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Biological membranes play an essential role in living organisms by providing stable and functional compartments, preserving cell architecture, whilst supporting signalling and selective transport that are mediated by a variety of proteins embedded in the membrane. However, mimicking cell membranes – to be applied in artificial systems – is very challenging because of the vast complexity of biological structures. In this respect a highly promising strategy to designing multifunctional hybrid materials/systems is to combine biological molecules with polymer membranes or to design membranes with intrinsic stimuli-responsive properties. Here we present supramolecular polymer assemblies resulting from self-assembly of mostly amphiphilic copolymers either as 3D compartments (polymersomes, PICsomes, peptosomes), or as planar membranes (free-standing films, solid-supported membranes, membrane-mimetic brushes). In a bioinspired strategy, such synthetic assemblies decorated with biomolecules by insertion/encapsulation/attachment, serve for development of multifunctional systems. In addition, when the assemblies are stimuli-responsive, their architecture and properties change in the presence of stimuli, and release a cargo or allow "on demand" a specific *in situ* reaction. Relevant examples are included for an overview of bioinspired polymer compartments with nanometre sizes and membranes as candidates in applications ranging from drug delivery systems, up to artificial organelles, or active surfaces. Both the advantages of using polymer supramolecular assemblies and their present limitations are included to serve as a basis for future improvements.

1. Introduction

Understanding and mimicking structures and functions found in nature for the design of novel materials and active supramolecular assemblies led to various methods and materials useful in domains such as materials science, chemistry, electronics, and medicine.[1-3] Fabrication of molecular bioinspired materials can be realized either by a 'top-down' approach, breaking down a complex structure into its components, or a 'bottom-up' approach, in which simple components are assembled to produce more advanced supramolecular structures. The latter approach, on which we will focus here, requires a deep understanding of individual molecular building blocks and their structures, assembly properties, and dynamic behaviours in order to manufacture nanomaterials. A step further involves the combination of biomolecules, such as enzymes, proteins, or nucleic acids with synthetic materials, for example block copolymers, in order to create new, complex bio-synthetic materials.[4] Specificity and efficiency of biological molecules in addition to robustness and the possibility of tailoring polymeric materials serve for the design of materials/systems with improved properties and functionality. In this respect, polymer supramolecular structures generated by self-assembly of amphiphilic copolymers are of particular interest because these architectures provide a large variety of topologies that permit the insertion/encapsulation/attachment of biomolecules.[5, 6] In addition, their properties can be adjusted by chemical modification to support the match with biological molecules, while preserving the characteristics of synthetic materials, such as stability and mechanical robustness.[7] The driving forces that bind building blocks together during self-assembly are weak and noncovalent interactions favoured by chemical complementarity and structural compatibility as key parameters. Amphiphilic copolymers, based on hydrophilic and hydrophobic blocks spontaneously self-assemble in solution in a manner similar to natural lipids, and generate 3D supramolecular assemblies, such as micelles, tubes, worm-like structures and vesicles,[8, 9] or 2D planar membranes.[7]

Of particular interest are vesicles, so called polymersomes, because they offer three topological regions for the location of

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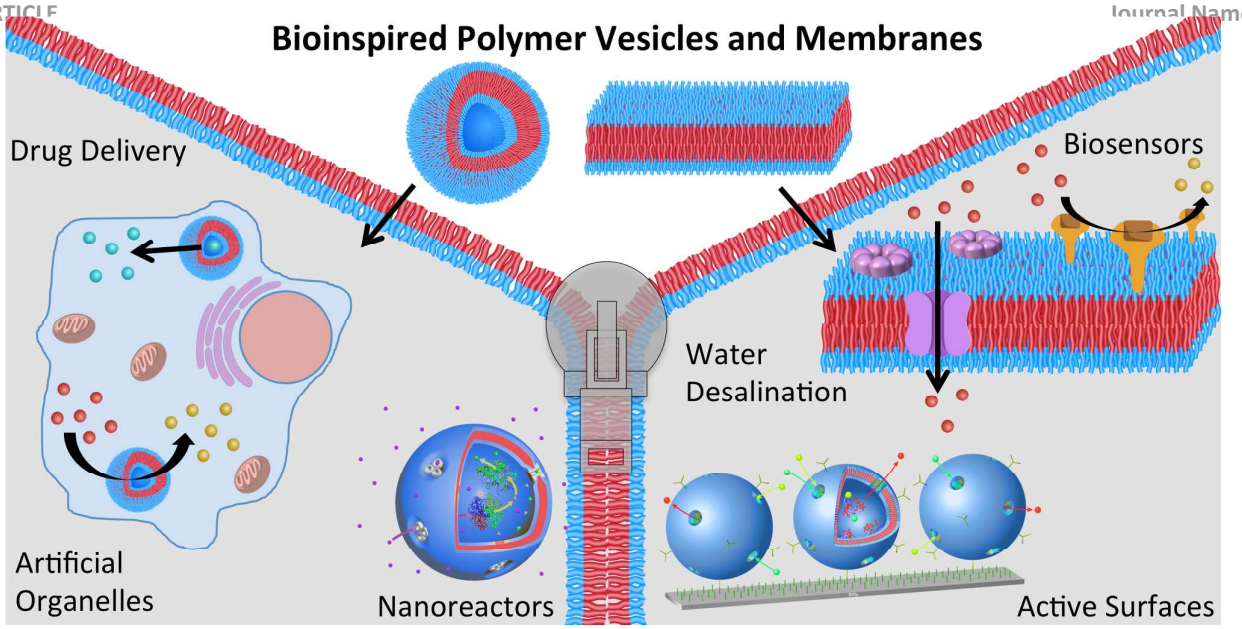

Fig. 1: Conceptual overview of bioinspired polymer vesicles and polymer membranes highlighting some possible applications of such assemblies. Modified with permission from ref. [16]. Copyright 2011 Wiley. Modified with permission from ref. [17]. Copyright 2012 The Royal Society of Chemistry

biomolecules: their inner aqueous cavity, the surrounding membrane, and the external surface exposed to the environment.[8] In the case of polymer membranes (free-standing films, supported membranes, membrane-mimetic brushes) the decoration with biomolecules can be achieved by physical adsorption, insertion, and covalent binding.[3, 7, 10]

In various natural metabolic-, signalling- or transport-processes, the presence of physical or chemical stimuli influence the whole pathway by blocking or unblocking specific molecules/reactions (*e.g.* in the cell cycle[11] or bacterial communication[12]). In addition, biopolymers such as proteins and nucleic acids are all basic stimuli-responsive components of living systems, and often remain stable over wide ranges of external variables, but undergo abrupt and drastic conformational changes at critical points. In this respect, a biomimetic approach is to design stimuli-responsive polymer assemblies that are able to change their architecture or properties in the presence of stimuli, and therefore to release a cargo, or to allow a specific *in situ* reaction "on demand".[13-15]

Here we present both polymersomes, and planar membranes with appropriate properties for developing multifunctional systems/materials by combination with biomolecules (enzymes, proteins, DNA, *etc.*); these potentially have a large variety of applications ranging from drug delivery systems up to artificial organelles, or active surfaces (**Fig. 1**). Our overview is focused on defining the biomimetic strategy to produce and modify such synthetic membranes to match particular bio-conditions for an overall functional hybrid system. Supramolecular assemblies that are stimuli-responsive complete the overview of biomimetic membranes and systems at the nanoscale. As this is an emerging nanoscience research field, we indicate both the advantages of using polymer supramolecular assemblies and membrane-mimetic brushes based on block copolymers, as well as their current limitations to serve as a driving force for future improvements. Selected examples from various fields of application, mainly in the medical domain, indicate how such biosynthetic systems/materials can bring new and advanced solutions to diverse problems.

1.1 Concept of bioinspired polymer membranes

The process of molecular self-assembly as a strategy for obtaining programmable colloidal nanostructures, is mediated by weak, noncovalent bonds, such as hydrogen bonds, hydrophobic interactions, van der Waals interactions, and ionic bonds.[18] These weak interactions act together, and govern the structural conformation of biomacromolecules, and the formation of synthetic supramolecular assemblies, as well as influencing their interactions. By observing the processes by which macromolecules are assembled in nature,[19] scientists are generating a variety of architectures by self-assembly of amphiphilic copolymers either as spherical polymer objects (3D) or as planar polymer membranes (2D). From these supramolecular assemblies (micelles, tubes, worm-like structures, vesicles, and planar membranes) we selected polymer vesicles (polymersomes), and planar membranes (free-standing films, supported membranes, membrane-mimetic brushes) to present here as bioinspired materials. Their particular architectures in combination with active compounds support a large variety of applications.

Polymersomes, as hollow spherical compartments delimitated by a membrane of block copolymer, have the advantage of a dual carrier role – they can serve as hosts to hydrophilic molecules inside their cavities or to hydrophobic molecules in their membranes.[8, 20] Due to the low entropy of mixing of polymers, polymersomes possess higher chemical and physical stability than their lipid-based compartments (liposomes), whilst low immunogenicity similar to liposomes can be achieved, thus meeting essential requirements for advanced technological applications.[20, 21] In addition, their chemical versatility makes it possible to tune properties, such as wall thickness, polarity, toxicity or stimuli-responsiveness.[22] In a

further biomimetic step to designing functional systems, polymersomes serve as compartments for *in situ* reactions at the nanoscale, and for the development of nanoreactors, nanodevices, and artificial organelles.[15] Compared to drug delivery systems, where the payload is released mainly by degradation of the polymersomes or by stimuli-responsive change of shape, the concepts of nanoreactors and artificial organelles require a preserved architecture to simultaneously protect the active compounds (enzymes, proteins, mimics), and allow their actions *in situ*.[15, 23] In this respect, reactions inside polymersomes, or multicompartment-polymersomes require the polymersomes to possess specific properties: (i) sufficient encapsulation of active compounds, (ii) membrane impermeability for encapsulated compounds, (iii) permeability for substrates/products, and (iv) stability in various environmental conditions characteristic for desired applications.

Two topological regions need to be considered for a polymer membrane to act as matrix for accommodating a biomolecule: the mono- or bilayer, and the surface exposed to the environment. Each domain has to mimic the properties of a biological membrane to serve as a template for biomolecules.[7, 24] Increased mechanic stability either in polymersomes or as planar membranes, results from the formation of thicker membranes, which can be 2 – 10 times that of phospholipid bilayers. This leads to a large mismatch between the membrane thickness and the size of the biomolecules, which could significantly affect the insertion, mobility and functionality of the biomolecules. Theoretical calculations have indicated that synthetic membranes are capable of adjusting their thickness to the size of the membrane inclusion / protein with a hydrophobic mismatch of 1.3 nm.[25] However, recent studies have shown that biomolecules (biopores or membrane proteins) remain functional in membranes up to 6 times thicker than the height of biomolecules.[26-31] Insertion of biomolecules, ranging from short peptides that self-assemble into pores[29] to large transmembrane ion channel porins[27, 31] represents a biomimetic approach for increasing membrane permeability that is similar to cell membranes. Moreover, very recently the properties of polymer membranes have been varied via polymer libraries in order to establish their effects on the lateral mobility of inserted biomolecules, and to understand which membrane properties are crucial for successful biomolecule insertion.[32]

The other topological domain of a membrane is its surface, the properties of which are essential for interactions with biological molecules via molecular recognition, or conversely, to avoid interactions that could lead to decreased circulation times of polymeric carriers in the blood stream. Molecular recognition at surfaces as a key biological process that is accomplished by specific affinity tags is now the focus for potential industrial and medical applications, such as the purification and immobilization of biomolecules,[33] labelling of proteins,[34] and 2D-crystallization.[35, 36] In order to study recognition processes at a molecular level, an efficient approach is to introduce simplified systems, as for example metals that serve as coordination centres with different ligands to provide open coordination sites to favour stable immobilization of biomolecules similar to those in nature.[37, 38] Specific molecules involved in molecular recognition interactions (biotin-streptavidin, antibody-antigen, Me-NTA-his tag proteins, *etc.*) have been used to decorate polymer membranes for targeting approaches or for immobilization of nanoreactors on solid supports.[17, 39]

In the next sections we describe how the decoration of polymer membranes/compartments with biomolecules is achieved to create hybrid membranes/systems with improved properties and functionality.

1.2 Properties of polymers forming bioinspired membranes

The chemical nature of the amphiphilic copolymers is a prerequisite for artificial membranes to support biomimetic activity by producing membranes/compartments with appropriate properties to allow preservation of the structure, integrity, and activity of biomolecules in a synthetic environment or to mimic biomembrane responses.[9, 10, 40, 41]

The molecular properties of each block, and of the overall copolymer chain, such as molecular weight, polydispersity and hydrophobic to hydrophilic block ratio, strongly affect the supramolecular assemblies. The most common amphiphilic copolymers used in combination with biomolecules consist of hydrophilic blocks, such as poly(acrylic acid), PAA, poly(ethylene oxide), PEO, poly(ethylene glycol), PEG, poly(2-methyl oxazoline), PMOXA, or poly[l-isocyanoalanine(2-thiophen-3-yl-ethyl)amide], PIAT, and a hydrophobic block, such as polystyrene, PS, poly(butadiene), PB, or poly(dimethylsiloxane), PDMS.[15, 42-44] Abbreviations of the polymers mentioned in this review can be found in Table 1. More details regarding the synthesis and properties of amphiphilic copolymers used to form supramolecular assemblies can be found in very recent reviews and book chapters.[43-45] Mechanical properties of polymersome membranes largely depend on the type of copolymer used to form the membrane and the length of the hydrophobic block and therefore membrane thickness plays a key role in the stability of the assembly.[46-49] Furthermore, addition of naturally occurring molecules, such as *e.g.* phospholipids into polymer vesicle membranes, further modifies mechanical properties of polymersomes,[50] whilst additional membrane protein insertion can increase membrane permeability.[27, 51] Therefore, the type of polymersome with optional biomolecules (*e.g.* phospholipids, proteins, peptides) can be carefully chosen to fulfil certain needs for specific applications. Ranges of some typical properties for purely synthetic polymersomes are summarized in Table 2 demonstrating that they can be specifically tuned using artificial block copolymer vesicles. It also highlights one main advantage compared to liposomes, namely physicochemical versatility. It has to be noted that many of these properties are measured on polymer-based giant unilamellar vesicles (GUVs) using *e.g.* micropipette aspiration.[52] For more details on physical properties of polymersomes, readers are referred to reviews on this subject matter.[48, 53, 54] In the case of polymersomes or

polymer membranes with stimuli-responsive properties, the selection of the polymers must either have the response associated with one of the blocks, or allow the introduction of specific molecules that reply to a stimulus, and therefore induce a change in the overall architecture/properties of the supramolecular assembly.[13] Various amphiphilic copolymers with stimuli-responsive properties are found in recent reviews,[13, 22] and selected examples are included in the next sections. Stimuli-responsiveness favours a better localization of the system in a desired biological compartment, and controlled release of a payload at the location of a pathological event, or rapid imaging of the pathological event.

Table 1: Common polymer blocks and their abbreviations.

Abbreviation	Polymer
PAA	poly(acrylic acid)
PB	poly(butylene)
PBD	poly(butadiene)
PBzMA	poly(benzyl methacrylate)
PCL	poly(caprolactone)
PDEAEM	poly(2-(diethylamino)ethyl methacrylate)
PDMAEMA	poly(2-(dimethylamino)ethyl methacrylate)
PDMIBM	poly(3,4-dimethyl maleic imido butyl methacrylate)
PDMS	poly(dimethylsiloxane)
PDPA	poly(2-(diisopropylamino)-ethyl methacrylate
PEG	poly(ethylene glycol)
PEGMA	poly(ethylene glycol) methacrylate
PEO	poly(ethylene oxide)
PEtOz	poly(2-ethyl-2-oxazoline)
PFMMA	poly (ferrocenylmethyl methacrylate)
PGA	poly(glutamic acid)
PGMA	poly(glycidyl methacrylate)
PHEMA	poly(2-hydroxyethyl methacrylate)
PIAT	poly(l-isocyanoalanine(2-thiophen-3-yl-ethyl)amide)
PLA	poly(lactic) acid
PMA	poly(4,5-dimethoxy-2-nitrobenzyl methyl methacrylate acid)
PMAA	poly(methacrylic acid)
PMCL	poly(*γ*-methyl-*ε*-caprolactone)
PMMA	poly(methyl methacrylate)
PMOXA	poly(2-methyl oxazoline)
PMPC	poly(2-methacryloyloxyethyl phosphorylcholine)
PNBA	poly(4,5-dimethoxy-2-nitrobenzyl methacrylate)
PnBMA	poly(*n*-butylmethacrylate)
PNIPAM	poly(*N*-isopropylacrylamide)
PNVP	poly(*N*-vinyl-pyrrolidone)
PS	poly(styrene)
PSA	poly(sulfobetaine methacrylate)
PSBMA	poly(11-mercaptoundecyl sulfonic acid)
PtBMA	poly(*tert*-butyl methacrylate)
PTMC	poly(trimethylene carbonate),
PVA	poly(vinyl alcohol)
PVP	poly(vinylpyridine)

Table 2: Specific membrane properties achievable with polymersomes and in comparison to some typical values for liposomes.

Membrane property	Polymersomes	Liposomes[48]
Membrane thickness [nm]	3[55] – 40[56]	3 – 5
Lateral diffusion coefficient [$\mu m^2/s$]	0.0024[57] – 6.0[49]	3.8[48] – 12.5[49]
Water permeability [μm/s]	0.8[27] – 526[51]	15 – 150
Bending modulus [kT]	25[58] – 74330[56]	11 – 30
Stretching modulus [mN/m]	15[59] – 2350[56]	250 ± 2

The requirements for bioinspired membranes/vesicles in the case of *ex vivo* applications are mainly restricted to enhancing system performance by optimizing the functionality of entrapped/encapsulated/attached active compounds in various environmental conditions (pH, ionic strength, temperature, *etc.*). A complex scenario of requirements characterizes *in vivo* applications, which start with the use of polymers that fulfil health safety standards by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) up to the biocompatibility and biodegradability of all the components of synthetic systems under biological conditions.[44] In addition, synthesis strategies for amphiphilic copolymer blocks and especially the preparation methods for the supramolecular assemblies should avoid organic solvents, which normally lower the enzymatic activity or denature proteins.

Properties, such as charge, flexibility, thickness and membrane density have to be tailored for a desired application. For example, a charged surface is required to attach biomolecules to polymer membranes by electrostatic interactions, and a factor that can influence the circulation time of systems inside the body.[60, 61] The flexibility of membranes plays an essential role in the insertion of biomolecules and preservation of their functionality[32] as it will be discussed in sections below. Therefore the selection of a particular amphiphilic copolymer and the supramolecular assembly generated by self-assembly has to match both the specificity of the biomolecules, and the intrinsic conditions of the desired application.

2. Bioinspired polymer vesicles

2.1 Fabrication of polymer vesicles and encapsulation procedures

The techniques available for formation of polymer vesicles consist of direct dilution (co-solvent method), bulk hydration,[62] thin-film rehydration, and reverse evaporation.[24, 63] All of these methods lead to the preparation of nanostructures (vesicles, micelles, rods, *etc.)*, but both the method and parameters to obtain hollow polymer vesicles need to be chosen carefully and controlled. In the case of the direct dilution method, the polymer is dissolved in an appropriate organic solvent and

added dropwise to an aqueous solution. Due to the high dilution of the organic solvent, the polymers assemble into nanostructures. Subsequent removal of the organic solvent by evaporation or extensive dialysis is a critical step, especially for intended usages in the biomedical domain. Similarly, vesicular self-assemblies can be formed by simply adding the polymer to an aqueous buffer solution under vigorous shaking (bulk hydration), and is often used when electrostatic interactions are the basis for vesicle assembly (PICsomes), *e.g.* when two oppositely charged block copolymers are mixed in buffer solution,[64] or when charged amphiphilic block copolymers are used.[65] In contrast, bulk hydration with uncharged amphiphilic block copolymers is often difficult to achieve due to a relatively large polymer hydrophobic fraction of 65 ± 10 wt%,[66] which is needed for successful vesicle assembly. The film rehydration technique is used to increase the surface area by drying the dissolved polymer under vacuum until a thin film is formed. Then rehydration of the polymer thin-film leads to the formation of nanostructures. This process is accelerated by stirring or shaking, but only when it does not affect the architecture of the supramolecular assemblies.[63] Supramolecular structures are significantly influenced by the fabrication method, which therefore constitute a modality to favour the formation of assemblies with a desired architecture. For stimuli-responsive polymersomes, the formation and storage conditions have to protect them from the trigger conditions so they are not affected before the intended application (*e.g.* site-specific cargo release). For example, protection from light is needed during the whole preparation, purification, and storage procedure for light-responsive polymersomes.

Encapsulation of molecules inside vesicles (**Fig. 2**) is usually achieved by performing film rehydration or solvent exchange methods with biomolecules dissolved in the solution prior to the self-assembly process, although solvent exchange methods are avoided with sensitive biomacromolecules, because the presence of organic solvents can induce their denaturation or degradation.[67] For example, horseradish peroxidase (HRP) and laccase added directly to the rehydration buffer, were successfully encapsulated during the vesicle-formation process, and preserved their activity.[68, 69] Furthermore, this process can be performed using multiple enzymes in order to load various reaction partners and finally achieve *in situ* cascade reactions.[6, 68] Alternative loading methods have been investigated recently to improve encapsulation efficiency, or for encapsulating specific cargo molecules. One possible method for cargo loading is to mimic cellular endocytosis, and it has been shown that under certain physicochemical conditions, the uptake of nanoparticles into the polymersomes follows a similar process to that observed in nature.[70] However, this method has not been yet applied for encapsulation of biomolecules. Another approach inspired by biotechnology is based on electroporation, as one of the standard methods for gene transfection into cells. Recently this method was applied to the loading of polymersomes.[67] By applying high voltage pulses, the membranes became porous and the polymersomes were loaded with a broad range of biomacromolecules, from proteins to siRNA and DNA. Furthermore, the surface charge of the loaded molecules was found to play an important role in the process; anionic molecules were loaded with the highest efficiency.[67]

In a bioinspired approach, impermeable polymer membranes of vesicles were permeabilised by insertion of membrane proteins and biopores. For the insertion of membrane proteins, two different procedures have been reported.[28, 31, 51, 71, 72] For example, slow detergent removal by using methods known from lipid membranes (dialysis, biobeads) have also been applied for membrane protein incorporation into block copolymer membranes.[5, 71, 73] Since insertion of membrane proteins into preformed membranes is energetically unfavoured, especially when the hydrophobic domain of the membrane protein does not match the thickness of the synthetic membrane,[74] preformed membranes have to be destabilized (by application of external electrical field[75] or addition of low amounts of detergent) to allow insertion of the membrane proteins.[28]

2.2 Intrinsic stimuli-responsive polymer vesicles

In order to preferentially release a specific cargo at a desired place in the body under certain specific conditions, polymer vesicles are being developed to respond to various internal biological stimuli (*e.g.* endolysosomal pH, reducing cytosol, monosaccharide concentration, enzyme concentration) or external physical stimuli (*e.g.* temperature, light, magnetic field, ultrasound). Responsiveness can most simply be integrated by using block copolymers with chemical groups that are intrinsically stimuli-responsive. After forming polymer vesicles with these specific block copolymers, the vesicles either disassemble[76, 77] or become leaky[78-80] under specific conditions related to the responsive property of the selected polymers. Upon applying a specific stimulus either a part of the block copolymer changes its property (*e.g.* from hydrophobic to hydrophilic),[77, 81-83] or it disintegrates into small parts;[84-86] both situations lead to vesicle disruption and release of the cargo. Importantly, the degradation products have to be evaluated carefully for undesired side effects. Polymer vesicles were first designed to respond to one specific stimulus, but now, more complex structures have been developed to respond to the presence of two or even more stimuli in order to increase spatiotemporal control of cargo release.[87] The following sections emphasize the broad access to specifically responsive polymer vesicles, which can be applied for desired bio-applications. In addition, recent approaches have introduced branched block copolymers instead of linear-copolymers for self-assembly into vesicular structures, but they still lack detailed characterisation to confirm a hollow architecture.[88] Another possible amphiphilic copolymers architecture is based on hydrophilic polymers (*e.g.* PEO) and hydrophobic supramolecular polymers. They assemble into a variety of shapes, including polymersomes, depending on the lengths of the hydrophobic and hydrophilic chains.[89, 90]

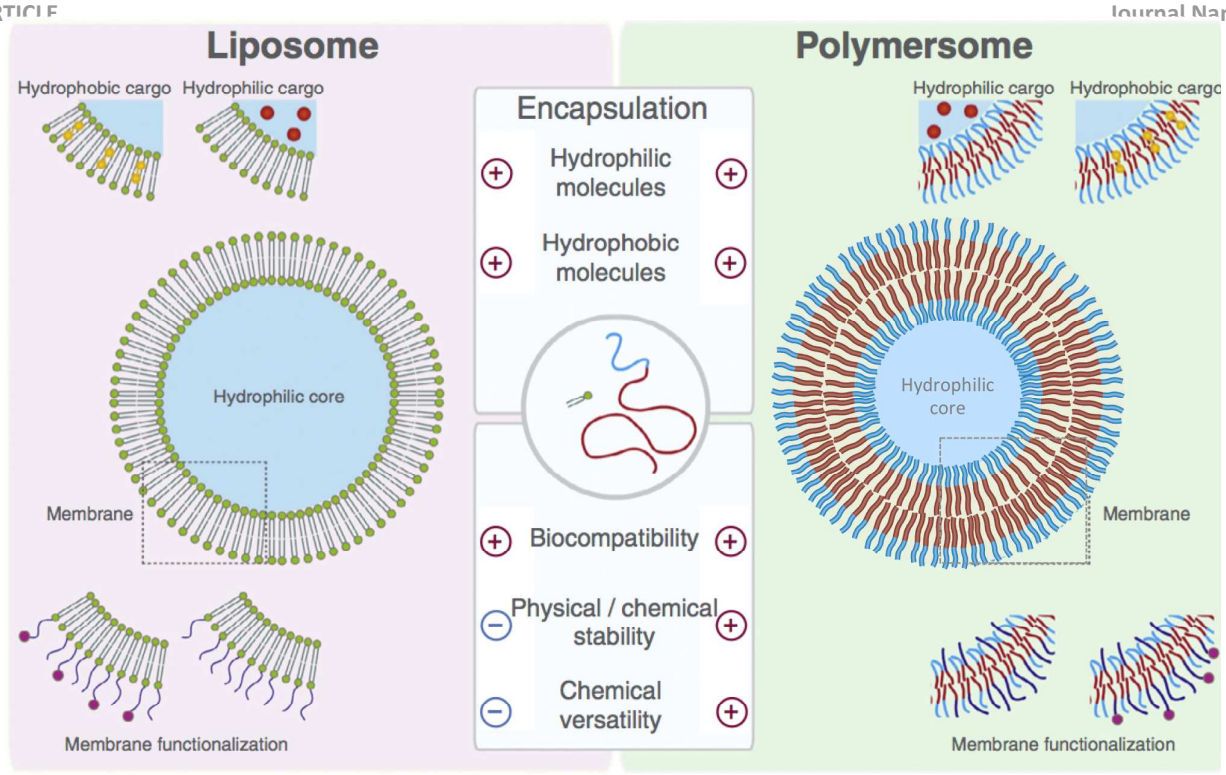

Fig. 2: Schematic presentation of the cargo-loading concepts in case of liposomes (left) and polymersomes (right). Both systems are able to transport and deliver hydrophobic cargo in their membrane as well as hydrophilic in the aqueous core. The membrane of both vesicles can furthermore be modified to enhance targeting and recognition. In contrast to liposomes, polymersomes exhibit increased physicochemical stability and offer more ways to modify its building blocks. Modified with permission from ref. [20]. Copyright 2014 Elsevier.

2.2.1 Single stimuli-responsive polymer vesicles

Biological gradients of certain molecules in a specific biological context (*e.g.* across cell membranes) are often utilized to change polymersome membrane stability or permeability based on the concentration-dependent responsiveness of the membrane to a specific ion/molecule (*e.g.* protons, glutathione or an enzyme). Additionally, external stimuli, such as light, temperature, or ultrasound, can be applied to biological systems to trigger release from polymersomes or an internal reaction in a time and space controlled manner.

2.2.1.1 Hydrolytic release of the payload

The simplest principle of responsive polymer vesicles for controlled payload release is based on hydrolysis of the membrane-forming block copolymers containing polyesters such as poly(lactic) acid (PLA) or various poly(caprolactone)s (PCL). This led to poration and finally disintegration of the corresponding polymersomes.[78] Release rates were tuned by blending hydrolysable block copolymers with different concentrations of non-degradable ones, the latter being responsible for longer retention of cargo in the carrier. Using this strategy, nanocarriers can be designed to reach target cells before hydrolytic release of therapeutics in the blood.

2.2.1.2 pH-triggered release of the payload

Inspired by the utilization of pH gradients in nature, for *e.g.* compartmented degradation of biomacromolecules (endolysosomes), research has been initiated to design pH-sensitive polymersomes, which react to endolysosomal pH after uptake. The formation of pH-sensitive polymersomes was achieved by using, a block copolymer, (poly(2-(methacryloyloxy)ethylphosphorylcholine)-*co*-poly(2-(diisopropylamino)-ethyl methacrylate, PMPC-*b*-PDPA) that changes its solubility as a result of protonation under acidic conditions.[76, 82] In this case, the vesicles fell apart at acidic pH below the pK_a of the PDPA block (pK_a = 5.8 for the block copolymer), due to a change from hydrophobic to hydrophilic character, and encapsulated plasmid DNA was released.[76]

Another possibility besides simple protonation of the hydrophobic block to render it hydrophilic[82] is to release small molecules from the hydrophobic block upon protonation (**Fig. 3**).[77] The doubly hydrophilic block copolymer poly(ethylene oxide)-*block*-poly(vinyl alcohol) (PEO-*b*-PVA) modified with 2-ethylidene-4-methyl-1,3-dioxolane (EMD) prior to self-assembly formed pH-sensitive polymersomes, which reacted to mild acidic conditions by cleavage of EMD-molecules.[77] As the resulting doubly hydrophilic PEO-*b*-PVA no longer form stable nanostructures, the whole structure fell apart, and the encapsulated hydrophilic (lysozyme) and entrapped hydrophobic (DOX) compounds were released.

Other copolymer architectures to form pH-sensitive polymersomes, which release cargo at acidic pH, include the use of asymmetric copolymers,[91] multiblock copolymers up to quintopolymer,[92] and brush copolymers.[93] In another attempt to achieve pH-dependent transport of biomolecules across polymersome membranes, the nucleopore complex – the gateway connecting the cytoplasm and cell nucleus – served as

inspiration for designing an artificial polymer pore complex in polymersome membranes. The polymer membranes with artificial pore complexes were closed at neutral pH and were opened under slightly acidic conditions (pH 5.5 - 6.5).[94] Introduction of CO_2/N_2 into aqueous solutions of pH-sensitive vesicles served for the traceless addition/removal of protons to the aqueous environment. This induced vesicle disassembly (after CO_2-induced proton production), and swelling/shrinking (alternating CO_2/N_2 cycles) of cross-linked vesicles, respectively, resulting in cargo release.[95, 96]

2.2.1.3 Redox-sensitive polymersomes

Redox reactions are major players in many cellular processes *e.g.* cellular respiration. Therefore redox chemistry was also integrated within polymersome membranes by incorporation of reduction or oxidation sensitive polymers. Commonly, during polymer synthesis *e.g.* reduction-sensitive disulphide groups are introduced between the hydrophilic and hydrophobic blocks of polymersome-forming block copolymers,[84-86, 97] or disulphide bonds are introduced within the hydrophobic blocks to obtain reduction-sensitive polymer vesicles.[98] Upon cellular uptake and endosomal escape, such reduction-sensitive polymersomes disassembled under the reducing environment of the cell cytosol, typically containing 2 – 10 mM glutathione (GSH) tripeptide, which is about 3 orders of magnitude higher than the concentration present in the extracellular environment.[99] An important aspect of reduction-sensitive polymersomes is the accessibility of the labile groups within the synthetic membrane for reducing molecules such as GSH.[100] Thicker membranes and structures formed by smectic liquid crystal disulphide-containing polymers lowered the release of cargo at typical GSH concentrations, which might impair their applicability in drug delivery.[97]

There have been fewer studies of oxidation-sensitive polymersomes, but they are particularly suitable for antigen-delivery to antigen-presenting cells (APCs).[101] The reason is the highly oxidative environment (high H_2O_2 concentration) within the endosomes of APCs, such as dendritic cells, which has been used for oxidation-triggered release.[102]

2.2.1.4 Small-molecule responsive polymersomes

Responses of polymersomes to specific molecules are of high interest for certain diseases. To this end, monosaccharide-responsive polymersomes have been proposed for the treatment of glucose-related human diseases, such as diabetes.[103] Glucose-triggered insulin release from polymersomes self-assembled from a boroxole-based block copolymer has been achieved, but the glucose-concentrations needed for disassembly (> 0.3 M) were too high to be of biomedical relevance; typical hyperglycaemia is defined with glucose concentrations of 11 – 20 mM.[103]

Other small molecule-responsive polymersomes have been designed for the detection of highly toxic chemical nerve agents, where the mode of detection is based on a vesicle-to-nanoparticle transition when the nerve agent is bound to a basket structure incorporated in the polymersome membrane.[104]

2.2.1.5 Light-triggered release from polymersomes

Plants use light as energy source, and this is converted to chemical energy by light-harvesting molecules (*e.g.* chlorophyll). Similarly, photosensitive moieties have been incorporated in or on polymersome membranes to achieve light-triggered release. Light-responsiveness is specifically interesting for spatiotemporal control of therapy due to the possibility of guiding light to a specific area and determining the time scale of light exposure. Strong UV or near-UV irradiation does not penetrate deep into tissue and is associated with lower biocompatibility than longer-wavelength irradiation. Light-sensitive polymersomes have been synthesized using a diblock copolymer with a photosensitive linker (irradiation with 365 nm UV light),[105] diblock copolymer and *meso*-to-*meso* ethyne-bridged bis (porphinato) zinc (PZn_2) fluorophore (488 nm, 515 nm, 543 nm, and 633 nm irradiation), incorporated into the hydrophobic membrane,[106] linear–dendritic block copolymers with azobenzene–aliphatic codendrons (350 - 400 nm irradiation),[107] and photodegradable dendritic polymers (dendrimersomes).[108] Coupling light-responsive groups to the hydrophobic end of a block copolymer allowed triggered release of payloads by controlled depolymerisation of the hydrophobic block upon irradiation

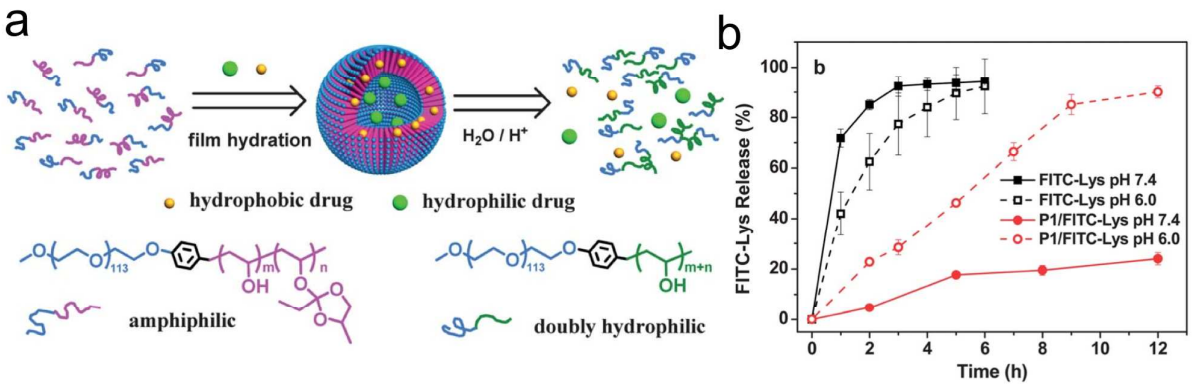

Fig. 3: (a) Schematic representation of polymersome formation and disassembly using a pH-sensitive block copolymer poly(ethylene oxide)-*block*-poly(vinyl alcohol) (PEO-*b*-PVA) modified with 2-ethylidene-4-methyl-1,3-dioxolane (EMD). Upon acidification, the small molecules (EMD) are released from the hydrophobic block, which turns the whole copolymer hydrophilic, resulting in polymersome disintegration. (b) Cumulative release of FITC-lysozyme (FITC-Lys) from acid labile polymersomes (P1) at pH 7.4 and 6.0 Modified with permission from ref. [77]. Copyright 2013 The Royal Society of Chemistry.

using visible (420 nm) or UV (365 nm) light (**Fig. 4**).[86]

2.2.1.6 Temperature-responsiveness of polymersomes

Temperature is another parameter that can be used for the introduction of specific polymersome responsiveness. The temperature-responsive homopolymer poly(*N*-isopropylacrylamide) (PNIPAM) is most often introduced into block copolymers to form temperature-labile polymersomes, because of its phase-transition at a biologically relevant temperature (32 °C). Such vesicles are stable at the normal body temperature of 37 °C, but readily disassemble and release cargo at temperatures below 32 °C.[81, 109-111] The disassembly process is based on a change of the membrane-stabilizing hydrophobic PNIPAM-block to a hydrophilic block when cooled below the transition temperature. Such a temperature responsive effect might be used to apply these polymersomes for triggered drug release in certain bio-domains inside body by *e.g.* locally using cryoprobes or cold packs.[81]

2.2.2 Multiple stimuli-responsive polymersomes

One of Nature's most intriguing features is its enormous complexity, which technically cannot yet be met with artificial systems, but is considered the fundamental basis for the development of more efficient and controlled systems. As a consequence, in attempts to approach the complexity of natural systems, dual or multiple responses, have been developed to act sequentially or simultaneously to fine tune the delivery vehicle, *e.g.* drug release profiles from polymersomes.[87]

2.2.2.1 pH and temperature/reduction/ultrasound responsive polymersomes

The sensitivity of polymersomes towards pH and temperature was introduced and tuned by synthesizing random diblock copolymers using various comonomer ratios.[112, 113] Another route to introduce this double pH and temperature sensitivity to polymersomes was achieved by the design of a supramolecular triblock copolymer using host-guest chemistry.[110] Acid-labile polymersomes were successfully formed and loaded by heating hydrophilic copolymers to 37 °C, at which temperature stable nanostructures were formed, loaded and maintained before subsequently releasing the cargo at slightly acidic conditions.[114] Two internal triggers, pH and reduction potential, were combined in polymersomes for the dual-response to low endolysosomal pH and reducing cytosol.[115, 116] A pH-sensitive triblock copolymer thiol derivative formed with disulphide-crosslinked polymersomes after oxidation at pH > 7.8, was then partially emptied at pH < 7.4, and completely disassembled when a reducing agent was added at the lower solution pH.[117] When pH-responsiveness (internal trigger) was combined with reactivity to ultrasound (external trigger), the release from polymersomes could be stimulated using only one or both triggers simultaneously.[116]

2.2.2.2 Temperature and reduction/monosaccharide/light responsive polymersomes

When a random terpolymer, made of monosaccharide-responsive styreneboroxole and oligo(ethylene glycol)-functionalized styrene, was combined with a PEG chain, the corresponding vesicular self-assemblies responded to temperature and glucose/fructose.[118] The combination of temperature and reduction potential triggers was designed for

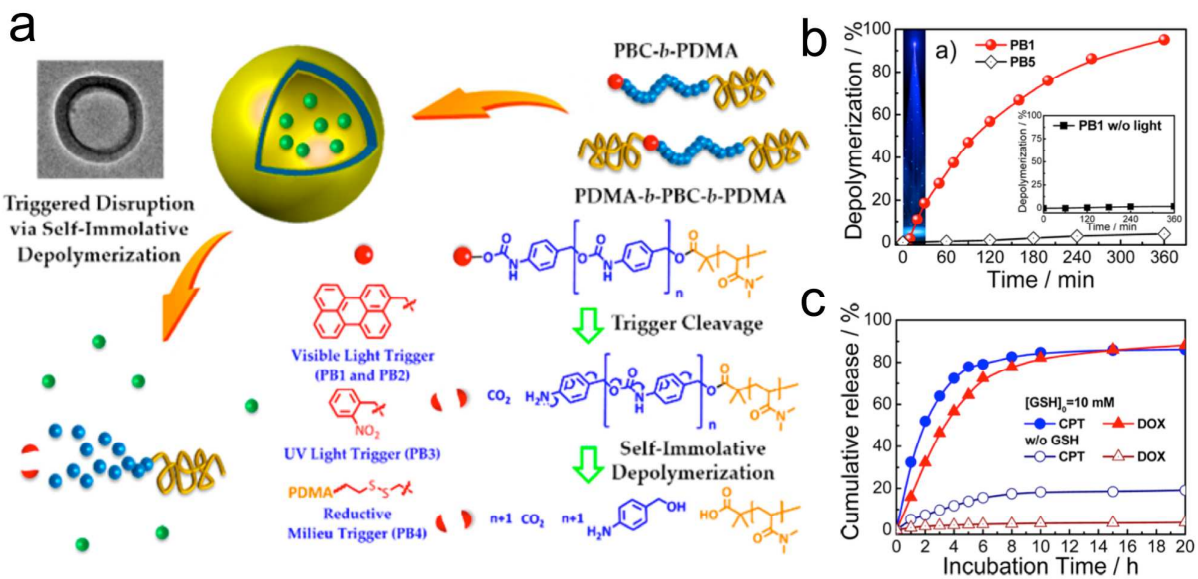

Fig. 4: (a) Schematic representation of self-immolative polymersomes based on poly(benzyl carbamate)-*block*-poly(N,N- dimethylacrylamide), PBC-*b*-PDMA modified with stimuli-responsive "capping" moieties. Light irradiation for the light-responsive group end-capped copolymer-based polymersomes or reducing milieu for disulphide-containing polymersomes leads to triggered self-immolative depolymerisation of the hydrophobic PBC block after cleavage of the responsive group yielding cargo release. (b) Depolymerisation profile for polymersomes with light-sensitive end-cap (PB1) and light-insensitive end-cap (PB5) with 30 min blue light (420 nm) irradiation and for PB1 without light irradiation (insert). (c) Cumulative release of camptothecin/doxorubicin (CPT/DOX) from co-loaded PB4 reduction-sensitive self-immolative polymersomes in the presence or absence of 10 mM reducing agent glutathione (GSH). Adapted with permission from ref. 86. Copyright 2014 American Chemical Society.

efficient protein loading and release under a reductive cytosolic environment.[109] Polymersomes were formed in the presence of proteins at pH 5.5 and at elevated temperature (40 °C), necessary to promote electrostatic protein-copolymer interactions for high loading efficiency. After crosslinking using cystamine the resulting polymersomes possessed high protein loading, stability at body temperature, and rapid release in a reducing environment. Using supramolecular amphiphiles based on a thermoresponsive pillar[7]arene and light-responsive azobenzene (365 nm), the corresponding vesicles released an encapsulated molecule by temperature change or UV-light irradiation.[119, 120]

2.2.2.3 Photo/magneto-thermal/oxidation responsiveness of polymersomes

Incorporation of light-sensitive molecules (*e.g.* hydrophobic ethyl eosin)[83] or metal nanoparticles in polymersome membranes led to the formation of polymersomes with optofluidic rupture properties,[83] photo- or magneto-thermal induced release of cargo,[121-125] and theranostic activity.[126, 127] Upon light irradiation the membrane-incorporated light-sensitive eosin molecules absorbed the photons, produced singlet oxygen, and oxidized the hydrophobic block.[83] In contrast, gold nanoparticles in polymersome membranes converted the photons to thermal energy, which heated up and disrupted the polymer membrane for cargo release,[122, 123] and for photothermal therapy (PTT).[126, 127] Alternatively, local high frequency alternating magnetic fields have been used to destabilize polymersomes with membrane-incorporated superparamagnetic iron oxide nanoparticles (SPION).[124, 125]

2.3 Biomolecule modified polymer vesicles

In addition to the use of intrinsic stimuli-responsive polymers, another strategy for designing responsive polymersomes is based on the incorporation of naturally responsive biomolecules (proteins, enzymes, DNA, *etc.*) into such synthetic matrices. Because of their similarity to cellular membranes, vesicles composed of phospholipids, called liposomes, have been the focus of research for decades.[128-130] However, despite good biocompatibility they lack long-term structural stability,[48, 129, 130] and these drawbacks have hindered their industrial use and limited their medical applications. In order to create biomimetic polymersomes, it is possible to incorporate enzymes, which can perform desired reactions in the interior compartment, and/or surface modifications to enhance molecular recognition.[7, 131] Furthermore, it is possible to reconstitute membrane proteins in the membrane, or to covalently bind biological moieties to membrane forming polymers. Since membrane proteins play a crucial role in fundamental cell processes, ranging from transportation, gradient formation, to signalling,[132-135] an improved understanding is required to create systems with complex functionalities, such as artificial organelles and nanoreactors. Furthermore, these biomimetic systems aim to mimic cellular membranes, its compartments or protocells. In the following sections, the current approaches to create biomimetic polymersomes with decoration of biomolecules are presented.

2.3.1 Modification of polymers with biological molecules

By chemically coupling biomolecules to block copolymers, systems have been achieved that can be triggered or possess enhanced stability in biologically relevant conditions. Further, recognition and targeting can be greatly improved when ligands are presented on the vesicle surface. Several techniques are known to attach and expose biomolecules on the surfaces of polymersomes, which can be categorized based on pre- or post-modification of vesicles.[136] Modification of polymers with biomolecules before self-assembly simplifies the procedure, but its impact on the self-assembly and cargo loading have to be evaluated carefully. In contrast, post-modification of vesicles adds additional steps to the vesicle preparation procedure and in certain cases the functional molecule serving for biomolecule attachment may be hidden in the membrane after the preparation procedure of vesicles and thus decrease the functionalization efficiency. For pre-modification of polymers, biomolecules are either attached to the hydrophilic block of block copolymers before self-assembly[136] or are used as one of the hydrophilic[65] or hydrophobic blocks.[137, 138] Examples of attached biomolecules are polysaccharides, such as dextran and heparin,[65, 139] polypeptides,[137, 140] and water soluble green fluorescent protein.[111] Modification of a hydrophilic polymer block with peptides has resulted in the production of a new class of chimeric polymersomes, called pepsomes (**Fig. 5**). Depending on the polypeptide, systems were responsive to stimuli, such as pH change, and the presence of glucose.[137, 138] Block copolymers composed of the thermoresponsive polymer PNIPAM and the green fluorescent protein variant amilFP497 assemble into polymersomes when heated above 37°C.[111] Combining this novel bioconjugate with the fluorescent anticancer drug DOX and the light harvesting protein phycoerythrin 545 (PE545), resulted in the generation of a system that allows spatial localization of the encapsulated cargo within the polymersome by using fluorescence lifetime imaging and Förster resonance energy transfer (FLIM-FRET).[111] Polypeptides with carbohydrate moieties have been developed for delivery with enhanced biocompatibility.[139, 141] Their exposed peptides are recognized by specific proteins and enable improved cellular recognition[141] and drug release due to enzymatic cleavage.[139]

Another polymeric platform that was introduced are polyion complexes composed of PEO-*block*-polypeptide, which are able to self-assemble into a vesicular structure (PICsomes). The PICsomes (**Fig. 6**) exhibited sufficient stability in physiological conditions even without crosslinking, and furthermore are sufficiently permeable for diffusion of small substrates through the membrane.[142] This allowed their use as a reaction compartment by encapsulating an enzyme for which the substrate and product could diffuse through the membrane.

Further improvements in the ability of polymersomes to interface with biomolecules have been demonstrated by the attachment of Cu(II)-trisNTA to PB-*b*-PEO.[38, 143, 144] The metal-

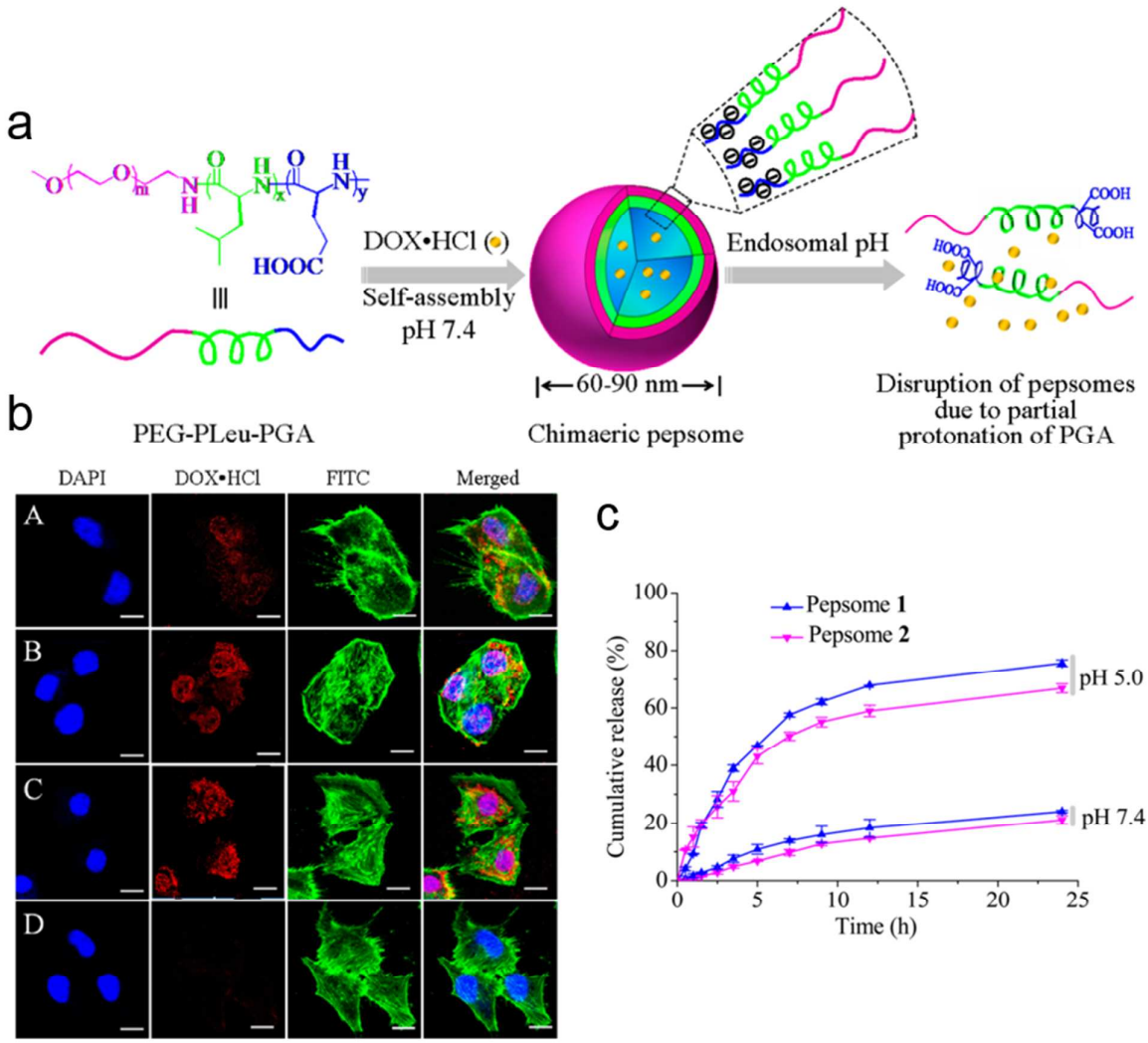

Fig. 5: (a) Assembly and loading of pepsomes. The release of the cargo is induced by the protonation of the PGA-peptide. Subsequently, the pepsome's membrane dissembles and releases its cargo (*e.g.* DOX) into the cytosol. (b) CLSM images of MCF-7/ADR cells incubated with DOX-HCl loaded pepsomes are presented. The rows A to C correspond to 1 h, 2 h and 4 h incubation with the DOX-HCl loaded pepsomes and row D to free DOX-HCl. (c) The release profile of encapsulated DOX-HCl upon different pH values is shown on the right. Modified with permission from ref. [137]. Copyright 2015 American Chemical Society.

functionalized polymers preserved their ability to assemble into vesicles, and allowed specific binding of His-tag modified proteins to the polymersome surface. [143] Because of the well-established protocols for His-tag modification of proteins, this approach could potentially serve as a platform for further protein decoration of polymersomes.

In contrast to the examples described above, targeting of colon cancer cells has been achieved by linking fibronectin mimetic peptides to vesicles after formation of vesicles in an aqueous environment.[145] In a similar study, tumour cell targeting was enhanced by linking a synthetic peptide to the polymer.[146]

2.3.2 Insertion of membrane proteins in the membrane of polymer vesicles

When polymersomes are designed to serve as reaction compartments, such as in the development of nanoreactors, nanodevices and artificial organelles, the permeability of their membrane is a crucial property. This should allow transport of reactants through (substrates and products) in order to fulfil the *in situ* reaction. There are various approaches to obtain polymersomes with permeable membranes: (i) use of polymer forming porous membranes,[68] (ii) use of polymer forming membranes with permeability to specific ions, such as oxygen species,[69] (iii) chemical treatment of membranes to induce pore formation,[147] and (iv) insertion of biopores or membrane proteins.[26, 30, 135]

The biomimetic approach involves insertion of transmembrane proteins and pores, as in cell membranes.[132, 133, 148] Successful insertion of the small pore forming peptide gramicidin in polymer membranes based on PMOXA-*b*-PDMS-*b*-PMOXA enables diffusion of protons, Na^+ and K^+ ions through it, whilst preserving the polymersome architecture.[30] Insertion of an ionophore, ionomycin with a size of 1.9 nm in the membrane of PMOXA-*b*-PDMS-*b*-PMOXA polymersomes with thickness up to 13.2 nm was used to engineer stable polymersomes with a membrane that is permeable only for calcium ions.[29] These

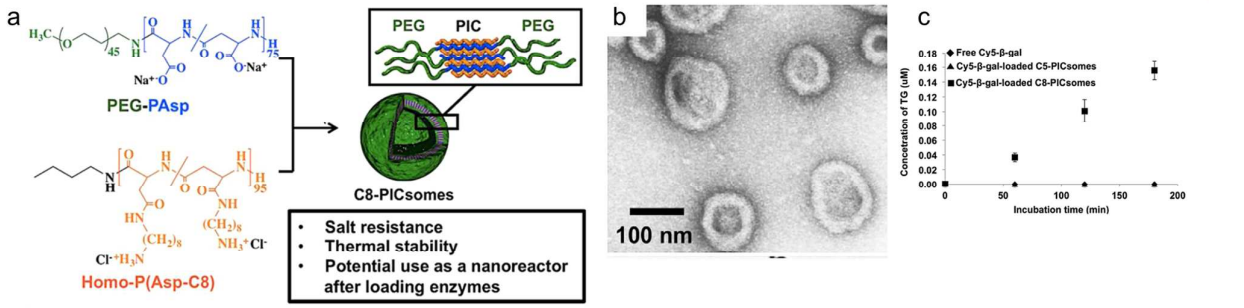

Fig. 6: (a) Schematic presentation of PICsome building blocks and their assembly into vesicles. Their enhanced stability makes them a potential candidate for nanoreactors. (b) Negative stain TEM pictures show the vesicular structure of the PICsomes and (c) the enzymatic activity of encapsulated beta-gal in PICsomes. Modified with permission from ref. [152]. Copyright 2015 American Chemical Society.

examples indicate that it is possible to functionally insert small biopores in thick polymer membranes, with thickness up to 6 times larger than the biomolecule if the membrane has appropriate properties.

Insertion of membrane proteins into membranes is a more delicate task, and successful reconstitution usually takes years to be developed, especially in the case of sensitive membrane proteins, which rapidly denature in any environment that is slightly different from the biological one.[148] First reconstitution trials are usually performed in lipid membranes because being significantly thinner (3-5 nm thickness), they are closer to the natural cellular membrane environment. Procedures to reconstitute a membrane protein have been in development for the last two decades and underlying mechanisms have been investigated.[134] The most common method for reconstitution of membrane proteins in lipid membranes relies on the use of detergents, which serve both as a stabilizer for the water-insoluble membrane proteins, and as a mediator during their insertion into the membrane.[149-151] Then, removal of the detergent by dialysis or addition of biobeads leads to re-formation of closed liposomes with the membrane protein incorporated. Procedures with respect to the lipids used, detergents, detergent removal, *etc.* usually have to be developed for each individual membrane protein.[134, 148]

The most obvious difference between lipid membranes and those formed by self-assembly of copolymers is the latter's higher molecular weight, which is usually in the range of two to five times greater,[48] and leads to much thicker membranes (Table 1). Therefore the properties of synthetic membranes and their interactions with detergent molecules are different. A bilayer based on diblock copolymers cannot relieve the tension induced by detergent integration in a flip-flop mechanism that is common with lipids.[74] Different stages of interaction between synthetic self-assembled supramolecular structures and detergents correspond to a co-existence of polymersomes and detergent micelles, whilst further increases of detergent concentrations induce membrane dissolution, as the most likely mechanism to relieve the surface tension.[74] Therefore, the procedures for membrane protein reconstitution in lipid membranes may not be appropriate for synthetic membranes, or at least not easily adapted. Moreover, there is still a shortage of extensive data on the interactions of different types of block copolymer (*e.g.* diblock, triblock) and different block compositions with detergent classes. The increased hydrophobic mismatch between the hydrophobic block of the copolymer and the membrane protein represents an additional problem, since the hydrophobic domains of membrane proteins are adapted to their lipid environments, and depending of the protein, are around 2-4 nm in size.[153] However, it has been shown that various membrane proteins can be successfully inserted into polymer membranes, if the polymer chains are sufficiently flexible to adjust the hydrophobic domain of the membrane near the protein to the size of the protein.[25, 27, 31, 32]

Adjustment of the thickness of synthetic membranes to the protein size is limited to a certain thickness as has been demonstrated by insertion of gramicidin (2 nm size) in PMOXA-*b*-PDMS-*b*-PMOXA membranes up to, but not greater than, 13.2 nm thickness.[29]

Membrane thickness and copolymer flexibility are key factors for successful membrane protein insertion.[29] Very recently, the lateral movement of various membrane proteins within GUV membranes of a library of PMOXA-*b*-PDMS-*b*-PMOXA triblock copolymers was found to be similar to their diffusion in lipid bilayers but at a timescale, which is an order of magnitude slower (**Fig. 7**).[32] When membrane proteins have to be inserted in thick synthetic membranes, and also preserve their functionality, the membrane thickness combined with its flexibility represents a crucial molecular parameter.

The first successful reconstitution of a membrane protein was that of the highly stable porin, outer membrane protein F (OmpF) into PMOXA-*b*-PDMS-*b*-PMOXA triblock copolymer membranes.[31] During the formation of vesicles OmpF was inserted into the membrane and allowed diffusion of molecules up to 600 Da into the inner cavity. Similarly, the alpha-helical model protein bacteriorhodopsin (BR) has been reconstituted in PEtOz-*b*-PDMS-*b*-PEtOz polymersomes during the self-assembly process.[135, 154] These examples are based on integration of the membrane protein during assembly of the synthetic membrane assembly, but a different approach has been used for the reconstitution of a very sensitive membrane protein, complex I, into polymersome membranes: the protein was inserted into preformed polymersomes by destabilization of the membrane with small amounts of detergent Triton X-100 followed by removal of detergent.[28] This approach allowed protein insertion with a desired orientation, and serving for electron transport from the environment of the polymersomes inside the membrane. Indeed, the final orientation of a protein

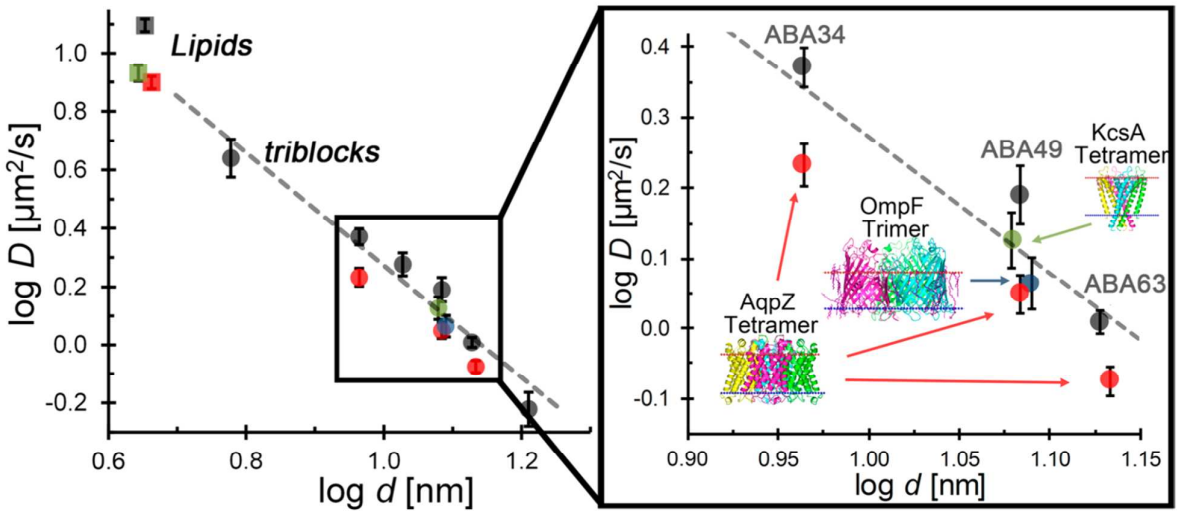

Fig. 7: Dependence of the diffusion coefficient *D* to the membrane thickness *d*. The power law dependence (dashed line) highlights the decrease of D with increasing molecular weight of the membrane building blocks and thus increasing membrane thickness. Depending on their thickness and thus rigidity, the diffusion coefficient can be close to phospholipid membranes. The membrane proteins AqpZ, OmpF and KcsA where tested in triblock copolymer membranes of different thickness and their lateral diffusion was measured. Compared to lipid membranes, their mobility in the membrane is decreased due to the increased membrane thickness. Reprinted with permission from ref. [32]. Copyright 2015 American Chemical Society.

in a membrane can be crucial for its functionality, as for example when electrochemical gradients are formed. In order to orient a protein, one can exploit structural features such as its shape[155] or large hydrophilic domains.[28, 154] For other proteins, as for example proteorhodopsin (PR), insertion can be guided by charges on the membrane surface.[156] In this respect, polymers with charged head groups may be used to guide protein reconstitution in polymersomes. An alternative way to guide proteins into membranes was shown by the use of asymmetric block copolymers (ABC type) together with the membrane protein aquaporin (AqpZ). By using two different hydrophilic blocks, the formed membrane induced a preferential orientation of the protein.[157] These examples demonstrate the possibility of tailoring block copolymers for controlling the orientation of membrane proteins, and helping to achieve functional reconstitution. However, investigations of the requirements for polymer membranes and their properties that allow membrane protein reconstitution is still at an early stage of research, and systematic studies on libraries of various copolymer types have not been performed.

2.3.3 Biomimetic reaction compartments

Polymersomes containing active compounds (proteins, enzymes, mimics) have been developed to serve as nanoreactors,[26, 31, 72, 158] or as artificial organelles inside cells.[5, 16, 154] For example, nanoreactors containing an enzyme were able to produce antibiotics "on demand" both in solution and when immobilized on surfaces.[17, 72] The catalytically active species are usually one or more enzymes, or mimics, which are encapsulated during the vesicle formation process. For example, HRP[6] and laccase[69] were shown to catalyse substrate conversion in the interior of polymersomes. Encapsulation of enzymes provides the advantage of working in a protected environment and avoids degradation by proteases or the influence of factors such as pH or ion concentrations. However, the greater the protection provided by the polymer membrane, the lower the exchange to the exterior, *e.g.* diffusion of substrates and products into and out of the polymersomes. In order to circumvent this hindrance, either permeable membranes are used, or they are permeabilised by various methods. The polymer membrane itself is either permeable towards the substrate in general due to its composition,[68, 69, 152] or permeability can be triggered by an external stimulus such as pH. Poly(N-vinyl-pyrrolidone)-*block*-poly(dimethylsiloxane)-*block*-poly(N-vinylpyrrolidone) (PNVP-*b*-PDMS-*b*-PNVP) based polymersomes are permeable towards reactive oxygen species (ROS), and allow their diffusion through the membrane.[69] Using PEG for the hydrophilic block, and a statistical mixture of a pH-sensitive poly(diethylaminoethyl methacrylate) (PDEAEM) and a photo-cross-linkable poly(3,4-dimethyl maleic imido butyl methacrylate) (PDMIBM) for the hydrophobic block, triggerable polymersomes were formed. Crosslinking of the PDMIBM blocks allows the structure of polymersomes to be preserved, and membrane permeability increased.[147] The drawback of these approaches is their non-specificity. Moreover, when a strong pH change (*e.g.* from 6 to 8)[147] is required, it can strongly influence or completely inhibit enzymatic activity.

The biomimetic approach is the encapsulation of desired enzymes together with the reconstitution of a membrane protein, which facilitates the transport of the substrate.[5, 72, 158, 159] In this case, substrate transport can be highly specific depending on the employed membrane proteins. However, fabrication of these systems gets more difficult as their complexity increases. Appropriate conditions must be chosen to ensure preservation of enzymatic activity during encapsulation, and at the same time to allow reconstitution of the membrane protein. So far, this has only been realized with model proteins, such as OmpF,[5, 16, 72, 158-160] FhuA[161] and biopores.[29]

Using more than one enzyme to facilitate cascade reactions in confined spaces increases the complexity of the systems.[147, 162] Cascades can be created by encapsulating one enzyme in a polymersome, which provides the substrate for one outside (or *vice versa*), co-encapsulation of both enzymes or encapsulation in separate polymersomes.[68, 147] Similar to co-encapsulation of two different enzymes, a three step cascade reaction has been realized in a single polymersome.[162] A more biological approach for immobilization of a protein on the membrane was developed by fusing the amphiphilic Cecropin A peptide to enhanced green fluorescent protein (EGFP) where Cecropin A serves as an anchor in the polymer membrane.[163]

3. Bioinspired planar polymer membranes

2D planar polymer membranes are useful: (i) as simple membrane-mimetic models (monolayer at air-water interface and free-standing membranes), and (ii) as suitable models for surface characterization due to enhanced mechanical stability compared to free-standing membranes. In recent years an increased demand for energy-efficient technologies (*e.g.* water purification) and novel medical applications (*e.g*. biosensing) is driving the development of planar biomimetic polymeric membranes in the direction of such applications, and not just as simple cell mimics models. Bioinspired polymer membranes are the basis of the design of more efficient systems for various technologies (*e.g.* highly selective transport devices, sensors, optical devices, *etc.*).

3.1 Fabrication of polymeric membranes

Various 2D model systems (monolayers at the air-water interface, free-standing, and solid supported) have been developed for mimicking biological membranes (**Fig. 8**).

3.1.1 Monolayers at air-water interface and free-standing membranes

Monolayers at the air-water interface are the simplest models of biological membranes that can be used for investigating interactions with biomolecules in various conditions (*e.g.* temperature, pH of the subphase, and surface pressure of the film).[165-167] Advantages of copolymer-based biomimicry of natural lipid bilayers include membrane fluidity, transmembrane water transport capabilities in specific conditions, and possible membrane protein reconstitution in more stable matrices.

For preparation of free-standing model membranes two

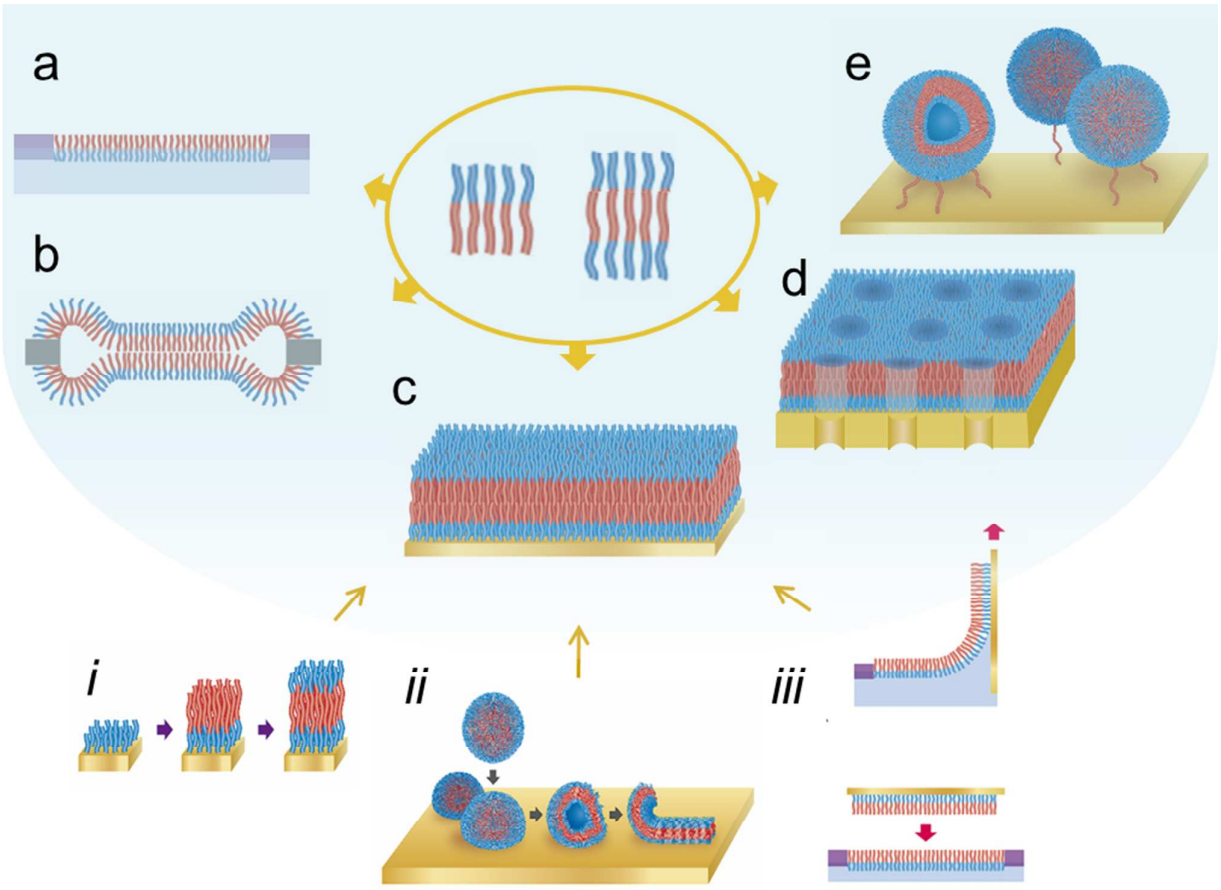

Fig. 8: Scheme of different planar polymer membranes. (a) Monolayer at water-air interface, (b) free-standing membrane, (c) solid-supported membrane, (d) nanoporeous solid-supported membrane and d) planer substrate immobilized vesicles. And scheme of the fabrication methods for solid-supported polymer membranes. (i) surface initiated polymerization, (ii) vesicle fusion and (iii) Langmuir monolayer transfer. Modified with permission from ref. [164]. Copyright 2011 The Royal Society of Chemistry

common methods are applied: pouring a polymer solution over aperture, and formation of folded bilayers. For planar free-standing membranes both sides of the membrane are accessible to aqueous solutions, thus mimicking a cell membrane in physiological conditions.[168] A major disadvantage of planar free-standing membranes is their low stability as a result of limited lateral tension and the presence of residual solvent, which may lead to membrane rupture.[169] Because of this instability, as well as difficulty in handling, planar free-standing membranes are of little technological interest, and their applications are limited to basic studies of membrane interactions with biomolecules. To solve the stability issue, additional crosslinking polymerization of individual block copolymers through covalent bonds may produce considerable strengthening of the membranes. If the polymerisable groups of the monomers are attached to the ends of the hydrophilic blocks, the hydrophobic middle block preserves some mobility within the membrane despite the crosslinking reaction. However, after the crosslinking process protein insertion is usually inhibited, and even some of the already reconstituted proteins may be expelled from the membranes.[170]

3.1.2 Solid-supported planar membranes

Planar solid-supported polymer membranes are obtained by physical or chemical attachment of polymer chains to a solid surface, resulting in improved mechanical stability compared to isolated free-standing membranes,[171] and the preservation of their structures even after drying.[172] Fusion of vesicles on solid supports is the simplest method for preparing solid-supported membranes. This method, which was originally developed for lipid systems, has been successfully adapted to copolymer vesicles. A colloidal solution is spread onto the solid support, and membranes are obtained by fusion of vesicles. The formation of membranes is strongly dependent on the composition and polydispersity in the size of vesicles, critical osmotic pressure, surface charge, and roughness of the solid substrate, in addition to environmental conditions, such as ionic strength and solution pH.[41] The disadvantage of the vesicle fusion method is the inhomogeneity and low reproducibility of the obtained membranes. Only a few articles on membranes prepared by vesicle fusion on a solid-surface have been reported,[172, 173] and to the best of our knowledge none of these were intrinsic stimuli-responsive membranes. Smart platforms resulting from vesicle fusion comprising bioactive moieties, and is described later in this review.

Another strategy for preparing homogenous solid-supported membranes is surface initiated polymerization, also called the "grafting from method".[174] Several polymerization techniques have been developed to control surface-initiated polymerization, including atom-transfer radical polymerization (ATRP),[175] reversible addition-fragmentation chain-transfer polymerization (RAFT),[176] nitroxide-mediated polymerization (NMP)[177] and ring-opening metathesis polymerization (ROMP).[178] The first generation of solid-supported polymer membranes are homopolymer brushes, which have the great advantage of providing a stable, easy to modify template for biomolecules anchoring. *S*urface-initiated polymerization enables good control of brush thickness and homogeneity. Suitable monomers can be selected to design polymer brushes that contain a grafting scaffold for the enzymes performing biomolecular transduction and, at the same time, introducing additional functional groups that facilitate the detection of the enzyme activity with a transistor. The ability to immobilize biomolecules with high binding capacities on surfaces while maintaining their activity is critical for protein microarrays and other biotechnological applications.

A better method used in fabrication of supported membranes is a combination of Langmuir-Blodgett (LB) and Langmuir-Schaeffer (LS) techniques. LB monolayers are formed when amphiphilic molecules interact at the air-water interface in order to minimize surface energy. Bilayers are obtained by transfer of the second layer using the LS approach. A LB-LS-transferred polymer tethered solid-supported bilayer membrane is more stable (more than two weeks in water; up to 12 h in air), than a free-standing polymer membrane (less than several hours in water). Such solid-supported polymer membranes can also be modified to allow attachment/insertion of active biomolecules, and thus generate "smart/active surfaces" with desired functionality.[7] The versatility of this method allows the preparation of asymmetric bilayers by tuning amphiphile compositions. These fabrication methods of solid-supported synthetic membranes are the same as those used for lipid bilayer fabrication.[174]

Biological molecules can be attached to polymer membrane surfaces or inserted within membranes either during the membrane formation process or after the membrane has been formed. Solid-supported membranes are asymmetric, and thus require specific strategies for insertion/attachment of biomolecules in/to membranes. The challenge for successful reconstitution of biomolecules on solid-supported membranes is related to inevitable prerequisites, such as hydrophilicity, balance between electrostatic repulsion and attraction, and the presence of a lubricating water layer between the substrate surface and the membrane. Voltage destabilization of the membrane is one approach to successfully reconstitute proteins in planer polymer bilayers.[75] Alternatively, controlled use of bio-beads to destabilize synthetic solid-supported membranes favours functional insertion of a membrane protein.[73]

3.2 Intrinsic stimuli-responsive polymer membranes

About a decade ago, a free-standing monolayer film using amphiphilic PMOXA-*b*-PDMS-*b*-PMOXA triblock copolymer was described.[179] However, to the best of our knowledge, no stimuli-responsive free-standing membranes and very few monolayers at the air-water interface can be found in the literature up to this day.

The bioinspired solid-supported membranes can be formed by stimuli-responsive amphiphilic diblock or triblock copolymers *via* grafting methods in which polymer chains are anchored to a surface, or an initiator molecule is coupled to the surface and

allows the growth of chains, *i.e.* brushes.[180, 181] At this point, it needs to be stated that there have been many investigations of responses of different brush architectures to environmental parameters such as ionic strength, temperature, light, pH, the presence of compounds that respond to adsorption (antifouling), or a combination of these. However, there are only a few examples of trigger biomimetic brushes based on amphiphilic block copolymers, and most have been focused on biosensing[182] or cell growth/adhesion oriented studies.[183]

For example, poly(ethylene glycol) methacrylate-*block*-poly(acrylic acid) (PEGMA-*b*-PAA) brushes have been used for the fabrication of a polymeric bioassay for the detection of antigens. A bilayer brush architecture that combined a PEGMA bottom layer (responsible for antifouling) with a PAA upper layer (enabling antibody loading) improved the antigen detection and suppressed FIB interference of antigen recognition compared to directly surface grafted PAA-IgG references.[184]

Enzyme-based biosensors require high sensitivity and thus controlled design is needed. Modified ITO-electrodes, poly(glycidyl methacrylate – glucose oxidase)-*block*-poly(ferrocenylmethyl methacrylate) (ITO-*g*-P(GMA–GOx)-*b*-PFMMA) and ITO-*g*-PFMMA-*b*-P(GMA–GOx), block copolymer brushes have been developed as an amperometric glucose biosensor, in which the block copolymer brush-functionalized ITO electrode with P(FMMA) as the inner block was more sensitive to glucose than that with P(GMA) as the inner block.[185] In another example, poly(11-mercaptoundecyl sulfonic acid)-*block*-poly(sulfobetaine methacrylate) (PSA-*b*-PSBMA) has been used for non-specific protein adsorption in human blood, and the influences of various polymer molecular weights and surface brush packing have been analysed. PSA-*b*-PSBMA brushes strongly resisted non-specific protein adsorption because of zwitterionisation of the surface. This surface anchored with zwitterionic copolymer brushes maintained excellent blood-inert properties on contact with human blood.[186]. Another example described pH-responsive poly(acrylic acid)-*block*-poly(vinylpyridine) (PAA-*b*-PVP) copolymer brushes that were swollen at extreme pH values, but collapsed at moderate pH due to a polyampholyte effect.[187]

Poly(2-hydroxyethyl methacrylate)-*block*-poly(*N*-isopropylacrylamide) (PHEMA-*b*-PNIPAM) block copolymer brushes were converted into the corresponding PSEMA-*b*-PNIPAM by esterification of the hydroxyl groups to produce a pH responsive behaviour. However, this resulted in a loss of thermal response of the PNIPAM at low pH values, and this was only recovered at high pH because of ionization of carboxyl groups.[188] Copolymer brushes based on PS-*b*-PNIPAM, poly(*N,N'*-dimethylacrylamide)-*block*-poly(*N*-isopropylacrylamide) (PDMA-*b*-PNIPAM), and polystyrene-*block*-poly(4,5-dimethoxy-2-nitrobenzyl methacrylate) (PS-*b*-PNBA) have been used to investigate the tuning of a dye release kinetics in the presence of three different stimuli: temperature, pH, and light. A photo-response was achieved by controlling the degree of photo-cleavage of photolabile *o*-nitrobenzyl groups. Complete photo-cleavage of *o*-nitrobenzyl groups converted the photosensitive PS-*b*-PNBA brush into a pH-sensitive polystyrene-*block*-poly(4,5-dimethoxy-2-nitrobenzyl methyl methacrylate acid (PS-*b*-PMA) brush, and pH-dependent dye release resulted from water solubility switching of the PMA outer layer between collapsed and extended states (**Fig. 9**).[189] In another approach, a PNIPAM-based block copolymer comprising a PNIPAM-*co*-hexafluoroisopropyl acrylate (HFIPA) brush has been used as a reversible chemo-mechanical switch by manipulating the temperature of the system. The system showed good water permeability at temperatures below 20 °C, and was water repellent above 40 °C due to the PNIPAM thermal response.[190]

Surface-grafted block copolymer brushes poly(poly(ethylene glycol) monomethacrylate) (P(PEGMA)) and PNIPAM chains with gradients based on continuous composition have been fabricated by a surface initiated ATRP. Investigations of *in vitro* cultures of HepG2 cells prepared on these "gradient surfaces" revealed that the cells adhered at 37 °C, but were detached at 20 °C. Introduction of the PEG chains as an underlying layer on the PNIPAM grafting surface resulted in a faster cell detachment compared to the direct PNIPAM grafting surface.[191] In a different example, a RGD peptide was grafted onto a PAA layer, and produced a PNIPAM-*b*-PAA-*g*-RGD brush. The immobilized RGD peptide accelerated cell attachment, whilst the underlying thermoresponsive layer effectively served to release the cells on lowering the temperature.[183, 192]

Block copolymer brushes have also been combined with the LS method.[193] Use of a block copolymer poly(2-(dimethylamino)ethyl methacrylate)-*block*-poly(methyl methacrylate) (PDMAEMA-*b*-PMMA) enabled preparation of tuneable interlayers by LS transfer at well-defined lateral chain densities. These transferred copolymer films remained stable over more than one week, and therefore could be used as pH-controlled solid substrates for the support of biological materials.[194] The weak polyelectrolyte PDMAEMA chains at the

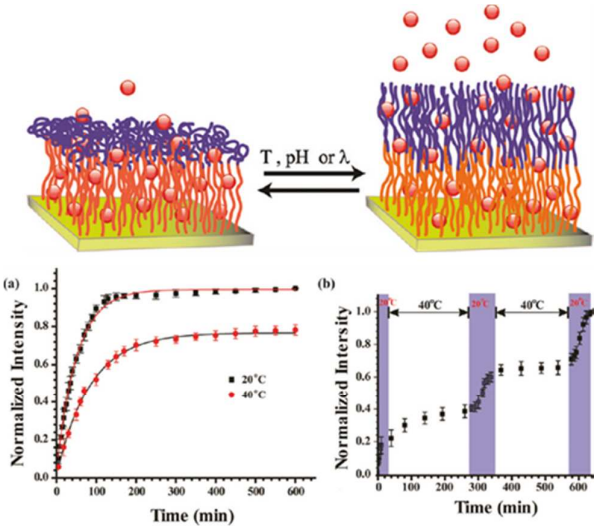

Fig. 9: Changes in normalized fluorescence emission intensity over time showing the dye release kinetic from PS-*b*-PNIPAM at two different temperatures. Reproduced with permission from ref. [189]. Copyright 2011 American Chemical Society.

solid/liquid interface are reversibly activated by pH changes to regulate electrostatic interactions at the interface, and thus to tune the thickness of the water reservoir between the membrane and the polymer film. This approach indicates the potential of such "tailored" polymer films for use in regulating cell-substrate interaction potentials via external stimuli.[195] Investigations of the effects of various pH values and ionic strengths on the surface micelle behaviour and morphology of the amphiphilic block copolymer poly(benzyl methacrylate)-*block*-poly(2-(dimethylamino)ethyl methacrylate) (PBzMA-*b*-PDMAEMA) at the air–water interface indicated that the balance between attractive hydrophobic interactions among PBzMA cores and repulsive electrostatic interactions between underwater PDMAEMA chains is significantly affected by pH, and suggested that LB films prepared under these experimental conditions form a variety of pH-dependent morphologies.[196]

3.3 Polymer membranes modified by biomolecules

Different types of planar polymer membranes have been tailored to accommodate biological molecules, whilst allowing transport of ions/molecules through the membrane, and facilitating signalling processes, or serving to sense changes in the membrane or its environment.

3.3.1 Monolayers at the air–water interface

Studies on monolayers with biomolecules provide an understanding of their interactions, which affect the combination of artificial membranes with biomolecules.[197] Decreasing the thickness of copolymer membranes is important for the realization of biomimetic membranes. In this respect it is essential to define the composite film fabrication parameters for the optimization of protein insertion. Amphiphilic triblock copolymers have been used for Langmuir film-based functionalization with OmpF at the air/water interface, and it was found that the initial surface coverage with the copolymer monolayer and the membrane thickness both playing important roles in determining the extent of protein integration.[198]

3.3.2 Free-standing membranes

Reconstitution of a channel protein in free-standing membranes is usually detected by a minor change in the conductance of the system.[199] Although planar free-standing lipid membranes (known as Black Lipid Membranes) have been widely used for investigating protein reconstitution, only a very few cases of artificial membranes have been reported.[179] The first example of such a synthetic free-standing membrane was based on amphiphilic PMOXA-*b*-PDMS-*b*-PMOXA copolymer, which forms a planar membrane with a thickness of 10 nm and a surface area up to 1 mm^2. Transmembrane proteins (OmpF and maltoporin) were successfully incorporated and their functions were fully preserved in these complete artificial polymer membranes.[170, 200] Based on the effects of membrane protein incorporation on the morphology of the resulting protein-polymer membrane, free-standing planar membranes are able to accommodate a higher density of proteins than vesicular polymer membranes.[71] The high protein densities in polymer membranes support orders of magnitude higher sensitivity or transport rates of such membranes, and should allow miniaturization or molecular recognition applications.[38] In addition, planar polymer membranes might be employed for membrane protein crystallization as it has been demonstrated in the case of two-dimensional crystals of the aquaporin-0 (Aqp0).[71]

3.3.3 Solid-supported membranes

The main advantage of using polymers for solid-supported membranes is their increased thickness (3–40 nm) compared with lipid membranes (1–3 nm),[201] which prevents strong interactions and frictional coupling between the solid substrate, and the incorporated biomolecules that could induce partial loss of functionality or complete biomolecule denaturation.[202] α-Hemolysin (αHL) has been successfully reconstituted in an amphiphilic PB-*b*-PEO solid-supported membrane by voltage destabilization of the membrane.[75] The combination of enzymes and solid-supported polymer membranes allows the generation of enzymatic reaction spaces by insertion or attachment of biomolecules at the polymer membrane surface, which acts as a template.[61, 203, 204] The reaction space is located either inside the polymer membrane if the enzyme is trapped inside, or at the interface between the polymer membrane and the environment if the enzyme is attached at the membrane surface.

Various reactions are used for immobilization of enzymes: esterification, amidation, and binding to nitrilotriacetate (NTA)-Cu^{2+} complexes. The impact of immobilization on the enzyme depends on many factors, including the enzyme itself, the physical and chemical characteristics of the support, the location of the enzyme on or within the support, or the method of immobilization. A range of binding chemistries, substrates, and techniques for immobilization has been reported, mainly for small molecules such as biotin, and for biomacromolecules (enzymes or antibodies). Various enzymes *e.g.* laccase,[205] RNase A,[206] HRP,[207] cholesterol oxidase,[208] uricase,[209] glucose oxidase (GOx),[210, 211] ascorbate oxidase,[212] catalase,[213] and invertase[214] have been immobilized on various polymer membrane surfaces *e.g.* poly(hydroxyethylmethacrylate),[205, 207] PAA,[206] polytetrafluoroethylene,[215] and polyaniline.[10, 209, 212, 214] Immobilization of biomolecules is able to increase their stability during storage,[207] broaden the optimum enzymatic activity conditions,[205, 207] and support biomolecule reusability.[215] For example, uricase immobilized on polyaniline brush completely retained its initial activity for the first 21 days, followed by a slow decrease for 56 days, whereas the free enzyme lost its activity within 35 days.[10] In addition, simultaneous immobilization of several biomolecules has been reported.[216, 217] An alternative approach is based on the use of graphene as a scaffold with special properties to produce copolymer brush functionalized graphene transistors. For example, solution-gated graphene field-effect transistors modified with acetylcholinesterase (AChE) and a transducing

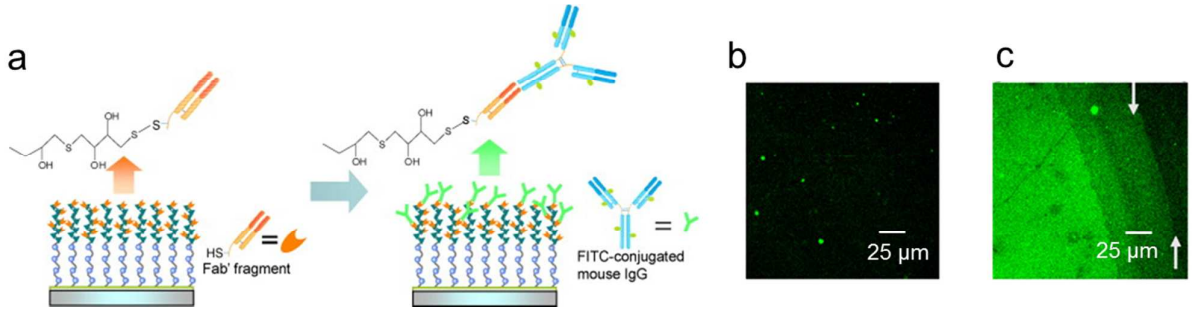

Fig. 10: (a) Scheme of Fab' fragment-immobilization on polymer brushes and reaction with FITC-labeled IgG Antibody Fab' fragments were immobilized onto these surfaces via thiol groups in Fab' fragments and pyridyl disulphide moieties in polymer brushes, which define the orientation of the antibodies. Pictures of block copolymer brushes observed with a fluorescent microscope (b) before and (c) after the immobilization of Fab' fragments and the subsequent reaction with antigen. Reproduced with permission from ref. [219]. Copyright 2008, Elsevier.

pH sensitive group, is an extremely sensitive detector for the neurotransmitter acetylcholine (**Fig. 10**).[218]

A further generation of solid-supported polymer membranes is using block copolymer assemblies based on various copolymers, *e.g.* PS-*b*-PMMA,[220, 221] PMPC-*b*-PGMA,[219] PFMMA-*b*-PGMA,[185] and PEG-*b*-PMCL-*b*-PDMAEMA. Di-/tri-block amphiphilic copolymers form different assembly domains on solid surfaces. The chemical nature of the block structure supports formation of membranes with properties closer to cell membranes than homopolymer brushes,[219] whilst the block architecture ensures good accessibility of the immobilized probe, and allows immobilization of biomolecules with a defined orientation, and higher activity **(Fig. 11**). The lower polymer segment is responsible for the binding the polymer chains to the solid surface, and in addition they may enhance the performance of the active system. For example, the enzyme-mediated ITO electrode exhibits high sensitivity, when a redox-PFMMA block is introduced as the electron-transfer mediator.[185] The asymmetry of a block copolymer membrane is a key factor, which favours the functionality of active surfaces with desired orientations. In this respect, a group of PEG-*b*-PMCL$_x$-*b*-PDMAEMA$_y$ amphiphilic copolymers, with different hydrophilic and hydrophobic domains was selected to generate "active surfaces" by solid-supported polymer membranes using the LB method: at the air–water interface, the films were oriented with PEG in the water sub-phase and PDMAEMA facing towards the air, which served for immobilization of enzymes. Laccase, used as a model enzyme was successfully attached to copolymer membranes by stable interactions and preserved its activity.[61]

Although attachment of biomolecules to solid-supported polymer monolayers was used successfully to study the properties of peripheral membrane proteins, membrane-integrated peptides, or the binding of fluorescent ligands to integral membrane protein receptors, it has limited scope for the study of membrane proteins. Solid-supported bilayer membranes with a hydrophilic-hydrophobic-hydrophilic wetting property that mimics cell structures represent a good candidate for addressing this issue. In a planar solid-supported polymer membrane, the bilayer couples to the surface only through the bottom shell, whereas the upper one is attached by hydrophobic interaction. The noncovalent interaction between these polymer layers allows a certain degree of membrane fluidity, which is essential for the insertion of peptides and membrane proteins.[73, 75, 173] Thus this favours the complex requirements necessary for functional insertion of a membrane protein, namely: (i) a homogeneous and stable membrane, (ii) sufficient membrane fluidity to host a protein, and (iii) the presence of a spacer between the substrate and membrane to prevent protein denaturation.[73] The first example of a successful insertion was reported for reconstitution of the water soluble protein αHL into a PB-*b*-PEO bilayer membrane (**Fig. 12**).[75]

The unique electrical properties resulting from the functional insertion of αHL in the synthetic solid-supported membrane was modelled by the Donnan potential caused by accumulation of ions in the inner, hydrophilic part of the polymer membrane. The water-insoluble membrane channel protein (nucleotide-modulated potassium channel from the bacterium *Mesorhizobium loti*, (MloK1)), which requires the presence of detergent for stabilization, has been reconstituted in amphiphilic PDMS-*b*-PMOXA solid-supported membranes after the removal of detergent using biobeads.[73]

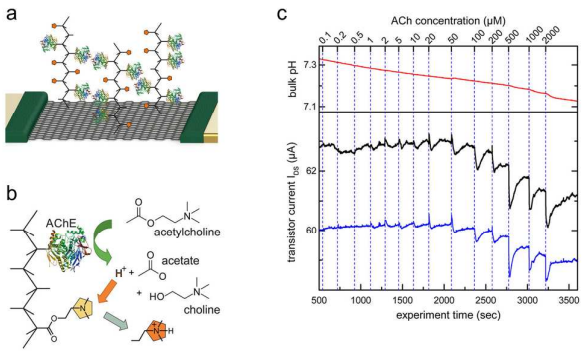

Fig. 11: (a) Schematic view of an enzyme-functionalized graphene transistor. The graphene sheet is contacted by insulated gold contacts from two sides. The graphene active area is modified with copolymers containing acetylcholinesterase and pH sensitive DMAEMA (orange pentagons) groups. (b) Sensing principle of the transistors. Acetylcholine is hydrolyzed to acetate, choline, and a proton with the help of the enzyme. This proton can react with the dimethylamino groups in the polymer, inducing a fixed charge close to the transistor's surface that results in a charge doping effect. (c) Drain-source current of two transistors and pH of the bulk solution are shown *versus* time. At the marked times, acetylcholine was added to the solution, which can be seen in the transistor current as a sudden decrease. Reproduced with permission from ref. [218]. Copyright 2014 American Chemical Society.

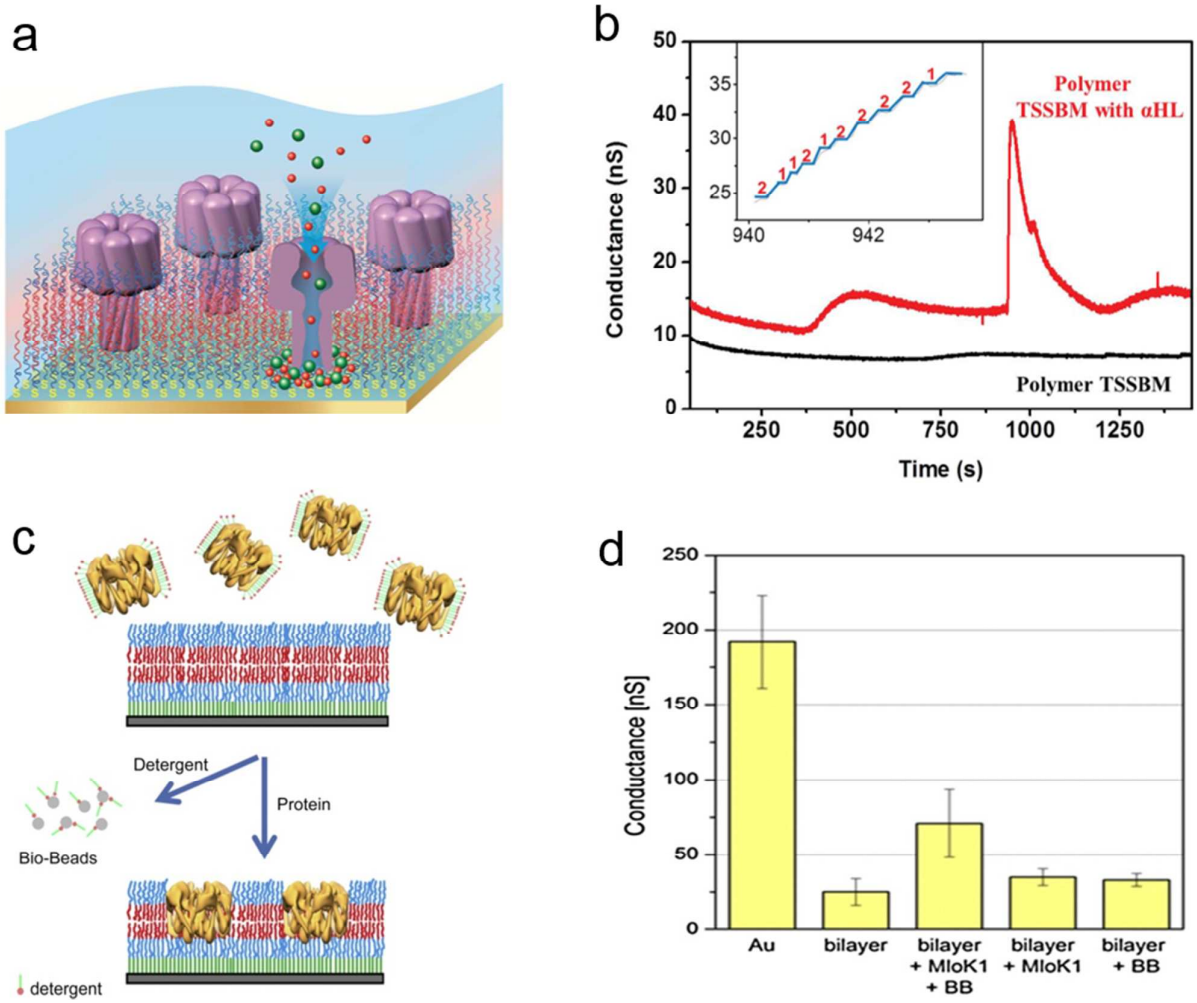

Fig. 12: (a) Schematic representation of the tethered solid-supported PB-*b*-PEO bilayer membrane, which is suitable for protein insertion. (b) Characteristic time course for conductance across the PB-*b*-PEO TSSBM before (black curve) and after (red curve) addition of αHL, at a voltage of 40 mV. Inset is an enlarged view of the stepwise increase in the characteristic time course of the conductance across the PB-*b*-PEO TSSBM with the addition of αHL. Reproduced with permission from ref. [75].Copyright 2013, Macmillan Publishers Ltd. (c) Schematic representation of membrane protein insertion into solid-supported polymer membrane with usage of biobeads. (d) Conductance measured at a constant applied voltage of 40 mV (Au=gold substrate, BB=biobeads). Reproduced with permission from ref. [73]. Copyright 2014, Elsevier.

3.3.4 Solid immobilized vesicles

A further step in the generation of cell mimics was realized by immobilization of nanoreactors on surfaces to serve as 2D enzymatic arrays.[222] Polymersomes containing trypsin[223] or acid phosphatase[222] were immobilized on glass substrates using electrostatic interactions[223] or specific biotin–streptavidin molecular recognition interactions.[222] Surface coverage of 12.8 - 99.8% was achieved by varying the ionic strength or pH. By changing the organization of the pattern of nanoreactors, it may be possible to support specific functionalities, *e.g.* simultaneous detection of various biological molecules.

3.4 Pore-solid-supported/pore-spanning membranes

A drawback of standard solid-supported membranes is that they do not allow investigations that mimic physiological conditions, such as the transport of matter, or determination of the mechanisms of ion fluxes through a membrane. An elegant approach to address this problem is to use pore-solid-supported membranes for insertion of proteins and to study transport processes. This represents a further step in synthetic membrane development, because pore-solid-supported membranes combine the mechanical stability of solid-supported membranes with the advantage of being free-standing over pores. This then enables the study of conformational changes in membrane proteins drawn by gradients, cargo transport, and external forces.[224] In addition, pore-solid-supported membranes offer unprecedented mechanical stability over periods of days, with mesh sizes between 20 nm and several micrometres and in defined geometric patterns.[225] Solid porous substrates can be classified into organic (porous polycarbonate film) and inorganic (porous alumina) membranes. The first reported example was based on insertion of AqpZ in the pore-solid-supported PMOXA-*b*-PDMS-*b*-PMOXA membranes, to generate a highly permeable membrane for water, but no solutes (**Fig. 13**).[71, 226] AqpZ is in its active form inside the planar membranes and serves as a tool in the development of nanofiltration membranes. A

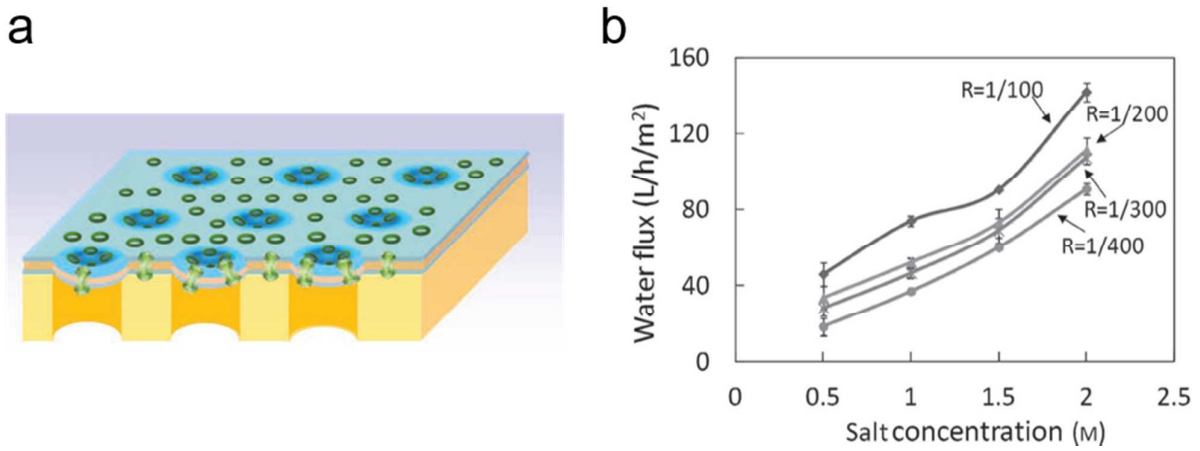

Fig. 13: (a) Schematic diagram of pore-spanning membrane with Incorporation of AqpZ in ABA block copolymer membranes. An ideal membrane must ensure that all substrate pores are covered by the AqpZ-ABA membrane. This can be accomplished by seamlessly covering all pores with a layer of vesicles and then causing the vesicles to rupture. (b) Plot of water flux versus salt concentration as a function of the molar ratio of AqpZ to ABA polymer (represented by R; n = 3). Reprinted with permission from ref. [226]. Copyright 2012 Wiley.

biomimetic membrane based on AqpZ inserted in PMOXA-*b*-PDMS-*b*-PMOXA membranes at a molar ratio of 1:50 possessed water permeability of 167 μm s^{-1} bar^{-1} ,[27] by far superior to the current state-of-the-art polymeric membranes based on osmosis. However, the key hurdles to be overcome in the design of such planar biomimetic membranes are: (i) the presence of defects due to the thin and fragile self-assembled amphiphilic matrix, (ii) low coverage of the porous substrate, and (iii) possible delamination at the interface between the selective layer and the substrate.[27] In addition, maintaining the functionality of AqpZ represents another challenge.

3.5 Hybrid lipid-polymer membranes

3.5.1 Lipid bilayers supported by intrinsic stimuli-responsive polymers

Hybrid lipid-polymer membranes are interesting because they combine both phospholipid and synthetic polymer properties, and thus provide more information on the behaviour of biomolecules in such hybrid systems. However, one challenging aspect of biomimetic membrane development is to understand the interactions between membranes and their supports, in particular when a porous support allowing mass transport across the membrane is involved.[227] Hence, the model hybrid system between lipid bilayers and responsive polymeric supports might represent a good approach for understanding the molecular basis of biological membrane transport. The simplest cell membrane mimic is obtained by direct deposition of a lipid monolayer on a solid support. Although a thin layer of water between the substrate and the lipid bilayer acts as a lubricant to preserve the fluid character of the bilayer, such systems have significant limitations in the case of membrane protein incorporation. To overcome these drawbacks different “soft” polymer layers have been developed, and these serve as intermediates between lipid bilayers and solid substrates.[228] They inhibit direct contact of proteins with solid supports, and consequently preserve their bio-functionality. Two main types of polymer supported lipid bilayer have been developed: (i) polymers, which serve as an independent (intermediate) support, and (ii) coupled bilayer–polymer systems. In the first case no direct binding between the polymer and lipid occurs, whereas in the second case the polymer is covalently bound to the lipid membrane. Independent supports can be prepared by various methods, such as spin-coating,[229] surface polymerization[174] or simple dip-coating,[230] followed by deposition of the lipid membranes. Coupling between the lipid bilayer and the polymer support is obtained by incorporating amphiphilic polymers, which serve as coupling points between the bilayer and the polymer support. The density of tethering points can be tuned by varying the composition of the polymer within the lipids, and the distance between the substrate and the lipid membrane.[231] However, only some chains of the lipid membrane are tethered to the substrate, and the function of the rest of the polymer layer is to serve as a cushion. The most common used design strategies for tethered lipid bilayers prepared on solid supports are shown in **Fig. 14**.

Supported lipid membranes formed on polymer cushions of alternating maleic acid copolymers were triggered by lowering the pH to 4. The stability of the lipid bilayer was not affected, but the hydrophilicity and swelling properties of the polymer cushions changed, and determined the kinetics of the bilayer formation. Such supported lipid bilayers on polymer cushions can provide good conditions for insertion of membrane proteins.[232] In a similar approach, surface-tethered polymer cushions based on cross-linked thermoresponsive PNIPAM polymer copolymerized with methacroylbenzophenone (MaBP) successfully supported two lipid model membranes. At moderate temperature changes the polymer swelled, and created a nearly aqueous cushion, which allowed exploration of fluctuations of the lipid membranes in a well-controlled manner.[233, 234] In a combined approach, polymer films of poly(N-isopropyl- acrylamide-*co*-carboxyacrylamide) (PNIPAM-*co*-carboxyAAM) with temperature and pH responsiveness served as cushions for lipid bilayers. Due to the weak bilayer-cushion coupling, the bilayer mobility was not affected by the swelling state of the cushion that was determined by changes of the stimuli.[235]

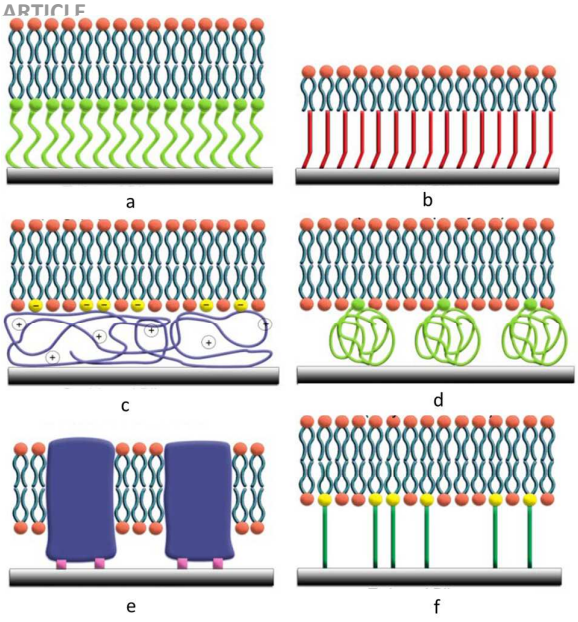

Fig. 14: Various strategies and units for tethered assembled lipid bilayer on solid support: a) Tethered bilayer (oligopeptide–tethered), b) Hybrid bilayer(alkane-lipid bilayer), c) Cushioned bilayer (polycation cushioned), d) Tethered bilayer (polymer-tethered), e) Protein-tethered bilayer and f) Tethered bilayer (thiolipid-tethered). Reprinted under the Creative Commons License from ref. [228].

3.5.2 Lipid-polymer hybrid membranes with incorporated biomolecules

The lipid-polymer monolayer has been used as the simplest hybrid membrane for investigating the behaviour of biomolecules. For example, the kinetics of the hydrolysis reaction mediated by lipase on monolayers of L-R-dilauroylphosphatidylcholine (DLPC) and poly(tert-butyl methacrylate) (PtBMA) served as a mimic of the DLPC/cholesterol system.[236] However, the polymer is not likely to exert the same retardation effect on monolayer dynamics that result in condensed complexes of phospholipids and cholesterol.

By increasing the complexity of lipid-polymer membranes, a better understanding of the underlying interactions with biomolecules can be achieved. For example, insertion of OmpF into a polymer-lipid thin film demonstrated that localisation of membrane proteins in a non-native, complex thin film environment can be regulated by the phase behaviour of film components.[237] Interestingly, the proteins were situated in the fluid polymer-rich phase, but not in the rigid lipid phase, which was mechanically unfavourable. Therefore, use of the phase behaviour of complex thin films as a 'trigger' to direct the insertion of biomolecules will lead to further development of complex and controlled multicomponent systems.

The formation of hybrid lipid-polymer free-standing membranes is difficult and also rare, although immobilization of an enzyme on conducting polypyrrole/lipid membranes is one example of such a system.[238]

Lipid bilayers supported by polymer-cushions (physically adsorbed) and -tethers (covalently attached) serve as good candidates for insertion of membrane-active peptides and membrane proteins.[40] The polymer support creates a space large enough (several nanometres) below the lipid bilayers, to allow membrane proteins to diffuse freely.[239] The lateral mobility of the membrane receptors in such polymer tethered lipid membranes can be controlled by the lateral density and length of the polymer spacers.[231] In addition, it is also possible to electrically manipulate the recombinant protein for local functionalization of solid surfaces when membrane-anchored proteins have different net charges.[240]

The polymer spacer between the lipid membrane and the solid surface is also essential for the investigating transport processes, such as the conduction of ion channels, or the transport of substrates through membrane proteins. Moreover, the characteristics of the polymer spacers enable the formation of hybrid membrane systems with new properties. For example, the presence of an anionic polymer cushion allowed successful reconstitution of membrane proteins within solid-supported lipid bilayers, and tuneable protein mobility and activity.[241] pH-responsive PAA cushioned lipid membranes serve the study of channel-mediated proton transport across the membrane bilayers.[242] Interestingly, when a conducting polymer PPy(DBS) was used as an electroactive polymer, and the alamethicin-bound bilayer lipid membrane as a bioderived material in a thin-film laminated device, the protein regulated the ionic concentration in the conducting polymer and the electrochemical doping/undoping process.[243]

In another example, micropatterned polymer-supported membranes have been used to confine diffusion through membrane proteins for single molecule studies,[244] whilst polymer hydrogels have served as supports for lipid bilayers and protein tethering.[245]

An elegant way to avoid interactions between solid supports and biomolecules, which might affect their structure and functionality, is to use nanopores in arrays of silicon chips. Indeed, nanopores in arrays of silicon chips functionalized with pH-responsive poly(methacrylic acid) (PMAA) brushes have been used as supports for pore-spanning lipid bilayers containing reconstituted membrane proteins.[246] The nanopore functionalization with pH-sensitive brushes allowed the opening and closing of the pores "on command". The polymer cushion can also affect the mechanical strength of the pore-suspending membranes. For example, a carboxylated PEG cushion significantly enhances the flexibility of a DMPC bilayer, and provides improved conditions for the reconstitution of AqpZ.[223] Reconstitution of AqpZ in a pore-containing planar membrane increases the energy barrier required for a normal force to punch through the membrane, and decreases the flexibility of the membrane.

4. Biological and medical applications of bioinspired polymer vesicles and membranes

Although still in its early stage of research, the development of artificial membranes that are able to respond to intrinsic stimuli or to be decorated with biomolecules represents a novel strategy with high potential for valuable nanometre

scale biological and medical applications (*e.g.* targeted drug delivery, theranostics). Here we introduce recent interesting examples of bioinspired polymer vesicles and membranes for biological and medical applications.[136, 247-251]

4.1 Polymer Vesicles

Due to their stability and possible stealth effects in the blood stream, vesicles assembled from block copolymers are considered to be an ideal candidate for targeted drug delivery,[136] nanoreactors,[15] artificial organelles,[21, 252] and simple cell mimics.[45] In addition to being able to reach their target, polymersomes used in bioapplications need to release their cargo "on site". Only certain triggers or combination of triggers have led to the design of polymersomes that are potentially applicable for biomedicine; others have not yet been evaluated, or their responsiveness was far away from relevant conditions in the human body. The designed polymersomes have to react to biologically relevant triggers or the introduction of a necessary triggering force, molecules or concentration of certain molecules has to be at least theoretically feasible. Depending on the type (anticancer, anti-infection, anti-inflammatory, *etc*.) and the route of application (intravenous injection, local injection, oral application, *etc.*), smart carrier systems have to fulfil a complex scenario of requirements: no/limited toxicity, biodegradability, sufficient blood circulation time (for systemic applications), ability to reach and be taken up by target cells, and release their therapeutic payload when desired.[252-255]

4.1.1 Cancer Imaging

In a recent attempt to design multifunctional diagnostic polymersomes, samples were loaded with a near-infrared (NIR) emitting dye and paramagnetic gadolinium (Ga(III)) cations.[256] *In vivo* imaging in mice after *i.v.* administration revealed successful dual-labelling of tumour regions, but the carrier system still has to be optimized in terms of reduced uptake by cells of the RES to gain longer blood circulation times for efficient targeting to tumours. Trimodal detection of tumours in mice was achieved using NIR fluorescence, thermal, and photoacoustic imaging by injection of gold nanoparticles- and photosensitizer Ce6-loaded polymersomes (**Fig. 15**).[126]

4.1.2 Cancer Therapy

Proposed applications of responsive polymersomes in cancer therapy have mainly focused on systemic delivery of smart loaded polymersomes, which can passively target tumour regions by the enhanced permeation and retention effect (EPR) known for solid tumours[257] or local intratumoural injection to circumvent the requirements needed for long blood circulation time.[126, 127]

Several pH-responsive polymersomes have been tested for drug/protein/nucleic acid encapsulation, release and subsequent killing of cancer cells, but extensive *in vivo* data are still lacking,[77, 80, 91, 92, 258-260] although pH-sensitive polymersomes have been proposed for *e.g.* head and neck cancer therapy.[261] This kind of polymersome loaded with two model drugs (DOX, Paclitaxel) was shown to penetrate deeply into a three-dimensional *in vitro* tumour model to reach cells in the middle of the cultured tumour spheroid.[261] This is one crucial parameter that has to be addressed in translation from cell experiments to real clinical applications.[253] Acid-labile PMPC-*b*-PDPA polymersomes have been used for delivery of functional antibodies against NF-κB to HDF cells, which were able to modulate cellular activity.[262] The effect of polymersome shapes on cellular uptake has also been studied. For example, tubular pH-sensitive polymersomes can deliver hydrophilic BSA (model protein) to different cell lines (primary human neutrophils and FaDu cells).[263] Delayed internalization kinetics and a different cell uptake mechanism were observed for elongated structures compared to spherical counterparts made from the same block copolymers. Therefore, shape is clearly an important parameter for defining circulation kinetics, biodistribution, targeting, and uptake mechanisms for nanostructures intended for various therapeutic approaches.[264]

Furthermore it is also possible to exploit the altered protein expression profile of tumour cells. Polymersomes based on a triblock copolymer composed of poly(trimethylene carbonate) (PTMC) linked to poly(glutamic acid) (PGA) using the peptide PVGLIG, have been used to deliver a cargo to tumour cells.[146] Also, dextran based polymersomes were shown to be able to deliver DOX and CPT by two distinct pathways: diffusion of DOX through the membrane, and assisted release by carboxyl esterase cleavage.[139]

Until now, the delivery of multiple drugs has been achieved by simultaneous encapsulation, and consequently they are released at the same time. A promising approach for a controlled series of release events has been demonstrated by using multicompartment polymersomes prepared by using a double-emulsion-template technique and a microfluidic device. Sequential release of the cargo was achieved by mechanical strain or osmotic shock.[265]

Polymersomes with asymmetric membranes exhibit higher biocompatibility, are more rapidly endocytosed, and escape faster from endosomes than similar symmetric polymersomes.[258] Cross-linked pH-sensitive polymersomes, which do not disassemble but swell upon acidification, have been proposed as delivery vehicles or artificial organelles.[79]

Redox responsive polymersomes have been applied to deliver DOX into breast cancer cells, and they showed minimal toxicity in mice and effective tumour suppression.[98, 266] In general, toxicity with respect to cultured cells, and intracellular release of drug/protein/nucleic acids have been extensively tested for various reduction-sensitive polymersomes,[84, 85, 97, 98, 100] and multiple-responsive polymersomes.[86, 109, 114-117, 124] However, extensive *in vivo* data comparing the applicability, and advantages of various triggered system are still missing.

4.1.3 Cancer Theranostics

A successful theranostic platform for photoacoustic imaging and PTT for possible tumour detection and reduction was introduced based on gold nanoparticle-polymersome hybrid structures.[127] To provide theranostic approaches in one

polymersome formulation, various components were incorporated to yield multi-functionality. Polymersomes loaded with gold nanoparticles and CeO nanoparticles have been used for trimodal tumour imaging, and efficacious synergistic photothermal and photodynamic therapy (PTT/PDT). This led to tumour regression upon irradiation with NIR light as a result of light-induced heating in the proximity of the gold nanoparticles (broad light absorption 650 - 800 nm), and the production of ROS by CeO (**Fig. 15**).[126]

Such complex nanoparticle-polymersome hybrid structures are often administered by local intratumoural injection, because the assemblies are too large (> 200 nm) to circulate for a sufficient time in the blood after intravenous injection.[126, 127] This limits the application of these metal nanoparticle decorated polymersomes to local theranostics; small tumours or metastases cannot be reached, although metastasis should be the main focus for targeted drug delivery.[253]

4.1.4 Disease-targeted drug delivery

Besides the triggered release of drugs from polymersomes, surface modification with targeting ligands offers the possibility of achieving disease targeted drug delivery. Receptor specific targeting to certain cell types requires immobilization of the ligand on the polymersome surface. However, depending on the nature of the ligand, tedious reconstitution procedures may be required to insert the hydrophobic region of the ligand into the membrane or to link it to the hydrophilic block. Nevertheless, intrinsic preferential uptake of non-functionalized pH-sensitive polymersomes has been demonstrated for melanoma cells, which internalized DOX-loaded polymersomes more efficiently compared to non-cancerous human fibroblasts, leading to possible melanoma targeted therapeutics.[259]

A hybrid approach has been designed with the amphiphilic antibacterial peptide Cecropin A as an “anchor”. By using EGFP as a water soluble fusion partner, the construct successfully anchored itself in the polymersome membrane.[163] Modification of the polymer itself with targeting ligands has also been successfully demonstrated. For example, PEO-*b*-PB diblock polymersomes were modified by “click” chemistry with GRGDSP peptides and the fibronectin mimic PR_b, both of which are able to target colon cancer cells. PR_b modified polymersomes loaded with doxorubicin were highly efficient and outperformed unmodified vesicles and GRGDSP modified polymersomes by roughly 80% and 40%, respectively.[145] The use of antibody-polymersome conjugates was furthermore applied to target and overcome the blood-brain barrier, a crucial obstacle for successful delivery of drugs into the human brain.[267] pH-responsive, apoptotic protein-loaded (granzyme B) polymersomes have been used to target lung cancer cells (H460 cells) by decorating their surface with anisamide moieties.[260] The anisamide molecules generated preferential uptake by sigma receptor over-expressing cancer cells, such as non-small lung cancer cells H460, which specifically

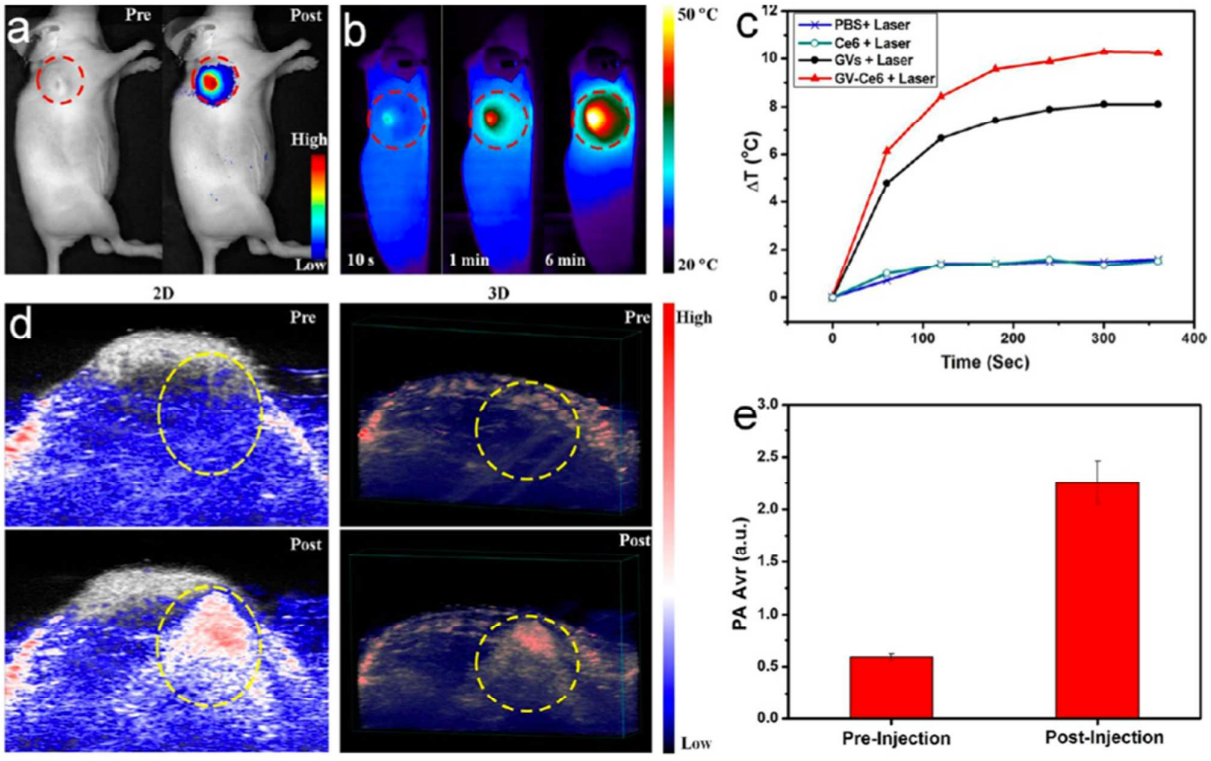

Fig. 15: (a) NIR fluorescence imaging before and after injection of vesicles made from polyethylene oxide-*b*-polystyrene (PEO-*b*-PS) tethered to gold-nanoparticles and encapsulated photosensitizer Ce6 in MDA-MB-435 tumour-bearing mice. (b) Thermal images after injection of same vesicles and exposing to 671 nm laser irradiation (red circle shows tumour region). (c) Tumour heating upon laser irradiation over time using various vesicles and controls. (d,e) Photoacoustic (PA) images and intensity at vesicle injection site (yellow circle). Modified with permission from ref. [126]. Copyright 2013 American Chemical Society.

endocytosed targeted polymersomes, released the apoptotic cargo, and induced cell death; this system forms the basis for possible future treatment of lung cancer, one of the most lethal malignancies.[260] Polymersomes might also be a valuable carrier alternative for targeting glioma.[268] The efficiency of cell surface targeting is not only dependent on the ligands used but also on the polymersome membrane composition. Mixing phospholipids HSPC together with PEO-*b*-PBD polymersomes resulted in the formation of hybrid vesicles with an intermediate elastic modulus, which led to significant improvement in their uptake by targeting the folate receptor overexpressed in tumour cells and by targeting the HER2/neu receptor.[50]

4.1.5 Other diseases

In addition to cancer therapy there are other possible biomedical applications using triggered polymersomes for diagnostics and therapy. pH-sensitive polymersomes have been tested for the detection of pathogenic bacteria,[269] and for possible intracellular antibiotic therapy.[93, 270] Hyaluronic acid-*block*-poly(ε-caprolactone) copolymers are prone to enzymatic degradation by bacteria and can be used for their detection. For example, upon enzymatic cleavage by hyaluronidase, which is common in *Staphylococcus aureus*, a reporter compound is released for detection.[269] Polymersomes based on peptide functionalized chitosan were able to encapsulate and release DOX upon proteolytic degradation and act antibacterial at the same time.[140] Thus this system might be applied in the future to deliver drugs and simultaneously protect from bacterial infections. An additional example in the combat of bacterial infection has been proposed for immobilized nanoreactors on implants. They provide the required antibiotic "on site", and only the precursor needs to be administered, thus minimizing side effects.[72] It was also shown that the number of intracellular *Porphyromonas gingivalis*, which infect oral epithelial cells, was reduced by intracellular delivery of metronidazole or doxycycline using acid-sensitive polymersomes,[270] and *Burkholderia pseudomallei*-infected murine macrophages were successfully treated using another type of pH-sensitive polymersomes (Ceftazidim-loaded) that disassembled in endosomes for efficient intracellular drug release.[93] More complex pathogens, such as malaria parasites *Plasmodium falciparum*, have been targeted using host cell mimicking polymersomes (nanomimics).[65] These nanomimics efficiently interrupted the reproductive cycle of the malaria parasites in human red blood cells by binding to the parasite surface after their egress from red blood cells (RBCs) and then blocking their subsequent invasion processes.

In another example, polymersomes functionalized with ganglioside GM1 targeting peptide and prion-targeting peptides efficiently crossed the blood-brain-barrier for treating Alzheimer's or Parkinson's diseases.[271, 272] A low molecular mass peptide was shown to be able to couple to the cell gangliosides GM1 and GT1b and mediated the transport of the nanocarriers *in vitro* and in mice.[272]

Oxidation-sensitive polymersomes have been introduced as a valuable vaccine delivery platform because antigen-cross presenting dendritic cells contain oxidative endosomes.[273] In addition, the advantageous architecture of polymersomes allows simultaneous encapsulation of hydrophobic and hydrophilic antigens and adjuvants, and therefore serves as an improved delivery system.[101] First, it was demonstrated that dendritic cells engulfed loaded oxidation-responsive polymersomes via endosomes, where they resided for more than 12 h, and then in a second step the payload escaped to the cytoplasm (**Fig. 16**). Endosomal escape of antigen is desirable for entering the 'cytosolic pathway' of antigen cross-presentation via MHC 1, which might be advantageous for adjuvant-induced activation and antigen presentation. Furthermore, enhancement of T cell priming was found when dendritic cells were tested for processing, and cross-presentation of a model antigen on MHC 1 when these polymersomes were used for antigen delivery compared to delivering free antigen to dendritic cells.[101] By combining oxidation-sensitive polymersomes with a photosensitizer (ethyl eosin), antigen release from the carrier and subsequent endosomal escape was dramatically speeded up upon light irradiation.[83]

By using ligands to specifically target diseased cells it has been demonstrated that diseases themselves can be targeted. For example, Glucose-responsive nanovesicles, which deliver insulin upon a pH change, have been reported. GOx was encapsulated in pH-sensitive diblock copolymer PEG-poly(Ser-ketal) vesicles, which can be hydrolysed to PEG-polyserine under acidic conditions. Upon entry of glucose in the compartment, GOx converses it to gluconic acid, which in turn lowers the internal pH, and leads to dissociation of the vesicles. The controlled release of insulin maintained the blood glucose level in diabetic mice at normoglycemic levels for up to 5 days, compared to only 1 day when insulin-only loaded vesicles were used.[274] Beside insulin being a possible target, this approach opens the way for targeting any disease in which glucose is an indicator. Recently, vancomycin was used as a model drug to be released from P(Asp-*co*-AspGA)/P(Asp-*co*-AspPBA) in the presence of glucose.[138]

Even though triggered delivery and release systems are still in an early stage of development, they hold great promise for future research and application.[275] Furthermore these systems can also be employed for live cell-imaging, visualizing uptake, and cargo distribution upon release.

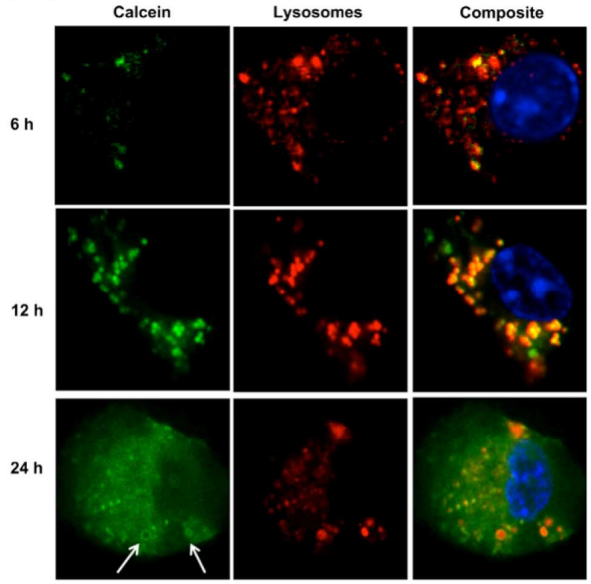

Fig. 16: Delivery of fluorescent model molecules to dendritic cells using oxidation-sensitive polymersomes based on poly(ethylene glycol)-*block*-poly(propylene sulfide) (PEG-*b*-PPS) as measured by confocal microscopy. Calcein (green) was delivered to endosomes and cytosol; release was not restricted to low pH compartments (lysosomes stained in red). Reproduced with permission from ref. [101]. Copyright 2012 Elsevier.

4.1.6 Reaction compartments

Cellular processes make great use of spatial separation for control of biochemical reactions. In a biomimetic approach various nanoreactors have been produced with biomolecules encapsulated/entrapped inside (**Fig. 17**).[5, 6, 31, 135, 154, 170, 276] PNVP-*b*-PDMS-*b*-PNVP triblock copolymers were used to encapsulate laccase from *Trametes versicolor*. The substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was co-encapsulated and served to allow *in situ* reaction. This process produced ROS, which diffused through the membrane, and oxidized ABTS in the vicinity of the polymersomes.[69] In order to detoxify the well-known ROS, peroxynitrites, and simultaneously function for oxygen storage, a nanoreactor has been designed based on encapsulation of hemoglobin (Hb).[160] In another example of a nanoreactor, an artificial metalloenzyme located in the inner cavity of polymersomes, permeabilised by the reconstitution of OmpF, was able to fulfil *in situ* its bioactivity.[158] The enzyme β-gal preserved its activity inside PICsomes, which allowed the diffusion of the substrates and products through their membrane.[142, 152]

Polymersomes have been used to build cascade reaction systems. For example, PS-*b*-PIAT compartments allow diffusion of small molecules, and have been used to encapsulate two enzymes, GOx and HRP.[68] The peroxide generated from GOx was then utilized by HRP for conversion of ABTS. A three-enzyme cascade reaction is also possible, by using the combination of CalB, GOx and HRP located in different regions of the polymersome.[162] Although these examples are still model systems, they demonstrate the feasibility of creating nanoreactors and the ability to conduct cascade reactions. Furthermore, recent examples have demonstrated the use of this concept for distinct applications.

ROS are generated in cells as a result of stress and lead to cell death if they reach critical concentrations.[5, 16] In order to protect cells from ROS, two enzymes SOD and LPO/catalase were encapsulated in a PMOXA-*b*-PDMS-*b*-PMOXA polymersome, with membranes permeabilised by the reconstitution of OmpF to mimic a natural peroxisome; the two enzymes acted in tandem to detoxify superoxide radicals and related H_2O_2.[16, 260] A completely different type of nanoreactor was used to generate ROS "on demand" for use in photodynamic therapy. The photosensitizer Rose Bengal-bovine serum albumin (RB-BSA) encapsulated inside polymersomes with oxygen permeable membranes produced ROS in a light-responsive manner. The nanoreactor acted like a Trojan horse as it was taken up by HeLa cells with no cell toxicity on its own. However, upon irradiation at a wavelength of 543 nm, it produced ROS, which then led to cell death.[5]

Nanoreactors have also been used for local antibiotic production to combat bacterial infections in implants. The encapsulated enzyme, penicillin acylase, was able to produce antibiotics under physiological conditions and to inhibit bacterial growth for up to 7 days (**Fig. 18**).[17, 72] However, the design of nanoreactors has been limited by the availability and compatibility of building blocks, especially regarding membrane proteins; mainly OmpF has been used, and this permits passive transport of molecules.[5, 6, 16, 31, 158, 160, 170, 277] Further studies on the development of membrane protein reconstitution might produce a higher specificity in terms of substrate/product selectivity, and the use of active transporters.

The development of biomimetic membranes with incorporated aquaporin appears to have reached a sufficient quality level for application.[27, 51, 71, 226, 278] Aquaporins are alpha-helical transmembrane pore proteins which allow the selective diffusion of water molecules through the membrane. These functionalized membranes have potential applications in water desalination, and comparative measurements have shown, that this type of membrane can outperform classical reverse osmosis (RO) membranes by a factor of approximately 750 times.[27] Recently, other members of the aquaporin family have been successfully reconstituted in membranes[278] and a framework has been proposed for quality assurance of reported methods and results.[51] Table 3 summarizes the reported reaction compartments and their potential application.

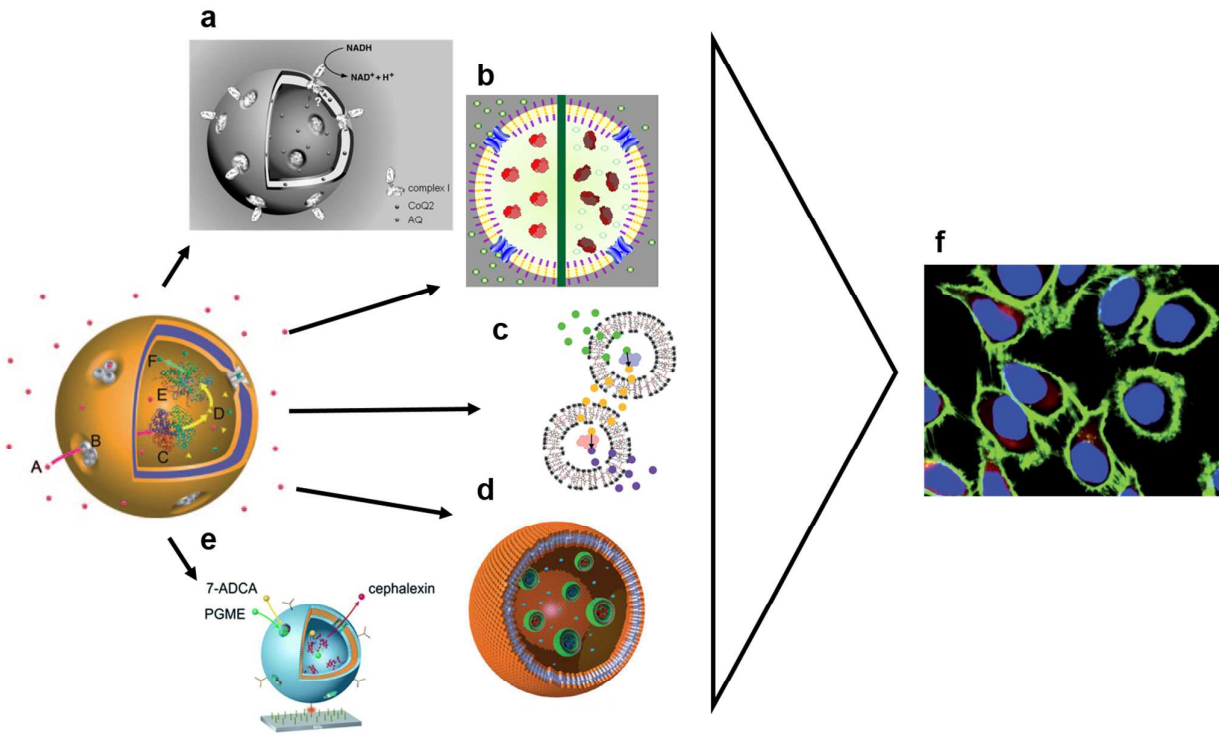

Fig. 17: Potential applications of nanoreactors that have been designed. Reproduced with permission from ref. [16]. Copyright 2011 Wiley. (a) Energy generation by electron gradient formation. Reproduced with permission from ref. [28]. Copyright 2010 Wiley. (b) ROS elimination and oxygen storage of Hb-containing nanoreactors. Reproduced with permission from ref. [160]. Copyright 2012 American Chemical Society (c) Reproduced with permission from ref. [147]. Copyright 2012 The Royal Society of Chemistry and (d) Cascade and multicompartment cascade reactions based on semipermeable polymers. Reproduced with permission from ref. [2]. Copyright 2014 Wiley (e) On-site production by surface immobilized nanoreactors. Reprinted with permission from ref. [17]. Copyright 2012 The Royal Society of Chemistry (f) Cell-uptake of light triggerable, ROS producing nanoreactors. Reprinted with permission from ref. [5]. Copyright 2012 The Royal Society of Chemistry

4.1.7 Synthetic biology & multicompartment systems

The bottom-up approach in the field of synthetic biology aims to recreate cellular processes, starting from simplified compartmentalization and ultimately leading to an artificial cell in the future.[45, 279] Within this perspective, cascade reactions in separate reaction compartments can be seen as a first step in mimicking cellular processes. The definition of a living entity contains not only reproduction but also the capability to form and maintain its own metabolism. On a cellular level this requires the formation of electrochemical gradients, which are used to power various processes. Among the first examples is the creation of an artificial organelle, which uses BR to form a proton gradient that is utilized by a co-reconstituted ATP-synthase. The resulting polymersomes were able to mimic one of the fundamental energy generating processes and provide ATP upon continued illumination.[135, 154] The bacterial respiratory enzyme complex NADH:ubiquinone

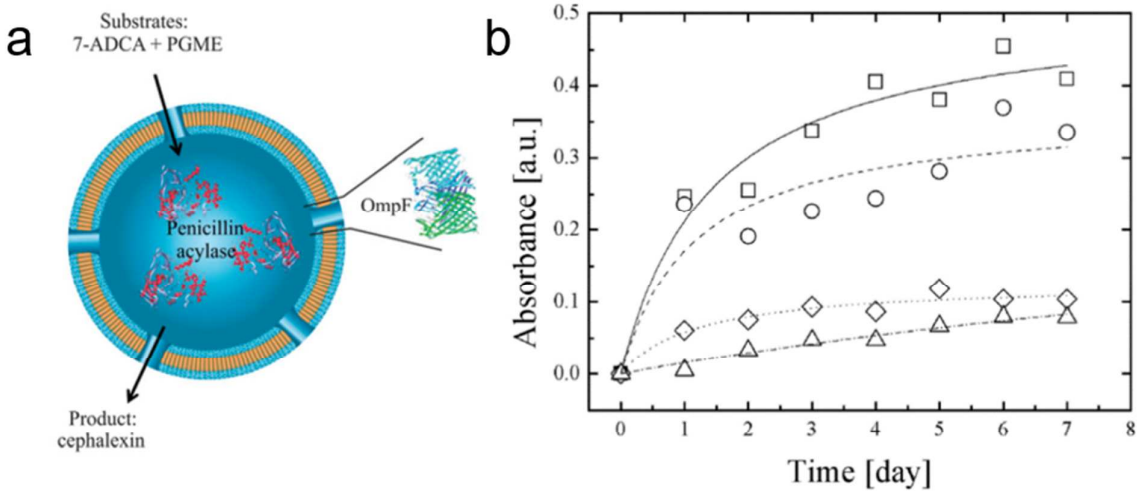

Fig. 18: Penicillin acylase nanoreactor, which catalyzes the conversion of its substrates into cephalexin (left). The reaction curves (right) show the catalytic activity of the encapsulated penicillin acylase over 1 week. Reproduced with permission from ref. [72]. Copyright 2013 The Royal Society of Chemistry.

oxidoreductase (complex I) translocates protons by a series of redox reactions from NADH to ubiquinone, and thus helps to generate and maintain the proton motive force. This principle was re-created in PMOXA-*b*-PDMS-*b*-PMOXA polymersomes, having complex I reconstituted in their membrane;[28] the protein maintained its activity in the synthetic environment.

4.1.7.1 Giant unilamellar vesicles used as cell models

Normally polymersomes for medical applications have sizes in the nanometer range and thus the visualization of these systems is barely manageable. So far, only electron microscopy can visualize these structures at a reasonable resolution, but it does not allow live-imaging of processes taking place. GUVs composed of either lipids or block polymers are advantageous for investigation via optical microscopy due to their size in the range of 10 – 50 µm.[2, 59, 279-283] By labelling of the polymers or lipids with fluorescent dyes, the formation of hybrid membranes could be observed which showed depending on the building blocks and their composition homogenous distribution of the lipids and polymers or domain formation.[284] Their cell-like size allows the investigation of their physical membrane properties via techniques like micropipette aspiration. [59, 280] Moreover this allows the detection of inserted pores[32, 281] and incorporation of lipids in the membrane by measuring the change of membrane elasticity.[50, 281]

A fundamental process in cellular activity and reproduction is the expression of proteins. GUVs can be used to encapsulate the expression machinery required to produce the protein MreB, a bacterial actin-like protein that is part of the cytoskeleton which defines the shape of a microorganism.[282] Successful expression of the fluorescent fusion protein MreB-RFP was visualized by confocal microscopy. Polymer stromatocytes can be loaded with platinum nanoparticles which function as a catalytic nanomotor, and the catalytic decomposition of hydrogen peroxide enables directed movement of the stromatocytes.[285] Furthermore, it has been demonstrated that polymersomes can possess an uptake mechanism that is similar to cell membranes.[70]

The cascade reactions described in the previous section show the concept of compartmentalized reactions, which are used in nature. An elegant approach has now been used to create multicompartments that are required for more complex systems.[265, 283] By encapsulating PS-*b*-PIAT nanoreactors in PB-*b*-PEO polymersomes a fully active multicompartment system has been introduced;[2] further details of multicompartment systems can be found in another review.[45] However, all of the above mentioned examples are still model systems and are extremely simplified compared to nature. They only recreate certain functionalities, such as gradient generation,[28, 135, 154] or protein synthesis in a confined environment[282] for the study of the underlying mechanisms. However, the goal of making these applicable to specific requirements has still not been achieved; nor has it yet been possible to recreate the high complexity required to mimic a cell.

Journal Name

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Table 3: Biomimetic reaction compartments and their potential applications.

Polymer	Enzyme	Transport	Context	Reference
Homo-P(Asp-C8)/PEG−PAsp	β-gal	Semipermeable membrane	Model, proof of principle	152
PEG-*b*-PDEAEM/PDMIBM	GOx, Myo	Triggerable, semipermeable membrane	Multicompartment cascade reaction model	147
PEtOz-*b*-PDMS-*b*-PEtOz	BR, F_0F_1-ATP synthase	-	Artificial organelle, ATP generation	154
PMOXA-*b*-PDMS-*b*-PMOXA	SOD, LPO	OmpF	Peroxisome, Artificial organelle	16,277
PMOXA-*b*-PDMS-*b*-PMOXA	Artificial Transfer Hydrogenase	OmpF	Artificial organelle	158
PMOXA-*b*-PDMS-*b*-PMOXA	Hb	OmpF	Dual functionality, Peroxinitrites elimination and oxygen storage	160
PMOXA-*b*-PDMS-*b*-PMOXA	RB-BSA	OmpF	“Trojan horse”, triggered cell death	5
PMOXA-*b*-PDMS-*b*-PMOXA	Penicillin acylase	OmpF	On-site cephalexin production	17, 72
PMOXA-*b*-PDMS-*b*-PMOXA	-	Aquaporin	Water desalination	27
PMOXA-*b*-PDMS-*b*-PMOXA	Complex I	-	Electron gradient formation	28
PNVP-*b*-PDMS-*b*-PNVP	Laccase	Semipermeable membrane	Biosensing, Oxidation	69
PS-*b*-PIAT	GOx and HRP	Semipermeable membrane	Cascade reaction model	68
PS-*b*-PIAT	CalB, GOx, HRP	Semipermeable membrane	Cascade reaction model	162
PS-*b*-PIAT, PB-*b*-PEO	CalB, ADH, PAMO	Semipermeable membrane	Multicompartment cascade reaction model	2

4.2 Planar membranes

4.2.1 Biosensors

Biosensors, which are able to detect small changes in physical properties or the presence of biological molecules with high precision, represent an important tool for detecting pathological conditions. Polymer membrane based biosensors have been applied both for detection in solutions and at surfaces, in biochemical arrays, and in the form of immobilized nanoreactors on prefabricated active surfaces. For example, micelles of poly(*n*-butylmethacrylate)-*block*-poly(*N*,*N*-dimethylaminoethyl methacrylate) (P*n*BMA-*b*-PDMAEMA), and choline oxidase were used to fabricate bilayer films on conductive surfaces at different pH-values. Sequential electrostatic adsorption of diblock copolymer micelles combined with the additional possibility of crosslinking enzymes within such films lead to well-defined, highly active, and stable biosensor coatings.[286] The assets of biosensors based on polymer–enzyme hybrids are high sensitivity, specificity, speed and accuracy; the exquisite selectivity and unique transport properties of membrane proteins can be harnessed for a variety of engineering and biomedical applications, and the modification of membranes with specific recognition sites[247-249] represents an elegant way to improving their interactions with specific molecules (proteins, enzymes, DNA).[6, 38]

In general, self-assembled polymer layers with an immobilized enzyme placed on an electrode is an established approach for fabricating biocompatible, sensitive, selective and stable implantable biosensors in medical diagnostics.[287] In this case, the enzyme catalyses the reaction of a biological compound to a specific side product, *e.g.* hydrogen peroxide is oxidized at the Pt surface to release an electron, which is detected amperometrically.[288]

Among the enzymatic biosensors, glucose sensors are of considerable interests because of the growing need for diagnostic analysis of diabetes.[185] Simple fabrication,

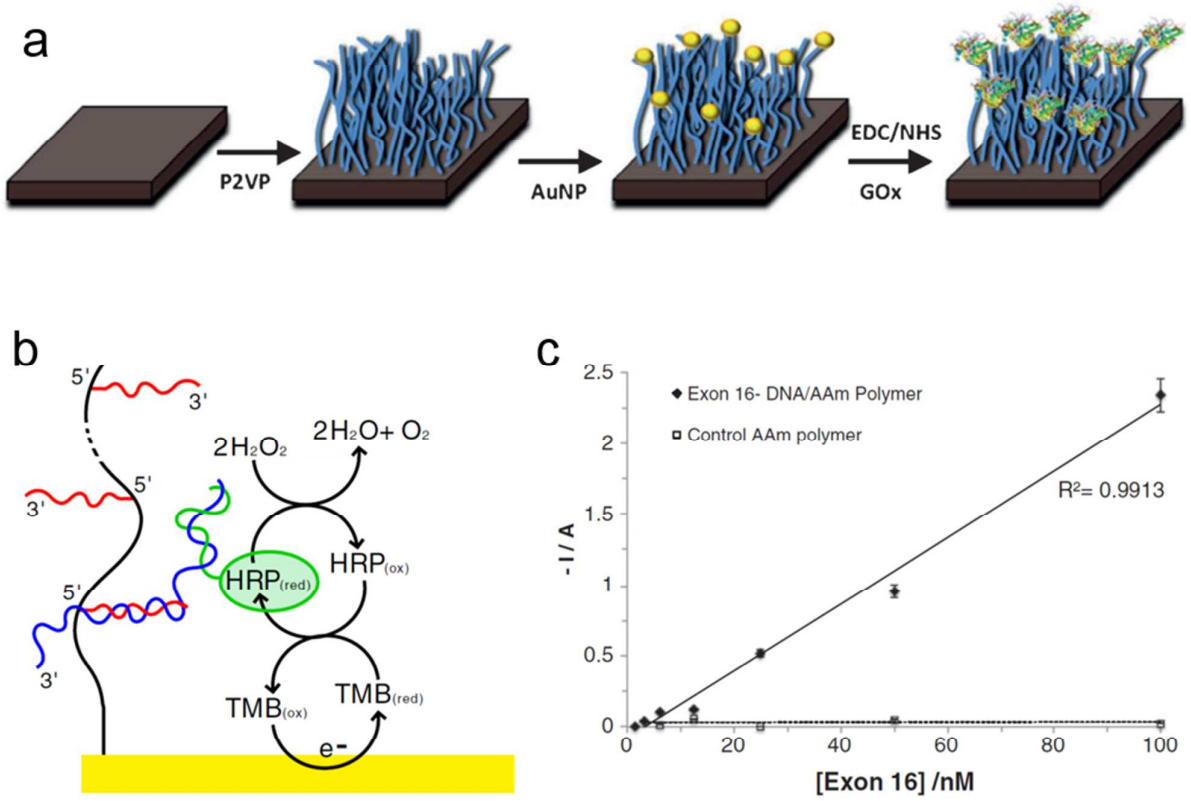

Fig. 19: (a) Procedure for the fabrication of a biosensor. Reproduced with permission from ref. [211]. Copyright 2014 Wiley. (b) Schematic of the acrylamide-co-Exon16 brush and principle of the electrochemical assay. (c) Calibration curve for the detection of Exon16. Reproduced with permission from ref. [290]. Copyright 2011, Elsevier.

biocompatibility, flexibility, low operational voltage, and the ability to function in aqueous environments make organic electrochemical transistors (OECTs) ideal to interface with biological media.[210] Generally, amperometric glucose biosensors are based on enzymatic oxidation mediated by GOx, a well-known biological sensing material for the quantitative determination of β-d-glucose in solution (**Fig. 19**). Utilizing biologically suitable polymer brushes in devices enables the covalent attachment of GOx to provide high sensitivity and stability of glucose sensing. The different block polymers provide additional features for biosensor surfaces. For example, a mixed brush comprising PGMA and PHEMA, has been shown to prevent non-specific adsorption, and thus confirm that this substrate selection is optimal for GOx attachment. In another amperometric glucose biosensor, an electrode with PFMMA as the inner (first) block was found to be more sensitive than one with PGMA as the inner block in surface-grafted copolymer brushes. This is probably associated with the fact that the electron-transfer mediating PFMMA block is attached closely to the ITO electrode, and facilitates electron transfer between the GOx redox sites and the ITO electrode surface. In addition, covalent bonding results in a remarkable stability over an extended time period: devices retained 100% of their response for 100 days, with only a small increase in standard deviation, from 14% on day 2 to 25% at 100 days.[210] In another glucose oxidase based biosensor it was possible to change the kinetic parameters of GOx operating in "on" and "off" states of the polymer brushes.[211] This biodevice can be used not only as a pH-controllable electrochemical biosensor to detect substrates, but also for further control or to modulate the electrochemical responses.

Uric acid measurement is important for the routine diagnosis and treatment of hyperuricemia and gout. Amperometric uric acid biosensors have been developed by immobilizing the uricase enzyme into the membrane of conductive polymer and the membrane of polyelectrolyte such as in combination between polyaniline (PANI) and poly (allylamine) (PAA). Compared to other measurement methods, these provide many advantages such as biocompatibility, selectivity and sensitivity.[289]

Sensitive, rapid and quantitative DNA testing is required in biological technologies and biomedicine. Poly(acrylamide-*b*-DNA) combed brushes on a gold electrodes was prepared *via* surface initiated atom transfer radical polymerization (SI-ATRP) in the presence of acrylamide modified DNA probes. Such monolayers of three-dimensional DNA polymer brushes were shown to be capable of binding their complementary 105-base DNA amplicon, which can be used for electrochemical detection of the breast cancer related marker Exon16.[290] This electrochemical genosensor exhibited an excellent sensitivity of 23.5 nA nM^{-1} and a limit of detection of 2.67 nM. Furthermore, the polymer brush prevented any non-specific binding of the enzyme labelled reporter probe and no cross-reactivity was observed with a non-related DNA sequence (Lymphotoxin α).

4.2.2 Biocomputing

Biocomputing/enzyme logic elements are able to effectively interface complex physiological processes and implantable biomedical devices to provide autonomous, individual, "upon demand" medical care, which is the objective of the new nanomedicine concept. In the chemical computing research area, novel horizons have been opened up by the introduction of biochemical systems and the formulation of biomolecular computing (biocomputing) concepts. Enzyme-based (*e.g.* GOx, esterase) logic gates are able to process biochemical input signals upon performing various Boolean operations (AND, OR, XOR, INHIB, *etc.*) and to generate a single output signal as a result of the biocomputing process.[291] As an example, a poly(4-vinylpyridine) (P4VP) brush modified electrode for electrocatalytic oxidation of NADH was developed using a pH-switchable redox-active group bound to the polymer (4,4'-dimethoxy-2,2'-bipyridine). Coupling between the enzyme logic systems and the bioelectrocatalytic interface was achieved by pH changes produced *in situ* by the enzyme reactions, resulting in different protonation states of the polymeric matrix associated with the electrode surface. Coupling of enzyme-based logic gates biocomputing systems with signal-responsive biocatalytic interfaces will allow the production of "smart" bioelectrochemical systems.

Self-immolative polymersomes with light or reduction triggers have also been tested for possible biocomputing (OR-, AND-, and XOR-type logic) applications by programmed enzymatic reactions using different mixtures of light- and reductive environment responsive polymersomes filled with various enzymes/substrates/inhibitors.[86]

4.2.3 Other biotechnical applications

Reconstitution of membrane proteins into PEG (with fatty acid moieties) brush supported lipid membranes enables the probing of isolated membrane protein diffusion and interactions.[292] On such a versatile analytical membrane platform, co-locomotion of individual ligand-receptor complexes has been detected, thus demonstrating its applicability for functional analysis of single biomolecules *in vitro*.

Solid-state NMR is a widely used method for determining the orientation and conformation of peptides embedded in oriented lipid bilayer membrane. Ultrathin polymer films made of Halar or polycarbonate represent an alternative to the widely used glass coverslips for preparing oriented membranes with large areas,[293] and provide a novel, efficient way for preparing protein–membrane samples for solid-state NMR.

Finally, the newly introduced concept of membrane channel proteins functionally inserted in polymer membranes can be expected to serve in the future as a promising tool for other biological studies and fine applications such as single molecule techniques, drug screening, trace analysis, *etc.*

5. Conclusions and perspectives

In a bioinspired strategy to design functional hybrid materials/systems, supramolecular assemblies based on amphiphilic copolymers represent stable and robust matrices with properties that can be adjusted for combination with biomolecules. These synthetic assemblies are expected to be able to cope better than lipid-based assemblies (liposomes, lipid membranes) with the complex scenario of requirements in bio- and medical applications, especially in terms of mechanical stability, chemical functionalization and modulation of permeability/accessibility. In particular, the architecture of polymersomes and planar membranes offers multiple choices for combination with biomolecules by encapsulation/insertion/attachment that are favoured by intrinsic membrane properties, such as thickness, fluidity, size, charge, stimuli-responsiveness, *etc.* The synthetic routes and specific conditions for the self-assembly process allow manipulation of the polymersome (size, shape, stability, responsiveness), and modulation of the membrane properties (thickness, fluidity, permeability). Molecular properties of such bioinspired synthetic membranes must offer a stable environment for the biomolecules, whilst allowing them to remain functional. In this respect, nanoreactors and artificial organelles represent an advance on conventional drug delivery systems because they produce specific activity without releasing the biomolecules, and thus overcoming the problem of uncontrolled delivery. The first examples of artificial organelles indicate that it is possible to have functional nanoreactors inside cells, acting as cellular implants, although their long-term activity and has not yet been studied. However, this research on functional nanosystems (nanoreactors, artificial organelles, multicompartments as cell mimics) is only at an early stage, and no *in vivo* tests have been performed. In addition, only a few studies have focused on improving such systems by varying molecular parameters, which prevents the production of a general overview of the properties of synthetic membranes for accommodating biomolecules, and explains why they have been mainly proposed as models and not as further therapeutic/diagnostic candidates. The lack of comparisons between different types of supramolecular assemblies in terms of functionality or stimuli-responsiveness makes it difficult to understand which of these systems are more appropriate for specific applications. In the case of reconstitution of various membrane proteins in planar membranes, the main critical point is the scaling up of their effective areas, while preserving their properties, in order to go toward applications. In addition, molecular parameters, such as thickness, fluidity and interaction with biomolecules have still to be varied in a systematic manner for understanding their combined effects on the properties of the resulting hybrid membranes.

It is expected that introducing multifunctionality in one single supramolecular assembly by an elegant choice of synthetic assembly properties and biomolecules will produce theranostic systems, or support targeting approaches, that are necessary for a patient-oriented medical strategy. In addition, such bio-nanodevices and -membranes will produce a better understanding of various processes in cells and the interactions with living cells that are absolutely necessary for the development of nanoscience-based solutions with desired space and time precision of the response. Even though

significant progress has been made in producing new hybrid materials, the interactions between biological molecules and self-assembling polymers represent an emerging field of science, and development is still needed to obtain greater insight into their behaviour, and thereby to increase the scope of their applications in medicine, environment protection or technology.

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