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Review

Synthetic Biology: Bottom-Up Assembly of Molecular Systems

Stephan Hirschi,* Thomas R. Ward, Wolfgang P. Meier, Daniel J. Müller, and Dimitrios Fotiadis*

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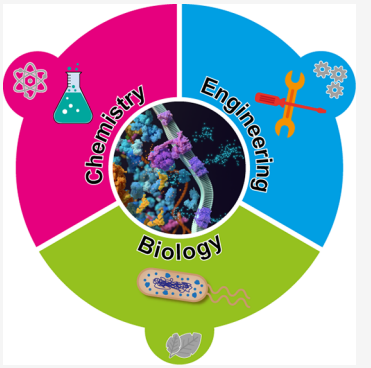
ABSTRACT: The bottom-up assembly of biological and chemical components opens exciting opportunities to engineer artificial vesicular systems for applications with previously unmet requirements. The modular combination of scaffolds and functional building blocks enables the engineering of complex systems with biomimetic or new-to-nature functionalities. Inspired by the compartmentalized organization of cells and organelles, lipid or polymer vesicles are widely used as model membrane systems to investigate the translocation of solutes and the transduction of signals by membrane proteins. The bottom-up assembly and functionalization of such artificial compartments enables full control over their composition and can thus provide specifically optimized environments for synthetic biological processes. This review aims to inspire future endeavors by providing a diverse toolbox of molecular modules, engineering methodologies, and different approaches to assemble artificial vesicular systems. Important technical and practical aspects are addressed and selected applications are presented, highlighting particular achievements and limitations of the bottom-up approach. Complementing the cutting-edge technological achievements, fundamental aspects are also discussed to cater to the inherently diverse background of the target audience, which results from the interdisciplinary nature of synthetic biology. The engineering of proteins as functional modules and the use of lipids and block copolymers as scaffold modules for the assembly of functionalized vesicular systems are explored in detail. Particular emphasis is placed on ensuring the controlled assembly of these components into increasingly complex vesicular systems. Finally, all descriptions are presented in the greater context of engineering valuable synthetic biological systems for applications in biocatalysis, biosensing, bioremediation, or targeted drug delivery.



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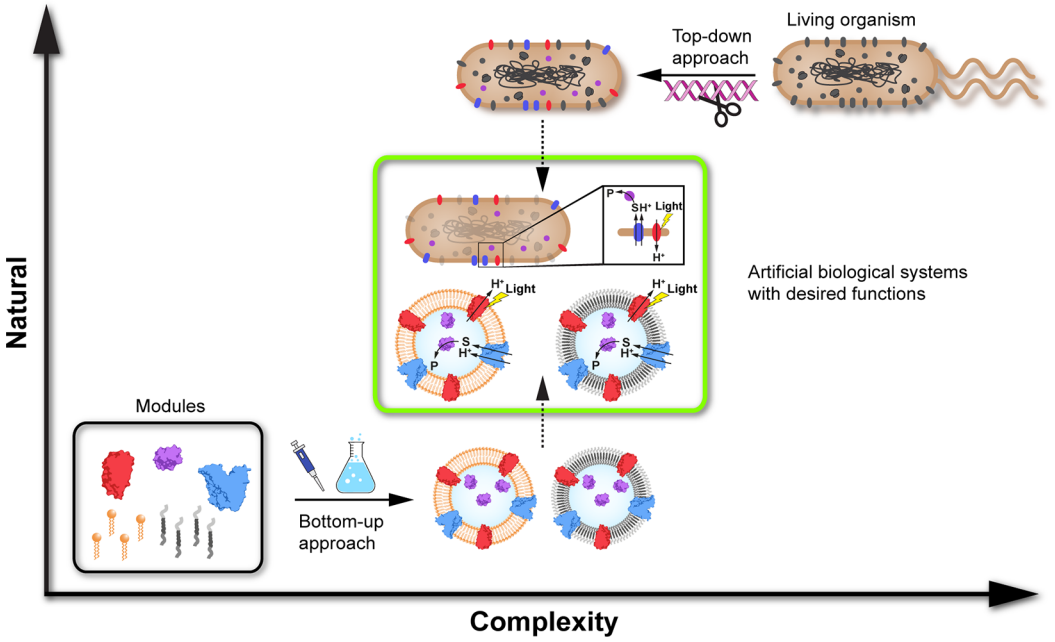

Figure 1. Schematic depiction of the core concepts in synthetic biology. Artificial biological systems with desired functions (green box) can be created by using two main methodologies. The top-down approach focuses on the modification of living organisms, usually through introduction of artificial elements by genetic engineering, whereas the bottom-up approach involves the combination of isolated biological and synthetic modules. The latter can include, but are not limited to, soluble (purple) or membrane proteins (red and blue) as functional modules and lipids or block copolymers (brown and gray) as scaffolds for vesicular systems. The example used to illustrate this concept is a simple reaction system including a light-driven proton pump (red), a proton-driven symporter (blue) that imports a specific substrate (S) using the established proton gradient, and an enzyme capable of converting the imported substrate into a desired product (P). These modules are either genetically introduced into a simplified host organism (top-down) or assembled from isolated components into functionalized liposome or polymersome systems (bottom-up).

1. INTRODUCTION

Synthetic biology is an interdisciplinary field striving to engineer artificial biological systems, which mimic selected cellular traits and processes or exhibit completely novel functionalities not found in nature.[1] The first documented use of the term "synthetic biology" in scientific literature was a description of genetically engineered bacteria in 1980.[2] In the past, its intended meaning was limited to describing the genetic manipulation of organisms and was mainly used as a synonym to bioengineering. With the progress of science and technology, the scope and toolbox of biology and chemistry complemented each other with their individual engineering approaches. The focus of chemists expanded from using synthetic molecules not only to mimic the behavior of biological components but also to integrate them into living systems to investigate, change, redesign, or control them.[3] The classical biological approach of analyzing natural processes by reduction and simplification was complemented by the engineering ideology of construction and combination. Together, these advances led to the multifaceted field of synthetic biology we know today. The diverse aspects and fields of synthetic biology can be broadly categorized into two themes: (i) the top-down approach, manipulating existing natural systems, and (ii) the bottom-up approach, assembling artificial biological systems from isolated components (Figure 1).[4] Both approaches focus on creating some type of artificial cell or cell-like system, which describes a range of constructs from minimal cells to particles that mimic only certain cellular traits, such as protocells or even completely artificial, nonbiomimetic cells.[5] Artificial vesicular systems, *i.e.*, vesicles prepared from natural or synthetic membrane-forming amphiphiles, provide the basis to assemble such systems from the bottom up. They are frequently used as models for simple protocells,[6] *e.g.*, to study the origins of life, and as biomimetic membrane systems to investigate the transfer of substrates or information between cellular compartments involving transmembrane proteins.[7−10] Furthermore, artificial vesicular systems such as proteoliposomes and -polymersomes, formed from either lipids or block copolymers, are appealing platforms for the discovery of new drugs targeting membrane transport proteins and receptors in a membraneous environment without the interference of a multitude of cellular factors.[11−13] In addition to their significant contributions to basic research, liposomes and polymersomes have gained increasing attention as valuable tools for biotechnological and biomedical applications.[9,14,15] This review provides an overview over the different technical and practical aspects of the bottom-up approach and explores selected applications and visions for the assembled vesicular systems. Top-down

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engineered systems are briefly introduced to illustrate the concept. For more detailed information, readers are kindly referred to the wide selection of recent reviews focusing specifically on this field.[16−19] The main focus of this review is to introduce the modular concept of bottom-up synthetic biology and to provide a diverse toolbox of biological and chemical modules, engineering methodologies, and different approaches to inspire future endeavors to assemble artificial vesicular systems. Modules in this context are defined as discrete biological or chemical building blocks that contribute structural or functional features to the final construct. In particular, we focus on the controlled assembly of artificial vesicular systems involving the use of membrane transport proteins and enzymes as functional modules and lipids or block copolymers as scaffold modules. This includes a detailed description of the engineering and reconstitution of membrane transport proteins into artificial vesicular systems composed of tailored lipid or polymer membranes. Special emphasis is placed on controlling membrane protein orientation during reconstitution into vesicle membranes. Different possibilities for the co-reconstitution of multiple membrane proteins and the encapsulation of enzymes are explored in the context of creating synthetic biological devices for customized applications. Finally, we discuss the implementation of modules dedicated to the maintenance and replication of the vesicular systems, enabling their continuous and autonomous function.

2. SYNTHETIC BIOLOGY: TOP-DOWN AND BOTTOM-UP

2.1. The Top-Down Approach: Engineering Living Systems

Advances in the synthetic production and recombination of DNA have dramatically increased the possibilities for the top-down modification of microorganisms, *e.g.*, introducing new biological elements (promoters, genes, and their products, regulators, *etc.*) to endow systems with a desired functionality.[17] Creating a synthetic biological system on the basis of modified living organisms offers the possibility of exploiting available cellular metabolic processes, including energy supply and protein production as well as the generation of chemical precursors. Whole metabolic or signaling pathways can be introduced into a host organism to produce high-value chemicals or to engineer cell-based biosensors, which can form the basis for bioremediation systems. However, there are intrinsic limitations to the introduction of genetic modifications. For example, alterations to the cellular metabolism can result in a significant energetic burden and the accumulation of inhibitory or even toxic products.[20,21] We will briefly explore the concept of the top-down approach illustrated by examples from the prominent fields of biocatalysis, biosensors, and bioremediation. Metabolic engineering is a powerful tool for the production of complex natural products, many of which are inaccessible with current catalysts. In addition, biocatalysis can offer alternative synthetic routes from inexpensive and renewable starting materials. 1,4-Butanediol is an important precursor for the synthesis of various plastics and polyesters. Currently, it is exclusively derived from finite resources such as oil and natural gas. To tackle this issue, an *Escherichia coli* strain has been equipped with an artificial metabolic network combining multiple native and heterologously expressed enzymes to generate 1,4-butanediol in high amounts from abundant sugars.[22] In addition to the engineered pathway, the *E. coli* metabolism was adapted for the production of this highly reduced chemical from metabolic intermediates, which places a significant strain on the cell. The metabolic burden, which expresses the proportion of host resources that are diverted to engineered pathways, represents a major challenge for the biocatalytic production of most industrially relevant high-value compounds, interfering with normal physiological processes, reducing growth rate and resulting in poor production rates and yields.[22−25] A recent review explores the cellular responses to metabolic burden and compiles strategies to resolve the underlying problems by introducing metabolic regulations, enhancing the supply of building blocks and energy and various other approaches.[26]

Biological macromolecules exhibit high specificity with respect to their molecular interactions, which makes them excellent recognition elements in biobased detection systems. Biosensors based on modified microorganisms are engineered to detect the presence of trace amounts of target molecules such as toxins, pollutants, or other dangerous chemicals and indicate their presence by an optical or electrical output.[27] An early bioluminescent sensor for the semiquantitative detection of mercury in environmental samples was created by cloning a promoterless luciferase operon downstream of a mercury resistance operon.[28] The optimized genetic components of the biosensor allowed highly specific and sensitive detection of bioavailable mercury, Hg(II), combined with a straightforward and semiquantitative read-out. Besides detecting and reporting the presence of harmful chemicals, microorganisms are also powerful tools for their remediation, *e.g.*, from sewage.[29−31] First successful experiments with microbial wastewater treatments were conducted over 100 years ago, exploiting the biological activity of soil bacteria for the removal of organic compounds and the oxidation of ammonium.[32] Better understanding of metabolic processes and development of genetic engineering unlocked the possibility of tailoring microorganisms to the bioremediation of highly toxic and persistent environmental pollutants, such as chloroaromatic compounds, which are manufactured on a global scale.[33] A variety of pathways in hybrid *Pseudomonas* strains were explored and combined to create complete degradation pathways of chloroaromatics, even providing the organism with an energetic benefit for the disposal of these pollutants.[34]

2.2. The Bottom-Up Approach: Building Artificial Biomolecular Systems

Isolating and studying individual biological and chemical components is the foundation of the bottom-up approach, with the goal to replicate biological processes from their essential molecular elements or repurpose them for unprecedented functions. In contrast to the top-down engineering of living cells, bottom-up assembled synthetic systems are less susceptible to unwanted crosstalk, metabolic burden, and toxic products as they are only comprised of the necessary components.[35] Artificial vesicular systems (see section 3), such as liposomes and polymersomes, are highly valuable model systems to study the function of isolated cellular processes and the interaction of their components in a controlled environment.[7,36−41] This approach was inspired by natural compartmentalization, an ubiquitous biological phenomenon required to separate biochemical reactions from each other and provide optimal reaction conditions for participating enzymes.[42] Furthermore, artificial compartments in the form of liposomes have long since served as model membrane systems to assess the transport of solutes and the transfer of information by

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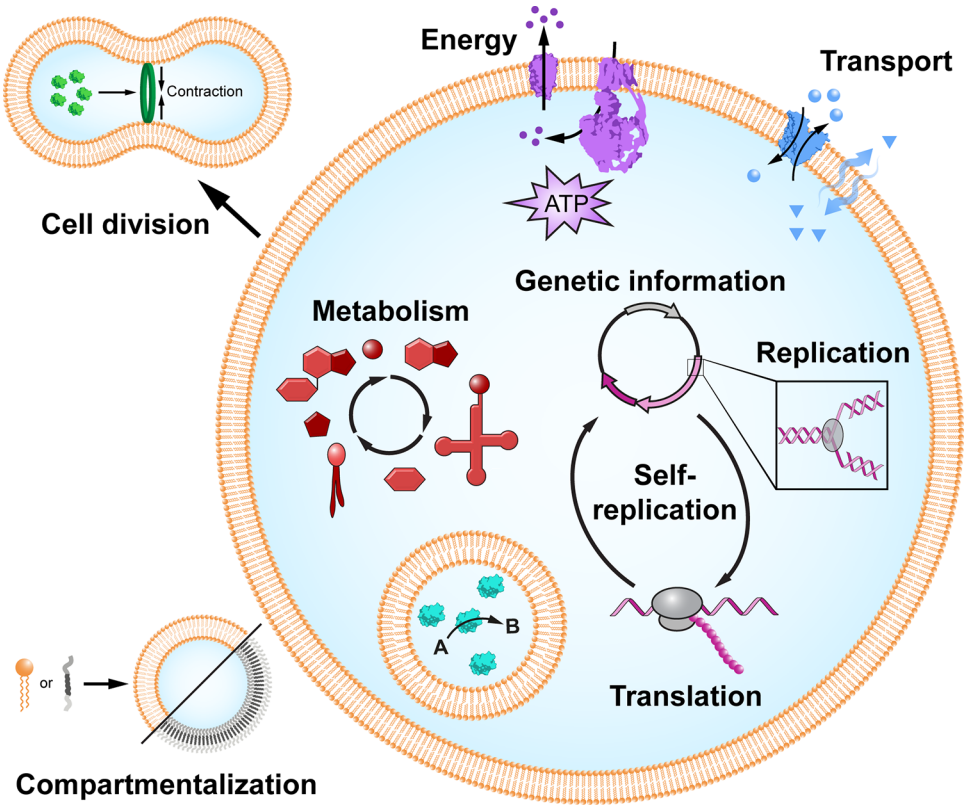

Figure 2. Bottom-up engineering of an ideal artificial cell. Isolated biological and chemical components are assembled in a modular fashion to create an artificial cell with desired traits and functions. The artificial cell is based on at least one main compartment and optional subcompartments that can harbor individual processes, each enclosed by lipid (brown) or polymer (gray) membranes. A compartmentalized process is exemplified by a reaction catalyzed by encapsulated enzymes (cyan). Energizing modules provide energy for energy-dependent modules; transport modules supply nutrients and building blocks and dispose of waste products, and a minimal metabolism (red) ensures replenishment of essential components. Replication, transcription, and translation (enzymes in gray) of genetic information enables continuous and autonomous function, while the ability to divide (component of a minimal divisome illustrated in green) ensures sustainable growth and proliferation.

membrane proteins across biological membranes. Considerable progress has been made since the first attempts to mimic fundamental biological processes in an artificial system, steadily generating more sophisticated cell-like constructs with increasingly intricate interactions. For complete autonomy and sustainability, ideal artificial cells require all essential features of biological cells, which include: (i) compartmentalization, (ii) supply of energy and (iii) protein production to perform fundamental functions, (iv) transport mechanisms across compartment boundaries and (v) a minimal metabolism, as well as (vi) the ability to replicate genetic information and (vii) a machinery capable of cell division (Figure 2). Based on a few selected examples, this chapter aims to introduce the concept and the methodology of the bottom-up approach. Readers are referred to the corresponding chapters for a more detailed description of how particular cellular processes are being recreated and repurposed in artificial environments.

Crucial cellular processes have been explored by isolating the principal components, mainly proteins, and studying their function in appropriate model systems, which ultimately facilitates their implementation as functional modules in bottom-up assembled systems (see section 4).[43] One of the main prerequisites to enable biological processes in artificial cell-like systems is a suitable power source. One of the first successful examples of recreating cellular energy generation *in vitro* was the isolation and co-reconstitution of purple membranes containing bacteriorhodopsin, a light-driven proton pump, and F-type ATP synthase-enriched membranes from bovine heart mitochondria into proteoliposomes, which mimics the light-dependent generation of ATP in phototrophic microorganisms.[44] More recent milestones in establishing a sustainable ATP supply for artificial vesicular systems are the assembly of a minimal respiratory system[45] and a catabolic pathway for the breakdown of arginine.[46] We explore the importance of energizing modules for bottom-up assembled vesicular systems and how they can be integrated with transport and metabolizing modules into complex metabolic networks in more detail later (see sections 4.1−4.3).

Recreating biological processes in artificial compartments is highly advantageous for the performance of biochemical reactions but also opens the possibility for autonomous function by incorporating the genetic information and machinery required for self-renewal and replication.[47] There are many aspects to the process of cellular self-replication, which encompasses not only the physical division of the artificial cell but requires replenishment of proteins, lipids, and DNA (see section 4.4). The first successful replication of genetic information within an artificial compartment was achieved by reconstitution of the polymerase chain reaction into liposomes.[39] This concept was expanded upon by using cell-free expression systems, which are usually composed of partially purified cell extracts containing the DNA replication, transcription, and translation machineries of a particular organism, enabling the replication of genetic material and

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production of necessary enzymes.[47,48] Lastly, a minimal cell division machinery is required to distribute the contents of an artificial vesicular system, including its genetic information, into daughter vesicles.[49]

The bottom-up approach provides powerful tools for synthetic biology with a vast number of combinations of small organic molecules and/or biomacromolecules at its disposal. Molecular systems with a wide range of tailored functions are conceivable, for example, with applications in bioremediation and biocatalysis, or as biosensors and pharmaceutical devices for the production and delivery of drugs.[15,29,50−53] Furthermore, synthetic biological systems have the potential to exhibit functionalities not found in nature due to the innumerable abiotic interactions and modifications that can be created *in vitro*. The possibilities of chemical biology and genetic engineering for the design and optimization of protein modules are addressed after the introduction of the various types of modules (see section 5). Lastly, approaches for the assembly of artificial vesicular systems and incorporation of functional modules are presented with an emphasis on reconstituting functional membrane proteins and controlling their orientation in the vesicular membrane (see section 6).

3. COMPARTMENTALIZATION AND SCAFFOLDS

3.1. Compartmentalization

Compartmentalization is a working principle that all living cells use to isolate biochemical reactions from each other and from external factors. It is thus not surprising to find that the same concept forms the basis of a majority of efforts to build synthetic biomolecular systems. This approach enables different pathways or reactions to proceed independently with individually optimized reaction conditions and without the risk of undesired crosstalk.[54] Furthermore, compartmentalization adds another controllable layer to optimize the features of artificial vesicular systems. In particular, the composition of the compartments can be controlled to adapt the activity of functional modules and interactions with the environment (see section 3.2). Combined with specialized transport modules or stimuli-responsive scaffold modules, compartmentalization allows for temporal and spatial control of transport processes (see sections 3.4 and 4.2). Introducing artificial organelles by means of multicompartmentalization provides subdivisions for artificial cells that can host components of reaction cascades, which require diverse and potentially incompatible reaction conditions (see section 6.5).

3.2. Lipid and Polymer Scaffolds for Synthetic Compartments

Biological membranes composed of a phospholipid bilayer form the boundaries of cells and their organelles and provide the basis for protein-mediated exchange of substrates and information in and out of the compartments. In artificial biological systems, these membranes are commonly mimicked by synthetic or extracted natural lipids or synthetic block copolymers to form vesicular compartments called liposomes and polymersomes (Figure 3a). Both liposomes and polymersomes can form vesicles of varying sizes depending on their method of preparation. They are usually divided into small unilamellar vesicles (SUVs) with diameters of 20−100 nm, large unilamellar vesicles (LUVs) from 100 to 1000 nm and giant unilamellar vesicles (GUVs) larger than 1 μm.[55] Nonuniform distribution of vesicle sizes can lead to potential complications for certain applications, as this results in various

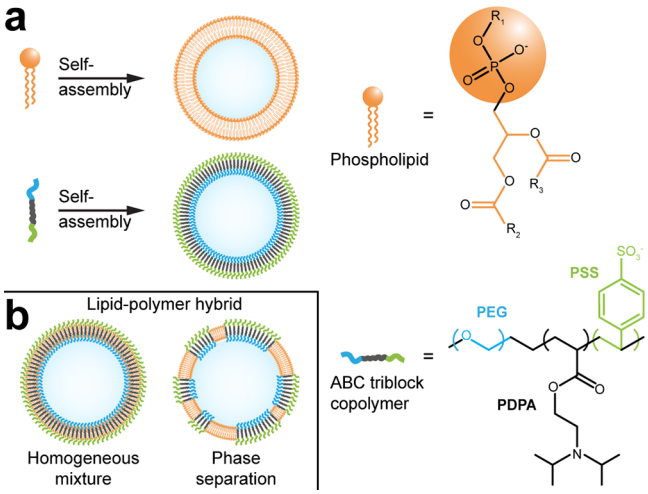

Figure 3. Lipids and block copolymers as scaffolds for vesicular compartments. (a) Self-assembly of amphiphilic scaffold molecules in an aqueous environment forming isolated compartments. The general structure of phospholipids (R_1 represents the hydrophilic headgroup, and R_2 and R_3 are fatty acids) and a selected example of an ABC triblock copolymer (PEG−PDPA−PSS) are displayed. (b) Hybrid compartments formed by mixing lipids and block copolymers can form homogeneously mixed membranes or exhibit phase separation, resulting in distinct lipid- and polymer-rich domains.

numbers of incorporated protein modules per vesicle (*i.e.*, reconstituted membrane proteins or encapsulated soluble proteins) and different internal volumes. In turn, this will affect transport kinetics across the membrane, enzymatic reactions within the compartment, storage capacity of the vesicles, and other factors. Vesicles can also have more than one membrane, arranged in a concentric manner, and are then referred to as multilamellar vesicles (MLVs). Additional membranes affect the physicochemical, mechanical, and functional properties of the vesicles, including their permeability and stability, their interaction with surfaces, and their encapsulation efficiency.[56−58] MLVs are frequently used as vectors for the targeted delivery of drugs,[52,59−61] transport and presentation of antigens,[62,63] or as nanoparticles for applications in biotechnology.[64] The membrane composition determines the ability of vesicles to encapsulate and release cargo as well as the potential to incorporate membrane transport proteins for selective permeability. Finding a suitable membrane composition for successful reconstitution of functional membrane proteins may be one of the most challenging obstacles, especially because every protein exhibits certain preferences.[65−68] While synthetic lipids or lipid extracts from biological sources closely mimic the properties of biological systems and the native environment for membrane protein reconstitution, amphiphilic block copolymers have gained interest due to their high chemical versatility. This provides access to a great variety of desired features such as tunable stability and sensitivity to certain stimuli (see section 3.4). The relative chemical instability of phospholipids mainly stems from their propensity for hydrolysis and the susceptibility of unsaturated fatty acids to oxidation. Due to their distinct chemical nature, lipid and polymer membranes exhibit fundamental differences. Lipid bilayers are commonly thinner (3−5 nm) than polymer membranes (5−50 nm) and display higher lateral fluidity and permeability due to their lower molecular weight.[55,69,70] Phospholipids are the most com-

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monly used lipid scaffolds for the preparation of liposomes. They are usually categorized by (i) the charge of their hydrophilic head groups at physiological pH, which can be either negative (*e.g.*, phosphatidylglycerol), positive (*e.g.*, *O*-ethylphosphatidylcholine), or zwitterionic (*e.g.*, phosphatidylethanolamine), and (ii) the length and degree of saturation of the fatty acids.[71] Depending on the phylogenetic origin of membrane proteins to be reconstituted, especially for most mammalian plasma membrane proteins, cholesterol, and its derivatives should be considered as essential membrane constituents. Block copolymers are categorized by the number of different polymer species into di- and triblock copolymers (*e.g.*, AB, ABA, or ABC), which are generally composed of hydrophilic (*e.g.*, polyethylene glycol (PEG) or poly(acrylic acid) (PAA)) and hydrophobic polymers (*e.g.*, polystyrene (PS) or polydimethylsiloxane (PDMS)).[55,72] The higher chemical versatility of block copolymers is mainly owing to the availability of a large variety of blocks and the possibility to control the overall molecular weight and ratios of individual blocks relative to each other. Furthermore, block copolymers are not only limited to linear architectures but can be star-branched with a number of different arms.[73,74] Even though block copolymer membranes are usually thicker than their lipid counterparts, which would suggest incompatibility with membrane protein reconstitution, their adaptability allows them to accommodate small membrane proteins by locally forming thinner membrane rafts.[75−77] In addition, the possibility of combining different chemical moieties into block copolymers makes it easier to mimic the asymmetric distribution of lipids found in the leaflets of natural lipid bilayers, which may be relevant for membrane protein function. Although successfully demonstrated for liposomes, the assembly of an asymmetric lipid membrane requires more sophisticated preparation methods.[78−80] The choice of suitable lipids or block copolymers depends on the properties that the assembled vesicle should display, including (i) desired size, (ii) membrane thickness, fluidity, and permeability, (iii) chemical and mechanical stability, (iv) surface charge and hydrodynamic properties, (v) compatibility with membrane protein reconstitution, and (vi) encapsulation of cargo.

Hybrid lipid−polymer vesicles have been explored to harness the advantages of both membrane systems and to compensate their individual drawbacks. Despite the great geometric mismatch, it is possible to create homogeneous hybrid systems with polymers of relatively high molecular weights.[81] By complementing polymersome membranes with lipids, the activity of reconstituted membrane proteins can be considerably increased.[82] Nevertheless, phase separation remains one of the main challenges when preparing hybrid vesicles, yielding vesicles with distinct lipid and polymer domains (Figure 3b), which can result in vesicle budding or coexisting pure liposomes and polymersomes.[83−85] Conceptually, homogeneously mixed membranes should optimally combine the advantages of both scaffold molecules, but the emergence of lipid- and polymer-rich domains can actually facilitate membrane protein reconstitution while maintaining the overall enhanced stability of the hybrid vesicles.[76] The preference of membrane proteins for lipid-rich domains seems to strongly depend on the phase of the lipid. In some cases, the reconstituted membrane proteins prefer the polymer-rich domains due to the lipids forming a solid-like gel phase.[86,87]

3.3. Alternative Compartmentalization Systems

In recent years, alternative compartmentalization systems such as proteinosomes, dendrimersomes, encapsulins, and niosomes have been developed. Similar to liposomes and polymersomes, these systems need to meet the same basic requirements. They should be semipermeable, chemically, and mechanically stable and be able to encapsulate functional modules in order to host desired chemical and/or biochemical reactions. Proteinosomes were developed by expanding on the concept of protein−polymer bioconjugates,[88] which can be regarded as giant amphiphiles, self-assembling into different nanostructures (Figure 4a). They were further engineered to create semi-

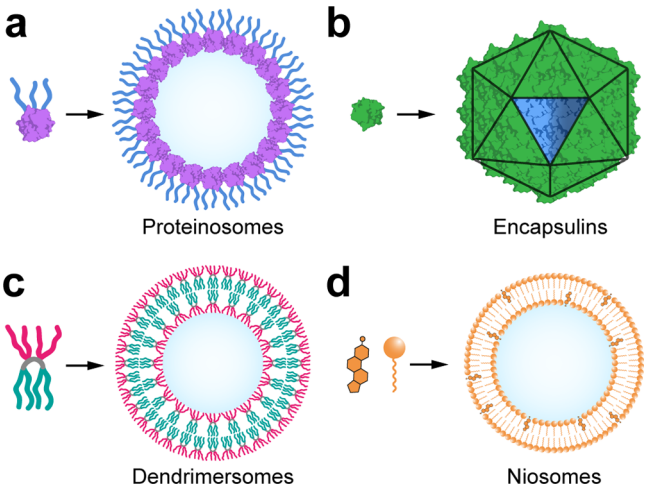

Figure 4. Alternative compartmentalization systems. (a) Proteinosomes are built by cross-linkage of aggregated protein−polymer conjugates.[89] (b) Encapsulins are naturally occurring microbial nanocompartments formed by shell-forming proteins.[91] (c) Dendrimersomes are assembled from Janus dendrimers, similar to block copolymers.[95] (d) Niosomes structurally resemble liposomes and are comprised of a mixture of single acyl-chain, nonionic lipids, and cholesterol derivatives.[99]

permeable microcompartments that can be stably dispersed in oil or water, are thermally stable, and can even be partially dried and rehydrated.[89] Importantly, the proteinosomes are capable of hosting different enzyme-catalyzed reactions as well as cell-free gene expression and have been extensively functionalized.[89,90] Encapsulins are capsid-forming proteins, which were discovered to assemble into large structures that can function as simple bacterial organelles (Figure 4b).[91] In recent years, encapsulin-based compartments have been engineered toward applications as drug delivery vehicles and scaffolds for nanoreactors.[92−94] Dendrimersomes were designed as an alternative to commonly used natural or synthetic vesicles with increased stability and more uniform size distribution.[95] They self-assemble from Janus dendrimers, synthetic amphiphiles with high chemical variety similar to block copolymers (Figure 4c). Dendrimersomes have been shown to exhibit superior stability, efficient encapsulation of compounds for drug delivery, the possibility to incorporate membrane proteins or to be decorated with proteins and nucleic acids, and to allow coassembly with lipids and block copolymers.[95−97] Due to their particular interactions with biological membranes they are even capable of engulfing living bacterial cells in a process mimicking endocytosis.[98] Niosomes are vesicles with a bilayer membrane composed mainly of a

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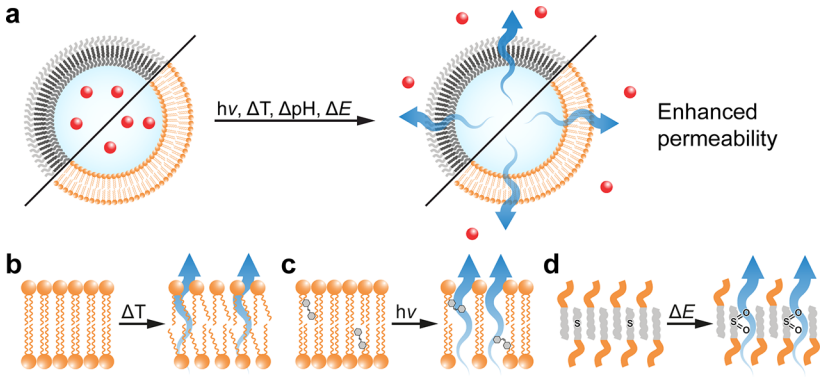

Figure 5. Controlled release of cargo from stimuli-responsive vesicles. (a) The release of molecules (red) stored within stimuli-responsive polymersomes (gray) or liposomes (brown) can be triggered by irradiation ($h\nu$), heat (ΔT), or by change in pH or redox potential (ΔE). (b) Heat-sensitive lipid bilayers exhibit a gel-to-liquid phase transition at a particular temperature, rendering the membrane permeable to hydrophilic molecules.[109] (c) Photosensitive molecules such as azobenzene derivatives of lipids undergo photoisomerization upon UV irradiation, which enforces significant changes in conformation and polarity, leading to permeabilization of the membrane.[106] (d) Redox-sensitive polymer membranes can change from hydrophobic to hydrophilic under oxidative conditions, resulting in increased permeability.[107]

combination of nonionic, usually single acyl-chained, amphipathic surfactants and cholesterol derivatives (Figure 4d).[99] They are primarily used as alternatives for liposomes and polymersomes in drug delivery, with similar stability and permeability for drug molecules.[100] Finally, the potential of bioresponsive and -compatible materials such as hydrogels should not be overlooked. They can find applications as heterogeneous compartmentalization systems to host locally confined enzyme catalyzed reactions.[101,102]

A major drawback of the alternative compartmentalization systems described above, with the exception of dendrimersomes, is their limited compatibility with the incorporation of membrane proteins as specific transport modules. While some of them can be tuned for permeability, most systems only allow exchange of solutes by passive diffusion through unselective pores formed by the material or across the membrane. Thus, systems that closely mimic the properties of biomembranes, such as liposomes, polymersomes, and to some extent, dendrimersomes, are the preferred choice when a selective and efficient exchange of solutes between the compartment and its environment is required. While they primarily serve as scaffolds in the context of this review, lipids, block copolymers, and other alternative building blocks can also have or be equipped with functional features (see section 5.4). Overall, a highly diverse pool of scaffold modules is available with the potential to be combined and tailored to specific applications with unique requirements for the molecular system.

3.4. Release and Exchange of Cargo

The capability of vesicular systems to import or export molecular cargo is a fundamental feature for a variety of applications. The passive or active uptake of external molecules into the compartment is required to replenish substrates in nanoreactor-type systems or to trigger a signal-generating cascade in biosensing devices. Conversely, the efflux of molecules can be exploited for targeted drug delivery systems but can also improve the efficiency of compartmentalized reactions by removing built-up product that may lead to product inhibition. The transport of solutes into and out of synthetic compartments can either be mediated by the incorporation of selective membrane transport proteins or by using lipids or polymers responsive to physical or chemical stimuli, which trigger the discharge of encapsulated molecules (Figure 5).[53] Release of cargo in response to an external stimulus results from increased permeability of the vesicle membrane due to chemical or conformational changes of the scaffold molecules. While the translocation of solutes by membrane transport proteins is very specific, stimuli-responsive release can be spatially and temporally controlled. In the following, we discuss developments exploiting some of the most accessible chemical and physical stimuli. The elementary control provided by stimuli-responsive building blocks allows the triggered assembly or disassembly of synthetic vesicles and release of their content upon a change in external factors such as pH, ionic strength, temperature, light, or redox potential (Figure 5).[103−108] Phototriggered or thermosensitive liposomes and polymersomes are appealing tools in the context of controlled localized drug delivery.[109,110] Vesicles responsive to very small changes in temperature were developed for targeted delivery of doxorubicin by exploiting local hyperthermia exhibited by tumors.[111] Addition of specific lysolipids (phospholipids with only one acyl chain), peptides, and polymers can be used to fine-tune the gel-to-liquid phase transition temperature of the lipid bilayer above which enhanced permeability is observed (Figure 5b).[112] Alternatively, incorporation of compounds which undergo photoisomerization or -cleavage, switch their hydrophobicity or generate heat upon irradiation, can endow liposome systems with light-triggered responses (Figure 5c).[53,110] One of the first examples used malachite green modified polyethylene glycol to generate vesicles that disassembled upon illumination with UV light.[106] A widely used example today are azobenzene derivatives of lipids, which exhibit reversible *trans*-to-*cis* isomerization upon UV irradiation, thus inducing permeability of lipid bilayer membranes.[113] AB diblock copolymer vesicles made from polyethylene glycol (PEG) and polypropylene sulfide (PPS) can be destabilized under oxidative conditions due to the conversion of hydrophobic PPS to the more hydrophilic polypropylene sulfone (Figure 5d).[107] Block copolymers, which inherently have a higher chemical diversity, allow the engineering of polymersomes that are responsive to a wide variety, and even combinations, of stimuli. Dual-responsive block copolymers represent versatile scaffold modules for induced cargo release, relying on a range of physicochemical triggers. Redox- and pH-responsive polymersomes were prepared by the combination of polyvinylferrocene

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and poly(2-vinylpyridine) blocks, which are sensitive to oxidation or protonation, respectively.[114] While releasing encapsulated cargo by triggered permeabilization of the vesicular membrane is very efficient, it involves a transient or permanent loss of compartmentalization. Membrane transport proteins, on the other hand, offer continuous and specific translocation of molecules across the compartment boundary but may prove challenging to incorporate. Nature provides a multitude of different transport proteins specific for a variety of solutes, ranging from inorganic ions to small organic molecules. In addition, they can be genetically engineered to tailor their stability, efficiency, and specificity (see section 5). The enormous potential membrane proteins offer as transport modules and the challenges presented by their reconstitution into liposomes and polymersomes are discussed in detail below (see sections 4.2 and 6).

4. PROTEINS AS FUNCTIONAL MODULES

Proteins are by far the biomolecules with the highest functional diversity and thus excellent tools for functionalizing bottom-up assembled biomolecular systems such as nanoreactors, *i.e.*, vesicular compartments in the nanoscale that are confined by some sort of membrane providing a defined reaction volume with optimized conditions for chemical or biological reactions. While there are promising approaches to functionalize biomolecular systems with other biomolecules such as DNA,[115,116] we will limit the discussion of functional modules to proteins according to the essential cellular functions discussed in section 2.2. Specifically, these include energy supply, transport, metabolism, and replication, with a dedicated subchapter describing each aspect. An important distinction is made between water-soluble and membrane proteins regarding the functions they can impart on the assembled system as well as the methods for their incorporation. Membrane proteins can mediate the exchange of molecules or information across the membranes of vesicular systems as well as interactions between them, whereas encapsulated soluble enzymes are especially suitable for catalyzing chemical transformations within the compartment.

Recombinant expression and genetic manipulation of bacterial protein homologues is usually found to be easier, cheaper, and more efficient than of their eukaryotic counterparts.[117−120] Prokaryotic proteins frequently provide higher yields when overexpressed in bacteria and tend to be more stable after extraction, a crucial parameter when building bottom-up systems from isolated biological components. Proteins from extremophilic organisms are especially suitable for industrial applications due to their resistance to challenging conditions such as high temperature, pressure, osmolarity, or extreme pH values.[121,122] A variety of enzymes derived from thermophilic organisms have proven to be extremely valuable in industrial applications such as in the food and pharmaceutical sectors, as well as for daily use in laundry detergents.[121,122] Furthermore, post-translational modifications of prokaryotic proteins are limited, which facilitates their heterologous expression in common bacterial expression systems, *e.g.*, *E. coli*.[120,123] In the following sections, different types of proteins are discussed that can act as energizing, transport, metabolizing, or replication modules in the context of bottom-up assembled biomolecular systems.

4.1. Energizing Modules

Energy is required for all active processes in living cells and is supplied by either photosynthetic mechanisms or cellular respiration. These reactions provide ATP or establish electrochemical gradients across biological membranes, which represent the two universally used cellular energy currencies. In addition, cellular respiration couples the regeneration of ATP to the recycling of metabolically important redox equivalents. One of the most central considerations when assembling a bottom-up biomolecular system is how to power essential processes. A synthetic cell requires energy for the sustained synthesis of genetic information and proteins, transport of substrates and waste products, maintaining a minimal metabolism and performing desired chemical transformations, as well as general homeostasis and stability of the system. The consumption of energy by different cellular processes has been quantified for *E. coli* and has revealed that the majority is used for protein synthesis and general maintenance, *i.e.*, regulation of pH, ionic strength, osmotic pressure, *etc.*[124,125] Besides maintaining basic homeostasis and replication, artificial cells are tasked with processes that put additional strain on the energy supply. Synthetic chemical transformations in the laboratory require significantly higher amounts of resources and energy compared to cellular reactions due to the step-by-step nature of the process and necessary separation and purification in between. In contrast, biotechnological productions in cells represent integrated processes, with cascade reactions that save time and energy compared to classical multistep reactions. However, a considerable proportion of the substrate and energy may be diverged to maintain the metabolism of the cell and grow the biomass.[126] Modern approaches strive to mimic the advantages of the natural processes in bottom-up assembled nanoreactors by forming synthetic metabolic networks (see section 4.3).

Synthetic biology aspires to mimic and utilize, in artificial systems, the various ways developed by nature to supply, convert, and store energy in form of energy carriers such as ATP or electrochemical gradients. The most promising approaches include (i) artificial photosynthetic systems, (ii) oxidative phosphorylation, (iii) arginine breakdown pathways, and (iv) decarboxylation pathways (Figure 6). The use of electrochemical gradients for the synthesis of ATP by F-type ATP synthases is central to the first two processes, whereas the latter two conserve energy of exergonic metabolic reactions. Phototrophic organisms convert solar energy into electrochemical gradients by using light-driven transport proteins such as microbial ion pumping rhodopsins or photosystem complexes.[127−132] Microbial rhodopsins are categorized into ion pumps and channels with different substrate specificities, including protons (*e.g.*, bacteriorhodopsin and proteorhodopsin), sodium (*e.g.*, *Krokinobacter eikastus* rhodopsin 2), and chloride ions (*e.g.*, halorhodopsin).[131,133] Photosystem II, a chlorophyll containing protein complex involved in photosynthesis, is also able to establish a proton gradient across the lipid bilayer by splitting water molecules into molecular oxygen and protons.[132] Heterotrophic organisms, on the other hand, use oxidative phosphorylation, which couples the proton translocation of terminal oxidases to the synthesis of ATP. Mimicking the natural energy conversion by utilizing recombinant light-driven ion pumps or photosystems as energizing modules is a highly efficient strategy for providing energy in synthetic biomolecular systems.[44,50,134−139] Similarly, components of the cellular respiration, such as the

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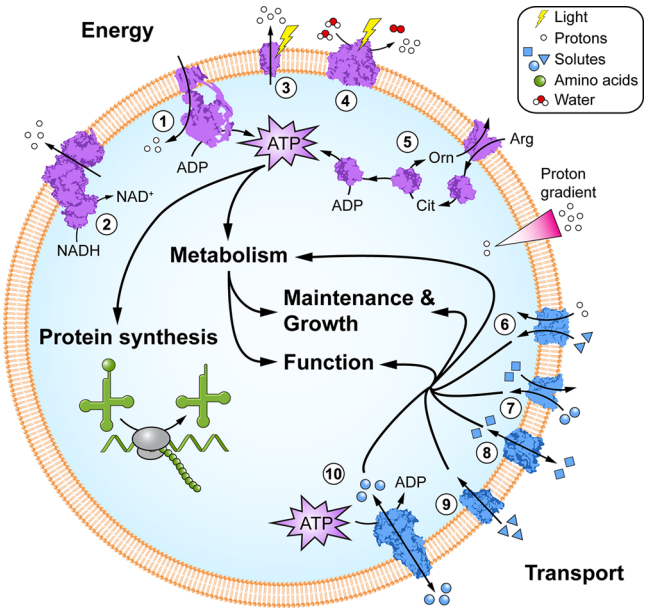

Figure 6. Schematic representation of possible energizing, transport, and metabolizing modules for the assembly of functionalized synthetic vesicles. F-type ATP synthase (1) is one of the central energizing modules, producing ATP by harnessing the energy stored in electrochemical gradients. Proton gradients (magenta) can be established by complex I (2), microbial light-driven ion pumps (3), or photosystem complexes (4). The arginine breakdown pathway (5) is an efficient and light-independent alternative for ATP synthesis. Transport modules for solutes include symporters (6), e.g., driven by the established electrochemical gradient; antiporters (7), coupling the import and export of two different substrates, pores (8) facilitating diffusion of small molecules, uniporters (9) allowing substrates to flow along their concentration gradient, and ATP-driven transporters (10), coupling hydrolysis of ATP to the active transport of their substrate.

NADH:ubiquinone oxidoreductase or bo_3 quinol oxidase can be employed to power proton-driven ATP synthesis in synthetic vesicles.[45,140−142] Importantly, without a coupled metabolic pathway that recycles the required redox agents, it is inevitable to provide an external source. On the other hand, artificial photosynthetic membranes with the same functionality can be achieved using a carotene−porphyrin−naphthoquinone molecular triad, capable of photoinduced proton transfer used to power coreconstituted F_0F_1-ATP synthase.[143] Depending on the type of application, the established electrochemical gradient is directly used to power secondary active transporters (see section 4.2) or is further converted into energy-rich molecules such as ATP (Figure 6).

In addition, energizing modules that conserve the energy of exergonic biochemical reactions such as deamination and decarboxylation can be directly integrated into metabolic networks. The arginine deamination pathway is a widely used means among bacteria to generate ATP and simultaneously protect the cells from acidification.[144] The breakdown of arginine occurs in three enzymatic steps, yielding ornithine, ammonia, and carbon dioxide, ultimately resulting in the formation of ATP from ADP. Researchers have recently exploited this pathway and assembled proteins of the *Lactococcus lactis arc* operon to establish a system capable of regenerating ATP in synthetic vesicles.[46] The system is composed of the arginine deiminase arcA, which hydrolyzes arginine to citrulline, the ornithine transcarbamoylase arcB, which then transforms citrulline and phosphate into carbamoyl phosphate and ornithine, and finally the carbamate kinase arcC, which forms ATP from carbamoyl phosphate and ADP. In addition, the arginine/ornithine antiporter ArcD maintains optimal reaction conditions by importing externally supplied arginine and exporting the product ornithine (see also section 4.2), while ammonia and carbon dioxide can freely diffuse out. This system enables sustained long-term synthesis of ATP, even when challenged with varying levels of ATP consumption by downstream processes. In comparison, decarboxylation of dicarboxylic acids does not yield enough free energy to drive direct ATP synthesis from ADP. Instead, two systems have been characterized that establish an electrochemical potential as a result.[145] Oxaloacetate decarboxylase directly couples the decarboxylation of its substrate to the export of sodium ions and can thus establish an electrochemical gradient in synthetic vesicles.[145] On the other hand, the malolactic fermentation pathway involves the decarboxylation of malate to lactate, which consumes a proton, and the subsequent electrogenic exchange of the substrate and product, resulting in a proton gradient and membrane potential.[125] Utilization of these different energy sources in artificial vesicular systems are discussed in the following chapters. In particular, the coupling of electrochemical gradients to the transport of solutes across the compartment membrane and consumption of ATP for maintenance of optimal operating conditions, protein synthesis, and replication. The core of all presented energy providing systems is formed by specialized membrane protein modules, whose correct orientation in the membrane of vesicular systems is a prerequisite for all dependent modules (Figure 6). Therefore, section 6.2 is dedicated to describe possible approaches to ensure the directed insertion of these modules into membranes.

4.2. Transport Modules

Translocation of solutes in and out of compartments can be mediated either by unspecific pores or substrate-specific transporters. Both can facilitate diffusion of solutes along their concentration gradients, while the latter can perform energy-dependent substrate translocation (Figure 6). The transporter classification system organizes membrane transport proteins into five major classes: (1) channels and pores (*e.g.*, voltage-gated ion channels and porins), (2) electrochemical potential-driven transporters also known as passive and secondary active transporters (*e.g.*, the major facilitator superfamily), (3) primary active transporters (*e.g.*, ATP- and light-driven transporters), (4) group translocators (*e.g.*, the phosphotransferase system), and (5) transmembrane electron carriers (*e.g.*, photosystems and transmembrane cytochromes).[146] Transport modules play an important role in the overall kinetics of biocatalytic reactions in compartmentalized systems as they can direct substrates toward the encapsulated catalytic components, *i.e.*, increasing the local concentration within the compartment, and remove products to minimize product inhibition. In fact, the influx and efflux of substrates are commonly the rate-controlling steps.[147] Employing a specific antiporter for the transport of product and substrate of a reaction is especially efficient because the product gradient will increase the driving force for the import of the substrate.[125] Similarly, small vesicles can be faced with a fast buildup of substrate gradients, limiting their maximal efficiency. In case of proton pumping microbial rhodopsins or ATPases (see section 4.1), addition of the ionophores valinomycin or nigericin in the presence of potassium ions

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can be used to dissipate the charge component of the membrane potential (*i.e.*, the buildup of positive charge due to the accumulation of protons), allowing the establishment of larger proton gradients.[148−150] Transport modules not only manage substrate fluxes for biochemical reactions but can fulfill crucial roles for the homeostasis of a system, *i.e.*, by regulating internal pH, ionic strength, and osmotic pressure.[151] Synthetic vesicles equipped with the arginine breakdown pathway (see section 4.1) were co-reconstituted with an ionic strength-gated ATP-driven glycine betaine transporter to counterbalance potential increases in internal ion concentration and maintain osmotic pressure and vesicle volume, all critical for enzyme function.[46] Another example of adaptive regulation was showcased by the reconstitution of the mechanosensitive channel MscL, which allows vesicles to sense and respond to changes in osmolarity.[152] Conceivably, pH and ionic strength could be regulated using light-driven or ionic strength-gated ion pumps and channels. In bacteria, cytoplasmic pH is regulated, among others, by the activity of the Na^+/H^+ antiporter.[153] This could be advantageous for artificial systems, as it allows adaptive adjustment of the pH while largely maintaining the membrane potential.

A milestone that illustrates the application of transport modules in nanoreactors was the incorporation of the outer membrane protein F (OmpF)[154] in polymersomes, facilitating the unspecific exchange of the substrate and product for an encapsulated β-lactamase.[155] In contrast, primary active transporters are able to catalyze the transport of specific substrates against their concentration gradient using either chemical energy (*e.g.*, ATP hydrolysis), redox energy (*e.g.*, mitochondrial electron transport chain), or light-energy (*e.g.*, light-driven ion pumps).[156] Similarly, electrochemical potential-driven transporters, also known as secondary active transporters, power the transport of solutes by coupling it to the thermodynamically favorable transport of a second substrate (*e.g.*, protons from a proton gradient established by a primary active transporter).[157,158] Porters, a subclass of electrochemical potential-driven transporters are further divided depending on their transport mechanism into uniporters, symporters, and antiporters (Figure 6).[157,158] Substrate specificities can range from bioorganic molecules such as sugars, nucleobases, amino acids, peptides, and lipids to inorganic ions and are reflected in classifications such as the solute carrier (SLC) families.[159] Transport proteins of certain classes display a significant substrate promiscuity and are able to accommodate molecules with marked structural diversity.[160] This includes in particular proteins involved in excreting various drugs or xenobiotics. Accordingly, these need to be able to constantly cope with new unencountered substrates.[161] Although the mechanism of their high adaptability is poorly understood, drug transporters offer significant potential for the engineering of artificial biological systems. Other ways of selecting membrane transport proteins with suitable substrate specificities are genome-wide knockout studies to identify new transporters or to engineer known transporters to specific needs by structure-based mutagenesis or directed evolution (see section 5).[147]

The definition of transport modules can also be expanded beyond protein components such as membrane transporters, channels, and pores. Vesicle fusion with SNARE-like proteins can be utilized as a means to resupply liposomes with reagents, nutrients, and even large components including enzymes or parts of genetic circuits.[162] This principle could also be exploited to bring together sequential parts of reaction cascades whose components are stored in individual liposomes. In addition, the supplying liposomes also represent an alternative source of lipids to their synthesis *in situ* (see section 4.4). Importantly, the respective SNARE-like proteins need to be correctly inserted and displayed on the outside of the liposomes in order to promote vesicle fusion. By linking membrane impermeable cargo molecules with a cell-penetrating peptide, even large molecules such as nucleic acids and proteins can be delivered across the lipid bilayer.[163−165] Both liposome fusion and cell-penetrating peptides are currently the most efficient ways of delivering larger synthetic molecules and biomacromolecules across liposomal membranes.

4.3. Metabolizing Modules

Metabolizing modules form the core of most bottom-up assembled biomolecular systems and define the overall function through their specific catalytic activity. In self-sustaining nanoreactors they can form a minimal homeostatic metabolism, providing building blocks for essential cell-mimicking functions such as nucleotides, amino acids and lipids from simple precursors. In addition, metabolizing modules are able to form integrated networks that connect the minimal metabolism and functions imposed on the artificial cell, such as desired chemical transformations of externally supplied substrates (Figure 6). The enormous functional diversity, high efficiency and specificity for catalyzed reactions make enzymes an obvious primary choice to fulfill the role of metabolizing modules in synthetic biological systems. Furthermore, enzymes can be tailored and improved by state-of-the-art genetic engineering techniques such as structure-based mutagenesis or directed evolution (see section 5.1).[166−168] Traditional small-molecule catalysts can also find applications in nanoreactor-type molecular systems. However, while the choice of homogeneous catalysts is quite vast, they can have certain drawbacks when compared to enzyme-based catalysts. These include: (i) limited solubility and stability in aqueous media, (ii) limited catalyst-control of selectivity and (iii) a substrate specificity that may be too broad for certain applications. A new and exciting possibility is the combination of metal-based catalysts with a protein scaffold to afford artificial metalloenzymes (see section 5.3).[169−175]

Inspired by natural biochemical pathways, synthetic metabolic networks are bottom-up assemblies of enzymes forming complex multistep reaction cascades in artificial systems.[176] These networks are assembled based on known chemical and physical principles and their components are then iteratively optimized. Two recent achievements illustrate the current possibilities and limits of synthetic metabolic networks. The crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle is a synthetic reaction pathway assembled using 17 enzymes from nine different organisms for the continuous fixation of carbon dioxide.[177] To highlight the ability to integrate the cycle with biological parts, it was combined with chloroplast membranes to create an artificial photosynthetic system.[178] Similarly, the pentose−bifido−glycolysis (PBG) cycle was designed for the biochemical production of bioplastics from affordable starting materials. The optimized reaction cascade efficiently converts glucose into the bioplastic polyhydroxybutyrate, requiring more than 20 reaction steps.[179] Both examples already employ components for regulation, maintenance, and proof-reading, which all remain key challenges of synthetic biochemistry. This can

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include regulating enzyme activities to control metabolite fluxes, the production and regeneration of cofactors, as well as prevention of adverse side reactions and reintroduction of dead-end metabolites into the cycles.[126,176−179]

4.4. Growth and Replication Modules

Self-replication of artificial cell-like systems is one of the most anticipated challenges to be overcome in synthetic biology. Equipping vesicular systems with the ability to replicate ensures autonomous and continuous execution of their functions. In addition to all modules involved in performing that specific function and providing the necessary energy, self-replication requires genetic material, transcription, and translation machineries as well as the capability of physical replication, *i.e.*, mimicking cell division. A major milestone toward this goal was achieved by creating a DNA-replicating liposome system that features the DNA replication machinery of the Φ29 virus and the PURE cell-free translation system, which is based on purified components from *E. coli*.[47] A complete autocatalytic DNA replication cycle was established by the *de novo* synthesis of self-encoded Φ29 proteins through translation by the PURE components. Major drawbacks of using the viral DNA replication machinery are the limitation to replicating linear genomes and the lack of regulatory mechanisms, *i.e.*, uncontrolled and continuous amplification that would drain the resources of the system.[180] Spatial separation of mother and daughter genomes is required to ensure symmetric distribution during the division of the vesicular system. Random partitioning has been proposed for synthetic cells containing a sufficiently high number of genome copies,[181] while entropy-driven segregation might be a feasible mechanism for larger genomes.[182] Alternatively, the necessary components from the active DNA segregation machinery could be isolated and reconstituted *in vitro*, such as a minimal mitotic spindle or the bacterial actin-like plasmid partitioning system.[183,184]

In addition to replicating the genetic material, synthesis of new proteins and lipids is required prior to the division of the synthetic cell. Fresh membrane components need to be supplied externally or produced *in situ* to grow the vesicles and maintain their size after division. Lipids and other constituents can be added to the extravesicular solution as monomers, micelles, and SUVs that can then fuse with the vesicle membrane.[185,186] However, only *de novo* synthesis of lipids satisfies the requirements for autonomous replication. Nonenzymatic approaches to synthesize phospholipids were demonstrated, using native chemical ligation of water-soluble and reactive thioester precursors[187] or click-chemistry using a membrane-bound, self-reproducing catalyst.[188] To mimic the natural cellular processes more closely, enzyme-catalyzed strategies for the biosynthesis of lipids have been developed. These approaches range from the single step reaction of forming phosphatidylcholine from lysophosphatidylcholine and acyl-CoA derivatives to recreating more complex synthesis routes such as the Kennedy pathway for the synthesis of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG).[189−193] These efforts have culminated in the assembly of a membrane synthesis machinery that is capable of synthesizing PE and PG in desired ratios, by means of transcriptional and metabolic regulation, using enzymes that are encoded and translated within the artificial cell.[193] Production of proteins is usually mediated by cell-free expression systems, which are a crucial component to establish self-replicating systems. The development of *in vitro* synthesis machineries, the optimization of individual components, and integration into synthetic cell-like structures have recently been reviewed.[194−197] *In vitro* protein expression systems have already been implemented to supply synthetic cells with protein components of major cellular functions, including DNA replication and transcription,[47] energizing modules,[139] lipid synthesis,[193] and cell division.[198]

Finally, the last step of cellular replication, the cell division process, involves separation of the components, deformation of the membrane, and membrane scission. Natural division machineries serve as inspiration to adopt this process into synthetic cells. Most prominently, the complex subject of bacterial cell division, in particular the role of FtsZ,[199] was investigated using bottom-up assembled systems.[41,200,201] Membrane-targeted FtsZ was shown to assemble into ring-like structures on the inside of tubular liposomes, which constricted the vesicles such as those required to initiate division.[41,202] Similarly, membrane deformation and blebbing could be observed in lipid vesicles reconstituted with actin and myosin, mimicking the actions of the cytoskeleton.[203] Reconstitution of a cell-free expression system that allows *de novo* synthesis of bacterial division proteins in giant lipid vesicles paved the way for autonomously dividing synthetic cells.[198] The final step in which the deformed liposomes are completely severed is the least explored. Nonetheless, promising results have been obtained by reconstituting the components of the eukaryotic endosomal sorting complexes required for transport (ESCRT) into giant liposomes, demonstrating constriction and eventual scission of nanotubes.[204] However, a manual setup was required for the deformation of the membrane by mechanical means, which does not yet allow autonomous replication of synthetic cells.

5. ENGINEERING OF PROTEIN AND SCAFFOLD MODULES

5.1. Rational Genetic Engineering and Directed Evolution of Protein Modules

Advances in the structural and functional characterization of proteins have paved the way for rational design and genetic engineering of artificial variants with optimized properties and functions. In addition, the rational design process receives increasing support from computational tools, such as sophisticated protein folding predictions[205] and exploration of genomic diversity to distinguish between mutants with neutral and affected phenotypes.[206] However, even with detailed structural information available, predicting the location and type of mutation rarely leads to the discovery of mutations remote from the catalytic site.[207] To tackle this challenge, novel molecular dynamics approaches are developed and distal mutation sites are correlated with enzyme activities in a new database.[208,209] More recently, artificial intelligence (AI) has been used to predict mutations that may lead to improving a targeted figure of merit of a given protein, such as its activity, stability, or selectivity.[210−213] All of these *in silico* tools are particularly versatile when combined with directed evolution strategies. Directed evolution is based on an iteration of random mutagenesis, protein expression, and screening for desired activity.[214] This enables the search for desired functionalities beyond known mechanisms but may require significantly more effort.[215−217] Rational and semirational, computer-aided designs, including AI, combined with site-

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directed mutagenesis can reduce the screening effort, leading to the identification of proteins and enzymes that meet a targeted figure of merit including: improved kinetics, substrate specificity, product ratios, and tolerance to chaotropic conditions. In addition to site-specific amino acid substitutions, introduction or substitution of prosthetic groups can change the initial recognition of substrates, stabilization of intermediate species, and overall reactivity and functionality (see section 5.3).[208,218]

Redesigning active sites of soluble enzymes to gain access to new functions has been a mainstream effort, driven by the demand for highly specialized industrial biocatalysts for the production of valuable secondary metabolites.[219,220] A majority of synthetic biology endeavors have targeted the biocatalysis of polyketides, nonribosomal peptides, and isoprenoids, which are appealing targets due to their modular structure and important bioactivity.[219] With the advent of synthetic biology, effort has also been directed to other protein modules required to assemble artificial cellular systems with biomimetic or new-to-nature features. Energizing modules, in particular light-driven proton pumps and channels, have moved into the spotlight of protein engineers due to their importance for applications in optogenetics and biotechnology.[50,221−224] Site-directed mutagenesis enabled the conversion of bacteriorhodopsin, a light-driven outward proton pump, into an inward chloride pump[225] and *Krokinobacter eikastus* rhodopsin 2 from a sodium into a proton pump[226] or even a non-natural cesium ion pump.[227] The engineering of substrate-specific secondary active transporters is less well elucidated, even though they play a vital role in whole-cell biocatalysis.[147,228] Highly efficient and specific transport modules are also required in bottom-up assembled systems to replenish the substrates for a biocatalytic reaction or to remove the final product. Functional and structural analysis of a proton-driven peptide transporter from *Yersinia enterocolitica* guided the mutagenesis of specific amino acid residues essential for ligand recognition and enabled tuning of the substrate specificity to different dipeptides and expanding it to β-lactam antibiotics.[229,230] In addition, it was demonstrated that the driving force, *i.e.*, the type of cosubstrate, for secondary active transporters can be engineered as well.[231,232] Lastly, the engineering of passive diffusion pores was demonstrated by a deletion mutation of the outer membrane protein F (OmpF) that exhibited a larger pore size, which was accompanied by a significantly increased diffusion rate for dissacharides.[233]

Directed evolution of enzymes is based on an iteration of random mutagenesis, protein expression, and screening for a desired activity. An initial enzymatic activity, albeit very small, is required as a starting point for a directed evolution campaign.[214] Improvements can include a number of parameters such as increased thermostability, resistance to inhibitory conditions, optimized or expanded substrate specificities, and access to non-natural catalytic mechanisms.[215−217,234,235] Phage or ribosome displays directly link the protein variants to their corresponding genes and facilitate identification of promising candidates from comprehensive gene libraries.[236,237] Compared to natural evolution, which results mainly from recombination that can cause deletions, insertions, and fusions, the *in vitro* process still mainly relies on point mutations.[238] Some substantial changes in terms of enzyme function require significant alterations to the secondary and tertiary protein structure, for which the relatively subtle side chain substitutions occurring during *in vitro* evolution might be insufficient. To access more catalytic activities, natural evolutionary mechanisms are mimicked by creating libraries through shuffling and reassembling randomly fragmented DNA or by novel transposon-based mutagenesis.[239,240] Notably, the extensive gene libraries generated by these strategies improve the chance to find a suitable candidate but can pose a significant hurdle in form of a resource- and time-intensive screening process.

5.2. Engineering of Fusion Proteins and Multienzyme Complexes

Domains are highly conserved structural and functional units of proteins, many of which are connected terminus-to-terminus, greatly facilitating their reorganization and recombination to create novel enzyme architectures and functions.[241] Protein engineers have mimicked this naturally occurring modular design by creating artificial fusion proteins using recombinant DNA technology or by conjugating individual enzymes using covalent modifications.[242] The most prominent examples of molecules fused to proteins are affinity purification tags including polyhistidine (His-tag) and streptavidin-binding peptide (Strep-tag) or small proteins such as glutathione *S*-transferase (GST-tag) or maltose-binding protein (MBP-tag).[243] Fluorescent proteins, *e.g.*, the green fluorescent protein (GFP), are frequently fused to proteins to monitor their expression levels, folding, functional state, and cellular localization.[244−249] Furthermore, pH-sensitive GFP mutants, so-called pHluorins, have been developed, which can be fused to proteins of interest to report local pH changes in cells or vesicles with greater sensitivity than commonly used pH-sensitive dyes such as ACMA (9-amino-6-chloro-2-methoxyacridine) or pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid).[250−253] Even though the level of sensitivity might not have reached its full potential, these tools are especially valuable for assessing the correct reconstitution and function of energizing and transport modules (see sections 6.1 and 6.2) in synthetic vesicles, a majority of which create or depend on established proton gradients. In addition, the fusion of soluble protein domains to integral membrane proteins has been proposed as an approach to create more water-soluble constructs and thus facilitate their expression and purification.[254] Similarly, the addition of fusion proteins to enzymes can alter their physicochemical properties and enhance their stability and solubility under nonphysiological conditions.[255−257] Combination with domains that act as artificial control switches provides another tool to modulate protein activity using exogenous signals.[258−261] In a biomimetic approach, phosphorylation-sensitive domains can be inserted into other proteins to build artificial switches and control their activity (Figure 7a).[262] Generally, the signal is recognized by the first and transferred to the second domain in order to regulate its function. The modular design, which allows coupling of desired signals and enzymatic activities, makes these hybrid enzymes versatile candidates for applications as biosensors.

Multienzyme complexes are composed of a number of noncovalently associated enzymes or a multidomain polypeptide that catalyze sequential chemical reactions. Fatty acid synthase contains seven distinct functional domains catalyzing the consecutive steps of the *de novo* synthesis of fatty acids from acetyl-CoA.[263] The close proximity of the individual enzymes allows an efficient catalytic cycle with a mechanism that does not rely on diffusion of the intermediates between reaction steps. This concept is mimicked by tethering

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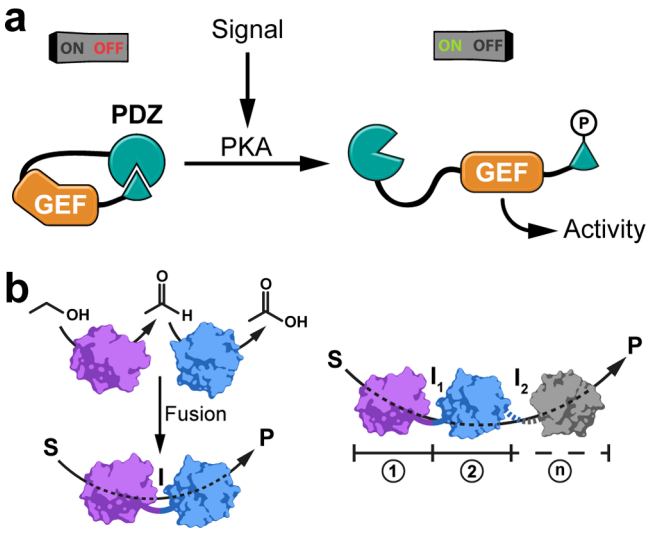

Figure 7. Engineering recombinant fusion proteins. (a) Engineering of a synthetic guanine nucleotide exchange factor (GEF), whose activity can be controlled *via* a phosphorylation-sensitive fusion domain.[262] The GEF is fused to a small peptide and a PDZ domain that recognizes the peptide, thus forming an autoinhibitory complex. The peptide is a substrate of protein kinase A (PKA) and is released from the PDZ domain upon phosphorylation. The catalytic GEF domain can thus be activated by an external signal that induces phosphorylation by PKA. (b) Fusion of an alcohol (purple) and an aldehyde dehydrogenase (blue) increases the catalytic turnover of the linked enzymes due to channelling of the intermediate aldehyde between the active sites without diffusion into the bulk solution.[265] Conceptually, any number of enzymes can be genetically fused terminus-to-terminus for efficient catalysis of a multistep reaction. The close proximity of active sites enables smooth channelling of intermediates (I_1 and I_2) to the next enzyme, thus increasing the overall production rate of the desired product (P).

individual enzymes to each other and bringing their active sites into close vicinity, thus creating highly efficient multienzyme constructs with limited diffusion of intermediates (Figure 7b).[264−266] Fusion of dihydroxyacetone kinase and fructose-1,6-bisphosphate aldolase resulted in a bifunctional enzyme, retaining the activities of both proteins.[264] Channelling the substrate in the fusion protein increased the overall reaction rate 20-fold compared to the individually expressed enzymes. An even more impressive improvement has been achieved by fusion of an alcohol and an aldehyde dehydrogenase, which increased the catalytic turnover 500-fold compared to the unlinked enzymes.[265] Alternatively, artificial assembly lines can be created by tethering individual enzymes to DNA or protein scaffolds.[267−269] This is a promising strategy, in particular for the multistep synthesis of biopolymers such as polyketides and nonribosomal peptides. In summary, the engineering of fusion proteins has become an essential tool for the production of optimized functional modules and their isolation using purification tags. Furthermore, the possibility of combining functional domains to proteins using recombinant DNA technology can significantly increase the versatility of any protein module for their application in artificial biological systems.

5.3. Chemical Modification of Proteins

In nature, protein activity can be controlled by (reversible) chemical modifications of specific amino acid side chains, such as through phosphorylation and dephosphorylation by protein kinases and phosphatases upon cellular signals.[270] In a similar fashion, chemical modification of specific amino acid residues can be exploited *in vitro* as a powerful tool to probe or control the function of proteins. The choice of targetable functional groups with suitable reactivity among natural amino acid side chains is limited and can be further restricted by a high abundance of that particular residue, resulting in low selectivity.[271] The thiol group of cysteines presents the best accessible nucleophile with a relatively low abundance, opening up the possibility of introducing cysteine residues by site-directed mutagenesis for selective chemical modification.[272] This approach was exploited to control the activity of the membrane proteins proteorhodopsin and AdiC after reconstitution into proteoliposomes (Figures 8a and 10a)[57,273,274]

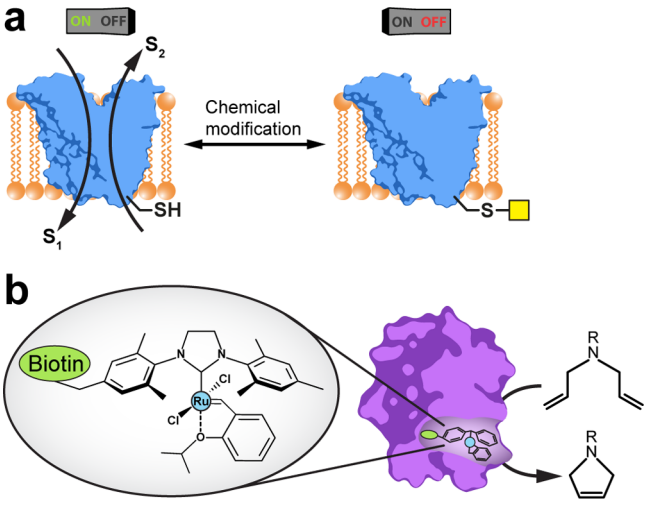

Figure 8. Modulating protein activity by chemical modification. (a) Controlling protein activity by reversible chemical modification of native or genetically introduced residues. Modification of an engineered cysteine residue with a sulfhydryl reagent reversibly inhibits the transport function of the arginine−agmatine antiporter AdiC.[273] (b) Engineering of an artificial metalloenzyme based on the biotin−streptavidin technology. A ruthenium catalyst (cyan) is coupled to biotin (green) and combined with streptavidin (purple) to create an artificial metalloenzyme for ring-closing metathesis reactions.[320]

and probe the activity of other transporters.[275−278] By deactivation of a selected population of transporters, a functional short circuit that results from a statistical distribution of the transporters in the vesicle membrane (*i.e.*, up and down oriented proteins), can be avoided (see section 6.2). In a similar fashion, a redox-sensitive variant of the outer membrane protein F (OmpF) was engineered by the introduction of cysteine mutantions and modification with a molecular cap that regulates the pore's activity.[279] The same concept of controlling protein activity can be transferred to other types of enzymes, targeting the same or different functional groups. Both the function of a protein as well as its accessibility for specific chemical modifications can be expanded by the introduction of unnatural amino acids (UAA).[280−282] Selenocysteine and pyrrolysine are two non-canonical amino acids used by nature, however, a much greater chemical variety is available for synthetic biologists. UAAs can be introduced using either solid-phase peptide synthesis, chemically acylated suppressor tRNAs, genetically engineered cell lines, or cell-free expression systems.[283−285] Encoding

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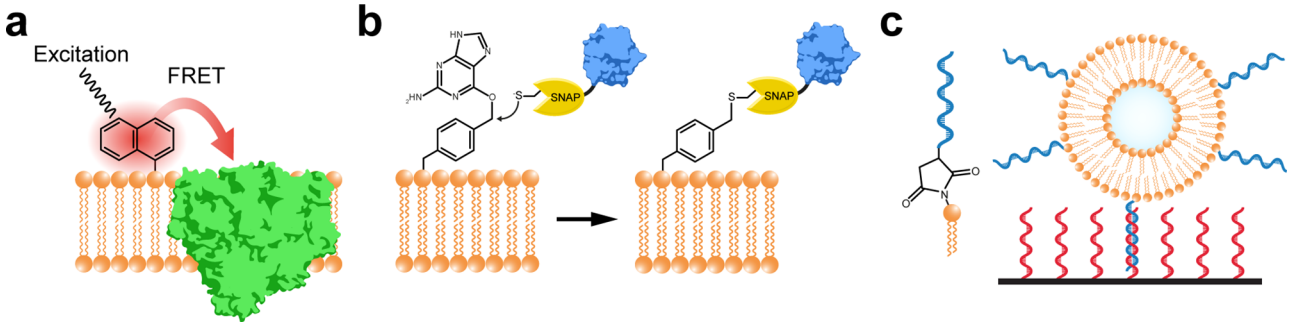

Figure 9. Increasing the versatility of scaffold modules. (a) Fluorescently labeled lipids expand the spectral absorption range of light-dependent membrane proteins (green) by fluorescence resonance energy transfer (FRET).[321] (b) SNAP-tagged proteins (blue with yellow SNAP fusion protein) can be localized to liposome membranes by covalent attachment to benzylguanine-functionalized lipids.[323] (c) Functionalization of lipids with maleimides enables conjugation to biomolecules such as DNA (red and blue).[324] Local immobilization of vesicular systems is achieved by specific interactions between complementary single-stranded DNA on the liposome and the targeted surfaces.

UAAs requires engineered aminoacyl-tRNA synthetases that recognize the artificial amino acid and are capable of loading it onto a designated tRNA.[286] Bio-orthogonal labeling of proteins was developed as one of the first applications for UAAs.[287] Through incorporation of non-native reactive groups, such as ketones, proteins could be selectively targeted even *in vivo* by suitable chemical probes, such as hydrazine derivatives of fluorescent dyes.[288] A wide variety of chemistries has since been introduced into proteins, including ring-substituted aromatics,[289−291] halogenated derivatives,[292−294] β- and γ-amino acids,[295−297] photo-[298−300] or redox-reactive[301−303] groups, metal binding moieties,[304−306] fluorescent probes,[307−309] and many more.[310] The introduction of *p*-aminophenylalanine into the *Lactococcus lactis* multidrug resistance regulator protein significantly increased the hydrazine and oxime formation and showcases how catalytic activities can be improved using UAAs.[311] Completely new functionalities can be engineered into proteins such as metal chelating sites, photoreactivity, redox centers, or fluorescence labels. In addition to affecting the catalytic mechanism, even the stability and preferred environmental conditions for optimal catalytic activity can be modulated by chemical modifications.[312] By conjugating cytochrome *c* with negatively charged polymethacrylic acid, which creates a more acidic microenvironment, its optimal pH range could be significantly shifted toward more alkaline conditions.[313] Similarly, modification of the haloalkane dehalogenase DhaA with inulin increased the resistance of the enzyme against harsh conditions, such as low pH, high ionic strength, and even organic solvents, by forming a protective hydration layer.[314]

Another opportunity to tailor the function of a protein is the introduction or substitution of prosthetic groups. An artificial heme enzyme capable of catalyzing the cyclopropanation of styrenes was created by introducing a hemin cofactor into the *Lactococcus lactis* multidrug resistance regulator.[315] The specific absorption maximum of light-driven proteins, such as proteorhodopsin and other retinal binding proteins, limit their application in synthetic biology. Slight red- or blue-shifts can be achieved by mutation of amino acid residues in the vicinity of the retinal cofactor.[316] However, by combining red-shifting mutations and retinal analogues, the absorption maximum of proteorhodopsin could be shifted into the near-infrared, enabling new applications in optogenetics thanks to the deeper penetration depth of longwave radiation in tissues.[317] Artificial metalloenzymes result from the incorporation of an abiotic metal cofactor into a protein scaffold.[172] Carboxypeptidase A was the focus of the first effort to alter the function of an enzyme by introducing a non-natural metal cofactor.[318] Substitution of the native Zn(II) by Cu(II) equipped the hydrolytic enzyme with the capability of oxidizing ascorbic acid. A more versatile approach was explored by introducing a biotinylated rhodium catalyst into avidin, yielding an artificial metalloenzyme with the properties of an asymmetric hydrogenation catalyst.[319] The protein scaffold provides a chiral environment for the catalyst, thus inducing potentially significant (enantio)selectivity for the reaction. This technology was further pursued to engineer enzymes capable of catalyzing reactions not found in nature. The first enzyme-catalyzed olefin metathesis reaction was achieved by combining a biotinylated ruthenium catalyst with streptavidin (Figure 8b), capable of performing the ring-closing metathesis of *N*-tosyl diallylamine.[320]

5.4. Increasing the Versatility of Scaffold Modules

Lipids and polymers are essential scaffold modules for assembling vesicle-based molecular systems. As structural basis for vesicular compartments, they provide a physical boundary toward the environment and a scaffold for integral or membrane associated protein modules. A range of molecules with different properties (sections 3.2 and 3.3) are available to form compartmentalized systems and facilitate particular applications, including the formation of stimuli-sensitive vesicles (see section 3.4). However, not all types of scaffolds are compatible with the incorporation of membrane-bound functional modules. Therefore, it is particularly important to expand the versatility of phospholipid scaffold modules, which are to date still the most reliable for the incorporation of membrane proteins.

Simple modifications such as the addition of fluorescent molecules to the head groups of lipids already increase the possible applications of proteoliposomes by facilitating tracking in different environments.[57] Furthermore, fluorescently labeled lipids can be used to create proteoliposomes, which enhance the spectral absorption range of light-absorbing proteins. The lipid-linked fluorophore Texas Red was used in proteoliposomes containing the light-harvesting complex II to broaden the absorption range by efficient energy transfer from the chromophore to the protein (Figure 9a).[321] Using fluorescent molecules with different spectroscopic properties, the absorption range of potentially any light-driven energizing module could be enhanced significantly. Aside from integrating

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membrane proteins into vesicle membranes, it is also possible to engineer targeted interactions between modified lipids and soluble proteins for their localization to the vesicle surface, providing a confined environment for enzymatic reactions.[322] Methylguanine DNA methyltransferase (SNAP-tag) catalyzes the covalent attachment of fusion proteins to benzylguanine-functionalized lipids and enables targeted localization onto liposome membranes (Figure 9b).[323] Bioconjugation mediated by maleimides, succinimides, or click chemistry can be used to tether any desired biomolecule with the corresponding reactive functional group to each other. By functionalizing maleimide-modified lipids with single-stranded DNA, it was possible to guide liposomes to a lipid bilayer surface decorated with the complementary DNA strand (Figure 9c).[324] This technology can be exploited to immobilize functionalized nanoreactors to surfaces or build assembly lines by connecting individual nanoreactors with different functionalities (see section 6.5). An increasing number of headgroup- or fatty acid-modified lipids for various purposes are commercially available, *e.g.*, for mediating attachment of biomacromolecules to vesicle membranes or other surfaces by covalent or high affinity interactions. Among such modifications, the most popular include maleimides, succinimides, thiols, and biotin. Surface modifications of liposomes are frequently used to optimize their application for targeted drug delivery and are a promising strategy to increase their circulation time as well as to direct them to specific tissues or organs.[325] Tailored scaffold modules offer a wide range of possibilities for the assembly of more versatile molecular systems, thus broadening their scope of application.

6. ASSEMBLY OF VESICLE-BASED BIOMOLECULAR SYSTEMS

6.1. Reconstitution of Membrane Proteins

Characterization and application of membrane proteins in bottom-up assembled biomolecular systems require biochemical purification and subsequent reconstitution into model membrane systems. The selection of a suitable membrane mimetic, *i.e.*, liposomes, polymersomes, or hybrid vesicles, is influenced by the desired application and dictates the required reconstitution procedure.[7,76,326] In addition, the compatibility of a target membrane protein with a specific membrane composition needs to be carefully evaluated for successful reconstitution.[65−68] Detergent-mediated reconstitution demonstrates the highest rate of success for the incorporation of functional membrane proteins.[326−329] Due to their amphiphilic nature, detergents are micelle-forming molecules and can act as substitutes for the native lipids, thus providing a hydrophobic environment for the membrane proteins in aqueous solutions after extraction. The choice of detergent for membrane protein purification depends on the physicochemical properties of the particular detergent and its compatibility with subsequent experiments (*e.g.*, reconstitution, crystallization, or functional assays). For the majority of applications, the critical micelle concentration (CMC) is the most important parameter. It affects the solubilization efficiency, the stability, and oligomeric state of proteins and determines the suitability of specific detergent removal methods for reconstitution.[330] Purification of membrane proteins using detergents involves the formation of lipid−protein−detergent micelles (ternary complexes) during the extraction process.[331] For reconstitution, additional lipid−detergent micelles or preformed detergent-destabilized liposomes are added before the detergent is removed, resulting in the eventual incorporation of the proteins in closed lipid bilayers. The most common strategies for the removal of detergents include controlled dilution or dialysis for high-CMC detergents (*e.g.*, deoxycholate or *n*-octyl-β-D-glucopyranoside) and gel filtration, cyclodextrins, or detergent-binding polymers for low-CMC detergents (*e.g.*, Triton X-100 or *n*-dodecyl-β-D-maltopyranoside).[332−334]

Natural lipid bilayers are asymmetric in regard to the lipid composition of their individual leaflets, a crucial property for membrane protein insertion, structure, and function. Even though it is possible to create asymmetric lipid vesicles, the preparation methods usually involve the use of organic solvents and sophisticated microfluidics setups.[79,335] Asymmetric block copolymers provide a more accessible alternative and additionally allow for more control and variety in regard to the individual leaflet components.[336,337] Mimicking the asymmetry found in natural biomembranes can also facilitate the unidirectional insertion of membrane proteins into synthetic vesicles (see section 6.2). Overall, methods for reconstituting membrane proteins into liposomes or polymersomes share many similarities but might not necessarily be transferable from one to the other.[76,338] To overcome frequently observed incompatibilities, in particular, the hydrophobic mismatch, of membrane proteins with synthetic polymer membranes, specifically designed block copolymers or engineered proteins might be required. The latter was demonstrated with a FhuA version exhibiting an increased hydrophobic surface, which facilitated its insertion into a thick polymeric membrane.[339]

Alternatively, detergent-free approaches aim at transferring membrane proteins from their host membrane to liposomes using either amphipathic polymers such as styrene maleic acid (SMA)[340] or cell-derived plasma membrane vesicles.[341] Both methods share the advantage that native lipids bound to the membrane protein, which may affect its structure and function, are not removed. In addition, fusion of cell-derived vesicles adds the possibility to simultaneously transfer cytosolic contents such as water-soluble proteins.[341] Instead of isolating membrane proteins from their expression hosts for subsequent reconstitution, cell-free expression systems perform the *in vitro* translation in a hydrophobic environment, *e.g.*, in the presence of detergents, amphipols, or lipid-like peptide−detergents to enable cotranslational solubilization.[342−344] To increase the chances of correct membrane protein folding, cell-free expression systems are combined with direct integration of target proteins into membrane mimicking structures such as bicelles, nanodiscs, or liposomes and recently even polymersomes.[345,346] While bicelles and nanodiscs are suitable scaffolds to study membrane protein structures by spectroscopy or electron microscopy, additional reconstitution steps are required to incorporate them into vesicular systems.[347,348] Due to the topological diversity of membrane proteins and their diverse insertion mechanisms, no universal membrane-embedding system exists. However, *E. coli* or wheat germ derived cell-free expression systems in the presence of liposomes have been successfully used for cotranslational insertions.[349] Cell lysates provide soluble chaperones that can facilitate the passive integration of membrane proteins into lipid bilayers but do not seem to be required in other cases.[350−352] Inverted membrane vesicles of bacterial or eukaryotic origin, *e.g.*, from *E. coli* inner membranes or endoplasmic reticulum (ER) membranes, represent more biomimetic approaches that take advantage of endogenous

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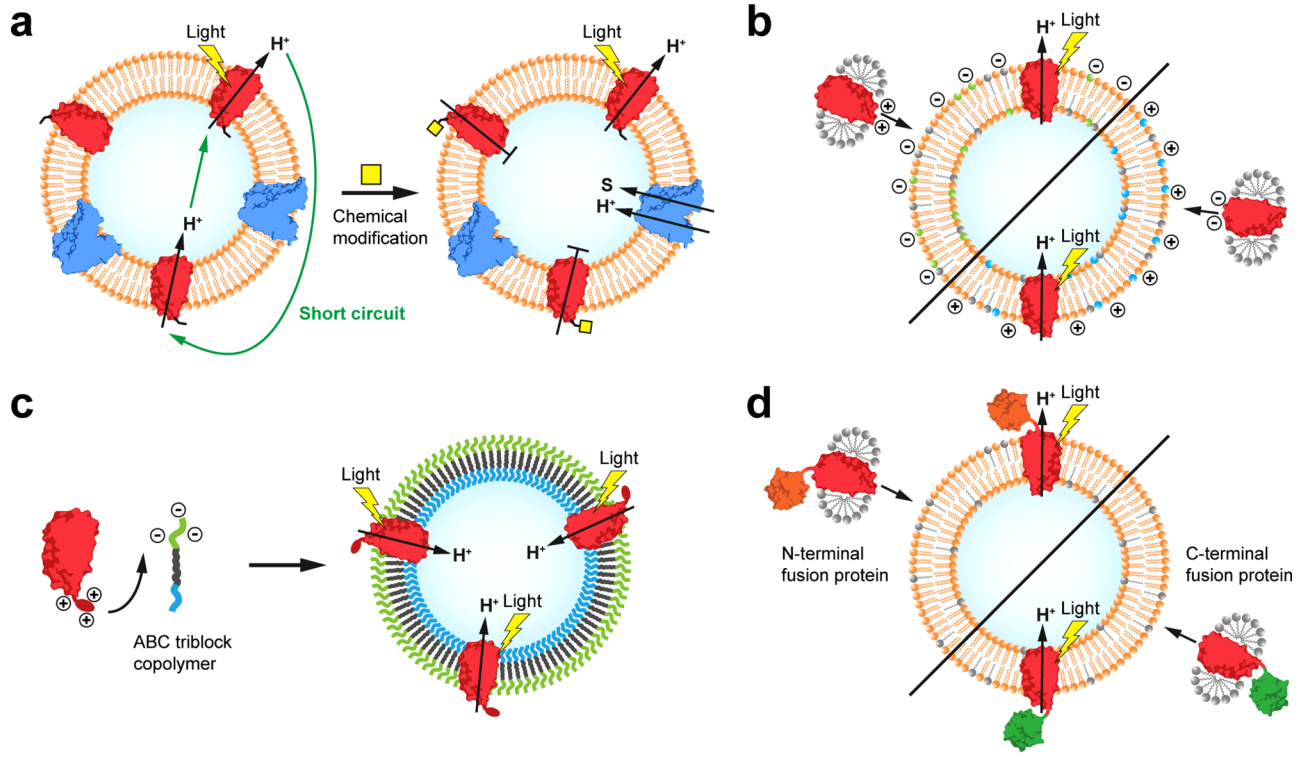

Figure 10. Controlling membrane protein orientation in bottom-up assembled vesicular systems. (a) Engineering a chemical switch into a light-driven proton pump (red).[274] Protein activity is controlled by reversible chemical modification of an engineered cysteine residue using a thiol-reactive reagent (yellow box). A functional short-circuit in proteoliposomes containing randomly oriented membrane proteins is prevented by selective deactivation of the undesired protein population. Establishment of a proton gradient enables translocation of a substrate by a proton-driven transporter (blue). (b) Incorporation of differently charged phospholipids (headgroups in green and blue) into liposomes creates preferential interactions and enables directed insertion of an asymmetrically charged membrane protein.[358] (c) Alignment of asymmetrically charged ABC triblock copolymers and light-driven proton pump (red) during reconstitution favors the directed reconstitution owing to preferential interactions.[337] (d) Soluble fusion domains at the N- (orange) or C-terminus (green) of a light-driven proton pump (red) guide its directed reconstitution into preformed liposomes.[359] The membrane protein integrates into the detergent-destabilized liposome with its unoccupied terminus facing inward, thus generating unidirectional protein insertion into the membrane. Phospholipids are depicted in brown and detergent molecules in gray.

translocon components.[353,354] Even though they provide the *in vitro* translated proteins with a natural environment, ER derived microsomes can require tedious processing for subsequent functional analysis.[355] Despite the continuously developed reconstitution methods, which can be advantageous for specific downstream applications, the established, classical detergent-mediated reconstitution methods are still widely used due to their accessibility and high success rate.

6.2. Membrane Protein Orientation During Reconstitution

The orientation of membrane proteins in synthetic membranes after reconstitution depends significantly on the reconstitution method, the membrane composition and the protein topology. Reconstitutions from solubilized protein−detergent and lipid−detergent mixtures commonly yield proteoliposomes with randomly oriented membrane proteins (*i.e.*, inside-in and inside-out) but may exhibit slight preferences.[38,356,357] The orientation of membrane proteins in vesicle membranes may be assessed by functional assays[38,57,274] or by exploiting the one-sided access to the protein. The latter can be done by analyzing proteolytic fragments or by evaluating the efficiency of a chemical labeling reaction before and after solubilization of reconstituted proteoliposomes.[337,358,359] Symmetric distribution of vectorial transport modules in the vesicle membrane results in a functional short-circuit, which prevents the establishment of a substrate gradient.[360] One possibility to rectify this type of malfunction is the selective chemical deactivation of one population of proteins with the same orientation (Figure 10a). This approach has been demonstrated for the light-driven proton pump proteorhodopsin[57,274] and the arginine−agmatine antiporter AdiC.[273,361] In both cases, a molecular switch in form of a genetically engineered cysteine residue was introduced, which was only accessible for chemical modification by water-soluble reagents from one side of the vesicle membrane. This enabled selective deactivation of a selected protein population. Reconstitution using preformed, detergent-destabilized liposomes can promote unidirectional insertion of membrane proteins due to preferential interactions with the liposome surface or steric effects.[38,362,363] The lipid bilayer composition and the resulting surface charge of liposomes can be utilized to create preferential interactions with the membrane protein during reconstitution to control the insertion orientation (Figure 10b).[358] Similarly, directed reconstitution of membrane proteins into asymmetric polymersomes from ABC triblock copolymers was achieved.[336,337] Electrostatic interactions induced the alignment of proteorhodopsin, which exhibits a slight polarity between its intra- and extracellular surfaces, and the asymmetric triblock copolymer during reconstitution, yielding unidirectional insertion (Figure 10c).[337] It has been frequently observed that membrane proteins tend to insert into preformed

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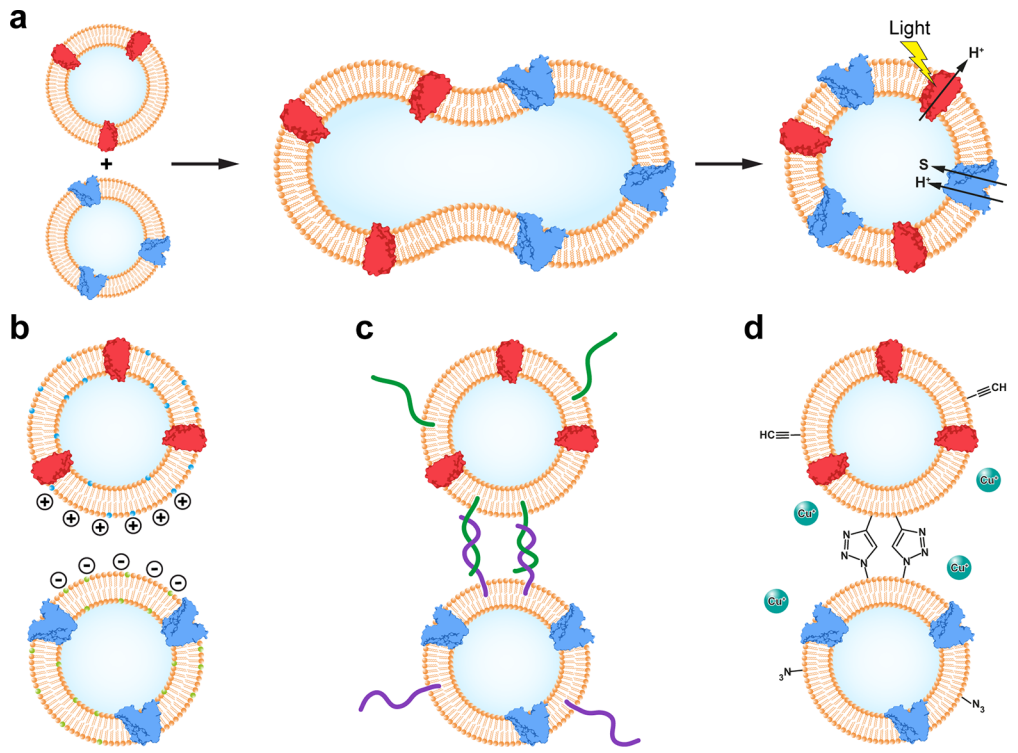

Figure 11. Co-reconstitution of different membrane proteins by fusion of preformed proteoliposomes. (a) Separate reconstitution of different membrane proteins into proteoliposomes, which are subsequently fused. Engineering functional nanoreactors by individual reconstitution of energizing modules, *e.g.*, a light-driven proton pump (red) and transport modules, *e.g.*, a proton-driven symporter (blue), followed by vesicle fusion. For (b–d) only a snapshot of the prefusion state is illustrated, all resulting in the final vesicle structure depicted in (a) following the same schematic mechanism. (b) Charge-mediated fusion of proteoliposomes prepared with cationic (blue headgroups) and anionic lipids (green headgroups) are based on electrostatic interactions.[379] (c) SNARE-mediated fusion of proteoliposomes is performed by components of the synaptic vesicle fusion machinery (green and purple).[381] (d) Click chemistry-mediated fusion is based on the copper-catalyzed chemical tethering of proteoliposomes functionalized with alkyne and azide groups.[383]

liposomes with their most hydrophobic domains first and that bulky hydrophilic domains, such as the head domain of ATP synthases, promote asymmetric insertion.[362,363] By mimicking the effect of naturally occurring hydrophilic domains on the reconstitution orientation of membrane proteins, directed reconstitution was achieved by attaching silicate beads to enforce insertion with the opposite terminus facing inward.[364] Alternatively, hydrophilic domains can be genetically added in the form of fusion proteins (see section 5.2). For example, fluorescent proteins were fused to either the N- or C-terminus of proteorhodopsin in order to promote directed reconstitution with the unoccupied terminus into the proteoliposomes (Figure 10d).[359] By exploiting the natural mechanism of protein translocation and with the help of soluble chaperones, cell-free expression systems are also able to achieve directed reconstitution into synthetic vesicles.[365−367] Importantly, controlling the reconstitution orientation of energizing modules is sufficient for the assembly of biomolecular systems with a directed transport function (Figure 10a). If transport modules depend on the establishment of an electrochemical gradient, energizing modules dictate the direction of substrate translocation by transport modules even if the latter were randomly inserted.

6.3. Co-reconstitution of Protein Modules and Vesicle Fusion

The assembly of functional biomolecular systems requires the reconstitution of multiple membrane proteins into the same membrane by simultaneous or subsequent insertion. The prerequisites for simultaneous co-reconstitution of several proteins are shared compatibility for detergents, lipid composition, and other buffer components. Even though the co-reconstitution of only two different membrane proteins can already be challenging, it has been demonstrated on several occasions.[44,368−371] However, reports of successful incorporation of more than two different membrane proteins, which will be necessary for more complex systems, are rare.[45,138] Alternatively, if the desired combination of membrane proteins do not share sufficient compatibility, subsequent reconstitution into the same vesicles or fusion of proteoliposomes carrying different membrane proteins might be required (Figure 11a). The fusion of proteoliposomes allows optimization of reconstitution conditions for each individual membrane protein and can thus significantly increase the chances of successful co-reconstitution. In case the mutually exclusive compatibilities for specific lipids prevent successful co-reconstitution, homologues of target proteins from different origins, with potentially different preferences for membrane composition, might need to be considered.[68]

Spontaneous vesicle fusion, although rare, can occur during prolonged close contact between vesicles with sufficient membrane tension, both of which can be facilitated by external energy or introduction of catalysts.[372] Membrane fusion can be promoted by external agents, so-called fusogens, which lower the energy barriers of intermediate steps during the fusion process.[373] Besides specific proteins and peptides, certain cations, in particular Ca^{2+} and Mg^{2+}, have been found to

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exhibit fusogenic properties. The binding of ions to membranes can shield lipid headgroup charges and modify surface polarity, which in turn leads to expulsion of water from the intermembrane space, *i.e.*, the gap between fusing membranes, allowing lipid mixing and finally membrane fusion.[374,375] A range of different approaches have been developed to fuse lipid vesicles that are based on mechanical stimulation, noncovalent interaction between lipids, chemical coupling between functionalized lipids, or are mediated by proteins. Mechanical stimulation such as sonication or cycles of freezing and thawing has been shown to induce vesicle fusion.[376,377] However, both methods are rather harsh and can have detrimental effects on the stability of incorporated membrane proteins. Furthermore, it has been demonstrated that subjecting proteoliposomes to sonication or freeze−thaw cycles can randomize membrane protein orientations in the vesicle membranes.[378] Other methods for the controlled fusion of proteoliposomes have since been developed, which largely maintain membrane protein orientation and integrity. Liposomes containing lipids with opposing charge interact to a degree that promoted vesicle fusion.[379] The interaction between charged liposomes was capitalized on as a means for functional co-reconstitution of bo_3 oxidase and F-type ATP synthase by charge-mediated proteoliposome fusion (Figure 11b).[380] SNARE-mediated proteoliposome fusion (Figure 11c) was developed based on the natural process of synaptic vesicle fusion and shown to enable the co-reconstitution of bacterial respiratory chain components.[381] The same approach could be successfully transferred to the fusion of polymersomes and hybrid vesicles.[382] In an effort to mimic the biological fusion process mediated by SNARE proteins, vesicles were endowed with lipids that can undergo chemical coupling. Liposomes prepared from alkyne- and azide-functionalized phospholipids could be tethered using Cu(I)-catalyzed click chemistry (Figure 11d), which promoted spontaneous vesicle fusion.[383] The extent to which the mechanisms of polymersome fusions have been studied is still limited compared to liposomes. Nevertheless, methods for the fusion of polymer vesicles are being developed, exploiting their unique chemistries and self-assembly mechanisms.[384−388] Creating hybrid vesicles by fusion of liposomes and polymersomes combines their compatibility with membrane protein reconstitution and increased stability (see section 3.2).[389,390] Simultaneous co-reconstitution of multiple transport modules can be strongly limited by the specific preferences of different membrane proteins for detergents, lipids, and polymers during purification and reconstitution, respectively. Therefore, vesicle fusion seems to offer the most promising approach for the bottom-up assembly of functional biomolecular systems.

6.4. Encapsulation of Protein Modules and Other Biomacromolecules into Vesicular Systems

Compartmentalization of metabolizing modules offers a range of advantages, including increased local concentrations of catalyst and substrates, as well as protection from detrimental external factors (*e.g.*, inhibitory molecules or proteases).[322] Such confinement can result in higher collision frequencies between encapsulated substrates and enzymes, thus increasing the catalytic turnover and lowering K_M.[391] An early study demonstrated the entrapment of lysozyme and glucose during liposome formation and found that the capture efficiency increased when using liposomes with a higher proportion of charged lipids.[392] It was suggested that a higher surface charge increases the aqueous interspace between lipid bilayers in polylamellar vesicles due to electrostatic repulsion. The possibility of encapsulating small molecules or enzymes in liposomes or polymersomes depends on their morphology (*e.g.*, size and lamellarity), which is related to the method of preparation and the chemical properties of the scaffold modules. Some encapsulation methods are optimized for chemically stable molecules, whereas milder protocols are required to maintain the structural integrity and function of proteins. Liposome preparations that involve apolar solvents, such as ether or ethanol injection and reverse phase evaporation, or methods that generate high amounts of local heat, such as sonication, have low compatibility with proteins due to their harsh nature.[51] A milder and highly efficient encapsulation procedure involving organic solvents, is the water-in-oil emulsion transfer. This method could be used to encapsulate enzymes and reaction mixtures capable of catalyzing processes such as reverse transcription polymerase chain reaction, transcription, and protein synthesis.[393,394] The lipid film hydration has emerged as a frequently used, nondenaturing method for encapsulating proteins into small and large liposomes.[395] The major drawback is the rather low encapsulation efficiency, which can be improved with increasing number of freeze−thaw cycles following the hydration step.[395] The final number of encapsulated enzymes determines the performance of the nanoreactor and can thus be tuned by scaling the amount of enzymes added, provided that the activity is preserved after encapsulation.[396] Multilamellar vesicles of varying size are formed by the hydration of lipid films, which can be converted to uniform unilamellar vesicles by a combination of freeze−thaw cycles, sonication, and/or extrusion.[397−399] The encapsulation efficiency decreases with increasing number of lamellae and protein size and can be as low as 5% compared to the highest reported values of around 40%.[400] Giant unilamellar vesicles (GUVs) prepared by emulsion transfer, electroformation,[401] or microfluidics devices[402] with diameters of several micrometers, open additional possibilities for the encapsulation of enzymes. Due to the large size of the vesicles, enzymes can be directly introduced with the help of microinjection devices and reactions can be conveniently monitored by light microscopy.[403] Liposomes prepared by microfluidics technology can be loaded individually with desired solutions or biomolecules using picoliter-injection at kilohertz rate.[404] The injection of subpicoliter amounts of fluid from a pressurized channel into the vesicles is triggered by an electric field, making this process highly controllable. Droplet-stabilized GUVs have been injected with proteoliposomes containing integrin or F-type ATP synthase, which fused with the lipid bilayer at the droplet periphery, generating biofunctionalized vesicles.[402] Similarly, an actin cytoskeleton and microtubules were assembled by injection of the microfilament-forming proteins actin and tubulin.[402] Finally, this approach facilitates the construction and intracellular application of cell-mimicking and non-natural organelles, such as synthetic peroxisomes, endoplasmic reticulum vesicles, and magnetosomes.[405] Encapsulation of biomolecules in synthetic vesicles plays a central role in biomedical research and the development of novel therapeutics, with several liposome-based formulations approved for the delivery of drugs, such as doxorubicin and amphotericin B.[406,407] The latest successful application of liposomes as delivery vehicles was the development of the COVID-19 vaccines by Moderna and Pfizer/BioNTech, which rely on

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encapsulating the fragile mRNA encoding for the SARS-CoV-2 spike protein and delivering it into the cytoplasm of host cells.[408,409] Polymersomes have been evaluated as alternatives to liposomes as drug delivery systems due to their improved chemical stability. Furthermore, they exhibit reduced membrane permeability, which is often linked to the thickness and low lateral fluidity of the polymer membrane (see section 3.2).[55] Whereas the integration of delicate membrane proteins into polymer membranes can be challenging due to the differences in chemical composition, polymersomes can display excellent encapsulation efficiencies for large biomacromolecules such as proteins and DNA. Biodegradable block copolymers were developed as valuable tools for the drug delivery of proteins with extraordinarily high encapsulation efficiency of over 90% for bovine serum albumin.[410] As an alternative to artificial vesicular systems, methods to generate cell-derived membrane vesicles provide an approach to encapsulate overexpressed and endogenous cytosolic proteins without purification, which can then be transferred by vesicle fusion (see section 6.3) to other vesicular systems.[341] Cell-derived extracellular vesicles have also been suggested as a novel drug delivery platform owing to their low immunogenicity, relatively high resistance to degradation, and efficiency in transferring their cargo to host cells.[411,412]

6.5. Assembly of Higher-Order Vesicular Systems

Individual liposomes or polymersomes can be combined into multicompartment vesicles, so-called vesosomes (Figure 12a), mimicking the hierarchical structure of eukaryotic cells and their organelles.[413,414] Individual vesicles with different content are fabricated to provide optimal reaction conditions for particular enzymatic reactions that ultimately make up a cascade (see section 7.3). The advantages of multicompartment systems can also be leveraged to mimic cellular signaling processes that result in triggered enzymatic activity, *e.g.*, through the release of enzymes from artificial organelles upon an external signal.[415] Vesosome structures are usually assembled in consecutive steps by encapsulating preformed vesicles into larger ones, which opens the possibility to employ vesicles of different compositions. An efficient way to generate polymersome vesosomes involves forming inner vesicles by nanoprecipitation and loading them into larger polymersomes by emulsion−centrifugation.[414] Multistep microfluidic systems were explored to endow GUVs with a variety of internal structures, including liposomes or even whole organelles such as isolated nuclei.[10] With the possibility of controlled step-by-step bottom-up assembly of functionalized vesicular systems, microfluidics provides the means for the fabrication of micron-sized reactors with a broad scope of application as well as cell-mimicking systems aimed toward designing artificial organelles and cells.[405,416] Another strategy to assemble liposomes or polymersomes into higher-order structures are organized clusters based on specific interactions. Polymersomes functionalized with single-stranded DNA were interconnected into 3D clusters by DNA hybridization (Figure 12b).[417] The physical proximity provided by encapsulation or tethering is a prerequisite for the functional combination of individual nanoreactors harboring parts of an enzymatic reaction cascade.

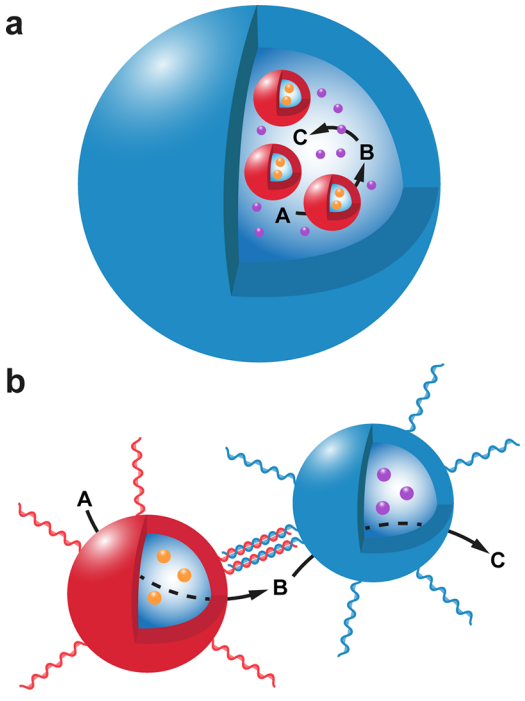

Figure 12. Assembly of higher-order vesicular systems. (a) Nested polymersomes as basis for cell-mimicking vesosomes (blue) containing artificial organelles (red).[418] (b) DNA-mediated organization of polymersomes (red and blue) modified with complementary single-stranded DNA into functional clusters.[417] Polymersomes in (a) and (b) can harbor different enzymes (orange and purple) able to catalyze individual steps of a reaction cascade. Similar multicompartment structures as in (a) and (b) have also been generated using liposomes.

6.6. Biophysical Analysis of Functionalized Vesicular Systems

A selection of biophysical methods for the characterization of assembled molecular systems is described below, offering a final quality control step. The general morphology of individual vesicles, such as size and lamellarity, and the overall assembly of vesicular systems (*e.g.*, vesosomes) can be assessed using variations of transmission electron microscopy (TEM).[419,420] Negative stain TEM involves adsorption of the sample onto a surface, commonly a thin carbon layer, embedding in heavy metal salts such as uranyl acetate and air drying.[421,422] This method allows fast and simple imaging of the sample with high contrast, but the morphology of the vesicles may be affected by the adsorption, staining, and/or drying process, and internal features are not visible. Cryogenic TEM, or cryo-TEM, enables analysis of samples at a high resolution in their most native state. Samples are prepared by vitrification in an aqueous film without the need for adsorption onto a surface. Currently, cryo-TEM is the most powerful microscopic tool to study vesicular systems at the nanoscale, yielding detailed information about their size, shape, and internal structure including lamellarity.[57,337,359,419] Statistical methods such as dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), and tunable resistive pulse sensing (TRPS) offer a more quantitative analysis without providing any morphological information.[423,424] DLS is an ensemble technique, which describes the average vesicle size, whereas NTA and TRPS measure on a vesicle-by-vesicle basis and yield accurate size distributions even for polydisperse samples. NTA can be combined with fluorescent labeling of lipids or proteins to track specific subpopulations of vesicles.[57,425] Analysis of surface charges by zeta potential measurements can shed light on the correct composition and orientation of lipids and

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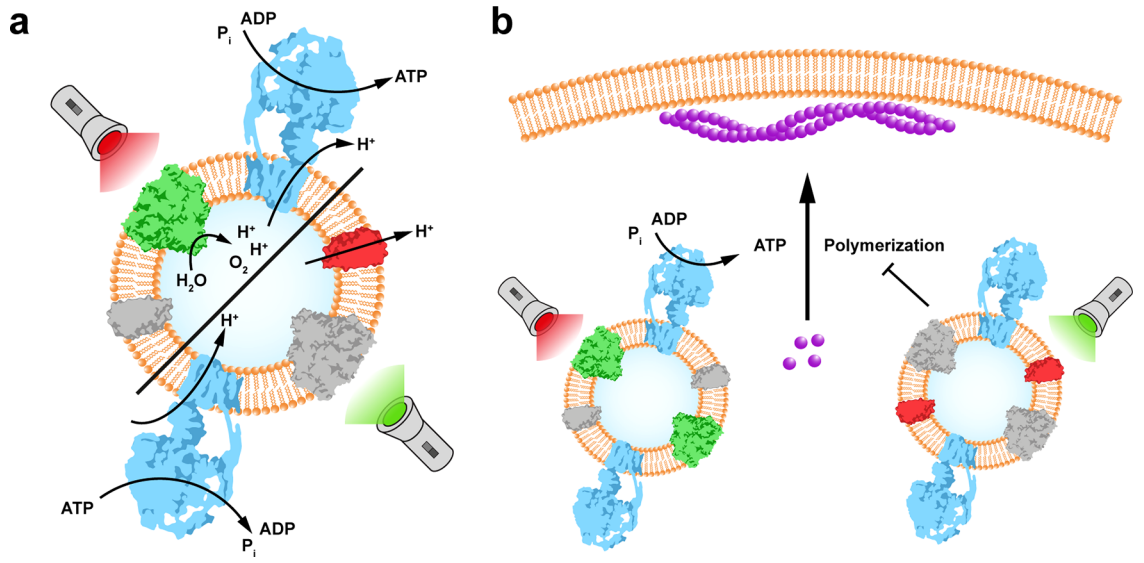

Figure 13. Artificial photosynthetic system converting light into chemical energy for cytoskeleton formation. (a) Proteoliposomes equipped with proteorhodopsin (red), photosystem II (green), and F-type ATP synthase (blue) produce or hydrolyze ATP depending on the wavelength of the illumination. Red light stimulates photosystem II and leads to the synthesis of ATP, whereas green light stimulates proteorhodopsin resulting in the hydrolysis of ATP. (b) Proteoliposomes containing proteorhodopsin, photosystem II, and F-type ATP synthase are encapsulated in giant vesicles. ATP synthesized during illumination with red light is used for polymerization of actin (purple) monomers at the membrane of the outer vesicle, while green light inhibits the formation of the actin filaments.[138]

polymers in the compartment membrane.[426,427] On the other hand, neither TEM nor the just mentioned quantitative measurements can handle micrometer-sized systems. For the analysis of cell-sized systems, light and fluorescence microscopy as well as flow cytometry are more suitable methods.[403,428,429] Atomic force microscopy (AFM) can be used to some extent to analyze the contents of proteoliposome membranes,[430−432] *i.e.*, to confirm the reconstitution of energizing and transport modules.[433−435] Specific detection was achieved when using AFM tips functionalized to recognize specific interactions. *e.g.*, between antibodies and antigens, or between polyhistidine-tagged molecules and nickel nitrilotriacetate.[436−438] Recent developments in electron microscopy are even paving the way for analyzing membrane proteins directly in proteoliposomes, highlighted by the structures of the multidrug efflux pump AcrB and the mechanosensitive ion channel Piezo1 determined in liposomes by cryo-TEM and cryo-electron tomography (cryo-ET), respectively.[439,440] Aggregation of membrane proteins during reconstitution into synthetic membrane systems is one of the most challenging obstacles, which can severely limit the number of functional proteins in the final vesicle system or may require removal of protein aggregates to avoid interference with downstream applications. Methods such as dual-color fluorescence cross-correlation spectroscopy and fluorescence anisotropy have been demonstrated to be valuable tools for optimizing the membrane protein reconstitution process due to their ability to assess successful incorporation and correct orientation of the proteins in the membrane.[66,441] To evaluate successful vesicle fusion and transfer of encapsulated contents, assays that monitor the mixing of lipids and contents are valuable tools. This requires initial liposomes to be prepared with either fluorescently labeled lipids or with encapsulated soluble fluorophores by means of which the fusion process can be observed.[380,442]

7. APPLICATIONS

Advances in the engineering of functional and scaffold modules, as well as improvement of processes for their assembly into molecular systems, have culminated in numerous significant milestones. Up to this point, we have presented an extensive set of modules and assembly methods, which serve as a toolbox for the creation of synthetic vesicular systems with tailored properties and functionalities. Applications can range from biocatalysis, drug delivery, biosensing, and bioremediation to replicating central cellular processes. Representative examples of different fields are described and discussed in more detail in the following section to highlight particular aspects of the bottom-up approach. Systems were chosen with a focus on the use of diverse energizing, transport, and metabolizing modules, versatile scaffolds with tailored functionalities, and elaborate higher-order assemblies.

7.1. Light-Dependent Energizing Modules in Artificial Photosynthetic Systems

Solar energy is an almost inexhaustible and universally available power source, whose conversion into chemical energy by photosynthesis forms the basis of most known life on earth. Developing artificial photosynthetic systems, including suitable energizing modules, able to exploit this efficient energy supply has been a goal of synthetic biology for a long time. In this first example, liposomes were equipped with light-powered transmembrane proteins that catalyze the conversion of light into chemical energy, which is then used to mimic cytoskeleton formation *in vitro* (Figure 13).[138] F-type ATP synthase was used in combination with proteorhodopsin and photosystem II to build artificial light-harvesting organelles, which were encapsulated in GUVs containing actin monomers, a microfilament-forming cytoskeletal protein. Illumination with either red or green light allows independent activation of photosystem II or proteorhodopsin, resulting in opposing proton gradients, which either induce or impede synthesis of ATP, respectively (Figure 13a). The chemical energy provided by

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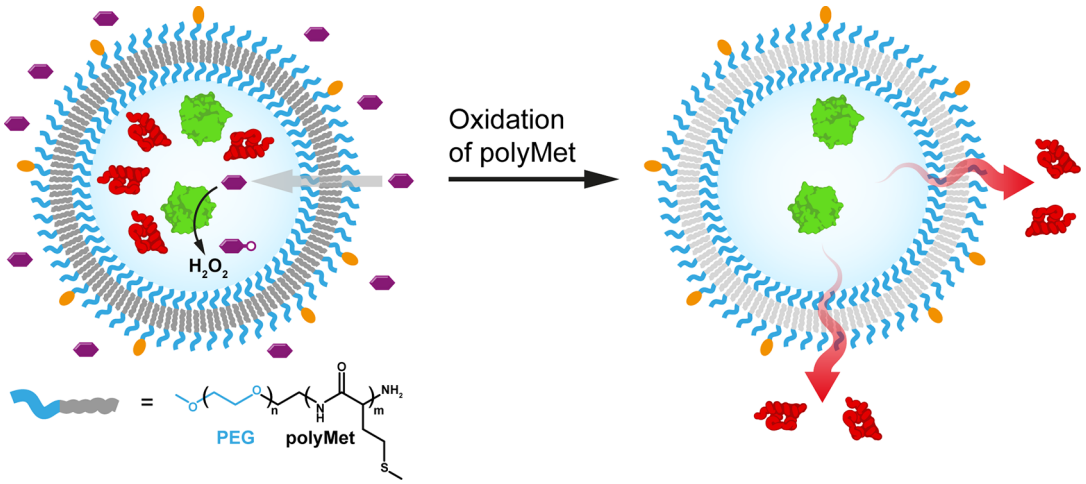

Figure 14. Liver-targeting and glucose-responsive insulin delivery vesicles. Polymersomes made from mPEG-polyMet block copolymer are decorated with liver-targeting peptides (orange) and encapsulate insulin (red) and glucose oxidase (green). At high external concentration, glucose (purple) diffuses into vesicles and glucose oxidase produces H_2O_2 as a byproduct of glucose oxidation. Subsequently, the methionine side chains of the polymer membrane are oxidized (illustrated by lighter gray coloring), rendering it hydrophilic and triggering the release of insulin.[443]

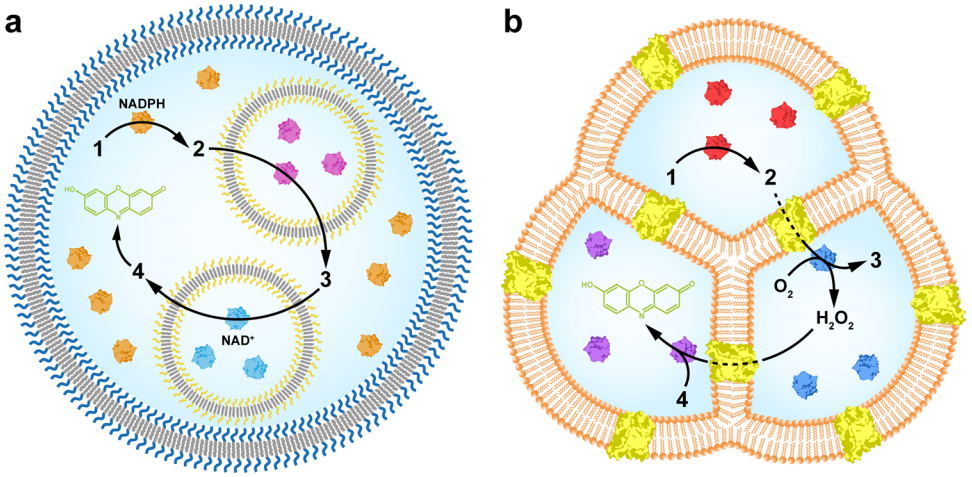

Figure 15. Multicompartment systems harboring enzyme-catalyzed cascade reactions. (a) Polymersome vesosome built from two different diblock copolymers containing the enzymatic components of a four-step reaction for the synthesis of resorufin (green) from nonfluorescent precursors (**1**−**4**). The individual steps are catalyzed by phenylacetone monooxygenase (orange), *Candida antarctica* lipase B (pink), and alcohol dehydrogenase (cyan), while the final reaction is spontaneous.[418] (b) Liposome-based multicompartment vesicle containing a spatially separated three-step enzyme cascade. Two-step transformation of lactose to gluconolactone (**1**−**3**) by lactase (red) and glucose oxidase (blue) generates H_2O_2 for the oxidation of Amplex Red (**4**) by horseradish peroxidase (purple) to fluorescent resorufin (green). Incorporation of α-hemolysin pores (yellow) enables diffusion of intermediates between the reaction compartments.[447]

ATP is then utilized for the polymerization of actin into microfilaments (Figure 13b). The interaction of the forming cytoskeleton structure with the proteoliposome membrane induced observable morphological changes of the vesicles, granting the artificial system almost cell-like behavior. Synthesized ATP could also be used in a similar setup to power a carbon fixation reaction. In the presence of acetyl-CoA and bicarbonate, pyruvate carboxylase catalyzed the ATP-dependent formation of oxaloacetate from pyruvate. Light-dependent energizing modules play a fundamental role in both examples by providing a sustainable supply of energy, an aspect with major relevance for future endeavors. The described artificial photosynthetic organelles exhibit essential traits for the development of synthetic cell-like systems requiring a basic metabolism or cellular behavior in the form of structural/morphological changes. The reproduction of two ubiquitous biological processes represents a milestone toward the development of artificial photosynthetic systems. In particular, it required the controlled assembly of multiple functional modules with specific relative orientation into a higher-order vesicular system by means of a multistep assembly process.

7.2. Engineered Stimuli-Responsive Scaffolds for Targeting Drug Delivery Vesicles

Besides applications in biocatalysis, vesicle-based molecular systems have great potential for treating a variety of pathologies *via* targeted drug delivery. Modules mediating specific molecular interactions are used for the delivery to a target tissue, where the desired drugs are locally released by either stimuli-responsive permeabilization or *via* specialized transport modules. The combination of these tasks was demonstrated by the creation of liver-targeted polymersomes that secrete insulin upon encountering increased external glucose concentration (Figure 14).[443] The vesicles were

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equipped with a peptide that binds to the ganglioside−monosialic acid receptor in the intestine, inducing transcytosis and eventual accumulation of the polymersomes in the liver. The ability to cross the intestinal epithelium enables oral administration of the therapeutic polymersomes. Vesicles were loaded with glucose oxidase, which produces hydrogen peroxide proportionally to the external concentration of glucose, which can diffuse through the semipermeable membrane. Subsequent oxidation of the polymethionine-containing mPEG-polyMet polymer membrane leads to permeabilization and release of the stored insulin cargo. The liver-targeted delivery and *in vivo* efficiency of insulin-releasing polymersomes was demonstrated in diabetic rats. Insulin delivery by the therapeutic vesicles normalized hepatic glucose utilization, enabling blood glucose levels to be maintained even after dietary administration. This example showcases a therapeutic biomolecular system capable of triggered cargo release by combining an engineered scaffold module sensitive to a stimulus (hydrogen peroxide) that was generated *in situ* by a specialized metabolizing module in response to an external signal (rising glucose concentration). Importantly, core concepts of different fields, namely biosensing and targeted drug delivery, have been integrated in this nanoreactor and were linked by a central compartmentalized reaction.

7.3. Multicompartment Systems for Cascade Reactions

The spatial separation of vesosome structures can be exploited to perform enzymatic cascade reactions with each individual step encapsulated in different subcompartments. To demonstrate the potential of multicompartmentalized systems, a three-enzyme reaction was assembled in cell-mimicking polymer vesosomes containing two types of artificial organelles (Figure 15a). In a first step, alcohol dehydrogenase and *Candida antarctica* lipase B were incorporated separately into polystyrene-*b*-poly(3-(isocyano-L-alanyl-amino-ethyl)-thiophene) (PS-*b*-PIAT) polymersomes. These organelle mimics were then encapsulated together with phenylacetone monooxygenase, mimicking a cytosolic enzyme and the required reagents in larger polybutadiene-*b*-poly(ethylene oxide) (PB-*b*-PEO) polymersomes. Due to the porous nature of PS-*b*-PIAT, reagents and products can freely diffuse between the artificial cytosol and organelles. The structural and functional cell mimic was capable of performing the consecutive enzyme reactions in successive compartments to produce fluorescent resorufin from a nonfluorescent precursor.[418] In a similar approach, multicompartment liposomes can be created by transferring a defined number of water-in-oil droplets into an aqueous medium using the phase transfer method.[444−446] The water droplets are injected into a lipid-in-oil phase and gradually move into the lower aqueous phase due to their higher density. The droplets coated by a lipid monolayer are enveloped by the monolayer of lipids at the oil−water interface to form a lipid bilayer membrane. In this way, the composition of the initial water droplets and thus the content of the final compartments can be fully controlled. A three-compartment liposome system was created with three consecutive but spatially segregated enzymatic reactions (Figure 15b).[447] The cascade reaction comprises the two-step transformation of lactose into gluconolactone, which produces hydrogen peroxide as a byproduct. Hydrogen peroxide is then used to generate resorufin by oxidation of Amplex Red, a nonfluorescent precursor. Transport of solutes between the compartments was mediated by incorporation of α-hemolysin pores into the lipid bilayer. Through sequential encapsulation of reaction components in vesosomes or by the phase-transfer of water-in-oil droplets, multicompartment vesicles with well-defined composition were created. The tight control that these methods offer over the assembled components is a prerequisite for the development of highly complex cell-mimicking structures. Two alternative approaches to regulate the exchange of solutes between compartments have been showcased, mediated either by semipermeable scaffolds or integrated transport modules. Finally, both examples illustrate the potential of multicompartment systems for *in situ* synthesis of chemical compounds or as biosensors that can create a chemical response to an environmental signal.

8. FINAL THOUGHTS AND PERSPECTIVES

The bottom-up assembly of functional molecular systems has significantly advanced in replicating cellular functions *in vitro* and has produced valuable tools for applications in biocatalysis, biosensing, bioremediation, drug delivery, and medicine. All resources invested into developing complex synthetic systems comprising cooperating modules also improve our fundamental understanding of the molecular and functional interactions between protein and scaffold modules. A variety of challenges still need to be addressed for the bottom-up approach to close in on the higher success rate of top-down engineered systems. The majority of bottom-up assembled systems depend on periodic external interventions and currently lack the ability for autonomous and self-regulated function. To this end, multiple aspects need to be addressed and integrated: (i) self-sustaining energizing modules using inexhaustible or renewable energy sources, (ii) regulatory mechanisms that ensure efficient reaction conditions and resource allocation (*e.g.*, regulating buffer conditions and controlling metabolic fluxes), (iii) proofreading and maintenance tools for functional and scaffold modules, metabolic networks, and replication processes, and (iv) self-replication modules to propagate genetic information and synthesize new proteins. Only with the incorporation of all these features are artificial biomolecular systems capable of continuous unsupervised function. Additional technical challenges include the development of more efficient and universally applicable assembly methods and achieving better control over the stoichiometry and relative orientation of functional modules, in particular membrane proteins. Many individual cellular processes have been successfully isolated and recreated in artificial systems, but combining and coordinating them into a functional system and optimizing their respective interfaces will be one of the greatest challenges in the future. Furthermore, there is an almost inexhaustible number of new-to-nature functionalities to be discovered by exploring the vast possibility of interactions between functional and scaffold modules. This plethora of functions will involve a wide range of optimal reaction conditions and limitations that need to be individually accommodated by careful compartmentalization. Consequentially, suitable means to exchange information, reactants, and intermediate products, confine or dispose of harmful metabolites, and potentially sequester final products need to be implemented.

In the future, artificial bottom-up assembled systems are sure to expand from the *in vitro* test ground to less explored areas of application including living and environmental systems, which will provide particular obstacles in the form of uncontrollable external factors. Every environment outside the laboratory poses specific challenges, including long-term stability issues

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and autonomous operation under resource-limited conditions. While these aspects can largely be addressed in industrial applications, *i.e.*, for scaled-up bioproduction, novel approaches are required for the deployment of synthetic biological systems in remote scenarios such as agriculture, developing nations, or even extra-terrestrial settings.[448] Biosensory devices for the detection of hazardous substances, *e.g.*, heavy metals, need to be equipped to adequately face the inherently deleterious conditions. Additional requirements arise for applications in biomedicine, such as the need for bioavailability, tissue or organ targeting, and immune evasion. In conclusion, the significant progress made in developing and optimizing new modules, networks, and compartmentalization systems will be followed by challenging but highly rewarding efforts to combine them into coordinated and autonomously functioning systems and apply them in different environmental settings.

AUTHOR INFORMATION

Corresponding Authors

Dimitrios Fotiadis − *Institute of Biochemistry and Molecular Medicine, University of Bern, 3012 Bern, Switzerland; Molecular Systems Engineering, National Centre of Competence in Research (NCCR), 4002 Basel, Switzerland;* orcid.org/0000-0001-9682-7931; Phone: +41-31 684 41 03; Email: dimitrios.fotiadis@ibmm.unibe.ch

Stephan Hirschi − *Institute of Biochemistry and Molecular Medicine, University of Bern, 3012 Bern, Switzerland; Molecular Systems Engineering, National Centre of Competence in Research (NCCR), 4002 Basel, Switzerland;* Present Address: S.H.: Department of Biochemistry, University of Oxford, South Parks Road, OX1 3QU Oxford, United Kingdom; Phone: +44-186 561 32 86; Email: stephan.hirschi@bioch.ox.ac.uk

Authors

Thomas R. Ward − *Department of Chemistry, University of Basel, 4056 Basel, Switzerland; Molecular Systems Engineering, National Centre of Competence in Research (NCCR), 4002 Basel, Switzerland;* orcid.org/0000-0001-8602-5468

Wolfgang P. Meier − *Department of Chemistry, University of Basel, 4056 Basel, Switzerland; Molecular Systems Engineering, National Centre of Competence in Research (NCCR), 4002 Basel, Switzerland;* orcid.org/0000-0002-7551-8272

Daniel J. Müller − *Department of Biosystems Science and Engineering, ETH Zürich, 4058 Basel, Switzerland; Molecular Systems Engineering, National Centre of Competence in Research (NCCR), 4002 Basel, Switzerland*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.chemrev.2c00339

Author Contributions

CRediT: **Stephan Hirschi** conceptualization, writing-original draft, writing-review & editing; **Thomas R. Ward** writing-original draft, writing-review & editing; **Wolfgang P Meier** writing-original draft, writing-review & editing; **Daniel J. Müller** writing-original draft, writing-review & editing; **Dimitrios Fotiadis** conceptualization, project administration, writing-original draft, writing-review & editing.

Notes

The authors declare no competing financial interest.

Biographies

Stephan Hirschi obtained his M.Sc. in Molecular Life Science in 2015 with a specialization in Biochemistry and Chemical Biology from the University of Bern, Switzerland. He then completed his Ph.D. in Biochemistry and Molecular Biology in 2019 at the Institute of Biochemistry and Molecular Medicine (IBMM), University of Bern, under the guidance of Prof. Dimitrios Fotiadis. After spending two years as research assistant at the IBMM, he received a Postdoc.Mobility fellowship from the Swiss National Science Foundation and moved to the Department of Biochemistry at the University of Oxford, United Kingdom. He is currently investigating the structural basis of erythrocyte invasion by the malaria parasite *Plasmodium falciparum* in the laboratory of Prof. Matthew Higgins.

Thomas R. Ward obtained his Ph.D. in Homogeneous Catalysis from the ETHZ (1991). Following a postdoc with R. Hoffmann (Cornell University, Theoretical Chemistry) and C. Floriani (University of Lausanne, Organometallic Chemistry), he initiated his independent career at the University of Bern in 1993. In 2000, he moved to the University of Neuchâtel as full professor of Bioinorganic Chemistry. There, he initiated his research on artificial metalloenzymes. He moved to the University of Basel in 2008. Since 2016, he is the director of the National Centre of Competence in Research (NCCR) 'Molecular Systems Engineering'. He was awarded the 2022 ACS Catalysis Lectureship for his work on artificial metalloenzymes.

Wolfgang P. Meier studied chemistry and received his Ph.D. degree in Macromolecular Chemistry (1992) from the University of Freiburg, Germany. In 1996 he was appointed as a lecturer in physical chemistry at the University of Basel, Switzerland where he habilitated later. In 2001 he accepted a position as professor at the International University of Bremen, Germany and came back in 2003 to the University of Basel as professor of chemistry. Wolfgang Meier was cofounder and director of the NCCR 'Molecular Systems Engineering'. He received several awards for his research such as the Ruzicka-Price (2001) and Hermann-Staudinger-Price (2006). His main research interests were in the fields of polymer−protein hybrid materials and hierarchical self-assembly of functional polymers.

Daniel J. Müller received his M.Sc. (Diploma) in Physics from the University of Technology Berlin, Germany, and his Ph.D. in Biophysics from the Biozentrum, University of Basel, Switzerland. He was a group leader at the Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden, Germany, and accepted a full professorship of Cellular Machines at the University of Technology, Dresden, where he acted as founding director of the Biotechnology Center. Currently, he serves as the Chair of Biophysics at the ETH Zürich, Switzerland, where he currently acts as director of the Department of Biosystems Science and Engineering. He colaunched several research centers including the Biotechnology Center, Dresden, the BCUBE Center for Molecular Bioengineering, Dresden, the Swiss NCCR 'Molecular Systems Engineering', Basel, and the Botnar Research Center for Child Health at University of Basel and ETH Zürich.

Dimitrios Fotiadis received his Diploma in Molecular Biology and Ph.D. in Biochemistry from the Biozentrum of the University of Basel, Switzerland. After a postdoc in the laboratory of Andreas Engel (Biozentrum, University of Basel) and a stay in Kris Palczewski's laboratory (University of Washington, Seattle, USA), he habilitated in Biophysics at the University of Basel (lecturer). He started his own laboratory at the University of Bern, Switzerland, in 2008 as a tenure-

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track assistant professor and was promoted to full professor. Since 2018, he is managing director of the Institute of Biochemistry and Molecular Medicine (University of Bern, Switzerland). In 2022, he joined the management of the Medical Faculty of the University of Bern, becoming vice-dean.

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