of CM–chitosan to crosslink both materials. The ketal group was stable at pH >7.5 and rapidly hydrolysed at pH <6.0 to cleave the crosslinker and consequently disassemble the proteinosome membrane and internal hydrogel. The acid-mediated controlled degradation of the CM–chitosan hydrogel and proteinosome membrane could also be triggered endogenously *via* encapsulated GOx activity. When glucose was introduced to the GOx and hydrogel-loaded proteinosomes, GOx converted it to gluconic acid, lowering the pH, hydrolysing the ketal crosslinkers, and consequently increasing the permeability of the protocells. The permeability could also be modulated by triggering the phase transition of the thermoresponsive polymer to control the diffusion of glucose into the colloidosomes. These acid-degradable protocells are a step towards the construction of a wide range of pH-sensitive smart materials and to engineer communication pathways between synthetic protocell communities that are triggered by programmable enzyme-mediated pH variations.

To further advance pH responsive protocells, Qin *et al.* developed modular proteinosomes with reversible permeability

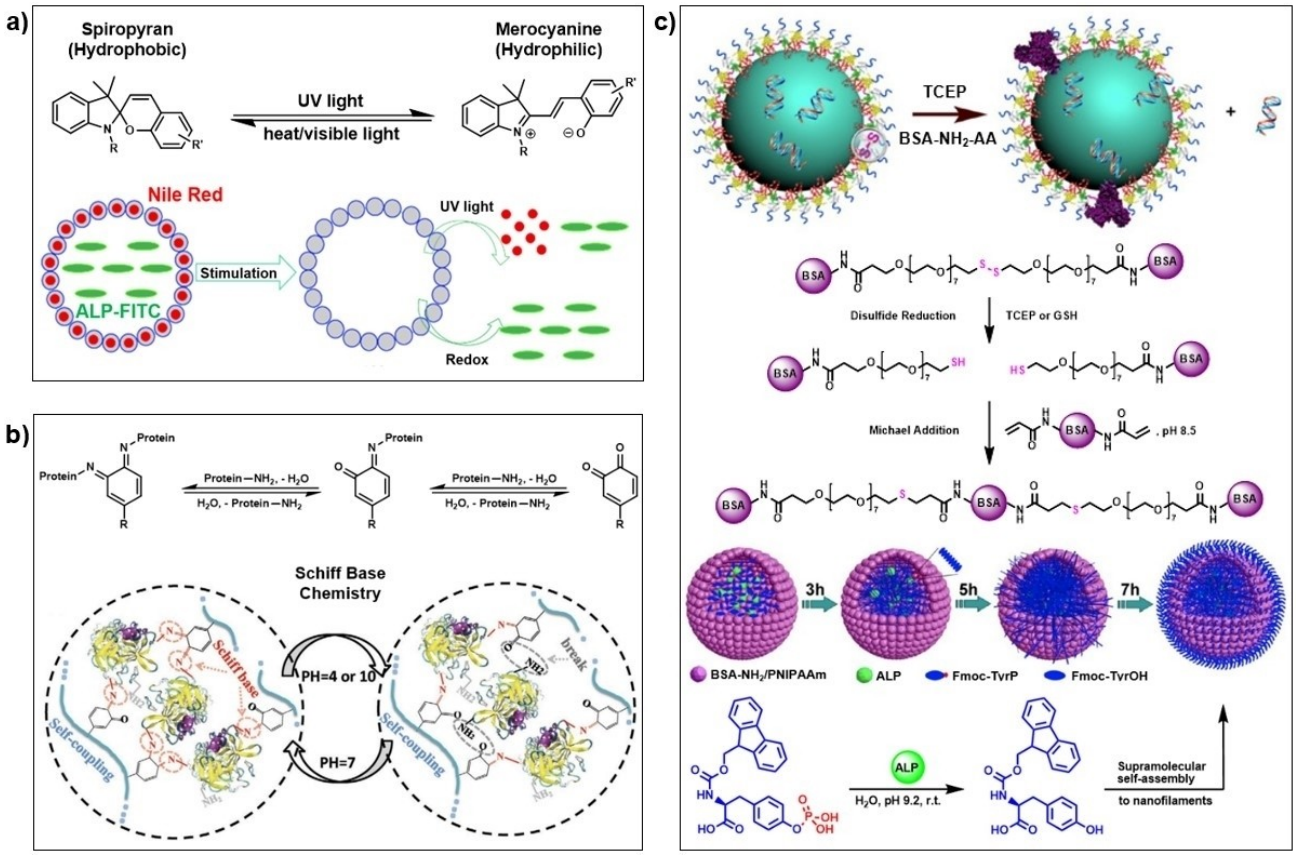


Figure 5. Examples of colloidosomes with permeability control using external stimuli. a) *Top:* Schematic depicting the photoisomerisation of a spiropyran derivative from the hydrophobic ring-closed isomer to the ring-open merocyanine form (hydrophilic). The merocyanine can isomerise back to spiropyran with heat or visible light. *Bottom:* Schematic showing the release of hydrophilic ALP-FITC (green) and hydrophobic Nile Red (red) dyes from the proteinosome upon exposure to UV light. Reprinted (adapted) with permission.[52b] Copyright 2018 American Chemical Society. b) *Top*: General Schiff base reaction showing imine hydrolysis through Schiff base chemistry to reversibly release protein-amines of the protocell membrane and increase the permeability of the proteinosome. *Bottom*: Schematic showing a possible mechanism for the change in permeability due to the reversible formation of a Schiff base at neutral pH. Reproduced (adapted) with permission.[29] 2020, Wiley-VCH. c) *Top:* Figure depicting multifunctional proteinosomes capable of interacting with TCEP and BSA–NH_2–AA to modify its permeability and release encapsulated DNA. *Middle:* Schematic showing strategy for TCEP or GSH induced reduction of the disulfide containing crosslinker and BSA–NH_2–AA resealing of the proteinosome membrane through Michael additions with the cleaved thiols. *Bottom:* Scheme showing time-dependent development of membrane compartments in hydrogel-modified proteinosomes *via* ALP dephosphorylation of Fmoc–TyrP. The dephosphorylation induces supramolecular self-assembly of the Fmoc–TyrOH into nanofilaments mainly driven by a decrease in electrostatic repulsion of the phosphate groups, an increase in hydrophobicity, and π-π stacking. Reprinted (adapted) with permission.[32] Copyright 2014 American Chemical Society.

control by conjugating several natural proteins with oxidised proanthocyanidins (oPC). oPCs are compounds with quinone moieties which are able to reversibly form covalent bonds with the protein amino groups *via* Schiff base chemistry (Figure 5b).[29] Thanks to the pH-responsive behaviour of oPCs, at a neutral pH the membrane was impermeable, but by altering the pH to acidic (pH = 4) or alkaline (pH = 10) the membrane became permeable to the target molecule, fluorescein-tagged dextran. This permeability change was repeated over multiple cycles. The imino groups that formed between protein and oPCs were unstable in acidic/alkaline environments, therefore by altering the pH, these bonds were reversibly cleaved and the permeability of the proteinosome increased. This work demonstrated the possibility to achieve pH-responsive proteinosomes that can be controlled by manipulating the chemistry of oPCs in acidic, neutral, and basic conditions pH.

Complex proteinosome architectures, responsive to a variety of stimuli were constructed by Zhou *et al.* to allow for greater tuning of their permeability.[30] This was achieved using multiple external triggers, such as temperature, disulfide bond reduction, and protein unfolding. To synthesise the building blocks of the proteinosome, BSA was cationised by a carbodiimide-activated reaction of aspartic and glutamic acid residues using 1,6-hexanediamine. Then, the cationised BSA was thiolated by using Traut's reagent (2-iminothiolane hydrochloride). Next, end-capped pyridine disulfide PNIPAAm was produced from the RAFT polymerisation of NIPAAm and a trithiocarbonate chain transfer agent (CTA) having a pyridine disulfide functionalised end group. Finally, BSA/PNIPAAm nanoconjugates linked with disulfide bonds were synthesised by reacting the thiolated BSA with end-capped pyridine disulfide PNIPAAm. These disulfide-containing nanoconjugates were used in a *w/o* PE to produce the proteinosomes. The permeability of these proteinosomes

was sequentially regulated by lowering the temperature from 40 °C to 25 °C, adding tris(2-carboxyethyl) phosphine (TCEP), a reducing agent, and then lowering the pH to 3.5. The temperature change triggered the LCST of PNIPAAm, where the membrane nanoconjugates were tightly packed at 40 °C but would expand at 25 °C and increase the MWCO of the proteinosome pores. TCEP reduced the disulfide crosslinks between the BSA building blocks to yield thiols and increase the pore sizes as well. Then, lowering the pH modified the structure of BSA from a heart-shaped N-form to an expanded F-form, further decreasing the permeability. In a further example, the same research group constructed fully degradable proteinosomes by incorporating multiple types of protein–PNIPAAm blocks into the membrane, including the so-called 'self-sacrificing' nanoconjugates.[58] In this study, a triple hybrid microcapsule using polymer-conjugated dextran, lysozyme and BSA as building blocks was synthesised. With the addition of TCEP, the MWCO of the microcapsules could be increased from 10 kDa to 22 kDa through cleavage of the internal disulfide bonds of the lysozyme/PNIPAAm building blocks. This allowed encapsulated lysozyme groups to leak out of the proteinosome membrane. The MWCO was then increased further to 71 kDa to release dextran by sacrificing BSA from the membrane through enzymatic degradation using a protease. Finally, the membrane was completely disassembled with the addition of lyticase to release loaded DNA. These ''self-sacrificing'' protocells demonstrated programmed stepwise release of loaded biomacromolecules through permeability control with external stimuli and have potential applications in precision drug delivery systems using biocompatible cell mimics.

Further studies have shown that the permeability of colloidosomes can also be controlled by adsorbing metal complexes onto the protocell membrane and removing the coating with metal chelation.[61] BSA/PNIPAAm nanoconjugates were used to form proteinosomes from a *w/o* PE. Polyphenol tannic acid was found to adsorb on the surface of the proteinosomes through hydrogen bonding, hydrophobic and electrostatic interactions with the BSA groups. Upon addition of Fe^{3+}, an external coating comprised of metal complexes with tannic acid was formed around the proteinosome. This decreased the permeability of the membranes and prevented the movement of charged substrates into the protocell. This shell was reversibly removed by addition of ethylenediaminetetraacetic acid (EDTA), which has a stronger affinity for Fe^{3+} compared to tannic acid, increasing the permeability of the protocell. In addition to electrostatic selectivity of the protocells' permeability, this work illustrated that the formation of external coatings around proteinosomes provides improved structural stability and prevents external damage (for example by proteases).[49]

In another sophisticated example, Huang *et al.* further advanced the fine-tuning of the membrane permeability in a colloidosome design. The authors regulated the colloidosomes' membrane permeability utilising proteases, reducing agents, Michael additions, and the formation of an intra-protocellular matrix and extra-protocellular hydrogel coating in DNA-loaded BSA/PNIPAAm proteinosomes. Importantly, the authors in this work introduced the possibility to heal the membrane after partial disassembly.[32] The primary amine groups of the BSA building blocks were crosslinked with PEG-bis(*N*-succinimidyl succinate) ester and another PEG-based ligand that contained a central disulfide group. TCEP and glutathione (GSH) were used to cleave the disulfide-containing crosslinkers and induce partial membrane disassembly by reducing the disulfide bonds to free thiols. This increased the permeability and released some DNA from the proteinosomes' interior. They also showed further release of DNA with the addition of proteases into the system to degrade BSA. The authors then synthesised acrylic acid-modified BSA (BSA–NH_2–AA) by coupling acrylic acid to the primary amines on cationised BSA with EDAC. The BSA–NH_2–AA was used to help reseal the membrane pores exposed by the reduced disulfide crosslinkers. This was achieved *via* Michael additions with the acrylamide moieties and the newly formed thiols from the reduced disulfide crosslinkers (Figure 5c). Alternatively, the permeability of these proteinosomes could be controlled with the formation of intra-protocellular matrices and extra-protocellular hydrogel coatings. This was achieved by enzyme-mediated amino acid dephosphorylation of *N*-fluorenyl-methyloxycarbonyl-tyrosine-(*O*)-phosphate (Fmoc–TyrP) using encapsulated ALP. The generation of the nanofilament-based hydrogel within and around the proteinosome membrane was dependent on the diffusion of Fmoc–TyrP across the proteinosome's pores. Within the membrane, Fmoc–TyrP was dephosphorylated by ALP, inducing the supramolecular self-assembly of Fmoc–TyrOH through a reduction in electrostatic repulsions from the removed phosphate groups, an increase in hydrophobicity, and additional π-π stacking.[54b] This increased the thickness of the proteinosome outer wall without disruption of the inner membrane such that micro-compartments formed within the intra-protocellular hydrogel matrix. This work demonstrated that the formation of an internal supramolecular structure and a protease-resistant outer coating is important not only because it increases the mechanical strength of the protocell, but it also allows for the achievement of higher-order behaviours, such as self-healing and compartmentalisation of interior structure.

3.1.2. Multi-compartmentalisation

The spatial organisation of a cell's internal components into microdomains is critical for many biochemical functions and pathways such as DNA and RNA synthesis, protein synthesis, ATP generation and degradation of unwanted material.[62] In analogy with the sub-cellular compartments of the biological cell, the development of hierarchical protocells with specialised sub-compartments (proto-organelles) can be seen as an effective strategy to increase the functional complexity and the processing ability of artificial cell mimics. The proto-organelles allow for specialised functions that collectively give the protocell spatiotemporal control over information and metabolic processing. In general, multi-compartmentalisation has been achieved *via* the formation of recursive PEs and through internal coacervate complexation.

Liu *et al.* were able to successfully synthesise multi-compartmentalised proteinosomes through a recursive PE method.[63] By controlling the concentration of the nanoconjugates in a constant water/oil volume fraction and the amount of shear stress applied during PE formation, the average diameter of resulting proteinosomes could be modulated between 10–200 μm. BSA/PNIPAAm building blocks were first assembled into small proteinosomes (*ca.* 15 μm mean size) through a *w/o* PE, crosslinked, and transferred into water. An aqueous aliquot of these proteinosomes was mixed with more BSA/PNIPAAm nanoconjugates and oil to perform an additional PE and form two-tiered nested proteinosomes after crosslinking and transfer to water. Aliquots of these two-tiered proteinosomes were then used in a final PE to create nested proteinosomes comprised of three levels of organisation. By regulating the encapsulation sequence, the numbers of confined guest proteinosomes could be controlled and functional macromolecules such as enzymes and DNA could be loaded within specific sub-compartments to produce chemically organised multi-tiered structures. These proteinosomes were partially crosslinked with PEG-bis(*N*-succinimidyl succinate) disulfide ester (NHS–PEG16–DS) which allowed these protocells to exhibit a modulated programmed release behaviour. The disulfide bonds were then reduced and the crosslinker was degraded when the proteinosomes were exposed to an aqueous protease solution or when subjected to stepwise addition of TCEP followed by protease. Then, three types of spatiotemporal response – retarded concomitant release, synchronous release, or hierarchical release of dextran and DNA – were demonstrated based on the sequential response of the host and guest membranes to external stimuli. In general, the fabrication of multi-tiered compartmentalised colloidosomes provide a step towards the construction of hierarchically structured protocells with chemically and spatially integrated proto-organelles.

Compartmentalisation has also been achieved by designing hierarchical polymersomes-in-proteinosomes inspired by pancreatic beta cells. These cell-like multi-compartmented structures were formed through a *w/o* PE of BSA/PNIPAAm nanoconjugates. The proteinosomes contained PEGylated-insulin-loaded (PEG–Ins) polymersomes, additional cationised BSA, dextran-aldehyde, and GOx within their membrane.[55a] The polymersomes were composed of a block-copolymer (BCP) with a hydrophilic PEG block and a hydrophobic, pH-sensitive 2-(*N,N*-diethylaminoethyl)methacrylate (DEAEMA) block. The hydrophobic block also contained units of 4-(3,4-dimethylmaleicimido)butylmethacrylate (DMIBMA) to crosslink the polymersome membrane. These DMIBMA monomers dimerise through a cycloaddition reaction under ultraviolet (UV) irradiation.[64] The BCP was synthesised with ATRP, self-assembled into polymersomes, and were then crosslinked with UV irradiation. Amines on the additional cationised BSA and dextran-aldehyde reacted together through Schiff base (imine) chemistry to produce a porous protein hydrogel-based proto-cytoskeleton inside the proteinosomes. This internalised polymeric network was key for increasing the proteinosome's structural stability, preventing polymersome aggregation, and allowing the even internal distribution of the polymersomes and GOx. The proto-cytoskeleton and polymersomes were both shown to exhibit synergistic, pH- responsive protocell swelling. The GOx enzymes that were permanently integrated into the proto-cytoskeleton generated a local acidic environment by converting glucose to gluconic acid. Acidic conditions increased the permeability of these materials as protonated imines in the proto-cytoskeleton are more susceptible to hydrolysis. The lowered pH also triggered the release of PEG–Ins from the polymersomes by protonating the membranes' amine groups, causing them to swell due to cationic electrostatic repulsions. This system successfully mimicked pancreatic beta cells by modulating glucose concentrations with the controlled release of stored insulin. These multi-compartment colloidosomes have high potential in the field of bottom-up synthetic biology to provide increasingly sophisticated eukaryotic cell mimics.

Proteinosomes with multiple coacervate micro-compartments have also been designed through chemically induced coacervation within the aqueous interior of pre-formed proteinosome-based protocells. Booth *et al.* fabricated nested coacervate-in-proteinosome protocells by encapsulating polyelectrolytes and adding counter-charged membrane-permeable small molecules to the external aqueous environment.[65] *In situ* complexation was then triggered by diffusion to form specialised coacervate sub-compartments (proto-organelles). This process was achieved with both positively and negatively charged polyelectrolytes composed of poly(diallyldimethyl–ammonium chloride)/adenosine 5′-triphosphate (PDDA/ATP) and carboxymethyl dextran/chlorhexidine (CMD/CHXD). The location and reconfiguration of the entrapped droplets could also be regulated by tuning the electrostatic interactions between the encapsulated coacervates and the surrounding negatively charged membrane. These hybrid, multi-compartmentalised colloidosomes allowed for the segregation of enzymes in both the protein membrane (HRP/PNIPAAm co-assembled with BSA/PNIPAAm) and coacervate interior (GOx), which provided a protocell model that more closely resembles biotic cells and yields a flexible platform for developing artificial metabolic reaction networks.

In a further example, Li *et al.* utilised chemically induced coacervation to synthesise coacervates-in-proteinosome protocells and showcased the enhancement of enzyme cascade reaction rates within catalytic proto-organelles that could work cooperatively to achieve information processing behaviours.[66] These architectures were composed of mPEG–BSA/PNIPAAm outer membranes and PDDA/succinylated dextran (Su–Dex) coacervates forming a proto-cytoskeleton within the proteinosome. The coacervate microdroplets within the proteinosomes exhibit effective enrichment towards biomolecules (uricase and HRP) to function as proto-organelles with enhanced enzyme cascade reactions. These hybrid protocells were shown to eliminate a toxic substance (uric acid) with *ca.* 6.5-fold improvement compared to analogous free uricase and HRP in proteinosomes due to the coacervate enhancement of the cascade enzyme reaction rates within the coacervate. The enriched condensates of biomolecules formed within these hybrid multi-compartment protocells can segregate enzymes

into specialised proto-organelles. These specialised and separated sub-compartments can work cooperatively to process information in multi-stepped enzymatic pathways, therefore producing increasingly realistic eukaryote-like models.

Mu *et al.* advanced the multi-compartmentalised coacervate-in-proteinosome design by including an elegant method to reversibly control the chemically induced coacervation within the proteinosome lumen using external stimuli such as light and pH.[67] This example is particularly interesting, as it shows how the use of synthetic functional molecules, such as pH- and light-switches could enable to build the next generation of complex, stimuli-responsive protocell-based systems. This was achieved by encapsulating diethylaminoethyl-dextran (DEAE-dextran), a pH-responsive cationic polyelectrolyte, into BSA/PNIPAAm proteinosomes, followed by the addition of a photoswitchable amphiphile [4,4′-glutamic acid azobenzene] (AzoGlu$_2$) into the external environment. AzoGlu$_2$ diffused into the membrane and initiated its electrostatic complexation with DEAE-dextran to form the coacervate proto-organelles. The azobenzene moieties undergo reversible photoisomerisation between a planar *trans*- and a nonplanar metastable *cis*-isomer by irradiation with UV and blue light, respectively (Figure 6a). Immediately after UV irradiation, the *trans*-AzoGlu$_2$/DEAE-dextran microdroplets underwent size shrinkage and eventually disassembled and could be reassembled by thermal relaxation of *cis*-azobenzene in dark or upon exposure to blue light. The assembly/disassembly was fully reversible for over 10 cycles. The mechanism was ascribed to the greater dipole moment *cis*-AzoGlu$_2$ has compared to the *trans*-isomer, which increases its polarity and lowers its tendency to complex with DEAE-dextran, thus inducing the disassembly of the coacervates. Furthermore, the *trans*-AzoGlu$_2$/DEAE-dextran proto-organelles were found to be highly pH sensitive, where coacervation occurred in a pH window ranging from 4.5 to 7.5. Finally, Boolean logic gates (NOR and NAND) were implemented within the protocell system by integrating coacervate formation/disassembly with enzyme cascade reactions. These tiered sub-compartments of the hybrid protocells were able to sense a variety of extracellular signals (*e.g.*, photons, protons, and small molecular weight compounds) and adapted their behaviours through a series of biochemical reactions.

A significant development in the design of multi-compartmentalised protocells was the fabrication of self-reconfigurable host-guest protocells. These hybrid constructs consisted of two protocell models arranged in a nested microstructure, derived from the urea-triggered confinement of guest BSA/PNIPAAm proteinosomes within individual fatty acid (myristic acid) micelle coacervate hosts.[55b] Synergistic (cooperative) and antagonistic modes of enzyme-mediated behaviour between the proteinosomes and micelle coacervates were used to achieve the host-guest protocell formation and self-reconfiguration of the system, respectively. (Figure 6b). Synergistic activity was demonstrated through the addition of urea to a population of proteinosomes (containing urease and GOx) and a dispersion of HRP-loaded fatty acid vesicles at pH 8.7. The diffusion of urea into the proteinosomes resulted in a gradual increase in pH, associated with the urease-mediated production of ammonia. The increased pH caused a proteinosome-mediated vesicle-to-coacervate transformation, immobilising the proteinosomes in the coacervate matrix. This synergistic activity of the proteinosomes allowed for the formation of the host-guest protocellular system by modifying the local pH and forming the host coacervates. Antagonistic enzyme behaviour was demonstrated next by the subsequent addition of glucose to the system, which produced a GOx-mediated pH decrease due to the formation of H_2O_2 and glucono-δ-lactone (GDL). The HRP within the coacervates utilised the H_2O_2 to convert Amplex red to resorufin and the increase in GDL concentration was associated with the decrease in pH. Lowering the pH caused the antagonistic disassembly of the coacervate droplets and establishment of the fatty acid vesicles-in-proteinosome structures. This is the first example where a multi-compartmentalised protocell system is capable of self-reconfiguration on the basis of the balance between the synergistic/antagonistic activity of its components. The emergence of higher-order behaviours from communities of protocell-based systems is a developing topic in the search for life-like systems and it is particularly important to shed further light on the spatially coupled processes occurring in living cells.

3.2. Networks of Interacting Colloidosomes

In the previous section, biomimetic functions were exhibited by a single population of colloidosomes to increase their biochemical complexity. This section will highlight several significant works that have contributed to the application of communicating colloidosome networks that are comprised of multiple, discrete populations of protocells interacting with one another. These works more closely resemble biological systems with different specialised protocell communities working in tandem to elicit higher-order biomimetic functions. Advancements in this field are expected to have great impact in biotechnological applications such as tissue engineering, biological therapies, biotic solar-to-chemical energy conversion, and the fabrication of multi-faceted, intelligent biomaterials, to name a few. These contributions can be categorised into three groups: replication, protocellular signalling, and predatory behaviour. Herein, we will discuss these categories and attempt to draw important insights on how synthetic chemistry can be used to advance these networks of micro-compartmentalised systems and further develop them for future applications.[50] The fabrication of networks of interconnected colloidosomes will also be discussed as the next frontier in the development of novel bioinspired materials from the controlled assembly of specialised protocell units.

3.2.1. Replication

A key advancement towards the development of communicating protocell models was the generation of colloidosomes which can autonomously grow and replicate. Chemical communication here can be intended as the transfer of internalised

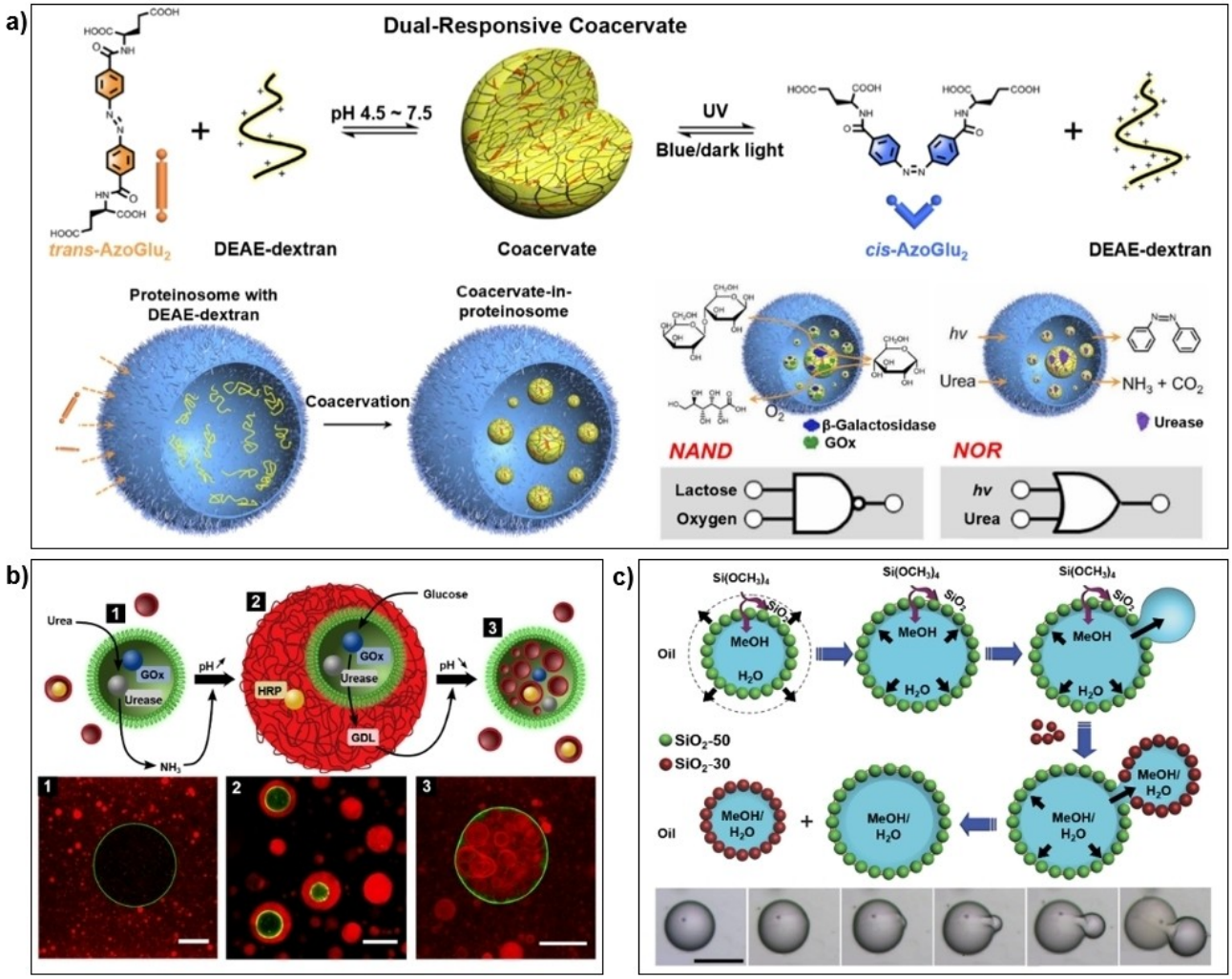


Figure 6. Colloidosomes with advanced biomimetic functions including sub-protocell compartmentalisation and replication. a) *Top:* Schematic of the coacervation of polyanionic, photoactive AzoGlu$_2$ and polycationic DEAE-dextran. Their complexation leads to microdroplet condensation *via* a liquid-liquid phase separation. The azobenzene photoswitch can control their coacervation with light irradiation due to polarity differences in the isomers of AzoGlu$_2$. Moreover, changes in pH below 4.5 or above 7.5 lead to disassembly by modifying electrostatic interactions upon protonation/deprotonation of either AzoGlu$_2$ or DEAE-dextran. *Bottom left:* Schematic depicting the diffusion of trans-AzoGlu$_2$ into DEAE-dextran loaded proteinosomes to trigger proto-organelle formation through coacervation inside the membranes. *Bottom right:* Schematic representation of Boolean NAND and NOR logic gates constructed from coacervate-in-proteinosome complex systems. The NAND gate incorporated β-galactosidase and GOx into the coacervates with lactose and oxygen as two inputs. β-galactosidase catalysed the conversion of lactose into galactose and glucose, and the GOx oxidised glucose into gluconic acid using oxygen. This reduced the solution pH, inducing the disassembly of the coacervates. Hence, the NAND gate depicted activity (coacervate formation) in the absence of either lactose or oxygen. The NOR gate consisted of urease-loaded trans-AzoGlu$_2$/DEAE-dextran coacervates within proteinosomes with the inputs as UV light and urea. UV light isomerised the azobenzene group and disassembled the coacervate microdroplets. The addition of urea initiated the hydrolysis of urea into carbon dioxide and ammonia, increasing the pH of the solution and causing the dissociation of coacervate microdroplets. This behaviour is characteristic of a NOR logic gate, where the activity (coacervate formation) was observed only in the absence of both signals (UV light and urea). Reproduced (adapted) with permission.[67] 2021, American Association for the Advancement of Science. b) *Top:* Self-induced capture of proteinosomes by enzyme-mediated vesicle-to-coacervate transitions. Graphic showing restructuration of a mixed population of proteinosomes (green) containing encapsulated GOx (blue circle) and urease (grey circle) in the presence of non-encapsulated fatty acid vesicles (red) containing HRP (yellow circle) (state 1) into a nested proteinosome-in-coacervate micro-architecture (state 2) after addition of urea, followed by transformation into a vesicle-in-proteinosome structure (state 3) after addition of glucose. Confocal fluorescence microscopy images of: *bottom left:* a mixed population of proteinosomes in the presence of non-encapsulated fatty acid vesicles (state 1, scale bar = 20 μm), *bottom middle:* nested proteinosome-in-coacervate micro-architecture (state 2, scale bar = 50 μm), and *bottom right:* vesicles-in-proteinosome structure (state 3, scale bar = 20 μm). Reproduced (adapted) with permission.[55b] 2018, Springer Nature. c) *Top*: Scheme showing silica-stabilised colloidosomes exhibiting growth and division by exploiting the generation of methanol at the *o/w* interface. The methanol was generated as a by-product of crosslinking the colloidosomes with TMSO and the newly formed colloidosomes are stabilised by partially hydrophobic silica particles in the oil phase. *Bottom*: Optical microscopy images showing the division of the silica colloidosomes over time. Scale bar = 100 μm. Reproduced (adapted) with permission.[15c] 2014, John Wiley and Sons.

chemical information from the parent to the generated offspring protocells. The achievement of protocell systems capable of spontaneous growth and division is both a primary focus in origin-of-life research and also has important technological applications for the synthesis of autonomously functioning microscale compartments that can multiply.[15c,68] Li *et al.*

achieved replicating silica-stabilised colloidosomes capable of increasing the internal volume of the micro-compartments until a critical volume was reached and the protocell split into two (Figure 6c).[15c] SiNPs with *ca.* 50% surface silanol and dimethylsilane groups (SiO_2-50) were used to form the 1st generation *w/o* PE. Replication was achieved by exploiting the generation of a hydrophilic by-product in the crosslinking reaction between the silica nanoparticles. The crosslinker used was TMOS, which generated methanol; as methanol is a hydrophilic molecule, it preferentially diffused into the water phase causing the aqueous microdroplet to grow until it reached a critical volume at which the silica membrane ruptured. The methanol/water droplet growth was stabilised at the *o/w* interface by the adsorption of SiNPs with 30% silanol and 70% dimethylsilane surface groups (SiO_2-30) present in the bulk oil phase. Eventually, the aqueous droplet separated from the parent colloidosome and both structures were sealed by further crosslinking and could be transferred into water. This protocellular system demonstrated communication between generations of colloidosomes through the transfer of biological material added to the aqueous phase prior to the PE. Self-replicating protocell systems based on self-assembled lipid membranes are common,[69] which is not the case of colloidosomes formed with the PE crosslinking method. The reason for this is that covalent crosslinking of the colloidosome does not allow for significant growth of the membrane. Finding a method to produce self-replicating colloidosomes in a single, aqueous phase system would be of great importance, especially for technologically relevant applications, where the robustness and stability of the capsule in various chemical environments are crucial.

3.2.2. Protocellular Signalling

For protocellular communication, diffusible chemical signals are transmitted from a population of sender protocells to a separate population of receiver protocells. These signals can initiate a variety of chemical responses in the receiver protocells. Cellular communication pathways are critical for biotic cells to exchange information in the form of diffusible signals leading to intricate biological processes like reproduction, differentiation, coordinated metabolism, cellular regulation and more.[2c,36] The implementation of biotic cell communication methods to colloidosomes allows for the development of a variety of complex chemical processes and automated protocell-based decision-making, triggered by chemical signals of remote protocells. Therefore, chemical reaction-diffusion networks between populations of communicating protocells could enable complex information processing tasks for future protocellular models of intricate biological processes.

Inter-protocell communication was shown for the first time using gene-directed signalling to elicit enzymatic activity in a binary system of proteinosomes and liposomes. Tang *et al.* successfully achieved communication between populations of receiver proteinosomes and sender liposomes. Triggering the expression of an enzyme in the liposome resulted in the release of a substrate, which in turn elicited a response from the proteinosome.[70] More specifically, the addition of a transcription inducer to a population of liposomes containing a cell-free gene expression system and gene coding for α-hemolysin, resulted in the expression of this macromolecule to form pores in the liposome membrane. As a result, glucose was released from the liposome and was oxidised at the proteinosome GOx/PNIPAAm membrane, resulting in the production of H_2O_2. The H_2O_2 was then used as fuel within the proteinosome for the HRP peroxidation of Amplex red to resorufin, generating a fluorescent output signal. Overall, the addition of a transcription inducer to liposomes facilitated the remote triggering of the enzyme activity of a population of proteinosomes, generating a fluorescent read-out through gene-mediated chemical signalling. Inter-protocellular signalling using the GOx/HRP enzyme cascade reaction in distinct colloidosome populations was also demonstrated in a few other examples.[48,56a,71]

In an important example of protocellular signalling, colloidosomes were engineered in order to be capable to produce a proton gradient with visible light. As a result, this chemical signal triggered ATP synthesis in a nearby population of liposomes.[31] To do this, Chen *et al.* engineered light-gated colloidosomes built from PDDA-coated Au–Ag nanorods (AuAgNRs) conjugated with a layer of bacteriorhodopsin-containing lipoproteins. The positively charged, PDDA-coated AuAgNR colloidosomes were ionically stabilised by the inherently negatively charged, bacteriorhodopsin-containing liposome coating and through the van der Waals forces between PDDA chains. The colloidosomes were able to produce a proton gradient from the interior of the membranes to the exterior upon light irradiation. After irradiation, the surface plasmonic resonance of the metal nanorods promoted the absorption of photons by the conjugated bacteriorhodopsins, which generated the proton gradient. The authors then successfully demonstrated protocellular signalling between these colloidosomes and a population of liposomes with membrane imbedded ATP synthases. Upon light irradiation, the protons produced by the AuAgNR colloidosomes acted as the chemical signals in the external medium and stimulated the synthesis of ATP within the liposomes. Control experiments showed that ATP was not generated in the absence of the colloidosomes or in the dark (Figure 7a). The fabrication of these light-gated colloidosomes is important in generating protocell models from metal-based stabilisers with unique properties that allow for intricate, chemical communication-driven functions and provides opportunities for developing alternative solar-to-chemical energy conversion systems.[31]

In a few key examples, highly programmable and specific DNA strand displacement circuits were implemented to build unprecedented consortia of proteinosomes capable of collective information processing behaviours. Joesaar *et al.* implemented a modular protocellular communication platform based on microfluidics using ssDNA. The proteinosome messaging system used internalised DNA-based molecular circuits to encode/decode information transmitted in the form of diffusible orthogonal chemical signals.[72] This was achieved *via* streptavidin-mediated encapsulation of DNA templates inside

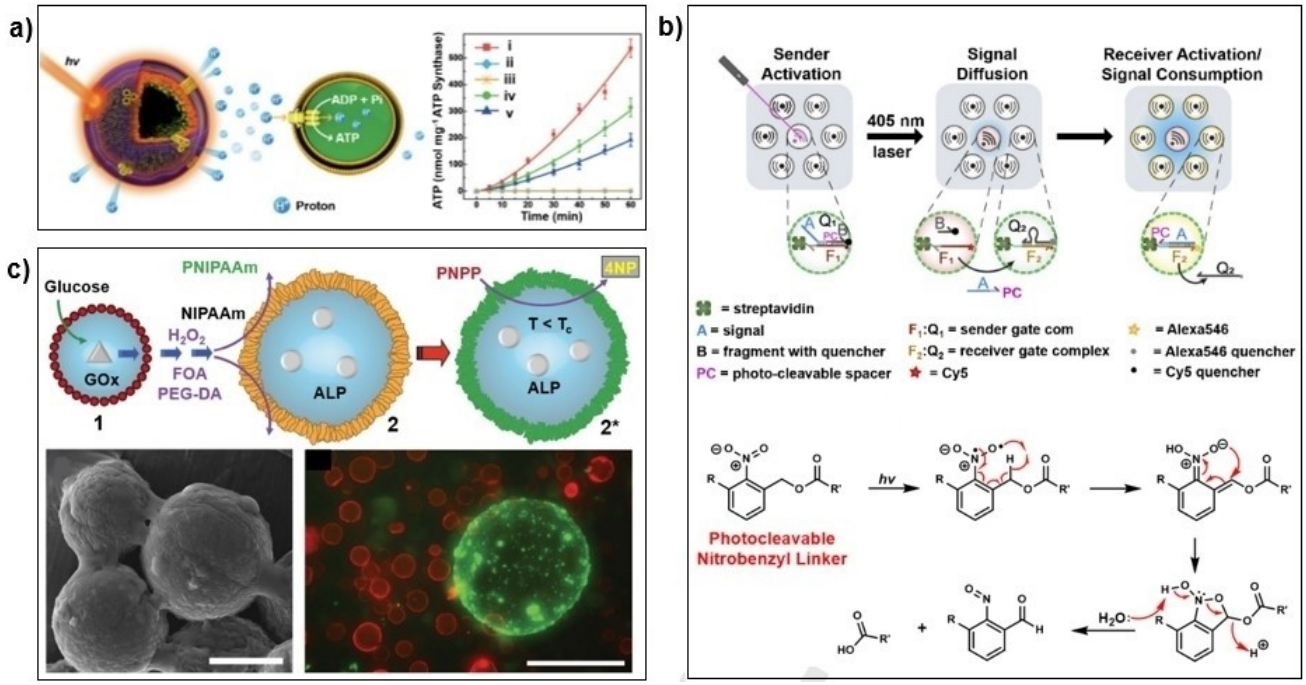


Figure 7. Examples of interacting colloidosome networks showing protocellular signalling behaviours. a) *Left*: Schematic showing plasmonic colloidosomes formed from AuAgNRs coated with bacteriorhodopsin-containing lipoproteins. This system pumps protons across the colloidosome membrane upon light irradiation. The increase in proton concentration outside the colloidosomes triggers ATP generation in target proteoliposomes, exhibiting communication between the two different protocell populations. *Right*: Graph showing the variation of ATP concentration over time synthesised under different conditions; i) colloidosomes and proteoliposomes under light irradiation, ii) colloidosomes and proteoliposomes in the dark, iii) proteoliposomes under light irradiation, without colloidosomes (iv and v show proteoliposomes with colloidosomes formed from silver and silicon-oxide nanorods under light irradiation respectively). In the absence of light, no ATP is generated, indicating that the proton pumping due to the light irradiation of the plasmonic colloidosomes is responsible for the synthesis of ATP in the proteoliposomes. Reproduced (adapted) with permission.[31] 2019, John Wiley and Sons. b) *Top:* Light-activated DNA-encoded sender-receiver proteinosome system. A single sender protocell and multiple receiver protocells are localised on a 2D spatial grid using a trapping array. Light-activated release of a ssDNA signal (A) from the sender protocell triggers a signalling gradient which activates nearby receiver protocells. The sender protocell contains a fluorescently quenched internalised gate complex (F1:Q1) anchored to streptavidin using a biotinylated DNA gate strand (F_1). Signal release from the sender protocell is triggered by laser irradiation resulting in photocleavage of strand Q_1, concomitant dissociation of the two cleaved parts (A and B) and Cy5 fluorescence. Signalling strand A activates the surrounding receiver protocells by displacement of a quencher strand (Q_2) from an internalised gate complex (F2:Q2) to produce an Alexa546 fluorescence output and consumption of strand A. Reprinted with permission.[74] Copyright 2020 American Chemical Society. Bottom: Reaction scheme for the photocleavage of a nitrobenzyl moiety into a carboxylic acid and a nitrosobenzaldehyde. c) *Top:* Scheme showing chemical communication and functional adaptation in colloidosome communities. Addition of glucose to silica colloidosomes (population 1, red) containing GOx (triangle) generates a H_2O_2 signal that diffuses to larger FeM-clay colloidosomes (population 2, orange), which then catalytically polymerise NIPAAm in the presence of fluorescein *o*-acrylate (FOA) and PEG-diacrylate (PEG-DA) to produce a green, fluorescent PNIPAAm shell specifically on the inorganic membrane (population 2*, green). Population 2 contains ALP (circles) and dephosphorylation of membrane permeable PNPP to 4NP becomes gated in 2* after signal-induced polymerisation if the temperature (T) is below the LCST (T_c). *Bottom Left*: SEM image of FeM-clay colloidosomes with temperature-sensitive PNIPAAm polymer coating. Scale bar = 40 μm. *Bottom Right*: Fluorescence microscopy image showing Rhodamine B-tagged silica colloidosomes (red) and the emergence of fluorescein o-acrylate tagged PNIPAAm/FeM-clay colloidosomes (green) after 24 h as the polymer shell is formed. Scale bar = 150 μm. Reproduced (adapted) with permission.[43] 2016, John Wiley and Sons.

proteinosomes. Enzyme-free, toehold-mediated DNA strand displacement (DSD) reactions were used to encode and decode molecular messages using membrane-permeable ssDNA. To minimise unintended cross-hybridisation, ssDNA with highly orthogonal base-pairing interactions was used. The high programmability and predictability of DSD reactions allow the design of molecular circuits exhibiting a wide range of dynamic functions including catalytic cascades,[16a] digital logic circuits,[22] Boolean networks,[23] transcriptional RNA signalling,[73] control algorithms[26] and oscillations.[33a] The use of proteinosomes in conjunction with microfluidic trapping devices to congregate the protocellular communities had several advantages. First, this allowed to study their behaviour at the single protocell level and the population dynamics could be easily tuned by controlling the compartment permeability. Also, high local concentrations of DNA gates inside the proteinosomes resulted in a considerable decrease in the DSD circuit timescale from hours to minutes. This work reported the first example of a protocell network capable of collective functions such as multiplex sensing, cascaded amplification, bidirectional communication, and distributed logic operations. This system was then further developed to be light-activated using a photocleavable nitrobenzyl linker inside the sender proteinosomes. Irradiation of the sender protocells degraded the linker and caused the release of a diffusible DNA strand signal.[74] A microfluidic trapping array localised a single sender protocell around receiver protocells to spatially control the signal release from the sender and monitor the signal diffusion using fluorescence

(Figure 7b). The sender proteinosome contained a ssDNA signal (A) bound to a streptavidin-anchored sender gate complex (F_1:Q_1) through a nitrobenzyl moiety. Upon light irradiation, the nitrobenzyl linker would undergo a radical rearrangement to yield an aldehyde at the benzylic position and cleave off a carboxylic acid. Consequently, signal (A) was detached from F_1:Q_1, allowing it to diffuse out of the sender proteinosome and turning on the red fluorescence of cyanine 5 (Cy5) bound to F_1:Q_1. The released signal (A) could then diffuse into a nearby receiver proteinosome and bind to a receiver gate complex (F_2:Q_2) through a DSD reaction. This turned on the yellow fluorescence of Alexa 546 bound to F_2:Q_2 and signified the consumption of the signal (A). Overall, the design and study of spatially controlled, DNA-encoded protocell systems allows for quantitative analysis of diffusion-based sender-receiver architectures to potentially uncover the design principles of biotic cell-cell communication modules.

Clays are an attractive material for the fabrication of primitive protocells and study of protocellular signalling. Large quantities of clay minerals are present in soils and sedimentary rocks that form the majority of the Earth's early surface and they are able to selectively adsorb and enrich in nucleotides, catalyse the synthesis of amino acids and RNA, as well as promote genetic transcription.[43] Sun *et al.* demonstrated protocellular signalling using the catalytic activity of ALP-loaded, Fe(III)-rich montmorillonite (FeM-clay) colloidosomes.[43] Partially hydrophobic FeM-clay microparticles were prepared by covalently coupling methyltriethoxysilane to the surface hydroxyl groups of the FeM-clay precursors. FeM-clay colloidosomes were then formed using these amphiphilic microparticles in *w/o* PEs. The colloidosomes were then crosslinked with TMOS *via* silylation of the individual FeM-clay microparticles. The stabilised colloidosomes could then be transferred into water with a series of dialyses with ethanol/water solvents. The structures generated were shown to encapsulate functional enzymes (ALP) and exhibit catalytic functionality to communicate with a population of GOx-containing silica colloidosomes. With the addition and diffusion of glucose into the silica colloidosomes, encapsulated GOx groups could enzymatically convert the sugar to H_2O_2. The H_2O_2 molecules could then be released from the silica membranes as chemical signals, triggering the peroxidative polymerisation of a temperature-responsive (PNIPAAm) wall around ALP-loaded and catalytically active FeM-clay protocells (Figure 7c). The PNIPAAm coating could then help reversibly regulate the catalytic activity of the ALP enzymes in the FeM-clay protocells when modulating the temperature. A decrease in ALP-mediated dephosphorylation of membrane-permeable *p*-nitrophenyl phosphate (PNPP) to 4-nitrophenolate (4NP) was exhibited when heated above PNIPAAm's LCST. The reason for the decreased activity is the contraction of the thermoresponsive PNIPAAm membrane above its LCST, inhibiting the diffusion of PNPP towards ALP in the colloidosome membranes. Therefore, this system demonstrated protocellular communication that resulted in the activation of thermoresponsive, biomimetic function in the FeM-clay colloidosomes. This work also highlighted the use of novel catalytically active, inorganic membranes that have potential applications in multiplex reactor networks or synergistic sensing systems.[43,75]

3.2.3. Predatory Behaviour

Another form of advanced biomimetic functionality based on interacting colloidosome populations is predatory behaviour, where a population of protocells are chemically programmed to disassemble another population of protocells or even biotic cells. Several strategies have been developed to achieve predatory behaviour, including phagocytosis-inspired ingestion,[15b] protease degradation of proteinosomes,[53a] acid mediated coacervate disassembly,[53b] bactericide release,[76] and hydrogelation,[77] to name a few. Implementing predatory behaviour to protocell populations is a further step up in the development of advanced synthetic constructs with emergent life-like properties. These colloidosomes show potential for various applications, such as in biological material transport,[15b,53a] cancer therapy,[76] and sanitation.[77]

In a key example, iron-based magnetic colloidosomes showing predatory behaviour have been used to degrade water-filled silica colloidosomes. The system developed by Rodríguez-Arco *et al.* was shown to mimic a primitive form of phagocytosis. In such research work, partially hydrophobic oleate-capped magnetite (Fe_3O_4) particles were used to stabilise a *w/o* magnetic Pickering emulsion (MPE), that were able to ingest silica colloidosomes.[15b] On addition of oleic acid, the iron oxide membrane of the MPE was destabilised by the adsorption of negatively charged oleate at the *w/o* interface. This event decreased the MPE interfacial tension and increased the fluidity of the membrane, generating fatty acid-stabilised apertures. Silica colloidosomes were spontaneously incorporated into the MPE, and their water-soluble payloads released into the MPE droplets. This system was further developed to include silica colloidosomes containing enzymes which on ingestion were able to trigger a range of chemical reactions. One example was where ALP-containing MPEs ingested silica colloidosomes loaded with non-fluorescent, fluorescein diphosphate molecules. Upon incorporation inside the MPEs, the silica colloidosomes disassembled, exposing the small molecules to ALP and triggered dephosphorylation reactions. The fluorescence of fluorescein, the catalytic product, was turned on and monitored using fluorescence microscopy and bright-field imaging. This system demonstrates the possibility of reversibly forming larger apertures in protocell membranes without damaging the colloidosomes' overall structure. The development of such complex behaviours in populations of synthetic protocells has potential uses in microreactor technology as well as biomaterial storage and delivery.[15b]

Alternatively, Qiao *et al.* reported a system where predatory behaviour was achieved through electrostatic interactions, followed by protease-induced lysis between populations of proteinosomes and coacervates.[53a] The coacervates (loaded with protease K) were synthesised by the coacervation of aqueous dispersions of positively charged PDDA polymers and negatively charged ATP molecules. These coacervates had an

overall positively charged membrane and were mixed with negatively charged BSA/PNIPAAm proteinosomes (loaded with dextran and ssDNA). Contact between the two different groups of protocells was achieved due to the electrostatic attraction of the oppositely charged protocell membranes. On contact, the protease from the coacervates induced the lysis of the protein–polymer membrane of the proteinosome. The 'killer' coacervates were also able to encapsulate the contents of the broken proteinosomes due to their ability to assimilate a wide range of molecular solutes, resulting in a rudimentary form of 'inter-protocellular trafficking.'[53a] This direct contact system displayed communication between cell mimics because the chemical information (dextran and ssDNA) contained within the proteinosome were eventually transferred into the coacervates.

This method of contact-dependent communication was developed further by using two different predation strategies and three different types of protocells, which were shown to exhibit response-retaliation behaviour.[53b] The protocells used in this system were: 1) GOx-containing proteinosomes that can acidify the local environment with the addition of glucose to the system, 2) acid-degradable poly-D-lysine (PLys)/adenosine 5′-diphosphate (ADP) coacervates loaded with protease K, and 3) pH-insensitive, counter-attacking PDDA/dextran-sulfate (DS) coacervates. The response-retaliation pathway began with the addition of glucose to the system. Glucose diffused into the proteinosomes to be processed by GOx, acidifying the local environment. The pH decrease induced the disassembly of PLys/ADP coacervates to release protease K, which was then encapsulated in the PDDA/DS coacervates. Then, the positively charged, protease K-loaded PDDA/DS coacervates electrostatically adhered to the negatively charged proteinosome surface. Contact-mediated degradation of the proteinosome membranes was then triggered through the enzymatic activity of the protease K moieties. This released the GOx moieties from the proteinosomes and the PDDA/DS coacervates sequestered the released cargo. These further developments in the communication and predation between populations of protocells could be very important in the development of communities of protocells with higher order behaviours, as coordinated behaviours between groups of biotic cells are key for regulating their growth, differentiation, death and assembly into tissues.[49]

Effective communication between protocells and living cells is a less developed area, but protocell communities have been shown to successfully capture and kill bacteria as well as cancer cells.[76–77] In a recent example, GSH-responsive BSA/PNIPAAm proteinosomes containing sodium alginate (NaAl) were used to kill cancer cells.[76] These proteinosomes were designed with disulfide-containing PEG crosslinkers to be susceptible to GSH reduction and cleavage because cancer cells are known to have higher concentrations of GSH.[78] Exposure to free calcium ions (Ca^{2+}) triggered the formation of alginate hydrogels within these proteinosome architectures. Once the protocells were encapsulated within cancer cells, the disulfide bonds in the proteinosome membrane were cleaved by glutathione present in the cancer cell to release the hydrogel, inducing cell death. More recently, Zhao *et al.* showed that positively charged, thermoresponsive proteinosomes were capable of capturing *E. coli* and inducing cell death by aggregation-triggered hydrogel disassembly.[77] Positively charged proteinosomes were synthesised from a *w/o* PE with BSA grafted with PNIPAAm and methacryloxyethyl dimethylethane ammonium bromide (MED-AB) copolymers. Cationised BSA, formyl benzoic acid-functionalised dextran (Dex-CHO), and arginine modified chitosan oligosaccharide (COS-Arg) were added into the aqueous phase prior to emulsification. The BSA amines, the Dex-CHO, and COS-Arg formed a hydrogel through acid-sensitive Schiff base linkages in the proteinosome interior. Upon increasing the temperature to 37 °C, negatively charged *E. coli* aggregated on the proteinosome membrane through electrostatic attractions. This resulted in a local acidic environment due to the metabolism of the *E. coli* that triggered the cleavage of the acid-sensitive Schiff base connections inside the proteinosomes. Antimicrobial COS-Arg was subsequently released from the hydrogel and diffused out of the membrane to kill the bacteria. Although there are very few examples of communication between biotic and artificial cells in the literature, more research in this field will have profound impacts in the medicinal and sanitation fields. Furthermore, protocellular communication with biological materials has only been demonstrated with protocell/cell communication, and protocells have yet to be combined with larger biological materials – *e.g.*, tissues or multicellular organisms. The study and advancement of protocellular interactions with biological cells and tissues will be crucial for the potential application of colloidosomes in drug delivery and regenerative medicine.

3.2.4. Networks of Interconnected Colloidosomes

The next frontier in the use of colloidosomes as protocell models is their spatial organisation into interconnected protocell networks with emerging and/or collective behaviours and capable of mimicking basic aspects of living tissues. Nevertheless, so far only a few examples of interconnected protocell networks are available in the literature.[79] This is due to the instability of the currently available tissue-like material models, which poses a major problem in their use for practical applications. A promising strategy that has recently emerged to overcome these problems is the use of interfacial organic chemistry for the generation of covalent (and consequently resilient) protocell-protocell connections. This covalent strategy has allowed for the design and fabrication of robust, free-standing "protocellular materials" (PCMs), characterised by complex three-dimensional (3D) architectures from the controlled assembly of individual protocell units.[80]

This first example of covalently bound protocell communities was reported by Gobbo *et al.*[21] They showed that spheroidal assemblies of proteinosomes (Figure 8a), called prototissue spheroids, could be fabricated by exploiting what we have coined interfacial strain-promoted alkyne-azide cycloadditions (I-SPAACs).[81] This bio-orthogonal reaction between azide- and bicylononyne (BCN)-functionalised proteinosomes crosslinked them together forming triazoles.[82] To achieve this, bio-orthogonally reactive proteinosomes in oil were fabricated

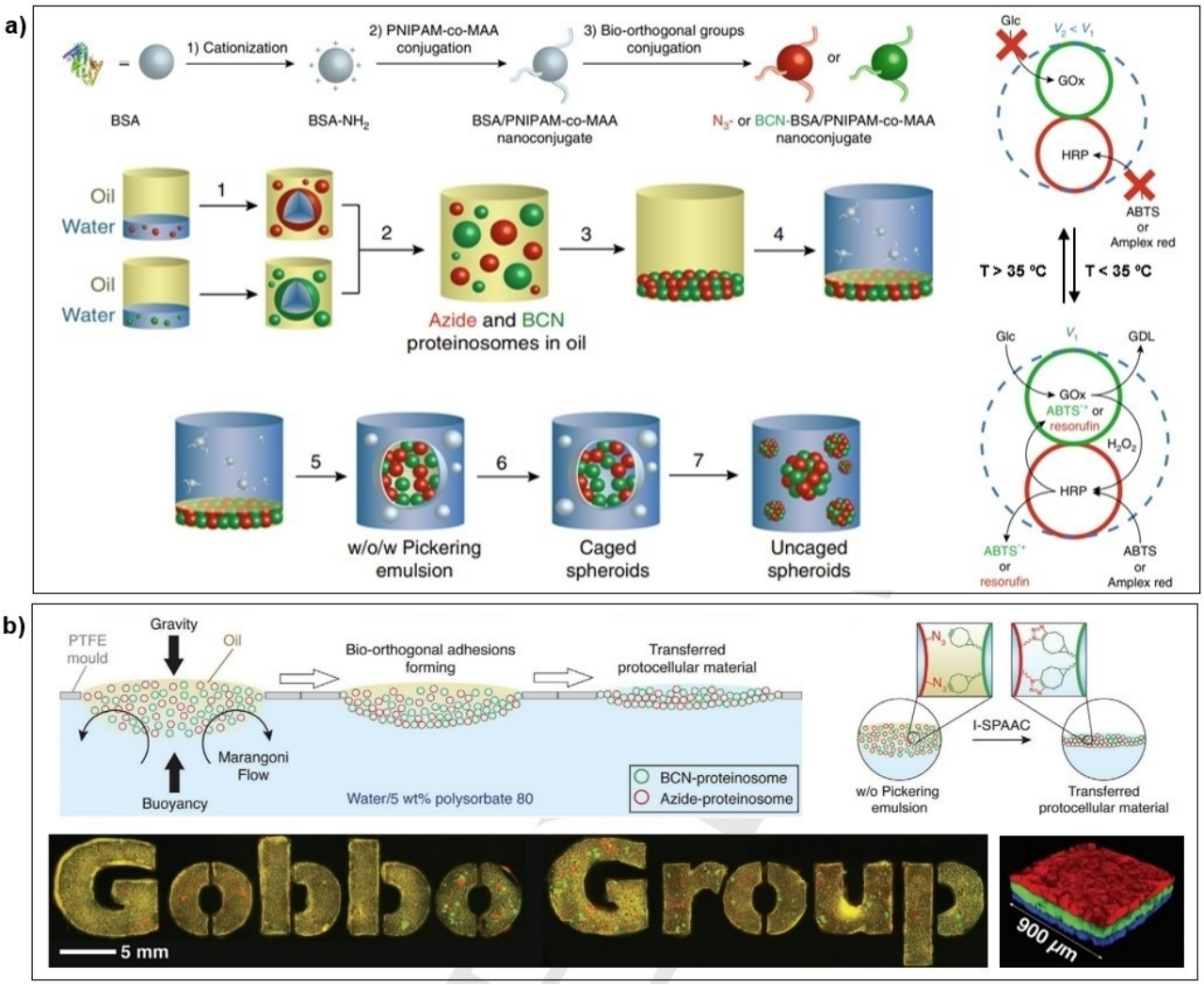


Figure 8. a) The fabrication of prototissue spheroids from *w/o/w* PEs of bio-orthogonal azide- and BCN-proteinosomes. *Top:* Scheme depicting the synthesis of azide- and BCN-nanoconjugates from BSA. First BSA is cationised and conjugated with PNIPAAm-*co*-MAA polymers. MAA groups on the polymer chains are then coupled to the amines of azide- and BCN–PEG ligands to generate the bio-orthogonal nanoconjugates. *Bottom:* Schematic showing the synthesis of prototissues from proteinosomes, by the formation of a double PE *(w/o/w)* and bio-orthogonal ligation. 1) An aqueous solution containing one of the bio-orthogonally functionalised nanoconjugates and a crosslinker was added to an oil phase to generate the chemically crosslinked and bio-orthogonally reactive proteinosomes. 2) 1 : 1 populations of the crosslinked functionalised proteinosomes were prepared. 3) A precipitate of the randomly arranged proteinosomes was formed by co-sedimentation. 4) The oil layer was removed, and an aqueous solution containing non-bio-orthogonally functionalised nanoconjugates was added. 5) Formation of a *w/o/w* PE, which was subsequently transformed into crosslinked spheroids by the addition of a water-soluble PEG crosslinker. 6) The removal of the oil and dispersion of the structure in water initiated the I-SPAAC reaction, bio-orthogonally linking the proteinosomes. 7) The outer proteinosome membrane was cleaved, leaving an uncaged prototissue spheroid. *Right:* Scheme representing coupling of the contractile behaviour to a GOx/HRP enzyme cascade reaction in a prototissue spheroid (blue dashed circle) consisting of GOx-containing alkyne-functionalised proteinosomes (green circles) and HRP-containing azide-functionalised proteinosomes (red circles). In the relaxed prototissue (V_1), glucose (Glc) and ABTS or Amplex red freely diffuse through the system, and are transformed into D-glucono-1,5-lactone (GDL) and green $ABTS^{\bullet+}$ or red fluorescent resorufin, respectively. In the contracted form (V_2), diffusion of the substrates towards the enzymes is hindered, due to hydrophobicity of the contracted prototissue spheroid membrane. Reproduced (adapted) with permission.[21] 2018, Springer Nature. b) *Top:* Scheme illustrating the mould technique used for PCM fabrication. Initially, a 1 : 1 binary population of azide- (red circles) and BCN-proteinosomes (green circles) in oil is cast inside a PTFE mould floating on a solution of polysorbate 80 in water (5 wt%). In this system the PE is subject to: 1) buoyancy, 2) gravity; and 3) Marangoni flow from the centre of the poly(tetrafluoroethylene) mould (PTFE) to the sides and into the bulk solution as highlighted by the curved black arrows. With time the effect of polysorbate 80 and Marangoni flow extracts the oil from the emulsion and brings the proteinosomes in contact allowing them to react *via* an interfacial strain-promoted alkyne-azide cycloaddition (I-SPAAC) reaction and assemble the PCM (top right). *Bottom left:* The generation of complex shape PCMs. Tiled epifluorescence microscopy images showing PCMs comprised of interlinked 1 : 1 binary population of RITC-labelled (red fluorescence) azide- and FITC-labelled (green fluorescence) BCN-functionalised proteinosomes internally crosslinked with PEG-diNHS and composing the "Gobbo Group" logo. *Bottom right:* 3D confocal fluorescence image of a 3-tiered stratified PCM ≈270 μm thick. All layers are composed of an interlinked 1 : 1 binary population of BCN- and azide-functionalised proteinosomes internally crosslinked with PEG-diNHS. Blue fluorescence: Dylight405; green fluorescence: FITC; and red fluorescence: RITC. The 3 PCM layers were perfectly attached to each other, and no delamination was observed. Reproduced (adapted) with permission.[80] 2021, John Wiley and Sons.

using the PE technique described earlier, with the exception that the protein–polymer nanoconjugates were made with BSA/PNIPAAm-*co*-methacrylic acid (MAA) nanoconjugates functionalised with either azides or strained alkyne (BCN) groups.

The azide- and BCN-proteinosomes were then spatially confined by enclosing them within a non-reactive proteinosome membrane, generating a *w/o/w* double PE. Upon removal of the inner oil phase *via* dialysis in ethanol/water, the interfacial azide and BCN groups covalently connected all the caged proteinosomes *via* the I-SPAAC. This formed the prototissue spheroids, which could then be dispersed in a continuous water phase. Significantly, individual prototissue spheroids exhibited reversible changes in volume due to collective contraction/relaxation of the ligated proteinosome units when transitioned above and below the LCST of the PNIPAAm-*co*-MAA. The authors then showed that the contraction/relaxation cycles could be sustained reversibly for tens of cycles at constant frequency and amplitude, and that the process could be switched off during the temperature cycling by internalised enzyme-mediated hydrogelation. Finally, for the first time this article demonstrated a mechanochemical transduction mechanism within a tissue-like material. This was achieved by coupling the emergent reversible contractile behaviour of the prototissue spheroids to the down-/up-regulation of a coordinated enzyme cascade hosted specifically within the I-SPAAC linked proteinosomes.

Recently, Galanti *et al.* made an important scientific breakthrough in this research area by developing a floating mould technique to assemble millions of azide- and BCN-functionalised proteinosomes into macroscopic, free-standing PCMs with complex 3D architectures.[80] In this technique, a poly(tetrafluoroethylene) (PTFE) mould was allowed to float on an aqueous solution of polysorbate 80, and was subsequently loaded with a 50/50 emulsion of crosslinked azide- and BCN-proteinosomes in oil. Due to a surface tension gradient between the bulk aqueous and oil phases contained in the PTFE mould, a Marangoni flow was generated. This effect in combination with the surfactant progressively removing the oil phase, brought the bio-orthogonally reactive proteinosomes in contact to react through the I-SPAAC reaction and assemble a PCM in water (Figure 8b). Significantly, this simple and versatile technique allows not only for the construction of PCMs of any size and shape, but also for the fabrication of stratified PCMs and PCMs with bi-dimensional patterns. These PCMs also displayed communication properties comparable to the prototissue spheroids and could be chemically programmed to sense the external environment for specific chemical triggers to coordinate an endogenous enzymatic metabolic response. These communication properties were then combined with the versatility of the floating mould technique to generate arrays of PCMs capable of collective non-equilibrium biochemical sensing. Particularly, these PCM arrays can allow the study of information encoded in propagating reaction-diffusion gradients of chemicals such as: (i) the direction of the diffusing front, (ii) spatiotemporal changes in chemical concentrations, (iii) estimation of the diffusion rates of chemical species, and (iv) the identification of rate-limiting steps of the PCM bioreactivity.

The generation of tissue-like materials based on the formation of covalent protocell-protocell adhesions through interfacial organic chemistry is remarkable. These paradigm shifting approaches provide unprecedented opportunities to design novel, robust, and multi-functional architectures for tissue engineering, personalised therapy, pharmacokinetics, micro-(bio)reactor technologies, and soft robotics.

4. Conclusions and Outlook

Colloidosomes are microcapsules formed by the self-assembly of amphiphilic colloidal particles at the interface of emulsion droplets. They are functional in a wide range of applications, from drug delivery[5,16b] and analytical chemistry (protein analysis[37] and surface-enhanced Raman scattering[38]), to biocatalysis,[33e,39] photothermal anti-cancer therapies,[40] water-treatment,[4b,41] and for anti-corrosion coatings.[42] In the past decade, colloidosomes have found a new type of application in bottom-up synthetic biology as a protocell model. The success and widespread use of colloidosome-based protocells is due to the flexibility of their design, which allows for the fine-tuning of their physical and chemical properties, yielding protocells with a vast array of biomimetic behaviours. This flexibility includes the type of colloidal material that can be used to assemble these robust colloidosomes and the introduction of specific functional molecules through organic synthesis to provide structural complexity and intricate responses to external stimuli.

4.1. Type of Colloidal Material

Protocell models based on colloidosomes have been successfully synthesised using colloidal particles such as proteins, protein–polymer conjugates, silica, clay, and metal nanoparticles. This wide-ranging use of different colloids yields protocells with a vast array of properties and behaviours. For example, we have discussed how magnetic, Fe_3O_4 nanoparticles could be used to create colloidosomes with a rudimentary phagocytosis behaviour,[15b] or how proteinosomes with membranes composed of enzyme/PNIPAAm nanoconjugates exhibited inherent interfacial, enzymatic activity.[43,72,74]

4.2. Functional Molecules

The various functional molecules and organic transformations that were successfully integrated within colloidosome systems to achieve biomimetic functions include:

1. thermo- or pH-responsive polymers,[45,48,57] Schiff-base chemistry,[29,55a,77] and supramolecular hydrogel assembly[32] to reversibly regulate membrane permeability,
2. molecular photoswitches to both reversibly control membrane permeability[52b] and form sub-protocell compartments,[67]
3. degradable crosslinkers *via* acid-mediated hydrolysis[28] or thiol redox chemistry[32] to add external control over membrane degradation,
4. photochemically degradable crosslinkers to release small-molecule, diffusible signals with spatiotemporal control,[74]

5. bio-orthogonal chemistry to enable covalent protocell-protocell adhesions and to form materials based on colloidosomes as building blocks.[21,80]

In addition to their flexible design, colloidosomes' peculiar permeability properties based on their tuneable MWCO has been exploited to engineer rudimentary models of advanced biological functions, such as inter-protocell communication,[48] protein synthesis,[45] phagocytosis,[15b] protocell growth and division.[15c] Moreover, the versatility of the PE technique also allowed to engineer novel protocell models endowed with specialised sub-compartments, providing basic replicas of organelles in living cells and showing advanced behaviours such as spatiotemporal regulation of enzymatic reactions[65] and information processing.[67]

4.3. Limitations and Opportunities

As has been shown in this review, a broad range of physical and chemical properties and biomimetic behaviours can be achieved by using colloidosomes in bottom-up synthetic biology. Nonetheless, several important challenges remain unaddressed in this field and the organic chemistry community has a crucial role to play in overcoming them.

One key area for development will undoubtedly involve the exploration and adoption of different types of colloids. While a substantial range of colloids has already been adopted for the assembly of colloidosomes (such as silica, clay, iron oxide and protein-based nanoparticles), we are convinced that there are other synthetic systems that can be used for this purpose, such as polymeric nanoparticles (*e.g.*, obtained by polymerisation-induced self-assembly),[83] or carbon nanomaterials.[84] Given that polymeric nanoparticles are potentially easier to functionalise than the existing materials applied so far, their use as colloids could allow for increased variation in the range of colloidosome properties through the addition of functional groups. As we have seen, the exploitation of the vast range of physical and chemical properties possessed by polymers has already allowed for the production of life-like systems. However, we believe that by using polymeric nanoparticles, we could explore more complex and alternative systems, whereas carbon nanoparticles offer different benefits. For instance, their adoption in making colloidosomes could reduce the cost of the starting materials, in turn, allowing for the upscale of their production.

The improvement of control over membrane permeability represents another crucial area for development in colloidosome-based protocell systems. One limitation of colloidosomes as protocell models lies in the fact that selective membrane permeability is based on the passive diffusion of molecular species through the interstices between the colloidal nanoparticles that compose the colloidosome. This restricts the extent to which species can be transported across a membrane. Practically, species that are larger than the MWCO of the colloidosomes' interstices must be encapsulated during the PE stage and are then immobilised inside the membrane and cannot be transported unless the colloidosome is dismantled. In turn, it is not possible to retain low molecular weight compounds inside the protocell lumen. Achieving control of small-molecule permeability would be a great improvement in such a design. This has already been partially addressed by using a macromolecular anchor connected with a photocleavable linker to a low molecular weight signalling chemical species.[74] Using this approach, the small molecule signal can be released with high spatial-temporal control. Moreover, the crosslinked and porous nature of colloidosome membranes also prevents the insertion of transmembrane proteins, which are essential to cell communication and selective transport of biomolecules. By investigating the issue of selective membrane permeability further, the organic chemistry community could make substantial advances to the design of colloidosome-based protocell systems.

Two further limitations that should be highlighted involve the biocompatibility of most colloidosome protocell designs and the challenging achievement of protocell replication. Even though the use of proteinosomes has helped to address the issue of biocompatibility of the colloid used to assemble the protocell, their membranes in most cases are still susceptible to protein unfolding and degradation by proteases. Although protective coatings were utilised to provide protease-resistant colloidosomes, this came at the cost of decreasing membrane permeability.[32,59] Concerning protocell replication, the one and only example demonstrating the replication of silica colloidosomes could only be achieved with non-crosslinked *w/o* emulsions.[15c] Protocell replication must become possible in all aqueous media for it to be applicable in biological environments, or to have any technological application. These challenges are not easy to address, but through the application of organic chemistry significant progress could be made in this area.

Although the field of bottom-up synthetic biology is reasonably new terrain for organic chemists, in this review, we have seen how organic chemistry could offer important developments in the bottom-up synthesis of novel life-like systems based on colloidosomes. There are two crucial areas where progress has already been made. First, the use of different types of colloids, and secondly, the improvement of membrane permeability. However, it is essential that we continue to explore alternative colloids and additional solutions to the problem of small molecule permeability. It is apparent that organic chemists can make significant contributions to this emerging and exciting research field as we work towards more complex, scalable, and advanced life-like systems and towards the development of unprecedented applications, not only in biotechnology but also in environmental and energy science.

Acknowledgements

JHP acknowledges funding from the Ontario Graduate Scholarship. AG acknowledges funding from the European Commission through the project Marie Skłodowska Curie IF "SAPTiMeC" (GAN: 101023978). MSW acknowledges NSERC. SR and PG acknowledge the EPSRC New Investigator Award (grant ref: EP/T01508X/1) and

the University of Bristol EPSRC Industrial and International Leverage Fund.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: Colloidosomes · Life-like systems · Protocells · Surface chemistry · Synthetic biology

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Manuscript received: August 17, 2022
Revised manuscript received: October 19, 2022
Accepted manuscript online: October 27, 2022