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Protocell Communication through the Eyes of Synthetic Organic Chemists

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The bottom-up fabrication of synthetic cells (protocells) from molecules and materials, is a major challenge of modern chemistry. A significant breakthrough has been the engineering of protocells capable of chemical communication using bio-derived molecules and *ex situ* stabilised cell machineries. These, however, suffer from short shelf-lives, high costs, and require mild aqueous conditions. In this Concept Article we analyse the chemistry at the heart of protocell communication to highlight new opportunities for synthetic chemists in protocell engineering. Specifically, we (i) categorise the main bio-derived chemical communication machineries in enzyme cascades, DNA strand displacement, and gene-mediated communication; (ii) review the chemistries of these signal transduction machineries; and (iii) introduce new types of bio-inspired, fully synthetic artificial enzymes to replace their natural counterparts. Developing protocells that incorporate synthetic analogues of bio-derived signal transduction machineries will improve the robustness, stability, and versatility of protocells, and broaden their applications to highly strategic fields such as photocatalysis and fine chemicals production.

1. Introduction

Communication between cells, or cell signalling, is a fundamental requirement for life; even the oldest unicellular organisms are known to have possessed systems designed to sense and respond to changes in their environment.[1] In fact, the ability to send and receive chemical signals may predate the evolution of multi-cellular animals.[2] In general, chemical communication can be seen as the movement of a signalling chemical species (signal) through space from a sender to a receiver. More specifically, in living cells, the sender will release a signal (usually in the form of ions, small molecules, or organelles) that will either passively diffuse through the medium or be actively transported against a chemical gradient (with a concomitant energetic cost) until it reaches the receiver.[3] This starts a process called signal transduction, a series of biochemical reactions involving different biomolecules and organelles within the receiving cell. At the end of this cascade reaction, a response (*e.g.*, either the promotion of a change within the receiving cell or the release of another signal to propagate communication) is triggered.[3a]

By drawing inspiration from Nature, the field of bottom-up synthetic biology has made significant progress towards the development of *protocells*, micro-compartmentalised artificial life-like systems that mimic at least one key aspect of living cells (*e.g.*, chemical communication, information storage, ability of growing and dividing, etc.).[4] To date, several rudimentary protocell models that mimic simple behaviours of living cells have been developed starting from small molecules, materials and chemical reactions. However, obtaining protocells with communication abilities comparable to their natural counterparts remains a considerable challenge. Achieving this can lead to the creation of new bio-inspired out-of-equilibrium technologies, which can have applications in the fields of medicine, material science, and micro-bioreactor technology. As such, recent efforts in the field have focused on the use of bio-derived systems to achieve communication in protocells.[5] This, in general, requires the use of a semi-permeable membrane which, based on a molecular weight cut-off, can hold the transduction machinery inside while allowing small signalling molecules to diffuse in and out freely. This Concept explores the state-of-the-art of the bio-derived transduction machineries used for the communication between protocells. We then discuss novel fully artificial alternative systems that could be used to move life-like systems towards unprecedented paradigms. This will highlight new challenges and opportunities for synthetic chemists.

2. Bio-Derived Transduction Machineries

To date, in general, protocell communication has been implemented by employing three categories of bio-derived communication machinery to emit and transduce chemical

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signals: (i) enzyme cascades, (ii) DNA strand displacement (DSD) reactions, and (iii) gene-mediated reactions.

2.1. Enzyme cascade reactions

Enzyme cascade reactions (Figure 1) are the most common type of bio-derived machinery used to implement protocell communication. The most established system is the glucose oxidase (GOx) – horseradish peroxidase (HRP) cascade, due to the high activity and stability of these two enzymes. In the presence of dioxygen, GOx catalyses the oxidation of glucose to *D*-glucono-1,5-lactone (which then hydrolyses to *D*-gluconic acid) and H_2O_2. The latter acts as a signalling molecule that is used by HRP to oxidise a non-coloured and non-fluorescent molecule to a coloured and fluorescent molecule, whose time-dependent production can be easily detected with a spectrophotometer. This enzyme cascade reaction was successfully used to achieve simple communication between distributed protocell populations,[6] and within tissue-like materials (termed *protot-issues* or *protocellular materials*) formed *via* the controlled assembly of a high number of protocell units.[7] Moreover, this enzyme cascade enabled the study of reaction-diffusion fronts of chemicals propagating through 2D arrays of protocells or protocellular materials.[8] To this classical cascade system it was also possible to add an extra enzyme, which worked in

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Stefano Valente is a synthetic and materials chemist. In 2021 at the University of Trieste (Italy) under the supervision of Prof. Lucia Pasquato he received his PhD in nanotechnology for his work on hybrid nanoparticle systems for theranostics. After one year of post-doc at the Joint Research Centre of the European Commission in Ispra (Italy), in 2022 he joined the group of Prof. Pierangelo Gobbo where he is currently working on the design and development of synthetic protocellular materials and their integration with living cells.

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 Prof. Pierangelo 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 as an EU Marie Skłodowska-Curie Postdoctoral Fellow.

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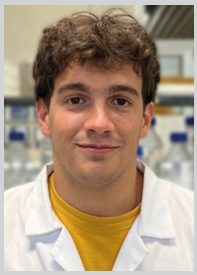

Francesco Vicentini obtained his Bachelor and Master degrees at the University of Rome La Sapienza (Italy). During his Master's degree he studied new organic synthesis pathways via asymmetric organocatalysis. In 2023 he joined Prof. Pierangelo Gobbo group at the University of Trieste (Italy) as a PhD student. His doctoral research is focused on the development of photocatalytic protocells.

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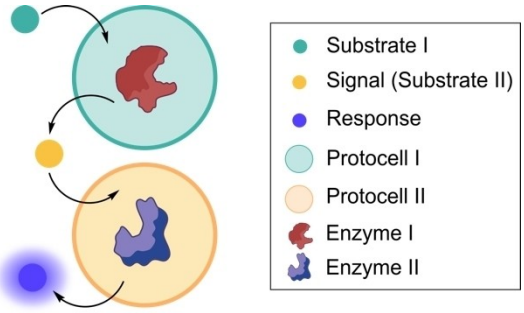

Figure 1. General scheme of enzyme cascade-based communication between two different protocells. The first protocell (green) utilises the substrate for the first enzyme to generate a signalling molecule (small orange circle) for the second protocell. The second protocell is loaded with an enzyme that uses the signalling molecule produced by the first protocell to produce a readable chemical response. In more complex systems, the end cascade reaction product may be used to propagate communication further by acting as a signalling molecule for a third protocell, and so on.

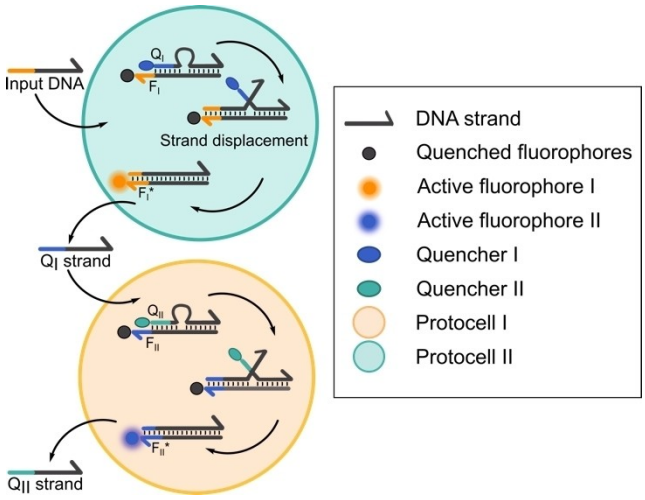

Figure 2. General DNA strand displacement-based communication. The input single-stranded DNA segment displaces the existing DNA strand from the duplex, releasing the previously-bound segment. The latter segment acts as the signal for the second protocell population. DSD cascades are monitored by the addition of fluorophores on the DNA strand with high affinity for the input strand, and quenchers on the displaced strands. Upon addition of the input strand, a fluorescence turn-on is observed, confirming the release of the low affinity DNA strand functionalised with a quencher.

combination with GOx and HRP to catalyse the hydrolysis of polysaccharides (*e.g.*, lactose[9] or starch[6a]). GOx has also been coupled with urease to create an oscillating pH-regulating system[10] or with alkaline phosphatase to polymerise *N*-isopropylacrylamide on the membrane of clay-based *colloidosomes* (protocells whose membrane is composed of colloidal particles).[11]

Protocell communication has also been achieved with other enzyme cascades. For example, Rozhkova *et al.* employed ATP synthase to produce ATP as a result of a light-fuelled proton gradient produced by another population of protocells loaded with bacteriorhodopsin.[12] More complex systems have also been demonstrated, such as a signal amplification mechanism based on an enzymatic cascade employing four different enzymes, Apyrase, Glycogen phosporylase b, Phospho-glucomutase and Glucose-6-PO_4 dehydrogenase.[13]

2.2. DNA strand displacement (DSD) reactions

By exploiting the specificity and predictability of Watson–Crick base pairing, DNA can be used as a powerful tool for protocell communication. To address this challenge, DSD has been implemented within protocells. DSD reactions involve the displacement of a single-stranded DNA segment from a duplex upon the addition of another DNA sequence having higher specificity. In turn, the released DNA segment can be used as a signal in DSD cascades (Figure 2). Joesaar *et al.* implemented this system within *proteinosomes* (protocells whose membrane is composed of a protein-polymer hybrid nanomaterial) to obtain complex DNA circuits capable of logic operations.[14] Yang *et al.* moved this technology further, and successfully used light to trigger the DSD cascades.[15] Light was also used to cause selective aggregation of different proteinosome populations allowing the DSD reaction to occur.[16] Di Michele and co-workers improved on simple DSD-based communication by assembling DNA-based protocells responsive to diffusible chemical signals. These protocells were capable of responding to the addition of DNA strands by forming distinct internal architectures, and establishing complex communication networks with living cells.[17]

Protocell communication can also be achieved by exploiting the specificity of DNA base pairing without involving the displacement of a pre-existing DNA strand. For example, Zembrano *et al.* showed that a population of proteinosomes containing a single strand of template DNA could respond to a signal by synthesising a copy of the input DNA and a response DNA strand. This response DNA strand could then be used as the substrate of an autocatalytic reaction by a second population of proteinosomes.[18]

2.3. Gene-mediated reactions

Gene-mediated communication has been achieved by developing protocells capable of gene expression and relying on the passive diffusion of chemical signals. This allowed protocells to interact with living cells using chemical signals coming from inside the sender protocell[19] or from the environment[20] (Figure 3). For example, Niederholtmeyer *et al.* fabricated intrinsically porous protocells that could communicate *via* the diffusion of proteins or transcriptional activators.[21] This was also done in non-porous micro-compartments using pore-forming proteins. In these works, protocells with a low permeability lipid bilayer membrane were loaded with protein monomers. These monomers were able to assemble into pore-forming proteins at the interface between two protocells, allowing for the diffusion of chemicals through the membrane.[22] These pore-forming proteins could also be synthesised in-situ through light-activated gene expression.[23] Furthermore, it was possible to implement genetic circuits in

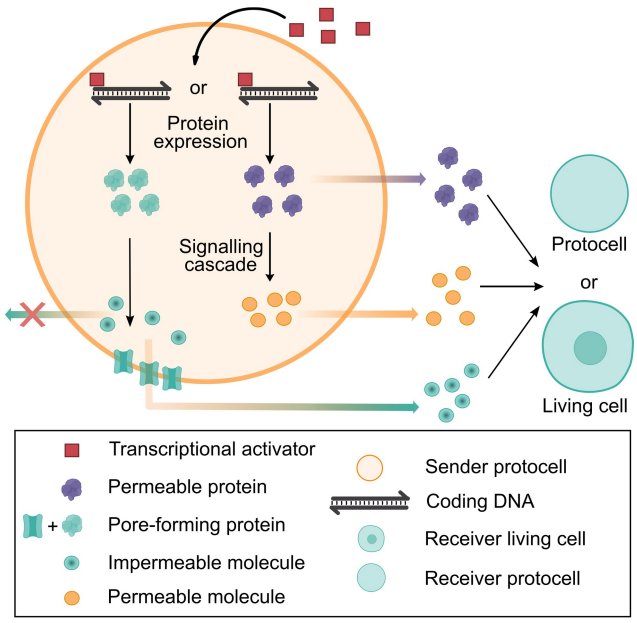

Figure 3. Gene-mediated communication. The incoming transcriptional activator allows the release of membrane-impermeable signals by triggering the expression of pore-forming proteins. Alternatively, the incoming transcriptional activator initiates the expression of a signal which can be sensed by both protocells and living cells either immediately or after an enzyme cascade.

different protocell populations yielding networks of complex signalling cascade reactions.[24] Notably, Lentini *et al.* demonstrated artificial cells capable of both receiving and sending chemical messages to living cells.[25] This was achieved by exploiting bacteria quorum sensing. Quorum sensing is the gene-regulated ability to sense and respond to cell population density mediated by small diffusible signals known as quorum molecules. In brief, Lentini *et al.* developed a type of protocell that through *in vitro* transcription–translation reactions could sense a quorum molecule generated by a first population of bacteria, and in response synthesise and release another quorum molecule utilised by a second population of bacteria. Through this process the artificial cell population was able to mediate communication between two living microorganisms that do not naturally communicate with each other. Enzyme cascade reactions, DSD reactions, and gene-mediated reactions are not mutually exclusive and can be combined to establish multi-step communication between heterogeneous populations of protocells,[26] or between protocells and living cells.[17,25]

3. Towards Bio-Inspired Fully Synthetic Transduction Machineries

While the utilisation of bio-derived machineries allowed for the achievement of important breakthroughs in the design and bottom-up construction of life-like systems, they unfortunately suffer from low shelf-lives, high costs, and can only work under mild operating conditions (*i.e.*, nearly neutral pH and temperatures between room temperature and 37 °C). We believe that the possibility of moving from bio-derived machineries to fully artificial and robust synthetic enzymes (also called *synzymes*) could provide invaluable opportunities in protocell engineering. Synzymes are fully synthetic systems designed to mimic the catalytic behaviour of an enzyme.[27] Synzymes boast higher stability across pH and temperature scales, improved substrate versatility, and lower costs compared to their bio-derived counterparts. Furthermore, they are soluble in a wider range of solvents and, in some cases, display multi-enzyme-like activity.[28] Synzymes can be synthesised from a wide range of starting materials to yield an extensive variety of molecules and materials endowed with enzyme-like activity (Figure 4).[29]

Synzymes can therefore provide unprecedented opportunities to construct enhanced life-like systems with stabilities beyond the physical-chemical conditions of life as we know it on Earth, allowing us to test modern hypotheses on both the molecular origin of life and system chemistry.

A prominent class of artificial enzymes are polyoxometalates (POMs).[30] POMs are metal-oxygen anionic clusters with remarkable reactivity, robust structure, and can have an excellent biocompatibility.[31] Many examples of POMs are reported, mainly composed of early transition metals (e.g., V, Mo, W), but occasionally with other metals (e.g., Mn, Co) as substitutes. Their tuneable chemical properties allow for the catalysis of several reactions (e.g., redox, acid-base, polymerisation), which can be exploited for the generation of complex reaction networks and life-like systems.[32] For example, Bonchio and Hill reported a ruthenium-based polyoxometalate complex, Ru_4POM, capable of performing H_2O_2 dismutation and water oxidation.[33] Recently, Gobbo *et al.* showed that Ru_4POM could stabilise coacervate microdroplets, yielding novel membrane-bounded protocells with catalase-like activity (Figure 5a).[34]

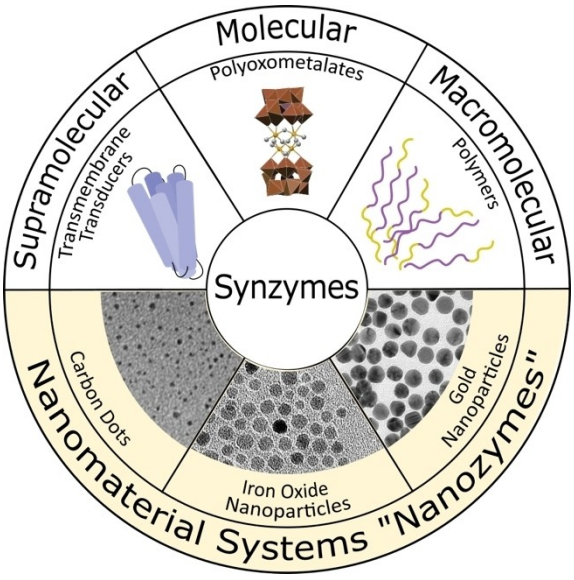

Figure 4. Examples of molecular, macromolecular, nanomaterial, and supramolecular systems that can act as synzymes. Nanozymes are highlighted as an emergent sub-class of synzymes, owing to the large volume and recent prominence of nanomaterial-based synthetic enzymes in the literature.

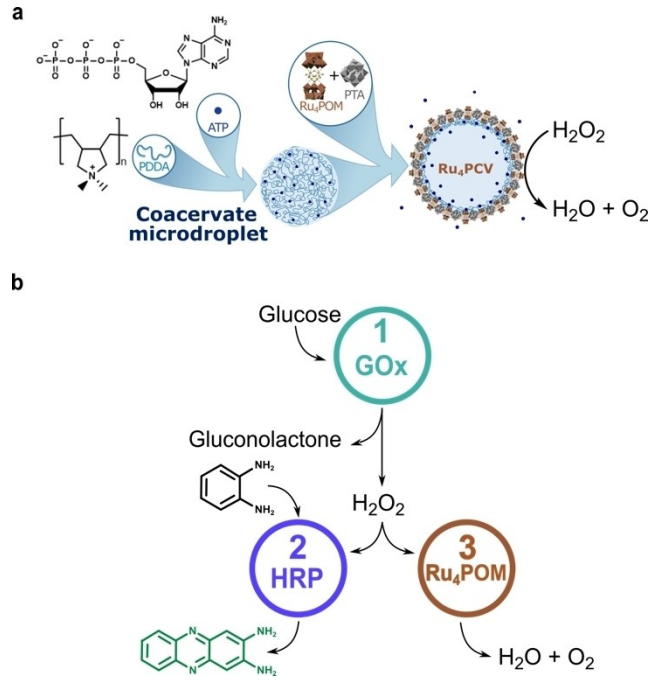

Figure 5. a, Incorporation of ruthenium-based polyoxometalate synzyme (Ru_4POM) into protocells to fabricate protocells with catalase-like activity; **b**, Schematic representation of chemical communication and competition for the signalling molecule within a ternary population of protocells. The first protocell population contains GOx and produces the signalling molecule H_2O_2. The second population contains HRP, and the third population contains the Ru_4POM synzyme. The HRP-containing protocell and the Ru_4POM-containing protocell compete for using the signalling molecule H_2O_2 for their own internalised catalytic processes. Schemes adapted from reference [34].

First, they formed membrane-free coacervate microdroplets by vigorously stirring equimolar aqueous solutions of poly(diallyldimethylammonium chloride) (PDDA) and adenosine triphosphate (ATP). Subsequently they added an aqueous mixture of sodium phosphotungstate and Ru_4POM, which structurally and compositionally reconfigured the coacervate microdroplets into three-tiered membrane-bound vesicles. The formation of the vesicles was the result of the complexation between PDDA and the POMs at the surface of the coacervate microdroplets, followed by the entry of water and the concomitant release of some of the ATP to the bulk solution.[34–35] This constituted the first example of synzyme integration within protocells and its successful exploitation to achieve both protocell communication and competition for a substrate, as shown in Figure 5b. Further implementations of POM-based synzymes inside protocells would greatly expand the versatility of these biomimetic systems, allowing them to mimic increasingly complex natural behaviours.

Polymers capable of acting as synzymes were first reported by Klotz *et al.* They demonstrated that methyleneimidazole-functionalised water-soluble polymers with dodecyl apolar binding sites could imitate enzyme binding sites and efficiently catalyse ester hydrolysis.[36] These fully-synthetic macromolecules are generally more stable than natural enzymes and their functional groups can be exploited to perform specific enzyme-like reactions. Furthermore, since polymers can be easily tuned and functionalised both during and after their synthesis, they are also more versatile alternatives to natural enzymes.[27] Importantly, polymer encapsulation within protocells has widely been reported owing to their high molecular weights.[37] Thus, given the potential intrinsic catalytic activity of some polymers,[36] the step forward is their incorporation and exploitation for engineering a new form of chemical communication.

Nanozymes, nanomaterial-based synzymes, are another prominent class of synzyme that stand out for their emerging features, which arise from their size and can be designed to mimic and replace natural enzymes.[38] Because of this, nanozymes can easily be coupled with other synzymes to replicate complex enzyme cascades. For example, gold nanoparticles (AuNPs) have been one of the first examples contributing to the affirmation of nanozymes because they are non-toxic, recyclable, and easy to prepare and functionalise.[39] Chen *et al.*[40] reported polyvinylpyrrolidone-covered AuNPs and demonstrated their GOx-mimicking mechanism[40–41] (Figure 6). Furthermore, AuNPs can also be incorporated within protocellular structures. For example, Gao *et al.* described caged coacervates surrounded by non-crosslinked Au/poly(ethylene glycol) nanoparticles. This yielded protocells with stimuli-responsive membranes, which could be assembled or disassembled to control the uptake of guest protocells.[42]

Iron oxide nanoparticles (IONPs) can also act as nanozymes to improve protocell communication. Gao *et al.* reported magnetite (Fe_3O_4) nanoparticles with intrinsic peroxidase-like activity, with the most reactive species – the Fe^{2+} ions – being located on the surface.[43] Since both AuNPs and IONPs have been successfully incorporated within protocells, yielding colloidosomes,[44] these examples showcase the possibility of designing nanoparticle-based protocells endowed with a specific enzyme-like activity, which arises directly from the constituent membrane building blocks.

Moving from inorganic catalytic nanoparticles towards organic systems, carbon-based nanomaterials offer a cheaper alternative with higher stability and ease of synthesis from inexpensive organic starting materials.[45] In this regard, for example, Wu *et al.* studied the superoxide-dismutase activity of carbon-based nanomaterials and showed that some exhibit comparable or even better activity to enzymes.[46] Using them in protocells would reduce the assembly cost, making them more suitable for industrial applications.

Within carbon-based nanozymes, Carbon Dots (CDs) have gained increasing interest due to their biocompatibility and enzyme-like reactivity.[39] The latter can arise from either metal doping or by exploiting their surface functionalities (Figure 7),[39] whereas their shell-like structure can be easily tailored to suit specific applications.[47] Since CDs-based nanozymes can mimic the activity of different enzymatic reactions, integrating them into protocells could be the key to achieving biocompatible and inexpensive biomimetic systems capable of replicating enzyme cascade reactions.

In conjunction with synzymes, stimuli-responsive supramolecular systems could be employed to regulate proto-

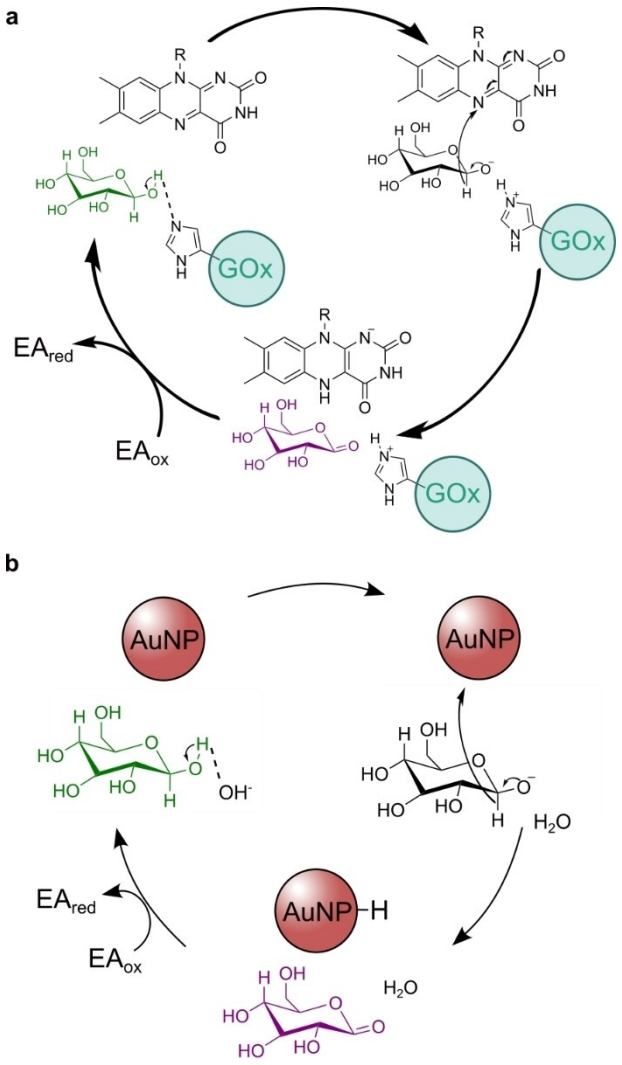

Figure 6. a, Scheme showing the catalytic cycle of the enzyme glucose oxidase (GOx); **b**, Scheme showing GOx-like mimicry by 5 nm polyvinylpyrrolidone-coated AuNPs (red spheres). In both cases, EA = Electron Acceptor in the oxidised (ox) or reduced (red) state. Adapted from reference [40].

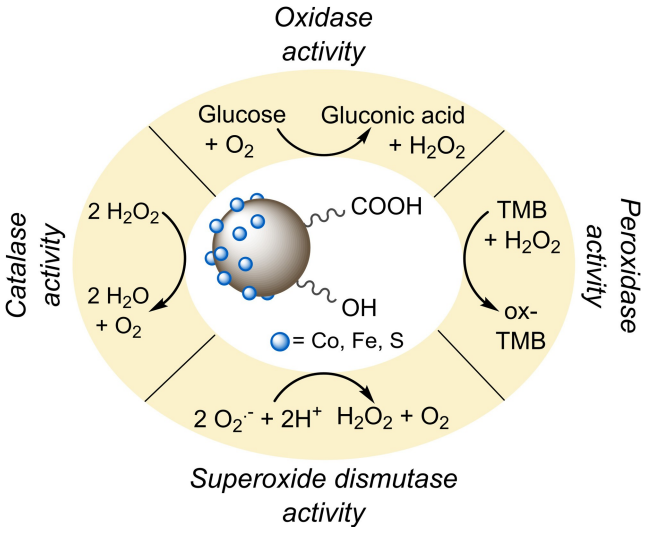

Figure 7. Schematic representation of enzyme mimicry examples performed by CDs either doped or with active surface functionalities.

cell-protocell communication. Supramolecular systems have been widely used in combination with phospholipid-based vesicles to perform signal transduction across the membrane.[48] Through-membrane communication could occur *via* different synthetic systems: transmembrane channels,[49] membrane translocators,[50] and transmembrane signal transducers.[51] Despite many efforts having been dedicated to the development of novel supramolecular systems for transmembrane signal transduction, so far, they have mainly been employed as a proof-of-concept of their operation. One of the future challenges in this field is surely the implementation of synthetic supramolecular systems to regulate protocell-protocell communication towards obtaining higher-order biomimetic behaviours.

The implementation of artificial, fully synthetic transduction machineries into protocells is still in its infancy. While much research has been carried out towards the development of membranes that allow protocell communication using bio-derived machineries,[4] the incorporation of synzymes in protocell models and their exploitation for chemical communication will require the development of new methodologies. For example, for synzymes that require being retained within the interior of the protocells, membranes with tuneable molecular weight cut-off may be better suited. Alternatively, synzymes may be used as the building blocks of protocellular membranes, as seen with POM-based coacervate vesicles.[34] The latter approach may yield less permeable protocells in which the signal is transduced without the need of permeating through the self-assembled structure.

Finally, while synzymes offer many benefits over the bio-derived machineries, these may not come without drawbacks. Specifically, fully artificial organic systems may present solubility limitations and be challenging to incorporate into aqueous systems. They may present a low biocompatibility and display a lower catalytic activity and/or specificity compared to natural enzymes. All these limitations represent challenges of bottom-up synthetic biology that need to be addressed to achieve fully functioning artificial protocells.

4. Summary and Outlook

Engineering forms of chemical communication between protocells has been one of the major challenges of bottom-up synthetic biology. So far, protocell communication has been achieved through the use of bio-derived transduction machineries. Successful enzyme cascade, DSD and gene-mediated types of chemical communication have represented a major stepping stone in the field, with examples showcasing methodologies of integrating these bio-derived systems within protocell structures. However, these bio-derived systems generally suffer from low stability and require narrow and specific ranges of pH and temperature to operate, limiting therefore the technological applications of the resulting communicating protocells.

With this Concept we aimed to highlight novel opportunities in protocell engineering for the synthetic chemistry

community. Specifically, the recent development of synzymes and the possibility of incorporating them within protocells offer the opportunity to expand beyond the limits of biological systems, simultaneously providing robust and tuneable tools to advance forms of chemical communication between protocells. The wide range of bio-inspired and synzyme-based signal transduction machineries discussed above provides a novel conceptual framework that bridges the fields of synthetic biology, nanotechnology, and synthetic chemistry in an original and synergistic manner.

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Conflict of Interests

The authors declare no conflict of interest.

Keywords: artificial enzymes · nanozymes · protocells · signal transduction · synthetic biology

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