



Membrane functionalization in artificial cell engineering

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Abstract

Bottom-up synthetic biology aims to construct mimics of cellular structure and behaviour known as artificial cells from a small number of molecular components. The development of this nascent field has coupled new insights in molecular biology with large translational potential for application in fields such as drug delivery and biosensing. Multiple approaches have been applied to create cell mimics, with many efforts focusing on phospholipid-based systems. This mini-review focuses on different approaches to incorporating molecular motifs as tools for lipid membrane functionalization in artificial cell construction. Such motifs range from synthetic chemical functional groups to components from extant biology that can be arranged in a 'plug-and-play' approach which is hard to replicate in living systems. Rationally designed artificial cells possess the promise of complex biomimetic behaviour from minimal, highly engineered chemical networks.

Keywords Artificial cell · Synthetic biology · Lipid vesicle · Responsive membrane · Compartmentalization

1 Synthetic biology: top-down versus bottom-up?

The past decade has seen accelerating progress towards an ambitious scientific goal—the development of a minimal cell. Two main approaches have been devised to achieve this: the top-down perspective aims to reduce the genetic composition of a single-celled organism until it contains the minimal number of genes necessary for cell survival [1, 2] whilst a second, alternative method has centred on the assembly of a minimal or artificial cell (AC) from its molecular building blocks. In such a bottom-up approach, reconstitution of aspects of cellular structure and function enables the study of specific processes decoupled from the complexity of biological systems, which to date has led to a better understanding of processes including membrane fusion [3] and fission [4]. Beyond generating fundamental understanding, the functions and behaviours

of engineered ACs (e.g. chemical/biochemical production, triggered release, information processing) have the potential to be utilised in applications across biotechnology.

Whilst the approaches of top-down and bottom-up synthetic biology possess many differences, each lead to the fundamental question: *what is a living system?*

The chemoton model [5] proposed by Gánti defines that for a system to be 'alive', it needs three components; a boundary that creates a separate chemical space separate from its environment; an information system or template and a basic metabolism. Whilst this model encapsulates the key physical components of cellular life, it has been argued that this framework can be restrictive and lead to an overly narrow definition due to its focus on existing Earth-based biology [6]. One alternative currently adopted by NASA as a working definition of life proposes that 'life is a self-sustaining chemical system capable of Darwinian evolution' [7, 8]. A third model proposed to adapt Turing's

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imitation game [9], defining the lifelike nature of an AC by how successfully the AC can mimic the behaviour of a living system [6]. Such a test has recently been implemented to test the efficiency of quorum sensing in a vesicle-based AC [10], indicating that such behavioural approaches may be especially useful until a universal definition of life has been reached.

Returning to the three-component chemoton model, the greatest amount of research to-date has focused on the structure and form of the boundary compartment. Due to the rapid development of different AC architectures and the central role of cell membranes in coordinating biological processes, this mini-review focuses on recent advances in lipid membrane construction/functionalization in bottom-up synthetic biology, and how these advances can be coupled with developments across biology, chemistry and nanotechnology.

2 Constructing an artificial cell chassis

A wide variety of materials have been used to create semi-permeable membranes, including phospholipids found in cell membranes [11, 12], synthetic polymer-protein conjugates that assemble at oil–water interfaces [13, 14], amphiphilic block co-polymers that form robust membranes [15–17], emulsion microdroplets stabilised via colloidal silica [18] and particles assembled layer-by-layer from oppositely charged polymers [19–21]. Membrane-free systems have also been utilised, including liquid–liquid phase separated coacervates [22, 23] and the use of microfabrication methods to define the cell boundary [24–26]. Whilst microfabricated boundaries possess the advantage of high spatial control of content the molecular components of the AC cannot easily be decoupled from its fabricated chassis.

Although a multitude of approaches have been developed over the last decade, phospholipids and fatty acids still represent the most commonly used structural molecule for building ACs. This is unsurprising when we consider their central role in the development of the cell. Fatty acids are often used in protocell studies which aim to mimic the prebiotic conditions of the early Earth, and the development of the first cell membrane [27]. Whilst these systems will not be discussed here due to their low stability in complex media, interested readers are directed to a number of excellent recent reviews on the development of protocells [27, 28].

Long-chain phospholipids are biocompatible and self-assemble into varied structures at extremely low (nM) concentrations [29] driven by the hydrophobic effect [30] (Fig. 1a). Many diacylglycerol phospholipids have been used to construct lipid vesicles in aqueous solution

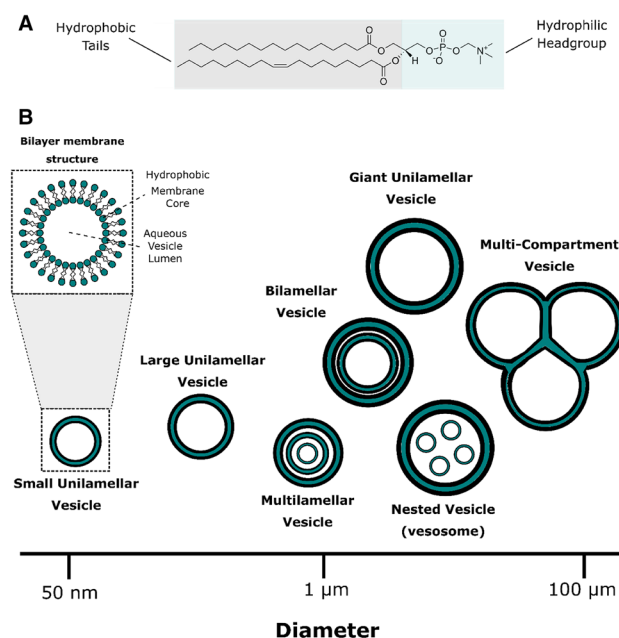


Fig. 1 Engineering vesicles across different length scales. **a** Structure of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine, a typical phospholipid. Lipid self-assembly into supramolecular structures is driven by the hydrophobic effect, minimising the interactions between hydrophobic tails and aqueous solution. **b** Vesicles can be generated from ~50 nm to ~50–100 μm. Traditional techniques such as sonication or extrusion can process hydrated multilamellar vesicles into large and small vesicles, whilst techniques such as electroformation and emulsion phase transfer can generate giant vesicles on the microscale. Smaller vesicles can be encapsulated inside to form nested vesicles, whilst bilamellar vesicles can be formed through sequential phase transfer processes. A cross-section of vesicular structure can be observed top left highlighting the bilayer structure wrapping around an aqueous lumen. Molecular content can be encapsulated within this lumen or within the hydrophobic core of the membrane depending on the physico-chemical properties of the encapsulant

encapsulating an aqueous core. The increased stability of lipid vesicles compared to fatty acid vesicles makes them more suitable for use in applications such as drug delivery as well as structural elements of ACs, which often require complex media for the successful functioning of biological components. Lipid self-assembly has been studied for over 50 years [31, 32], and from this work multiple production techniques have been refined that enable the user to create vesicles of controlled size and lamellarity (Fig. 1b). Nanoscale ‘large’ unilamellar vesicles (LUVs) can be assembled through thin-film hydration and extrusion [33] or solvent-based approaches [34] or hydrated vesicles can alternatively be processed below 100 nm through sonication to form ‘small’ unilamellar vesicles (SUVs) [35], whilst cell-size ‘giant’ unilamellar vesicles (GUVs) can be assembled via electroformation [36] or emulsion phase-transfer [37–39].

Electroformation uses the application of an alternating electric current to swell and bud off GUVs from a hydrated lipid film contained between two indium tin oxide (ITO) plates [36]. Whilst electroformation has been used extensively in biophysical studies to create vesicles with differing lateral phase behaviours [40–42], the mechanism of vesicle budding in electroformation prevents the encapsulation of large molecular-weight content, making it less suited for the development of lipid-based ACs as discussed below.

Emulsion-based methods are more promising for cell construction, offering users the ability to generate ACs with monolayer or bilayer compartment structure. Lipid- (or surfactant-) stabilised water-in-oil (w/o) droplets generate containers where the monolayer interface acts as a barrier, isolating water-soluble cargo within the droplet. Droplet systems have been utilised to perform chemical and biochemical assays in high-throughput microfluidic formats [43]. If two monolayer-stabilised w/o droplets are brought together, the monolayers will combine upon contact to form droplet-interface bilayers (DIBs) (otherwise known as the contracting monolayer method) [44, 45]. AC models can be created in droplets as well as in DIBs, using the lipid monolayer to demarcate the boundaries of the cell [46]. In DIB networks, each DIB acts as a semi-permeable membrane that allows the transport of molecular information between droplet compartments, opening the possibility for the construction of artificial tissues with controlled information pathways [47–49].

If lipid-stabilised microemulsions are transferred across a second w/o interface, GUVs are readily formed in a process known as emulsion phase-transfer (EPT) [37]. One major advantage of the EPT method compared to electroformation is its ability to form asymmetric lipid membranes more akin to biological systems [50]. This increases the biomimicry and functionality of the AC by controlling the structure of each monolayer individually, enabling the production of vesicle bilayers with different physicochemical properties and molecular interactions on each face. One downside however is the use of oil in the production process. Whilst vesicles produced via EPT form stable membranes and can be used to reconstitute membrane pores such as the *Staphylococcus aureus* toxin alpha hemolysin (α HL) [51–53], the amount of oil included in such membranes varies [37, 54] and is yet to be quantified. This contrasts with electroformation where the aqueous production method ensures oil contamination of GUV membranes cannot occur.

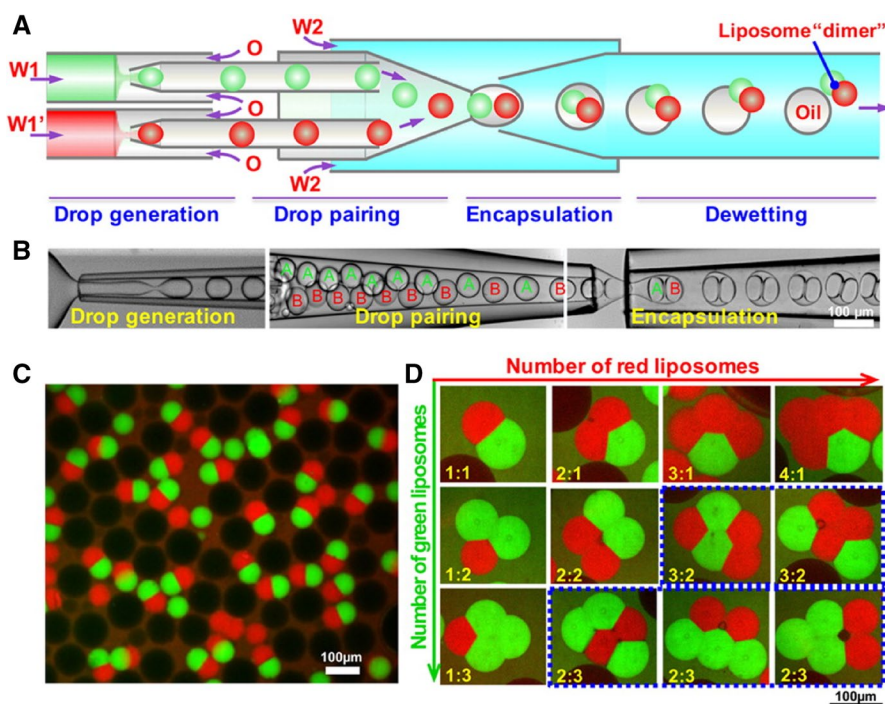
The other primary advantage of EPT over electroformation is in its encapsulation behaviour. EPT is able to encapsulate water-soluble molecules size-independently with very high encapsulation efficiencies [37], whilst electroformation struggles to obtain such levels due to the mechanism

of vesicle production. Bilayer swelling is reported to occur outwards from the multilamellar lipid sheets during electroformation [55], preventing the permeation of large and/or charged compounds. This advantage is extremely significant when building an AC as multiple components need to be encapsulated simultaneously with greatly differing molecular weights. This could include anything from small co-factors to ribosomes to plasmid DNA (< 1 kDa, ~ 2,600 kDa [56] and ~ 3–60,000 kDa [57] respectively). Successful reconstitution of cell-free expression has been carried out numerous times in GUVs generated via EPT which is testament to the high encapsulation efficiencies across a wide range of molecular weights [11, 58, 59], boding well for the future production of ACs with increased complexity.

Smaller vesicles can also be utilised as components in an AC [60]. Enveloping vesicles within such a chassis creates nested vesicles (also known as vesosomes), which show promise in triggered release applications where the external lipid membrane acts as a boundary preventing degradation and content loss from the encapsulated vesicles within [61, 62]. Such structures can be quickly generated via EPT and possess considerable potential as cell mimics with the encapsulated vesicles functioning as artificial organelles. One disadvantage to using nested vesicles as AC models lies in the poorly defined spatial organization of internal compartments. Encapsulated vesicles can also lack connectivity between compartments, however this can be overcome through membrane functionalization as discussed below.

Beyond its encapsulation abilities, EPT has also been used to create microscale ACs with varying numbers of compartments [52, 63]. Such multi-compartment vesicles (MCV) can be created through the deposition of multiple lipid-stabilised w/o droplets that subsequently create GUVs linked by a single bilayer membrane. This approach yielded vesicles with multiple compartments, and an enzyme cascade was successively segregated within each internal space of a three-compartment vesicle [63]. Similar multi-compartment vesicles have been created from w/o/w double emulsions using microfluidics, where the vesicle size and compartment number can be tightly controlled [64]. This was achieved through the use of an additional surfactant, which modulates the interfacial energy and helps control the dewetting of MCVs from the oil phase. Using this approach MCVs of varying compartment number could be controllably produced (Fig. 2). Such microfluidic approaches reduce the difficulty in MCV assembly associated with manual liquid handling as well as increasing the throughput of vesicle production.

Fig. 2 Forming multicompart-ment vesicles via microfluidics. Multi-compartment vesicles are controllably formed through the co-encapsulation of two different droplet species into w/o/w emulsions. Addition of Pluronic F-68 to aqueous solution triggers de-wetting of the oil from the droplets, generating oil-free multi-compartment vesicles with controlled compartment number. Reprinted with permission from [64]. Copyright 2016 American Chemical Society



3 Using molecular motifs to drive artificial cell function

Advancements in membrane engineering have led to the creation of different molecular architectures that can be constructed and combined to form higher-order structures [47, 63, 65, 66]. After these significant developments, new questions arise: *how can the cell architecture be networked with the other components of the AC*, and *how can the cell chassis be incorporated as an active element in cell function*?

Whether considering the creation of artificial cells or ‘smart’ soft materials for application, in each case the construct needs to become programmable. This can be achieved at a molecular level using chemical, physical or biological motifs, including the use of different chemical functional groups embedded in the cell mimic, functionalising the cell with the repertoire of biomolecules found in nature or alternatively, using the phase behaviour of lipid membranes as responsive elements in an artificial cell wall. Thinking more ambitiously, one could imagine engineering hybrid systems which use whole cells or organelles as components of a complex material [67–69].

Integrating molecular motifs into lipid membranes is a powerful way to generate artificial cell function (Fig. 3). Many efforts have focused on generating synthetic lipids that provide drug delivery formulations with a specific trigger that can respond to disease states in vivo, including pH [70, 71], enzymes [72–75], temperature [76, 77] or redox

state [78]. These can be repurposed as elements in artificial cells, where instead of releasing a drug in response to a disease trigger, the membrane acts as a responsive part of a larger ensemble. Alternative motifs such as nanoparticles or polymerizable lipids can be included to generate vesicle-based systems that respond to exogenous stimuli such as light [79, 80], ultrasound [81] or applied magnetic fields [82, 83].

In each case, the responsive element can act reversibly or irreversibly. Many nanomedical triggered-release systems are irreversible, designed to release their entire payload over time [84]. Examples of reversible systems are rarer, generally focusing on the response of the membrane to transient heating events. This includes gel-phase liposomes that have been shown to leak cargo as they undergo a phase transition from the gel to fluid states at a characteristic melting temperature (T_m) [85], and more recently three-component (ternary) compositions were shown to possess content release properties dependent on the mixing temperature (T_{mix}) of the formulation [86]. Leakage occurs at these temperatures due to incompatibilities in membrane packing and hydrophobic matching at forming/melting domain boundaries [87], temporarily increasing membrane permeability.

Once activated, some functional groups act by destroying the entire membrane [88] whilst other create pores in an otherwise stable membrane [79, 89, 90], facilitating the size-selective passage of solutes across the membrane. When considering derivatisation of vesicles for building

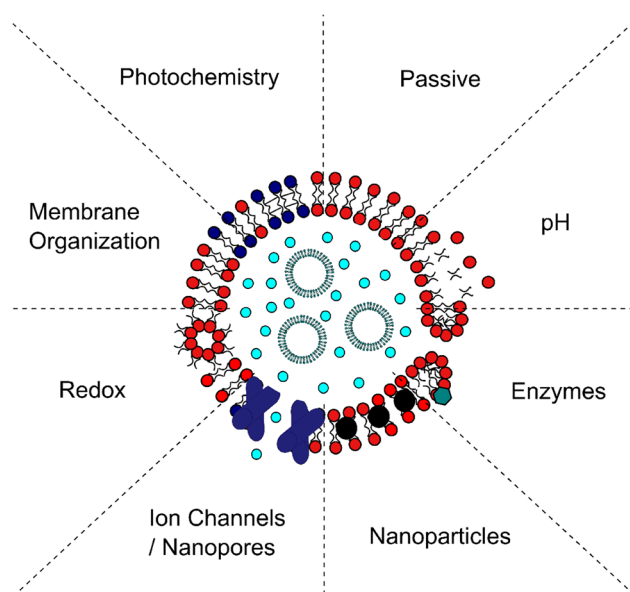


Fig. 3 Generating responsive artificial cells using molecular functionalization. A variety of molecular motifs can be used to generate stimulus responsive permeability changes in lipid ACs. This includes; tailoring the lipid composition to respond to enzymes such as phospholipases; tailoring the pKa of lipids to fashion pH-response; the inclusion of nanoparticles to generate optically- or magnetically-induced membrane fusion; ion channel or DNA nanopore reconstitution to generate chemical/mechanical-response; dithiol reduction to modulate membrane composition and hence permeability; lateral phase separation or gel-liquid melting transitions to generate membrane discontinuities at composition-dependent temperatures and photopolymerisation to trigger temporary membrane defects. Vesicle membranes are encapsulated alongside small molecules to illustrate that functional membranes can be incorporated to trigger intra-cellular events as well as content exchange across the external membrane of the AC

artificial cells, an ideal system would provide the transient generation of nanopores in a stable membrane. This enables intra- and inter-cellular communication through content mixing without destroying the benefits of compartmentalization within the artificial cell. This is particularly true when considering functionalisation of the external boundary of the cell, as membrane breakdown here will lead to loss of cell contents (and hence cell function).

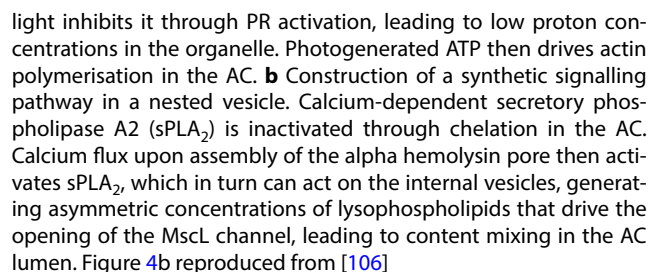
One example of a nested vesicle that uses membrane biophysics to control reactor function was developed by Vogel and co-workers [90, 91]. In this work, triggered release of different enzyme substrates occurs from two populations of encapsulated vesicles that are activated at successively increasing T_m , triggering reactor function in a programmed fashion [91]. One disadvantage to using temperature for control of chemical reactions in ACs is the lack of orthogonality: in order for reaction 2 to activate, reaction 1 must also be activated. The same disadvantage is present for other endogenous stimuli such as pH; such stimuli are generally best used to control

singular processes in ACs. Alternatively, exogenous stimulus response (e.g. light) enables selective activation of different processes leading to the creation of vesicle modules capable of orthogonal function. Optical control over AC reaction processes has been recently demonstrated by various laboratories, from the use of diacetylene photopolymerisation for controlling enzyme catalysis in nested vesicles [92] to using photocaged RNA polymerase to control cell-free transcription-translation in a minimal tissue [48].

Purified components of biological systems represent another rich source for generating functional membranes [93–96]. Proteins represent an obvious candidate for vesicle functionalisation due to their ubiquitous presence across biology. In one example, synthetic chemistry was combined with enzyme catalysis by Devaraj and co-workers to generate vesicle in situ [97]. In this work, the soluble enzyme FadD10 was used to produce adenylated fatty acids that react with amine-functionalised lysolipids, generating membrane forming phospholipids. Such efforts highlight how synthetic chemistry can be used to reduce the number of biochemical steps necessary for function (1 catalytic step in the minimal system versus 3 in the Kennedy cycle [98]) whilst maintaining the same functional effect (in this case phospholipid production and membrane growth).

Another set of proteins studied in bottom-up systems is the Min system from *Escherichia Coli*. The system consists of MinC, D and E which together positions the bacterial cell division machinery at the centre of the cell [99]. The Schwille group has successfully reconstituted MinD and E into surface-attached lipid bilayers (SLBs) [100], micro-emulsion droplets [101] and giant vesicles [102] in order to study system function ex vivo. This enabled observation of oscillatory protein complex assembly at membrane interfaces, generating multiple oscillatory behaviours in giant vesicles including ‘breathing’, ‘circling’ and sphere to dumbbell morphological changes based on vesicle size and encapsulated protein concentration [102]. The Min system represents an excellent example of how a single two-protein complex can dynamically affect membrane morphology, whilst simultaneously showing the utility of model membrane systems in understanding fundamental cell biology.

The assembly/disassembly of the Min complex is just one of many energy intensive processes essential for biological homeostasis. The generation of energy in artificial cells is therefore essential for long-term cell function. Inspired by photosynthetic energy generation found in nature, light-responsive artificial cells have been generated using different photosynthetic reaction complexes to functionalise membrane compartments [103–105]. Recent work by Shin and Parker used a nested vesicle containing



bottom-up synthetic biology over genetic engineering approaches: the ability to ‘plug-and-play’ with molecular components. By taking PSII from plants and PR from bacteria/archaea, two photoconverters have been combined that do not normally exist together in biology [104]. Another example of the ‘plug-and-play’ approach was recently developed by our laboratory, combining detergent-mediated reconstitution of membrane proteins with EPT to generate a nested (vesosome) system containing a synthetic signalling pathway (Fig. 4b) [106]. This pathway was constructed by encapsulating a previously developed interaction between secretory phospholipase A2 (sPLA₂) and vesicles functionalised with the mechanosensitive channel of large conductance (MscL) [107] to generate a nested system. sPLA₂ activity was inhibited via calcium chelation, and the pathway was completed by functionalising the external vesicle membrane with the alpha hemolysin membrane pore. This allowed the nested vesicles to respond to an external calcium flux through sPLA₂ activation and subsequent sPLA₂-M-MscL

communication, leading to increased AC fluorescence upon the release of a self-quenching calcein dye through activated MscL channels. Whilst further developments to the engineered pathway are necessary to produce ACs that can respond to specific molecules in the local environment, such systems represent a new tool for controlling AC processes and highlight the potential of plug-and-play approaches for de novo pathway design in ACs.

4 Future perspectives: interfacing functional membranes with biology and chemistry

As detailed above, a wide variety of approaches exist to create functional lipid membranes that can be exploited in AC design. The biomimetic nature of such lipid systems facilitates active protein reconstitution [108] as well as a high degree of biocompatibility [109], enabling membranes to be interfaced with (and generated by) biochemical machinery such as cell-free transcription-translation systems obtained from the lysates of living cells [110]. This allows the creation of ACs with the ability to express proteins in the cell lumen [11, 111–113], and has been exploited in a variety of contexts as detailed by a recent review [114].

Unlike living systems which rely on proteins for almost all functions, ACs are readily compatible with the wide range of molecules generated by synthetic chemistry and nanotechnology. These include synthetic block polymer ion channels [115] and DNA origami nanopores [116, 117] to replace the use of membrane protein pores, the creation of hybrid copolymer-lipid membranes for patterning and increased membrane stability [118, 119], nucleic acid cytoskeletons [120], synthetic molecules capable of signal transduction across the AC membrane without the use of protein components [121, 122] and nucleic acid strand displacement networks to program cell functions [123–126]. One future challenge to be considered if using synthetic molecules instead of biologically derived components in ACs is how to integrate regeneration of such components in situ without destabilising the cell itself. Solutions could be found by using green chemistry [127] as well as protein engineering approaches [128] to create new biosynthetic routes to abiotic molecules initially produced in the chemistry laboratory.

The next step in the development of ACs will require the researcher to creatively combine elements from the vast range of molecular motifs available to generate robust, switchable functions that can operate in increasingly complex environments. By employing frameworks from systems chemistry [129–131], molecular networks capable of the complex behaviour displayed by genetic circuitry

operating in living systems [132] could be utilised in ACs. This would enable the creation of ACs with longer operating times capable of multiple activation cycles. In order to achieve this, developments in engineering AC architecture and replication [133, 134] needs to be married with significant developments in generating a protometabolism that enables cell regeneration. Just as it has evolved in living systems [135], it seems likely that membrane organization and composition in ACs will play a vital role in conducting the multiple processes necessary for life-like function.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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