POLYMER CAPSULES AS MICRO-/NANOREACTORS FOR THERAPEUTIC APPLICATIONS: CURRENT STRATEGIES TO CONTROL MEMBRANE PERMEABILITY

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Abstract

Polymer capsules, fabricated either with the aid of a sacrificial template or via the self-assembly of block copolymers into polymer vesicles (polymersomes), have attracted a great deal of attention for their potential use as micro-/nanoreactors and artificial organelles for therapeutic applications. Compared to other biomedical applications of polymer capsules, such as drug delivery vehicles, where the polymer shell undergoes irreversible disruption/rupture that allows the release of the payload, the polymer shell in polymer micro-/nanoreactors has to maintain mechanical integrity while allowing the selective diffusion of reagents/reaction products. In the present review, strategies that permit precise control of the permeability of the polymer shell while preserving its architecture are documented and critiqued. Together with these strategies, specific examples where these polymer capsules have been employed as micro-/nanoreactors as well as approaches to scale-up and optimize these systems along with future perspectives for therapeutic applications in several degenerative diseases are elucidated.

Keywords: polymer capsules, permeability, therapeutic applications, microreactors, nanoreactors

1. Introduction

The reorganization of matter from single disoriented molecules to highly organized structures is the process that drives the life in us and around us. In general, self-assembly and the compartmentalization strategies are important aspects of the cellular and subcellular environment. They serve as a source of inspiration, from which similar concepts/processes can be translated into artificial systems, thus using a biomimetic approach to develop new tools for therapeutic applications [1]. Such artificial systems, like layer-by-layer (LbL) capsules, polymersomes, PICsomes (i.e., described in [2] as those polymersomes prepared with a pair of oppositely charged block copolymers that form a polyion complex (PIC) membrane), dendrimers/hyperbranched polymers, or spherical micelles have been intensively investigated as potential candidates for providing defined synthetic reaction spaces [3, 4]. Among these, layer-by-layer (LbL) capsules, polymersomes, and PICsomes deserve special interest. LbL capsules are based on a sacrificial template to form the hollow structure. They employ either the coating/grafting of a single polymer or LbL coating of multiple polymers on a sacrificial template, allowing the control of size, surface charge, morphology, etc. of the resulting capsule [5-7]. These systems have been widely employed as drug/protein/gene delivery vehicles, but their use as micro-/nanoreactors for therapeutic applications has been limited, mainly because their polymeric shell generally undergoes irreversible dissociation/rupture during their application. Meanwhile, polymer vesicles are the result of block-copolymer self-assembly into aqueous solutions that offer ease of preparation and controllable sizes [8-10]. Their inner cavity accommodates active biological macromolecules (e.g., enzymes, enzyme mimics, oligonucleotides) inside and

their membranes are mechanically stable, with easily adjustable properties (thickness, flexibility, stimuli-responsiveness) and permeability [11].

Strategies that permit the selective diffusion of reagents and reaction products across the membrane while maintaining its mechanical integrity and the protective role of their active payloads are of major importance for the successful design of polymer capsules to act as micro-/nanoreactors. Their design simulates the functionality of biological systems and enables the understanding of bio-chemical metabolic compartmentalized reactions, which have already opened numerous pathways for the development of stimuli-responsive nanocarriers [12], smart nanofactories [11], artificial organelles [13], synthetic cells [14], and other promising *in vitro* and *in vivo* applications [15].

The present review considers current strategies to control the permeability of the polymer capsules shell/membrane, as a crucial requirement for their development as micro-/nanoreactors or artificial organelles for therapeutic applications. Together with the adopted strategies to control shell or membrane permeability, specific examples where polymer compartments have been employed as micro-/nanoreactors for therapeutic applications are provided. Specific examples for scaling-up and optimizing the micro-/nanoreactors such as microfluidics are also briefly introduced.

2. Polymer capsules prepared employing a sacrificial template

Fabrication of polymer capsules employing a sacrificial template has attracted a great deal of attention for therapeutic applications because of the possibility of controlling the structural characteristics of the polymeric shell such as shell thickness, surface charge, mechanical strength, size and permeability [16, 17]. Moreover, the polymer shell can be

decorated with membrane lipids to increase the circulation time of the capsules *in vivo* and with recognition molecules such as antibodies or membrane-bound proteins, to confer additional functionalities to the polymer capsules that hold particular interest for several biomedical applications (e.g., biomarkers, cell isolation, targeted drug delivery, etc.) [18, 19]. In most of the cases these capsules are fabricated using the layer-by-layer (LbL) technique [20, 21], which involves the deposition of polymer layers through electrostatic [22, 23] or covalent interactions [24], hydrogen bonding [25-29] or single polymer assembly [30] on a sacrificial core followed by its dissolution and subsequent purification (Figure 1).

Polymer capsules have potential utility in controlled release of various biomolecules such as nucleic acids [31-37], proteins/peptides [38, 39] and drugs [40] both intra- or extracellularly for therapeutic applications. For their use as intracellular delivery vehicles, polymer capsules first have to be uptaken by cells, a process that is influenced by their size, shape, surface chemistry and mechanical stiffness, as well as by the cell line [41-46]. However, their use as micro-/nanoreactors for therapeutic applications has been limited mainly because their polymeric shell generally undergoes irreversible dissociation/rupture upon a stimulus (e.g., pH, temperature, glucose, etc.) and consequently only a few examples are reported in the literature [47] (Table 1).

Herein, strategies that permit the control of polymeric shell permeability while maintaining the structural integrity of the polymer capsule are comprehensively analyzed and schematically represented in Figure 2.

2.1. Commonly employed sacrificial templates

The sacrificial template plays a pivotal role in the fabrication of polymer capsules since it determines the size [48] and shape [49, 50] of the resulting polymer capsules and also the encapsulation method that can be used for the active component (that is, pre- vs. post-loading of the active component) [51] (Table 2). Many of the templates considered in the literature do not allow for the preloading of the active component, which severely hampers the application of the resulting capsules as micro-/nanoreactors for therapeutic applications due to the low encapsulation efficiencies of the active component and the limited reproducibility associated with the diffusion process involved in the post-loading method. Extreme changes in solvent composition [52], salt concentration, pH [22] and/or temperature [53] have accordingly been employed to increase the permeability of the polymer capsules. However, these conditions are not suitable for loading sensitive biomolecules such as enzymes. Among those templates that do not allow the preloading of the active component, melamine formaldehyde (MF), poly(methyl methacrylate) (PMMA) and polystyrene (PS) colloidal particles have been widely employed. All of these templates are commercially available with a wide range of sizes (500 nm-12 μm for MF; 100 nm-300 μm for PS; 100 nm-200 μm for PMMA) and low polydispersity values, which permits the fabrication of monodisperse polymer capsules of different sizes.

The MF cores are typically dissolved in HCl solutions at pH values below 1.6, or removed in N,N-dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) solutions. During MF core removal, the capsule swells because of the osmotic pressure difference created by the decomposition of the core which results in its decomposition if the process is not adequately controlled (gradual adjustment of the pH is recommended to prevent the

disruption of the capsules) [54]. Moreover, some degradation byproducts of the MF core remain irreversibly adsorbed on the internal wall of the capsule, making the complete removal of the sacrificial template almost impossible [55]. Despite this, several biomolecules (including peroxidase [56], α-chymotrypsin [57], and horseradish peroxidase [55], have been satisfactorily encapsulated in polymer capsules fabricated from MF sacrificial cores, with relatively high encapsulation efficiencies while retaining the activity of the encapsulated entities. Thanks to a well-established synthetic process, the PS and PMMA cores can be fabricated in a wide range of sizes and surface functionalities (mainly carboxyl, hydroxyl, sulfate or amine groups), facilitating the subsequent deposition of polymer layers [31, 32, 34-36, 38, 39]. However, the need for organic solvents [including tetrahydrofuran (THF), chloroform and dichloromethane (DCM)] to remove the core represents a significant drawback for the use of the resulting polymer capsules for therapeutic applications which affects the stability and/or activity of the encapsulated entity.

To overcome the drawbacks associated with the post-loading process described above, the use of porous inorganic templates including mesoporous silica and calcium carbonate micro- and nanoparticles have been considered. Because of their porous structures, high amounts of the active components can be adsorbed in these templates. Additionally, they are easily dissolved to form low molecular weight ions, avoiding osmotic stress and ensuring the complete removal of the template. Mesoporous silica particles with controllable size, porosity and pore size/structure have been satisfactorily employed for the encapsulation of several enzymes (including catalase, peroxidase, cytochrome C, lysozyme) [58-60] and DNA [25]. Unlike mesoporous silica templates, which need hydrofluoric acid

to be completely removed, which may limit their use in biomedical applications, calcium carbonate templates are dissolved in very mild conditions (such as ethylenediaminetetraacetic acid (EDTA) solutions). The main challenge associated with calcium carbonate templates is, however, the poor control over the size of the synthesized particles and the difficulty of obtaining nano sized particles. A potential solution to this might be achieved by passing the different sized capsules through an extruder with a controlled pore size, but this leads to the loss of efficiency in the number of generated capsules. Changes in process parameters (such as salt concentration, stirring speed and time) [61, 62], incorporation of polymers that inhibit or stabilize specific crystal structures [63, 64] (such as poly(vinylsulfonic acid)) and/or the use of alternative methods (such as atomization, high-gravity reactive precipitation) [65, 66] have been proposed to produce calcium carbonate particles with a controlled size and reduced polydispersity values. Significant efforts have been made to control the shape (spherical, elliptical and square). [50] and the pores [67] of the calcium carbonate templates.

Hydrogels, including calcium alginate and dextran, have been considered as potential sacrificial templates in which the active component can be preloaded. Hydrogel microspheres are commonly produced by an emulsion process, allowing the incorporation of several entities including enzymes [68] or nanoparticles [69] during the fabrication process. However, this process results in a wide polydispersity of the particles obtained due to poor control over the particle size and their distribution [70]. Moreover, the complete removal of the template is challenging. Removal is achieved by treatment with an EDTA solution in the case of calcium alginate hydrogels or by hydrolysis of the carbonate esters that connect the dextran chains in the dextran hydrogels. In fact, the negative charge

associated with the remaining alginate after the incomplete removal of the template has been used to drive the diffusion of positively charged molecules into multilayered capsules [71]. Moreover, a high swelling pressure that results in the rupture of the surrounding membrane is created during the removal of the template [70]. Even though this property has been exploited to create self-rupturing microcapsules suitable for pulsed drug delivery [72, 73], this is far from ideal when the capsules are intended to be used as microreactors. In addition to the commonly employed sacrificial templates for the fabrication of polymer capsules described herein, other alternative templates can be found in the literature. These include carbon-based templates, metal-based templates and natural materials. For further details about the synthesis and properties of these alternative templates, readers are referred to recently published review papers on the topic [74, 75]. The advantages and disadvantages of the commonly employed sacrificial template to fabricate polymer capsules is provided in Table 2.

2.2. Strategies to limit permeability of LbL capsules

The diffusion of entities across the membrane of polymer capsules is driven by the concentration difference between the capsule interior and the external medium following the Fickian diffusion release mechanism [76, 77], where the multilayer polymeric shell acts as a barrier. Accordingly, changing the intrinsic properties of the multilayer polymeric shell or increasing/decreasing its thickness represent the easiest strategies to tune the permeability of the membrane. Increasing the number of deposited polyelectrolytes results in an increase in the thickness of the polymeric shell [78-80]. Consequently, the diffusion of the entities along the membrane is limited. As an illustration, the thickness of chitosan/alginate (Chi/Alg) membrane increased linearly from 15 to 25 nm when the

number of layers was increased from three to seven. As a result, the release of a model drug (Rhodamine B) can be adjusted via the control of the number of layers: 27% of the encapsulated dye was released in one hour when the number of layers was three, whereas four hours was necessary to release the same amount from the capsules containing seven layers. Even though this strategy is time consuming, it avoids the use of alternative processes (e.g., chemical or UV-based crosslinking), which could have a detrimental effect on the activity of the encapsulated entity, to limit the permeability of the membrane. For the encapsulation of enzymes in $CaCO_3$ sacrificial templates and their corresponding use as microreactors, this strategy proved to be very efficient. It was reported, accordingly, that increasing the number of bilayers from one to three, significantly improved the retention of enzymes within the polymer capsules, particularly for high molecular weight enzymes (e.g., encapsulation efficiency for IgG was ~58% for one bilayer, whereas it was ~70% for three bilayers) [81].

Occasionally, the permeability of the membrane needs to be further decreased, limiting the diffusion of low molecular weight molecules. To achieve this, lipid layers have been adsorbed on multilayer polymeric shells [23]. This external layer turned the shell of poly(styrene sulphonate)/poly(allylamine hydrochloride) (PSS/PAH) capsules non-permeable to small (376 g mol^{-1}) water soluble molecules. In the case of polymer capsules acting as microreactors, the diffusion of encapsulated macromolecules across the polymeric shell needs to be totally restricted, allowing only the transport of reagents/reaction products through the membrane. In a reported study, catalase (CAT)-loaded microcapsules were fabricated LbL by the alternative assembly of PSS and protamine (Pro), employing $CaCO_3$ as a sacrificial template [82]. To limit the release of CAT from the polymer capsule, an

inorganic silica layer was constructed via a biomimetic mineralization process induced by the outer Pro layer. PSS/Pro capsules were suspended in sodium silicate (Na_2SiO_3) solution and, due to the positively charged Pro molecules on the external layer, negatively charged silicon precursors were adsorbed by electrostatic interaction. Then, Si-O-Si bonds were formed by condensation, finally resulting in the formation of a silica shell of low permeability. Leakage of CAT was reduced by three-four fold when an external silica layer was constructed on PSS/Pro capsules; in those capsules containing more than eight layers and with the external silica layer, leakage of CAT was as low as 4-5%. Importantly, the encapsulated enzyme was able to maintain its activity as demonstrated by the decomposition of hydrogen peroxide into water and oxygen.

Additionally, crosslinking of the polymeric shell has also been reported as a promising strategy to limit the permeability of the resulting polymer capsules [83]. For example, polymer capsules composed of hyaluronic acid (HA) and a polycationic polymer [PAH or poly(lysine) (PLL)] were deposited LbL on $CaCO_3$ sacrificial templates. Uncrosslinked polymer capsules were highly permeable to FITC-labelled dextran molecules as demonstrated by confocal microscopy. However, upon reaction of activated carboxylate groups of HA with amine groups of the polycation to form amide bonds by EDC and NHS crosslinking, the resulting capsules proved almost totally impermeable to FITC-labelled dextran of low molecular weight (4000 g mol^{-1}). Limiting the permeability of the polymeric shell while still allowing the diffusion of low-molecular weight molecules, offers the possibility of employing polymer capsules as micro-/nanoreactors for therapeutic applications. In this respect, glucose oxidase (GOx) was satisfactorily encapsulated in polymer capsules composed of PSS and diazoresin (DAR) [84]. DAR improves the stability

of the multilayer shell by converting weak interactions between neighboring layers into covalent bonds upon UV irradiation. Before crosslinking, GOx was able to diffuse into the polymer capsules. But upon UV irradiation and the corresponding formation of covalent bonds in the polymeric shell, permeability was reduced, resulting in the retention of encapsulated GOx. Interestingly, GOx was able to maintain high activity as demonstrated by reaction of the polymer capsules with glucose. It has to be highlighted, however, that some of these processes may have a detrimental effect on the activity of the encapsulated entities [68]. Therefore, a balance between the amount of encapsulated entity and the preservation of its activity should always be considered.

In addition to the LbL approach, coating of a single polymer on a sacrificial template has also been employed for the fabrication of polymer capsules with potential uses as microreactors for therapeutic applications. Promising results have been obtained in the particular case of polydopamine-based polymer capsules. Polydopamine is a dopamine derived synthetic eumelamin polymer that contains both catechol and amine functionalities in its backbone and has been satisfactorily employed to coat several substrates [85, 86]. By simply submerging in a slightly alkaline solution of dopamine hydrochloride, a stable layer of polydopamine was deposited on the surface of the $CaCO_3$ sacrificial template, which was previously loaded with enzymes [e.g., CAT [87], GOx [88], Candida Rugosa Lipase (CRL) [89]] via the coprecipitation method. After removing the $CaCO_3$ template with EDTA, polymer capsules loaded with the enzyme of interest were obtained. Interestingly, shielding the enzyme with a polydopamine layer resulted in the enhanced stability of the activity of the enzymes against storage time, thermal denaturation and acidic conditions.

2.3. Polymer capsules designed with stimulus driven permeability of the membrane

In the LbL approach, the use of polymer layers that respond to specific stimuli such as pH, temperature, light, electromagnetic field, etc. gives rise to polymer capsules with stimulus-driven permeability. Additionally, this responsiveness can be achieved by the fabrication of nanocomposite capsules, where nanoparticles are incorporated on the surface or within the polymer shell. In the present review, stimulus responsive polymer capsules have been categorized into two groups. In the first group, those capsules that respond to biologically relevant stimulus (e.g., pH, temperature, glucose, etc.) that cannot be remotely controlled are analyzed. In the second group, the control of polymer shell permeability via an external energy source (i.e., light, electric field, magnetic field, etc.) is considered. In both cases, particular attention is paid to those capsules that show reversible transitions and thus find potential application as micro-/nanoreactors.

2.3.1. Local stimulus-responsive polymer capsules

Fabrication of polymer capsules LbL employing one or two relatively weak polyelectrolytes gives rise to pH-driven permeability of the resulting capsules. For example, the pH responsiveness of the polyelectrolyte pair PSS and the relatively weak PAH has been widely studied in the literature [22, 90, 91]. In this system it is possible to tune the capsule walls between open and closed states, providing an efficient tool to control the uptake and release of different entities (drugs, biomacromolecules, etc.). As a proof of concept, the exclusion properties of polyelectrolyte capsules composed of eight PSS/PAH layers for FITC-labeled dextran of various molecular weights (75,000 or 2,000,000 g mol^{-1}) were studied [22]. At pH values above eight, high and low molecular weight dextran could not diffuse across the membrane. However, when the pH value was decreased to three, the

interior of the capsules became fluorescent upon exposure to FITC-labeled dextran of 75,000 g mol^{-1}. In the case of FITC-labeled dextran of 2,000,000 g mol^{-1}, the transition between the closed and open state occurred at pH values between 1.5 and 2. A possible explanation of this behavior in the former experiments is that PAH was not adsorbed in the fully protonated state under the assembly conditions. Accordingly, reduction of the pH increased its charge density, leading to partial segregation of the polymer and an increase in permeability of the shell. In another study, the permeability of polymer capsules composed of sixteen PSS/PAH layers for fluorescein-labeled poly(acrylic acid) (PAA) of 50,000 g mol^{-1} was investigated [91]. At neutral pH, the shell of polymer capsules was non-permeable for PAA. However, when the pH was increased above 11.8, the size of the capsule swelled to double and the permeability increased, allowing the diffusion of PAA across the membrane. Considering that the pK_a of PAH in the PAH/PSS layer is approximately 10.5, at pH=11.8 PAH is significantly deprotonated. Thus, polymer capsules accumulate negative charges due to PSS, which is a strong polyelectrolyte (charge density is not affected by pH), causing repulsion between the polyelectrolytes and increasing the permeability of the polymeric shell. Adding HCl to reduce the pH results in a reversible contraction and closing of the capsule. Considering that each enzyme has its optimal pH-range in terms of enzymatic activity, this approach cannot be considered as a universal method for the encapsulation of every enzyme and an optimization process for each enzyme will be required.

Similar behavior has also been reported when two weak polyelectrolytes were employed [92-95]. In one study [94], the pH dependency of microcapsules containing PAH and poly(methacrylic acid) (PMA) was investigated. According to titration experiments, the

pK_a of PAH and PMA was 10.8 and 3.9, respectively. In the pH range of 2.7-11.5, the capsules showed great stability with a diameter of ~3.7 μm. Below pH 2.7, the capsule diameter increased to 8.1 μm due to the protonation of carboxylate groups of the PMA and the resulting repulsion between the positively charged PAH and the neutral PMA. In order to increase the stability of the polymer capsules under extremely acidic or alkaline conditions, crosslinking of the polyelectrolytes has been proposed. In the case of PAH/PMA microcapsules, crosslinking with EDC results in a reaction between amino groups of PAH and the carboxylic acid groups of PMA, forming an amide bond. Additionally, functionalization of PMA with chemically stable benzophenone (BP) permits the crosslinking of PAH/PMA capsules via UV irradiation, due to the reaction of BPs with unreactive C-H bonds [92]. In another example, stable weak polyelectrolyte microcapsules were prepared by assembly of poly(ethylenimine) (PEI) and PAA followed by glutaraldehyde (GA) crosslinking [95]. The pH tunable permeability of polymer shells composed of two weak polyelectrolytes was employed as a strategy to encapsulate FITC-labeled BSA in polymer capsules composed of PAH and PMA [93]. In this particular example, $CaCO_3$ templates loaded with PSS were first fabricated and subsequently coated with PAH and PMA at a pH of 6. Finally, the $CaCO_3$ template was removed by exposing the particles to EDTA. At pH=7, FITC-labeled BSA could not be loaded due to the impermeable nature of the membrane at this pH. However, when the pH was reduced to 3, $2.03x10^8$ BSA molecules per capsule were satisfactorily encapsulated. The charge reversion of BSA from positive to negative (isoelectric point of BSA is 4.8) and the corresponding enhanced interaction with encapsulated PSS, together with increased permeability of the PAH/PMA polymeric shell at lower pH, resulted in the high loading capacity of BSA in the polymer capsules.

Recently, a new strategy to prepare polymer capsules with pH driven permeability was reported [24]. As an alternative to the LbL approach, the membrane can be constructed by a single polymer layer via surface-initiated reversible addition fragmentation chain-transfer polymerization. In this particular case, a copolymer composed of pH-responsive polydiethylaminoethylmethacrylate (DEAEM) and crosslinkable pyridyldisulfide ethylmethacrylate (PPDSM) was polymerized on silica nanoparticles, which were used as a sacrificial template. Subsequently, the stability of the membrane was enhanced by crosslinking with dithiol molecules of different molecular weights. The resulting permeability of the membrane was dependent on the pH and on the length of the dithiol molecule employed during the crosslinking. The PDEAEM chain underwent physicochemical changes going from an unprotonated, entangled state at a high pH to a protonated, repulsive state at low pH. As demonstrated via dynamic light scattering measurements (DLS), this transition resulted in an increase in the diameter of the polymer capsules. Additionally, the diameter change and the corresponding permeability can be controlled by employing crosslinkers of different molecular weights, attaining the size and the maximum permeability when higher molecular weight crosslinkers are employed. As a proof of concept, myoglobin (Myo) was encapsulated at a pH of 4 in those polymer capsules prepared with the highest molecular weight crosslinker, and the enzymatic activity was checked at different pH values in the presence of guaiacol and H_2O_2 as substrates. At a pH of 8, low enzymatic activity was observed due to the closed state of the polymeric shell and the corresponding low diffusion of guaiacol and H_2O_2 across the membrane. However, the activity was clearly enhanced at a pH of 6, when the capsule membrane was in a swollen state, allowing the guaiacol and H_2O_2 to reach the Myo. Moreover, the transition

was totally reversible as demonstrated by the low enzymatic activity observed when the pH was increased back to a pH of 8.

Fabrication of polymer capsules displaying responsiveness to two or more stimuli has generated increasing interest. In a recently published work, reversible pH- and temperature-responsive polymer capsules were prepared by employing PSS and poly(urethane-amine) (PUA) as polyelectrolytes [53]. In comparison to other temperature-responsive polymers such as poly(N-isopropylacrylamide) (PNIPAm) or poly(diallyldimethylammonium chloride) (PDADMAC), PUA shows improved degradability and cytocompatibility, and is thus more attractive for biomedical applications. PUA was synthesized under compressed CO_2 using salen-Mn complex as the catalyst. The use of this catalyst results in an increased amount of urethane units in the polymer, leading to a decreased lower critical solution temperature (LCST of 48 ºC). Furthermore, the salen-Mn complex effectively decreases the stereoregularity of the polymer and so the resulting PUA shows a sharp hysteresis and excellent reversible thermal-tunable properties. The diameter of the PSS/PUA capsules at a pH of 4.5 decreased from 3.5 to 2.5 µm when the temperature was switched from 55 to 37 ºC, showing that the transition is completely reversible. The pH- and temperature-responsiveness of PSS/PUA capsules was proved by studying doxorubicin (DOX) release at different pH values and temperatures. After 36 hours, only 44% of DOX was released from capsules at a pH of 8.0, whereas 78% of DOX was released at a pH of 2.1. On the other hand, the release of DOX after 36 hours was 20% higher at 55 ºC than that at 37 ºC due to the coil-to-globule transition in aliphatic PUA. Responsiveness to multiple stimuli can also be elegantly achieved via the precise combination of polymersomes and LbL approach [96]. Following this strategy, polymersomes of poly(ethylene oxide)-*b*-

poly(dimethylaminoethyl methacrylate) (PEO-*b*-PDMAEMA) were deposited by hydrogen bonding together with tannic acid on silica sacrificial templates, giving rise to LbL capsules. After sacrificial template removal, the resulting polymer capsules displayed pH- (due to the protonation and deprotonation equilibrium of tannic acid) and ionic strength-responsiveness (due to the ionic strength-dependency of the polymersome structure). Thus, sequential release of several entities can be achieved by encapsulating one entity into the polymersomes (that will be released upon a change in ionic strength of the media) and a different entity within the LbL polymer capsules (that will be released upon a change in pH).

Apart from the aforementioned synthetic polyelectrolytes, the use of natural derived polymers has gained considerable interest for the development of polymer capsules due to their improved biocompatibility, non-cytotoxicity, biodegradability and intrinsic bioactivity. In these cases, enzyme-driven degradability has to be considered, since it plays a major role in the degradation of polymer capsules fabricated from natural polymers [97]. Among natural polymers, natural polysaccharides such as HA, Dex, Alg, Chi, cellulose, etc. have been widely employed as pH sensitive capsules [98-101]. Chitosan is of particular interest for therapeutic applications because it shows a pK_a value close to the physiological pH (pK_a ~ 6.5). Accordingly, the permeability of polymer capsules where one of the components is Chi can be switched from the open to the closed state in the pH range of 6-8. As an illustration, polymer capsules composed of Chi and Dex were fabricated and their permeability toward various molecular weight (M_w= 4000, 20000, 250000 g mol^{-1}) FITC-labeled dextran molecules was thoroughly investigated at different pH values (pH values of 5.6, 6.8 and 8.0) [98]. At pH<6.8 none of the dextran molecules was able to diffuse into the

polymer capsules, indicating the impermeability towards these molecules of the polymer shell. In contrast, at pH>8 all the dextran molecules were able to cross the polymer shell as demonstrated by confocal microscopy. At this pH, amine groups in Chi are deprotonated. Therefore, electrostatic repulsion between the polyelectrolytes occurs, resulting in an increased permeability of the polymeric shell. Interestingly, this transition between the open and the closed state was demonstrated to be reversible and was satisfactorily employed to encapsulate macromolecules such as growth factors (basic fibroblast growth factor). Additionally, precise combinations of natural- and synthetic-derived polymers can result in the development of polymer capsules that respond to multiple stimuli [102]. For instance, multi-responsive polymer capsules were prepared by the combination of LbL of chitosan/alginate and Ce(IV) initiated grafting polymerization of PNIPAm. PNIPAm is a thermoresponsive polymer that undergoes a transition from random-coil conformation at temperatures below ~32 ºC to collapsed-globule conformation at temperatures above ~32 ºC. Thus, the resulting polymer capsules displayed pH/ionic strength and temperature driven permeability due to the presence of Chi /Alg multilayer and PNIPAm, respectively, allowing fine control of the release of a model hydrophobic drug [dipyridamole (DIP)].

Change in solvent composition has been reported as another stimulus to induce the reversible transition between opened and closed states within the polymeric shell [52, 103]. Polymer capsules composed of PAH and PSS were prepared via LbL deposition on a melamine formaldehyde core, which was subsequently removed at low pH. As demonstrated by confocal microscopy, urease was not able to permeate into the capsule when only water was present in the media. However, by adding ethanol to the aqueous solution, the permeability of the polymeric shell was increased, allowing the diffusion of

urease across the membrane. Additionally, the transition from the opened to the closed state was reversible by changing the media back to pure water. As a result, urease was satisfactorily encapsulated in PAH/PSS polymer capsules, and was able to maintain its enzymatic activity as demonstrated by the hydrolysis reaction of urea in the presence of the capsules. The main drawback of this strategy is that the activity of the encapsulated compounds, particularly enzymes, may be negatively affected by the presence of harmful solvents such as ethanol.

The development of polymer capsules that respond to glucose levels has also been achieved by employing phenylboronic acids which form covalent complexes with polyol compounds such as glucose [104]. In a particular study, 3-acrylamidophenylboronic acid was copolymerized with dimethyl aminoethyl acrylate, acting the resulting copolymer (denoted as PAD) as positively charged polyelectrolyte in the LbL approach. Subsequently, polymer capsules composed of poly(N-amidino)dodecyl acrylamide (PAD) and PSS were fabricated and their permeability toward FITC-labelled BSA (FITC-BSA) was analyzed at different pH values in the presence or absence of glucose. In the absence of glucose, FITC-BSA could not diffuse across the membrane at a pH of 9. In contrast, when 5 mg/ml glucose was incorporated, capsules were gradually filled with the FITC-BSA, indicating an enhanced permeability of the membrane in the presence of glucose. The pK_a of phenylboronic acids, which is usually between 8 and 9, drops by 2-4 units upon complexation with glucose. Accordingly, at a pH of 9 and in the presence of glucose, the uncharged phenylboronic acid groups became anionic, strongly interfering with the polyelectrolyte multilayer: first, there is an intermolecular repulsion between PSS and anionic phenylboronic acid; second, there is an intramolecular complexation between the cationic ternary amino groups from PAD

and the anionic phenylboronic acid. Both contribute to the repulsion between the polyelectrolyte layers resulting in an increased permeability of the polymeric shell and even in the disassembly of the polymer capsules.

2.3.2. Remote control of permeability

Remote control of permeability of polymer capsules is generally achieved via the incorporation of ultrasound-, magnetic- or light-sensitive nanoparticles within the polymeric shell and this has gained interest in recent years. Two general routes exist to incorporate nanoparticles in the polymer shell of polymer capsules. On the one hand, prefabricated nanoparticles can be deposited into the polyelectrolyte shell via electrostatic, covalent or hydrogen-bonding interaction [105, 106]. This represents a facile approach but the control over the distribution of nanoparticles on the polymer shell is challenging. On the other hand, nanoparticles can be incorporated *in situ* in polyelectrolyte capsules [107]. This is the case, for example, with the *in situ* growth of TiO_2 needle-like nanoparticles in polyelectrolyte capsules via the hydrolysis of titanium butoxide that give rise to hybrid capsules with UV- and ultrasound-responsiveness. In most of these cases, however, the disruption of the nanoparticle-containing capsules is non-reversible, limiting their potential as micro-/nanoreactors for therapeutic applications. In a particular example, SiO_2-TiO_2 nanocrystals were deposited on the surface of polymer capsules composed of PSS and poly(diallyldimethylammonium chloride) (PDDA) [108]. The permeability of these capsules was remotely controlled upon UV irradiation due to the photocatalytic effect of TiO_2, which is able to decompose the polyelectrolytes. Incorporation of gold nanoparticles into the polymeric shell is of particular interest for biomedical applications because their absorption within the polymer capsules is shifted to the near-infrared region, where most

tissues show only weak light absorption [109]. In this sense, two-compartment polyelectrolyte capsules were fabricated via a shell-in-shell strategy, being the polymeric shell of the internal capsule loaded with gold nanoparticles [110]. Upon irradiation with near-infrared laser light, the internal capsule was disrupted, allowing the intermixing of the contents of the two compartments. The present system can enable the mixing of two different reagents and, thereby, the start of a reaction by an external trigger. However, it does not represent a permeabilization strategy since the disruption of the polymer shell is non-reversible.

Magnetoresponsive capsules have also been developed by the incorporation into the polymeric shell of nanoparticles that respond to a magnetic field, such as magnetite (Fe_3O_4) [111] or gold-coated cobalt [112] nanoparticles. Additionally, via the incorporation of these nanoparticles, polymer capsules can be magnetically guided to the target disease area and tracked by magnetic resonance imaging (MRI). For example, magnetite nanoparticles were deposited on polymer capsules composed of PAH and PSS. An external lipid bilayer was also incorporated to limit the release of low molecular weight drugs. Magnetite nanoparticles are able to generate heat upon irradiation with an alternating magnetic field due to hysteresis loss and/or Néel relaxations. Accordingly, membrane permeability towards two model dies (calcein and phenol red) was clearly enhanced after irradiating the polymer capsules with an alternating magnetic field due to the "gel" to "liquid crystalline" phase transition of the lipid bilayer caused by the local heat generation.

However, the potential toxicity of metal nanoparticles may limit the application of the aforementioned polymer capsules in the biomedical field. In addition, as mentioned before, in most of the cases the shell disruption is non-reversible, limiting their application as

micro-/nanoreactors and restricting multiple loading/unloading cycles of the capsules. This is the reason that great efforts are being made to develop polymer capsules with remotely controllable permeability in the absence of metal nanoparticles [113-115]. In a particular example, polymer capsules composed of PSS and PAH were decorated with triphenylsulfonium triflate, which releases protons upon irradiation with UV light [113]. Because of this, the pH of the capsule solution is locally reduced and the permeability of the polymeric shell enhanced after UV irradiation. In another example, the dual cross-linking concept was employed to prepare UV-responsive capsules in the absence of nanoparticles [116]. In this approach, the ability of cyclodextrin to form non-covalent, reversible interactions with guest molecules that undergo structural changes upon UV-irradiation is exploited to prepare polymer capsules that display light-responsiveness. More precisely, azobenzene moieties were employed as guest molecules due to their *trans* to *cis* isomerism after UV-irradiation. Accordingly, the cyclodextrin-azobenzene complex, which only occurs with azobenzene *trans* isomer, is disrupted upon UV irradiation. This results in a decreased cross-linking density and a higher permeability of the polymer shell as demonstrated by the release of encapsulated FITC-dextran (500 kDa) initiated by UV irradiation. Additionally, thanks to the presence of irreversible covalent cross-links provided by the continuous assembly of polymers mediated by ring-opening metathesis polymerization (CAP_{ROMP}), the transition occurs without the complete degradation of the particles. Finally, the incorporation of a star polyelectrolyte as one of the layer components, together with the use of salts with photochemical properties, also represents a promising strategy to develop polymer capsules with remotely controllable permeability in the absence of nanoparticles due to the high sensitivity of star polyelectrolytes to the ionic environment [115]. Using this, polymer capsules based on PSS and the star polyelectrolyte

poly[2-(methacryloyloxy)ethyl] trimethylammonium iodide (PMETAI) were prepared by the LbL technique. The addition of multivalent counterions, such as trivalent hexacyanocobaltate(III), to the star polyelectrolyte solution results in retraction of the arms of the star polyelectrolyte. This transition from retracted to extended state of the star polyelectrolyte arms can be reversed upon irradiation with UV light and corresponding transformation of trivalent hexacyanocobaltate(III) into a mixture of mono- and divalent ions. Taking this into consideration, the permeability of PSS/PMETAI polymer capsules with a different number of bilayers (n=5, 8 or 11 layers) to FITC-dextran molecules of different molecular weights (M_w= 70, 250, 500 or 2000 kDa) was thoroughly studied via confocal microscopy. The addition of trivalent hexacyanocobaltate(III) ions to the capsule solution dramatically reduced the permeability of the polymeric shell, avoiding even the diffusion of lowest molecular weight FITC-dextran molecules (70 kDa) across the polymer capsule with lowest number of bilayers (five). However, after UV irradiation and corresponding decomposition of trivalent hexacyanocobaltate(III) into a mixture of mono- and divalent ions, the permeability of the polymer capsules was enhanced. This reversible state permitted loading-unloading of molecules triggered by UV light as demonstrated in those capsules composed of eight bilayers: first, 500 kDa FITC-dextran permeates into the PSS/PMETAI capsules; then, trivalent hexacyanocobaltate (III) was added so that the capsule wall is closed and the FITC-dextran is encapsulated; finally, FITC-dextran is released upon UV-irradiation and due to the transition between collapsed and stretched state of the arms of the star polyelectrolyte.

3. Spherical polymer compartments prepared without a sacrificial template

Preparation of spherical polymer compartments, or polymer vesicles, in the absence of a sacrificial template is achieved by self-assembly of amphiphilic block copolymers: diblock (AB), symmetric triblock (ABA), or asymmetric triblock (ABC) copolymers, where at least one of the blocks is hydrophobic, the other(s) being hydrophilic [117-123]. Although vesicles made from multiblocks copolymers have also been reported, they will not be discussed here [124]. Self-assembly of copolymers can be achieved in various media, but for therapeutic applications only those that self-assemble in aqueous solutions will be considered because traces of organic solvents might impact on the stability of biological molecules involved in the preparation of micro-/nanoreactors. As a result of the self-assembly process in aqueous media, the hydrophobic blocks are oriented towards the center of the membrane, whereas the hydrophilic blocks are oriented towards the aqueous environment, forming a bilayer membrane. Due to their similarity with the bilayer of liposomes, these structures have been coined as "polymersomes" [125]. Other terms are also encountered in the literature, depending on the macromolecules used for the formation of the final vesicular architecture for particular types of nano-compartments, such as peptosomes (i.e., peptide based molecular assemblies) [126], PICsomes (i.e., cationic and anionic amphiphiles) [2], hybrid lipo-polymersomes (i.e., made of a combination of lipids and copolymers mixtures) [127], or branched-polymersomes (i.e., from hyperbranched polymers) [128].

There are several factors that dictate the self-assembly of amphiphilic block copolymers into vesicles, spherical or worm-like micelles or, as recently reported, more complex systems such as assemblies with gated nanopores [129, 130]. Accordingly, the effect of

copolymer composition and concentration, the nature of the polar head group and the method of preparation, temperature, etc. on the self-assembly process have been widely studied and reviewed in the literature [130-132]. Among them, the copolymer composition, which determines the hydrophilic fraction and is directly related to the dimensionless packing parameter, plays a major role in determining the self-assembly process of amphiphilic block copolymers. In general, hydrophilic fraction > 50% yield micelles; between 35-10% yield polymersomes and < 25% yield inverted structures. Worm-like micelles are formed in a small region when the hydrophilic fraction is around 50%. However, these values are only estimative and various architectures can be obtained for these hydrophobic to hydrophilic fractions because the self-assembly process depends on the complex scenario involving the parameters mentioned above.

For biomedical applications, polymersomes are of considerable interest than other self-assembled structures. For drug delivery purposes, hydrophilic and hydrophobic entities can be simultaneously encapsulated in aqueous cavities and hydrophobic membranes [133-139]. In addition, surface attachment of various ligands, or surface topology modification allows a tunable and directed internalization in cells [11, 140].

On the other hand, polymersomes can serve to develop nanoreactors and artificial organelles, where the active compounds (e.g., enzymes, proteins, nanoparticles, etc.) are protected from the environment and, are at the same time, able to react with the reagents that penetrate into the aqueous cavity (Figure 3) [141-144].

Considering the high molecular weight of amphiphilic block copolymers (up to 100 kDa) in comparison to the building blocks of liposomes (below 1 kDa), the resulting polymersomes are physically and chemically more stable but have a membrane that is less permeable and

fluidic [145]. Although a low permeability is sometimes needed (see viral capsides) [146], quite often this may limit their use in several biomedical applications because it blocks the molecular exchange with the environment. Herein, different strategies have been adopted to control the membrane´s permeability of polymersomes, but not all of them were demonstrated as further applicability for micro-/nanoreactors development.

3.1. Intrinsic permeability of the polymersome membrane

The level of molecule diffusivity through the polymersome membrane varies depending on the type of copolymer used for the self-assembly. Some block-copolymers have reduced or no permeability for small molecules while others can self-assemble into membranes that allow specific molecules to pass (Table 3). There are two membrane permeability scenarios: i) porous membrane and, ii) membranes permeable only to specific ions, molecules. The category of copolymers forming a porous membrane are amphiphilic block copolymers containing a poly(styrene) (PS) tail and a charged helical poly(isocyanide) headgroup [147]. First, isocyanides were polymerized from dipeptides [isocyano-L-alanine-L-ananine (IAA) and isocyano-L-alanine-L-histidine (IAH)] resulting in the formation of helical peptide polymers. Then, a series of poly(styrene)-*block*-poly(isocyanide) copolymers was prepared employing poly(styrene) derivatives of well-defined length (40 repeating units). Finally, the protected block copolymer was deprotected by treatment with a 1 M aqueous NaOH-toluene mixture that removed the ester functions of the copolymers, yielding a superamphiphile with a negatively charged helical headgroup in the case of PS-*b*-PIAA and a superamphiphile with a zwitterionic headgroup in the case of PS-*b*-PIAH. The synthesized amphiphilic block copolymer self-assembled into various morphologies

(e.g., spherical or rod-like micelles, vesicles, superhelices, etc.) depending on the length of poly(isocyanide) block, pH and the anion-headgroup interactions.

Similarly, synthesis of rod-coil block copolymers that consist of 40 styrene and 50 3-(isocyano-L-alanyl-amino-ethyl)-thiopene (PS-*b*-PIAT) have been widely reported in the literature to self-assemble in water into polymer vesicles (polymersomes) [148-150]. The membrane is composed of PS-*b*-PIAT in which the PS blocks are oriented towards the center of the membrane and the poly(isocyanide) blocks are orientated toward the solvent. The potential of these polymersomes to serve as compartments for the development of nanoreactors was proved via the encapsulation of *Candida antarctica* Lipase B (CAL B) and subsequent reaction with DiFMU octanoate [150], which fluoresces when hydrolytically cleaved by CAL B. The increase in fluorescence intensity confirmed that the enzymes were active upon encapsulation in the polymersome and that the substrate passed through the polymersome membrane.

The possibility to encapsulate enzymes within polymersomes with a permeable membrane has been employed to perform single reactions of permeable substrates with specific enzymes, as for example ring-opening polymerization of 8-octanolactone and dodecalactone to yield oligomers via the reaction with encapsulated CAL B [149]. In another study, *Caldariomyces fumago* (CPO) was encapsulated within PS-*b*-PIAT polymersomes and the reaction with pyrogallol and thioanisole was investigated [148]. In the case of pyrogallol, the reaction with CPO is limited by the diffusion of the substrate molecules along the membrane, whereas for thioanisole the limiting factor is the reaction with the enzyme.

Cascade reactions have attracted a great deal of attention due to the possibility to mimic simple metabolic reactions occurring in living organisms. As an illustration, GOx and horseradish peroxidase (HRP) were independently encapsulated within PS-*b*-PIAT polymersomes [151]. Because of the unique property of PS-*b*-PIAT polymersomes namely that of being sufficiently porous to enable diffusion of low molecular weight molecules while retaining large biomolecules such as enzymes inside, reaction between the enzymes and the corresponding intermediate reaction products was achieved. First, by reacting with GOx, glucose is converted into gluconolactone and H_2O_2, which further reacts with HRP and ABTS to form the ABTS radical cation ($ABTS^{\cdot+}$). Cascade reactions within a single polymersome have also been reported with different enzymes strategically positioned in separate domains within the polymersome (i.e., inner cavity, polymersome membrane or polymersome surface) [152, 153]. In a particular case, HRP was covalently bound to the surface of polymersomes via click chemistry between acetylene-functionalized anchors on the surface of polymersomes and azido functions of HRP; CAL B was located in the membrane, and GOx was located in the lumen [152]. Thus, a three-enzyme cascade reaction was conducted within a single PS-*b*-PIAT polymersome: first, glucose acetate is deprotected by CALB to form glucose; second, glucose is oxidized by GOx yielding gluconolactone and H_2O_2; third, ABTS is oxidized to $ABTS^{\cdot+}$ in the presence of H_2O_2.

PS-*b*-PIAT polymersomes containing Cu/Zn superoxide dismutase (SOD) and catalase (CAT) in their lumens have been proposed as model devices to combat overproduction of reactive oxygen species (ROS) within the cellular environment [154]. Although high encapsulation efficiency and confirmation of a low probability for empty or single enzyme-loaded capsules is reported, the activities of the final PS-*b*-poly(ethylene glycol) (PEG) or

PS-*b*-PIAT nanoreactors are still less efficient when compared with the enzymatic cascade reaction in bulk. In addition, no activity inside cells was reported to establish the *in vitro* functionality of the nanoreactor.

Poly(N-vinylpyrrolidone)-*b*-poly(dimetheylsiloxane)-*b*-poly(N-vinylpyrrolidone) (PNVP-*b*-PDMS-*b*-PNVP) polymersomes were employed to encapsulate ceria nanoparticles, which are known to act as superantioxidant particles [155]. However, ceria nanoparticles were also reported to participate in a Fenton-like reaction with H_2O_2, resulting in the production of $HO^{\cdot}$ and $O_2^{\cdot -}$ radicals that are cytotoxic. Their encapsulation inside polymersomes was intended to decrease their toxicity while still benefiting from their super-antioxidant property. Indeed, due to the specific permeability of PDMS to $O_2^{\cdot -}$ and impermeability to H_2O_2, the encapsulation of ceria nanoparticles within PNVP-*b*-PDMS-*b*-PNVP polymersomes served as a promising strategy to take advantage of the super-antioxidant properties of ceria nanoparticles while avoiding their related cytotoxicity. Impermeability to H_2O_2 and scavenging of $HO^{\cdot}$ and $O_2^{\cdot -}$ of these polymersomes was demonstrated via UV-vis spectroscopy and electron paramagnetic resonance (EPR), respectively. Ceria loaded polymersomes were uptaken by HeLa cells and, as assessed by MTS assay, they were not cytotoxic after 72 hours of exposure. Finally, HeLa cells were exposed to oxidative stress induced by the addition of paraquat. Interestingly, the viability of HeLa cells exposed to paraquat was reduced to 60% after 24 hours of incubation, whereas those cells pretreated with ceria-loaded polymersomes maintained a viability of 96%. This indicated that ceria nanoparticles-loaded polymersomes act as an efficient ROS detoxification system.

The intrinsic permeability of PDMS to $O_2^{\cdot -}$ has also been exploited to design nanoreactors for PhotoDynamic Therapy (PDT) under various irradiation conditions (for example, mild illumination with a light dose: 23.7 - 70 $J \cdot cm^{-2}$, illumination time < 25 min) [156, 157].

This has been achieved by encapsulating conjugates of a protein with photosensitizers to serve as a source of singlet oxygen "on demand": only upon irradiation, encapsulated photosensitizers produce singlet oxygen, otherwise the system is not active. Rose Bengal (RB), a photosensitizer known to produce singlet oxygen with a high quantum yield upon irradiation with specific wavelength, was conjugated to bovine serum albumin (BSA) and encapsulated in polymersomes self-assembled from a library of poly(2-methyl-2-oxazoline)-*b*-PDMS-*b*-poly(2-methyl-2-oxazoline) (PMOXA-*b*-PDMS-*b*-PMOXA) and PNVP-*b*-PDMS-*b*-PNVP triblock copolymers. RB was therefore protected by the cavity of polymersomes, eliminating the inherent systemic toxicity typical of photosensitizers. RB conjugation to bovine serum albumin served to increase its solubility and encapsulation efficiency inside polymersomes, without affecting the photosensitizing activity. RB–BSA nanoreactors uptaken by Hella cells and irradiated induced formation of blebs and initiation of apoptosis (Figure 4). Due to the polymersome formation at neutral pH, PNVP-*b*-PDMS-*b*-PNVP copolymers (pK_a~ 6.8) assembled in polymersomes with an overall negative charge, which induced a low encapsulation efficiency of the RB-BSA conjugate due to electrostatic repulsion (pK_a for BSA ~ 4.7). This issue was solved by using neutral PMOXA-*b*-PDMS-*b*-PMOXA based nanoreactors of approximately 200 nm in diameter, which were better candidates in terms of size, high encapsulation efficiency of the photosensitizer, cellular uptake, permeability towards ROS and thus, high activity inside the cells.

Another polymer assembly generating polymer vesicles, known as PICsomes, is formed of a pair of oppositely charged block ionomers [2]. In this case, an anionic and a cationic block copolymer are prepared from PEG-poly(β-benzyl-L-apsartate) (PEG-PBLA) with

identical molecular weight and composition. Anionic PEG-poly(α,β-aspartic acid) [PEG-P(Asp)] is prepared via alkali hydrolysis of PEG-PBLA, whereas cationic PEG-poly(poly([2-aminopentyl]-α,β-aspartamide) [PEG-P(Asp)-AP] is prepared from PEG-PBLA by aminolysis. After mixing separate aqueous solutions of anionic and cationic block copolymers, hollow spherical assemblies are formed. They show selective permeability toward small molecules (M_w= 443.5 g mol^{-1}) but are impermeable to molecules with higher molecular weight (M_n= 40000 g mol^{-1}) at physiological pH [2, 158, 159]. Very recently, crosslinked PICsomes were prepared by encapsulating β-galactosidase and acted as nanoreactors (NRs) for the conversion of a model prodrug (HMDER- β Gal) into a strongly fluorescent product (HMDER) [15]. Moreover, 100 nm in diameter PICsome-based NRs were systematically injected in C26 tumors, subcutaneously inoculated into mice. After injection, the NRs accumulated in the tumor due to the enhanced permeability and retention (EPR) effect, and retained their activity for four days after administration, as demonstrated by a combination of *in vivo* and *ex vivo* fluorescence imaging techniques (Figure 5). Even though, there is still room for improvement - a very low encapsulation efficiency of β-galactosidase inside NRs of only 0.9% was achieved - this study is a solid proof of the importance of designing polymer based NRs for therapeutic applications.

3.2. Polymersomes with a membrane rendered permeable by chemical modification/reactions

PMOXA-*b*-PDMS-*b*-PMOXA is known to generate polymersomes of controlled size thickness and a membrane permeable to oxygen species (O_2 and $O_2^{\cdot -}$ [160]) or specific molecules, such as GdnHCl that can diffuse through, whilst being impermeable for other

small molecular weight molecules, as for example H_2O or H_2O_2. In the particular case of $O_2^{\cdot-}$, its diffusion can be tuned via precise control of copolymer composition. With this in mind, three $PMOXA_n$-*b*-$PDMS_m$-*b*-$PMOXA_n$ copolymers were synthesized with various lengths of hydrophobic blocks (i.e., m=22, 55, 165) and the permeability of $O_2^{\cdot-}$ toward the membrane was analyzed [160]. For that purpose, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-(2H-tetrazolium)) was encapsulated within PMOXA-*b*-PDMS-*b*-PMOXA polymersomes and its reduction to formazan due to the presence of $O_2^{\cdot-}$ was analyzed by spectrophotometric assays. As expected, longer lengths of hydrophobic blocks resulted in a decrease in permeability. A potential biomedical application of these selectively permeable polymersomes is their use as NRs for the regulation of ROS species [161]. SOD was successfully encapsulated within PMOXA-*b*-PDMS-*b*-PMOXA polymersomes, with its structural and functional integrity unaffected upon encapsulation. Accordingly, due to the permeability of the membrane toward $O_2^{\cdot-}$, these polymersomes were able to scavenge $O_2^{\cdot-}$ and act as efficient antioxidant nanoreactors, whilst keeping H_2O_2 inside.

Another recently reported versatile strategy consists in the photoreaction of a photoinitiator with the polymeric membrane [162, 163]. In this approach, a water-soluble type I photoinitiator (i.e., 2-hydroxy-4´-2-(hydroxyethoxy)-2-methylpropiophenone (PP-OH)) is first added to a solution containing PMOXA-*b*-PDMS-*b*-PMOXA polymersomes which are then irradiated with UV light. As a result, ketyl and alcohol radicals are produced that further attack the membrane of the polymersome causing an increase in its permeability. To demonstrate the enhanced permeability of PMOXA-*b*-PDMS-*b*-PMOXA polymersome upon photoreaction with PP-OH, HRP was encapsulated and the reaction with four

molecules of different hydrophilicity (ABTS, AEC, pyrogallol and TMB) was monitored. For those polymersomes not photoreacted with PP-OH, the reaction between the aforementioned molecules and HRP did not occur, demonstrating the low permeability of the polymersome membrane. In contrast, the UV-induced photoreaction rendered the vesicles permeable and, accordingly, the reaction between HRP and the substrates occurred satisfactorily. By using this approach, poly(ethylene glycol) methyl ether acrylate (poly(PEGA)) was successfully polymerized by enzyme-catalyzed atom transfer radical polymerization (ATRP) within PMOXA-*b*-PDMS-*b*-PMOXA polymersomes [162], demonstrating the potential application of these photo-reacted polymersomes as nanoreactors.

3.3. Biomimetic membrane permeabilization by insertion of biomolecules

An elegant way to render permeable an impermeable polymersome membrane is to use a biomimetic strategy of insertion of membrane proteins (MPs) and biopores/ion-carriers (Table 4). Such biomolecules-decorated membranes with a selective or partially selective diffusion through of specific molecules offer the possibility of inducing stimuli-responsiveness for a non-permeable synthetic membrane (Figure 6).

Since their introduction by Meier *et al*. [164], various channel proteins (e.g., AqpZ, OmpF, Tsx, FhuA, etc.) have been incorporated in the membrane of polymer vesicles to tune their permeability. Although a few examples exist in the literature reporting the incorporation of channel proteins in the membrane of other block copolymers [e.g. polybutadiene-*b*-polyethyleneoxide (PB-*b*-PEO) [165], polyisobutylene-polyethyleneglycol-polyisobutylene (PIB-PEG-PIB) [166]], PMOXA-*b*-PDMS-*b*-PMOXA has been the copolymer of choice in most cases [167, 168]. Considering that the thickness of the membrane in synthetic block

copolymers is 2-10 times that of phospholipid bilayer, there is a large mismatch (in the range from 3.3 to 7.1 nm) between the membrane thickness and the size of the protein channels that makes the insertion of channel proteins within the membrane of block copolymers difficult, if not impossible. It has been shown that the hydrophobic mismatch may be formed either by a contraction of the block copolymer macromolecules in the vicinity of the channel protein, by the arrangement of smaller block copolymer chains around the protein or by a combination thereof. Mean-field analysis derived models or molecular dynamics simulation studies have focused on adaptability of the polymer membranes due to the insertion of membrane proteins [169, 170]. They indicated that a large hydrophobic mismatch (more than 1.3 nm) can be overcome and a successful insertion of MPs is possible. Indeed, despite significant hydrophobic mismatch (up to 5 times), membrane proteins have been successfully inserted and, surprisingly, they maintained their functionality. As demonstrated by a combination of cryo-transmission electron microscopy (cryo-TEM) and z-scan fluorescence correlation spectroscopy (z-FCS), this hydrophobic size mismatch can be overcome by a high flexibility and fluidity of the hydrophobic domain (for example PDMS block within PMOXA-*b*-PDMS-*b*-PMOXA block copolymers) [168]. It has been demonstrated using (PDMS)-containing amphiphilic block copolymer membranes (9-13 nm) that the relative diffusion coefficient (diffusion of membrane protein, D_{MP}, divided by the effective diffusion coefficient of the corresponding membrane, D_{eff}) decreases with an increasing hydrophobic mismatch. This is due to the flexible arrangement of block copolymer chains forming domains around the protein, which guide the protein through the membrane, and slows it down once the polymer membrane thickness increases.

The ability of PMOXA-*b*-PDMS-*b*-PMOXA membranes to accommodate their hydrophobic domain to allow insertion of biopores has been reported in specific domains of thickness, up to 12.1 nm. Ion selective PMOXA-*b*-PDMS-*b*-PMOXA polymersomes were developed by incorporation of gramicidin (gA) in their membrane [171]. gA has a length of 2.6 nm and an inner diameter of 0.4 nm and allows the controlled passage of protons and monovalent cations across the membrane. Due to the small size of the biopore, the incorporation of gA in the membrane of synthetic block copolymers was challenging. Thus, a library of PMOXA-*b*-PDMS-*b*-PMOXA copolymers was developed, with their membrane thickness being in the 9.2-16.2 nm range. Successful incorporation of gA was achieved when the thickness of the membrane was below 12.1 nm. For thicker membranes, the thickness mismatch and the corresponding perturbation energy of the membrane limited the insertion of the biopore. As demonstrated via fluorimetric and stopped-flow device experiments, polymersomes containing gA allowed the influx of protons, Na^+ and K^+, this being the influx controllable with the amount of inserted gA in the membrane. The authors took the concept even further and they replaced the gA forming pore peptide with an ion-carrier (ionomycin, 1.5 nm in diameter) in order to selectively permeabilize the PMOXA-*b*-PDMS-*b*-PMOXA polymersome membranes with different thicknesses (up to 13.4 nm) for calcium ions [172, 173]. Ionomycin was capable of moving calcium ions from one side of the membrane to the other without disturbing the architecture of the polymersomes, and offered more insight into the flexibility and versatility of these types of membranes for the feasible reconstitution of membrane proteins and carriers.

Another strategy to favor biopore insertion is to increase the size of the pore with respect to the membrane thickness, thus to increase the hydrophobic region of the protein in order to

overcome the mismatch with the hydrophobic domain of the synthetic membrane. For instance, the FhuA protein (4 nm in length) was extended by increasing the number of hydrophobic amino acids in the structure and therefore increasing its length by 1 nm more, being able to match the 5 nm thickness of the polymersome membrane assembled from the PIB_{1000}-*b*-PEG_{6000}-*b*-PIB_{1000} triblock-copolymer [166]. Although this strategy might affect the pore functionality, in this case the modified FhuA was able to keep its functionality once inserted in the membrane, as proved by a classic TMB/HRP assay. Even FhuA in its natural form has been inserted in synthetic membranes as well [174]. PMOXA-*b*-PDMS-*b*-PMOXA polymersomes incorporating FhuA were designed and their ability to selectively recover a product or perform enzymatic reactions was evaluated. On the one hand, positively charged PLL was encapsulated within the polymersomes to act as an effective trap for negatively charged molecules, such as sulforhodamine B. On the other hand, HRP was encapsulated within the polymersome and the oxidation of TMB by the enzyme was confirmed.

Outer membrane protein (OmpF) is the most extensively reported channel porin for the design of nanoreactors and artificial organelles because it allows passive, concentration-driven transport, where molecules with molecular weight above 600 Da are sterically excluded. OmpF is a homo-trimeric channel protein found in the outer membrane of Gram-negative bacteria such as *E. coli* [175, 176]. Additionally, OmpF channels can be closed above a critical transmembrane voltage of about 100 mV [177], allowing the protein channel to act as an on/off switch that can be controlled externally. PMOXA-*b*-PDMS-*b*-PMOXA based polymersomes incorporating OmpF channel proteins in their membranes have been employed as nanoreactors with various functionalities, as for example to perform

enzymatic reactions for production of therapeutically relevant products such as antibiotics [178], or drugs [179]. For instance, nanoreactors containing OmpF permeabilized membranes and encapsulated penicillin acylase inside their inner cavity were shown to locally produce the cephalexin antibiobic at 37°C, over a period of seven days, in the presence of 7-aminodesacetoxycephalosporanic acid (7-ADCA) and phenylglycine methyl ester (PGME) substrates [178].

Of particular interest in the biomedical field are those nanoreactors that are able to scavenge reactive oxygen and nitrogen species (ROS and RNS) and, thereby, prevent oxidative stress [13, 180, 181]. They have been developed either by encapsulation of proteins naturally involved in the detoxification of $O_2^{\cdot -}$ (SOD) [161], peroxinitrites and oxygen transport (hemoglobin, Hb) [181], or by encapsulation of enzyme mimics with a dual role, highly active against both superoxide radicals and H_2O_2 (Cu^{II}ENZm) [182].

A step further was realized by the co-encapsulation of two antioxidant enzymes [SOD+lactoperoxidase (LPO)/SOD+CAT] within PMOXA-*b*-PDMS-*b*-PMOXA polymersomes containing OmpF channel protein in their membrane [13], which introduced the first mimic of a natural cell organelle, the peroxisome. The membrane of these polymersomes allowed the passive transport of substrates/products of the cascade reaction while avoiding the escape of the enzymes from the vesicle cavity. As a result, the reaction between $O_2^{\cdot -}$ and SOD and the subsequent reaction between H_2O_2 and CAT to finally release O_2 and H_2O happened in the interior of the polymersomes. According to MTS assay, successfully uptaken by cells, artificial peroxisomes (up to 500 $\mu g \cdot ml^{-1}$) exhibited minimal *in vitro* cytotoxicity in HeLa cells for a period of up to 48 hours. In the last step, HeLa cells were exposed to paraquat mediated oxidative stress in the presence or absence

of antioxidant polymersomes. Viability of those cells pretreated with artificial peroxisomes was 26% higher than that of untreated cells, suggesting a peroxisome-induced protective effect to oxidative stress. This was further demonstrated when pyocyanin was employed to induce oxidative stress. Pyocyanin is known to induce oxidative stress mediated by ROS generation and to suppress the host antioxidant mechanisms. As demonstrated by monitoring ROS detoxification employing ROS fluorescent probes, only the cells pretreated with artificial peroxisomes were able to detoxify ROS.

PMOXA-*b*-PDMS-*b*-PMOXA polymersomes incorporating OmpF protein channel have also been employed to develop active surfaces by immobilization on solid support. The immobilization of polymersomes on solid surfaces has been achieved for example via a receptor-ligand pair, biotin-streptavidin [183]. With an awareness of this, streptavidin-structured glass surfaces were incubated with biotinylated vesicles to allow the specific binding of the polymersomes on the solid substrate. Polymersomes were loaded with acid phosphatase to perform a model dephosphorylation reaction with the fluorogenic substrate ELF97. As demonstrated via time-resolved fluorimetric studies, this strategy permitted reactions to be performed on surfaces at precise locations. In another study, PMOXA-*b*-PDMS-*b*-PMOXA polymersomes incorporating OmpF protein channel were employed to develop active surfaces to prevent bacteria-associated medical implant infections [178, 184]. In this case, the immobilization strategy was based on Schiff-base formation between aldehyde groups on the outer surface of polymersomes and amino groups on the silicon surface, followed by reductive amination: first, hydroxyl-terminal groups of the polymer were oxidized to aldehyde by Dess-Martin oxidation; these polymersomes were then incubated with aminated silicon surface to form imine bonds; finally, upon reductive

amination, strong and stable amino bonds were formed between the polymersomes and the silicon substrate. The enzyme penicillin acylase was encapsulated in these polymersomes. Due to the presence of OmpF in the membrane of the polymersomes, the substrates 7-ADCA and PGME were able to diffuse into the polymersome and react with the enzyme, resulting in the release of the antibiotic cephalexin. Hence, those surfaces where nanoreactors were immobilized inhibited bacterial growth and prevented biofilm formation up to seven days, a process far more efficient than the free enzyme already inactive after less than two days.

The transport across the OmpF channel protein was also turned from passive to pH-driven functionality via an engineered OmpF channel in which all six amino acids were substituted to histidines (OmpF 6His) [185]. Channel cross-section of OmpF 6His increased from 0.9 x 1.3 nm at a pH of 5 to 1.2 x 1.4 nm at a pH of 7, resulting in an increased release of a model cargo (acridine orange) from 33.6 to 79.2% when the pH was increased from 5 to 7. Very recently, the OmpF channel was chemically modified by embedding a pH sensitive Cy5-hydrazide molecular cap to control the passive diffusion of molecules once it is inserted in the membrane of PMOXA-*b*-PDMS-*b*-PMOXA polymersomes [186]. This strategy allowed the restriction of molecule diffusion through at a pH value of 7.4, while at pH 5.0, the cap was released from the pore, which opened and allowed the passage of molecules. The triggered permeability of the membrane has been demonstrated by the encapsulation of HRP model enzyme inside polymer vesicles with and without modified OmpF inside their membrane, followed by monitoring of the formation of fluorescent or colored products upon addition of two specific substrates: TMB and Amplex Red. When using the TMB chromogenic substrate, in presence of H_2O_2, the formation of a colored product can be

monitored if it encounters the encapsulated enzyme. TMB and H_2O_2 indeed could not pass through the PMOXA-*b*-PDMS-*b*-PMOXA membrane, and they could only reach the protected enzyme when the Cy5-hydrazide molecular cap of the successfully reconstituted OmpF inside the polymersome membrane was cleaved due to a drop in pH, from 7.4 to 5.0.

Selective permeability of the polymersome membrane can serve for development of water purification nanodevices. For example, Aquaporin Z (AqpZ), a homotetrameric protein that allows the diffusion of water across biological membranes has been successfully incorporated within the membrane of PMOXA-*b*-PDMS-*b*-PMOXA polymersomes [187, 188]. Permeability measurements of the membrane conducted via stopped-flow light-scattering experiments revealed that the incorporation of AqpZ into PMOXA-*b*-PDMS-*b*-PMOXA polymersomes resulted in a 90 times increase (from 0.8 μm/s to 74 μm/s) in the permeability of the polymeric membrane. Furthermore, AqpZ incorporated in the membrane was highly selective to water, allowing the diffusion of water while avoiding the transport of small solutes such as salts, glucose, urea and glycerol. This opens new bio-nanoscience based solutions for water purification processes.

A completely different application was intended when Tsx, a substrate specific small (31.4 kDa) channel protein involved in the transport of nucleosides and nucleotides that serve as carbon and nitrogen sources and as precursors for nucleic acid synthesis, was inserted into synthetic membranes [176, 179]. The specific transport of several substrates (e.g., inosine, adenosine, guanosine, 2-fluoroadenosine, thymidine, deoxyuridine) along PMOXA-*b*-PDMS-*b*-PMOXA polymersomes incorporating Tsx has been demonstrated in a few studies [179, 189]. In this sense, *Trypanosoma vivax* (TvNH) was encapsulated in PMOXA-*b*-PDMS-*b*-PMOXA polymersomes that contained Tsx in their membrane [179].

The apparent kinetic constants [$(k_{cat})_{app}$] of the reaction between encapsulated TvNH and inosine, adenosine, guanosine or 2-fluoroadenosine were evaluated and compared with the $(k_{cat})_{app}$ of the same enzyme encapsulated in PMOXA-*b*-PDMS-*b*-PMOXA polymersomes incorporating OmpF instead of Tsx. Due to the specificity of Tsx to nucleosides and nucleotides, the diffusion of the substrates and thus, the $(k_{cat})_{app}$ of polymersomes containing Tsx was much higher with respect to polymersomes containing OmpF. For example, $(k_{cat})_{app}$ of the reaction between inosine and TvNH in polymersomes containing OmpF (in a protein channel-to-polymer ratio of 1:100) was 2.28 s^{-1}, whereas the $(k_{cat})_{app}$ for polymersomes containing Tsx (in a protein channel-to-polymer ratio of 1:100) was 24.09 s^{-1}. PMOXA-*b*-PDMS-*b*-PMOXA polymersomes incorporating Tsx in their membrane has been proposed to treat mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) disease [189]. In this disorder, loss of function of thymidine phosphorylase (TP) results in the systemic accumulation of thymidine and deoxyuridine. As a proof of concept, TP was encapsulated in PMOXA-*b*-PDMS-*b*-PMOXA polymersomes. Due to the presence of Tsx in the membrane of the polymersome, thymidine was able to diffuse into the polymersome and react with TP, resulting in the formation of thymine. Moreover, the developed polymersomes did not affect the viability of primary hepatocytes nor did they cause stimulatory effect on macrophages and, therefore, may be useful to control thymidine levels in MNGIE disorders.

An interesting approach has been introduced when LamB was incorporated in the membrane of PMOXA-*b*-PDMS-*b*-PMOXA polymersomes [190]. LamB forms a trimeric channel in biological membranes and is involved in the transport of maltose and maltodextrines. Additionally, it also serves as a receptor for λ phage. Phages were able to

bind to LamB via their tails and, afterwards, they were able to inject their genome into the polymersomes, demonstrating that the functionality of LamB was preserved in the artificial block copolymer.

While various membrane proteins allow passive bidirectional transport, others serve for unidirectional active transport and so their orientation might affect the functionality and compromise the final objective of their intended application. For instance, the model proton pump, bacteriorhodopsin (BR), successfully inserted in the membrane of poly(2-ethyl-2-oxazoline)-*b*-poly(dimethylsiloxane)-*b*-poly(2-ethyl-2-oxazoline) (PEtOz-PDMS-PEtOz) polymersomes, pumped protons inwardly or outwardly based on protein fractions with different orientations [191]. Therefore, a random orientation inside synthetic membranes can be avoided by adopting a proper method of preparation [191], protein modification [192], or by ensuring specific charges on the membrane surface [193].

A strategy worth mentioning consists in using an asymmetric triblock copolymer to force a controlled interaction of the protein with a desired directionality. This is the case with a PEG-*b*-PDMS-*b*-PMOXA triblock copolymer able to self-assemble into polymersomes and which provided a membrane with the right conditions to orient an Aquaporin type pore [194]. Of course, the use of one single strategy might not be sufficient, and most often a combinatorial approach is necessary to obtain the correct orientation of the MPs, which have a final impact on the efficiency of the desired system. In the case of NRs, their efficiency will also be affected in addition by other parameters, such as the self-assembly process of copolymers and proteins, MPs incorporation efficiency, vesicular membrane thickness, and interaction with the polymer membrane etc. Therefore, to fully understand

all these interaction factors, more detailed investigations are necessary both of the synthetic membranes and of the MPs.

3.4. Stimulus-driven permeability or activity without compromising the architecture of the polymer compartment

Small changes in the environmental conditions induced by external stimuli such as light, pH, temperature, presence of a biological molecule (e.g. glucose) affect those compartments self-assembled from "smart" block-copolymers which are specifically designed to change their properties in the presence of a stimulus (Table 5) [195]. Therefore, they cannot be used to obtain polymersomes with both stimuli-responsiveness and preserved architecture. Other approaches should be used to design polymer compartments with a membrane, which, upon a specific stimulus, has an increased permeabilization without affecting their overall architecture over a certain range of conditions. For example, a feasible solution for a concomitant increased stability and permeability has been reported by an appropriate structural design of block copolymers containing motifs triggered by specific stimuli [196, 197]. Following this approach a so-called light-regulated "traceless" crosslinking strategy has been developed (Figure 7, left) [197]. This consists in triggering motifs using amphiphilic block-copolymer based on poly(ethylene oxide)-*b*-poly(2-nitrobenzyloxycarbonyl aminoethyl methacrylate) PEO-*b*-PNBOC containing photolabile carbamate-caged primary amine moieties. These moieties will initially be part of the hydrophobic domain, which under UV irradiation will release the primary amine, initiate amidation reactions, and induce vesicle crosslinking directly correlated with a hydrophobic-to-hydrophilic transition within the membrane. This strategy allows encapsulation of hydrophilic such as doxorubicin hydrochloride (inner cavity) or hydrophobic entities, Nile

red (within the hydrophobic part of the membrane) inside polymersomes, which after the UV-initiated chemical reaction, have been released due to an enhanced permeability of the membrane. By encapsulation of alkaline phosphatase (ALP), these crosslinked polymersomes served to design stable and stimuli-responsive nanoreactors. Once permeabilization of the crosslinked bilayer occurred under UV irradiation, water-soluble non-fluorescent phosphate-caged fluorescein substrate diffused across the membrane and was converted by ALP into a highly fluorescent fluorescein product, which has been monitored by a spectrophotometer (Figure 7, right). The release of a dye labeled dextran (DLD), with five times lower molecular weight than the molecular weight of the enzyme, indicated no ALP leakage from the inner cavity of the vesicles upon membrane permeabilization.

However, once their permeability is increased, it cannot be reversed. Other strategies have been reported to achieve a reversible permeability of the polymer membrane. In order to increase the stability of pH-responsive polymersomes and to prevent their dissociation upon a pH change, incorporation of a crosslinkable comonomer has been proposed. The crosslinkable 3-(trimethoxysilyl)propyl methacrylate) (TMSPMA) was copolymerized with the pH-responsive 2-(diethylamino)ethyl methacrylate (DEAEM) using a poly(ethylene oxide)-based macro-initiator and a standard ATRP protocol, resulting in an amphiphilic, self-crosslinkable, pH responsive block copolymer: PEO-*b*-P(DEAEM-*stat*-TMSPMA) [198]. Although this pH responsive block copolymer did not find application for the design of NRs, a similar system was proposed by employing the polymerizable photo-crosslinker 3,4-dimethyl maleic imidoethyl methacrylate (DMIEM) in the hydrophobic part of the block copolymer, resulting in a PEG-*b*-PDEAEM-*stat*-PDMIEM structure [199]. The

optimal amount of photo-crosslinker that avoids polymersome disassembly upon a pH change was ascertained after thorough investigation. After being irradiated with a mercury lamp for two hours in basic conditions, those polymersomes with <10 mol.% of DMIEM underwent dissociation when the pH was above 7 due to the protonation of the tertiary amine in the DEAEM block. However, those polymersomes with 10 and 15 mol.% of DMIEM were able to reversibly undergo swelling/deswelling when the pH was turned from acidic to basic. What causes the swelling of polymersomes for pH values below 7 is the protonation and subsequent repulsion of the cationic chains in the PDEAEM block. The attribute these polymersome have to swell/deswell at a particular pH was employed to load a hydrophilic dye (rhodamine B). The dye was loaded at a slightly acidic pH, when its diffusion across the membrane is facilitated due to the protonation of the PDEAEM block. Finally, by changing the pH from high to low values, the dye was successfully released. The proposed polymersomes might find application in the biomedical field due to their cyto-compatibility and mechanical stability [200-202]. These polymersomes have been employed as NRs where single [203] or cascade [204] enzymatic reactions have been conducted. 3,4-dimethyl maleic imidobutyl methacrylate (DMIBM) was employed as a photo-crosslinkable unit instead, because of the reduced time required for crosslinking when compared to the aforementioned DMIEM (60 minutes vs. 30 seconds). The reduced time will accordingly protect the functionality and biological activity of the biomolecules encapsulated within the polymersome. As a proof of concept, GOx and myoglobin (Myo) or HRP were encapsulated within the PEG-*b*-PDEAEM-*stat*-PDMIBM polymersomes. GOx turned D-glucose into D-glucono-δ-lactone and hydrogen peroxide, which acted as a co-substrate for Myo to oxidize guaiacol and for HRP to oxidize ABTS, respectively. At a pH of 8, no enzymatic reaction was observed, indicating that at this pH the substrates (D-

glucose and guaiacol) cannot diffuse along the membrane. However, at a pH of 6, when the tertiary amine of DEAEM is protonated and the block turns hydrophilic, while maintaining the stability, the substrates were able to permeate into the membrane initiating the enzymatic reactions. Interestingly, the reaction was abruptly stopped at pH 8, describing a switch between an off (pH 8) and on (pH 6) state of the polymersomes.

Following a similar concept, synthetic nuclear envelope-like vesicles (NEVs) have been proposed [205]. The strategy to encapsulate biomolecules within polymersomes was based on mimicking the gateway connecting the cell nucleus and the cytoplasm in eukaryotes (nuclear envelope-like structures). Photo-crosslinkable coumarin (CMA) was employed to yield PEO-*b*-PDEAEM-*stat*-CMA polymersomes. Due to the nanophase segregation during the polymersome formation, the resulting membrane consists in a CMA-rich matrix with CMA-poor patches. The CMA-poor patches act as giant valves, mimicking the nucleus pore complex and facilitating the diffusion of biomolecules [GOx and hemoglobin (Hb)], by tuning the pH. The cascade enzymatic reaction within the polymersome was confirmed as follows: first, glucose reacts with GOx to yield H_2O_2 and gluconic acid; second, H_2O_2, together with Hb, oxidizes o-phenylenediamine (OPD) forming 2,3-diaminophenazine (DAPN), easily detected via UV-Vis spectroscopy. The proposed approach represents a promising strategy to encapsulate fragile biomolecules (e.g., proteins, RNAs, enzymes, etc.) in pure aqueous solutions triggered by slight pH changes.

PICsomes were also proven to be characterized as pH-responsive, and undergo a reversible structural transition [206]. Upon reduction of the pH of the PICsomes solution, COO^- groups of PEG-PAsp were gradually protonated, resulting in a lower association force between the cationic and anionic counterparts. As a consequence, the permeability of the

membrane was increased and high molecular weight molecules such as myoglobin, Myo (M_n= 70000 g mol^{-1}), have been encapsulated at low pH values (pH=5.8) [207]. After addition of $Na_2S_2O_4$ to Myo-loaded PICsomes, metmyoglobin was reduced to deoxymyoglobin, which was subsequently transformed into oxymyoglobin by the injection of O_2 gas. Interestingly, oxymyoglobin returned to deoxymyoglobin after bubbling the solution with Argon gas, since this oxygenation/deoxygenation is completely reversible and demonstrates the possibility of these systems to act as an oxygen carrier.

Carbon dioxide (CO_2), CO_2/NO_2 and/or pH driven permeability has also been reported in the literature [208-210]. In a particular case, an amphiphilic block copolymer of PEG and CO_2-sensitive poly(N-amidino)dodecyl acrylamide (PAD) was synthesized via ATRP. The hydrodynamic radius (R_h) of the resulting polymersomes increased at a rate of 2.5 nm·min^{-1} when CO_2 was passed through the solution at a flow rate of 1 ml·min^{-1} due to the transition of part of the PAD blocks from unprotonated, entangled state (polyamidine) to a protonated, stretched state (polyamidinium). This enabled the tuning of the thickness and corresponding permeability of the membrane upon CO_2 bubbling, allowing the separation of molecules of different size. Myo was encapsulated in PEG-*b*-PAD polymersomes, and its reaction was monitored over 5 minutes in CO_2 presence, inducing swelling of the vesicles and "opening" of their pores. Opening of the pores allowed the diffusion of glutathione across the membrane. Incubation of oxymyoglobin with tripsin results in complete disappearance of the O_2-binding activity. However, due to the larger size of trypsin compared to glutathione, 15 min of CO_2 bubbling was necessary to allow the reaction.

Amphiphilic dendritic star-block terpolymers consisting of a hydrophobic poly(ε-caprolactone) (PCL) block, an intermediate CO_2/pH-sensitive bridging PDEAEMA block, and a hydrophilic PEG end block self-assemble into vesicular nanostructures which undergo reversible morphological transitions (vesicles, swelled vesicles, macroporous vesicles) upon alternating CO_2/N_2 stimulation [210]. It will be interesting to see a future design of a NR based on this special self-assembly, with the promise it holds for encapsulating biomacromolecules and controlling the passage of subtrates/products via a CO_2-adjustable size of the inner cavity and membrane poration.

A very fruitful strategy for NRs design is to mix conventional amphiphilic block copolymers with stimuli-responsive block copolymers, the latter being the component which upon a certain stimulus poration is induced in the final self-assembled architecture, whilst maintaining its integrity. The use of phenylboronic acid derivatives permits the development of glucose responsive polymers due to the strong binding between boronic acid and monosaccharides through the formation of reversible covalent bonds [211]. PEG-*b*-PS amphiphilic copolymers have been blended with glucose-responsive PEG-b-poly(styrene boronic acid) (PEG-b-PSBA). At a PSBA concentration of ~10%, phase-separated PSBA domains were dispersed along the PS matrix in the membrane. These moieties are disassembled upon increase of the pH to 12.6 or by exposure to D-glucose or D-fructose, generating pores in the membrane of the polymersome and increasing, accordingly, its permeability. For the proof of the concept, CAL B was encapsulated within the aforementioned polymersomes and its reaction with DiFMU octanoate was allowed only after the disassembly of PSBA moieties. Additionally, the permeability of the membrane can be tuned by changing the weight ratio between the two block copolymers.

Of course the stimulus does not necessarily have to influence the permeabilization of the membrane, but can influence the overall activity of the nanoreactor as is the case with the light-responsive NRs containing the encapsulated photosensitizer RB-BSA. This NRs becomes active upon light irradiation and produces ROS *"on demand"*, able to pass through the intrinsic permeability of the PMOXA-*b*-PDMS-*b*-PMOXA membranes for these species (see section 3.1) [156, 157].

3.5. Towards more complex decorated membranes

The membrane of a synaptic vesicle appears as a very crowded environment. It consists of a multitude of attached or inserted macromolecules, each having its own specific functionality (Figure 8). Moreover, each individual macromolecule is subjected to its own conditions for reconstitution, therefore in a biomimetic approach trying to adapt the natural conditions using synthetic polymer membranes is challenging, and perhaps even impossible. Examples of single type membrane proteins inserted in the membrane of polymer vesicles have already been achieved, but only one example for simultaneous insertion of two different MPs has been reported. The insertion of bacteriorhodopsin, a light-driven transmembrane proton pump, and F0F1-ATP synthase, motor protein inside a membrane of PEtOz-PDMS-PEtOz polymersomes has been introduced as a preliminary study [212]. However, to understand the factors favoring a membrane proteins co-insertion, and even to predict the conditions needed to go one step further by insertion of multiple membrane proteins or complexes, an advanced biomimetic approach is needed.

4. Microfluidics as a rapid methodology for optimizing and scaling-up the fabrication of polymer capsules

Conventional fabrication of polymer capsules involves multiple synthetic steps, limited encapsulation efficiency, difficulty in attaining the desired structural and compositional complexity for specific applications and ultimately challenges in scaling up the capsules[75]. This applies particularly to the traditional template-free fabrication of polymer capsules or capsules fabricated using hard or soft sacrificial templates (droplets [213], calcium carbonate [214], silica [215], or biological-based [216]. To overcome these challenges, microfluidics offers the benefit of a mature technology for rapid, monodisperse, highly reproducible and efficient material use for the controlled and high-throughput manipulation of the fabrication of supra-molecular polymer capsules [217-219].

By employing microfluidic devices, the polymer assembly can be driven based on colloidal particles, interfacial condensation, and electrostatic interaction to generate microcapsules with engineered stimuli-responsiveness, tuneable stability, high degree of monodispersity and porosity [220], but also with a high degree of kinetic control over the polymer self-assembly [221].

A typical time of four to five hours or even longer is required for the conventional preparation of templated LbL capsules with five to six polyelectrolyte layers. In contrast, the fabrication of PAA/poly(N-vinypyrrolidone) (PVPON) microcapsules containing six deposited controlled thickness-layers of polyelectrolyte can be achieved in an impressive period of less than three minutes by employing a droplet microfluidic pinball device[222]. The crucial multiple washing steps involved in the conventional LbL technique is also reduced to a single step, considerably contributing to a reduction in the preparation time,

with the only limitation being the limited efficiency in terms of the number of microcapsules generated.

Another huge advantage of microfluidics is the ease of random chip design simulation and functional implementation based on specific applications with minimal training requirements (for a useful library database, see: http://random.groverlab.org) [223]. Although microfluidics has proven to be a versatile technique for the fast generation of polymer capsules, its technological scalability remains a limitation. The technology is not yet fully controllable. This is because complete removal of the template from the shell is not always satisfactorily achieved especially in the particular case of conventional microfluidic approaches, such as single (O/W) or double (O/O/W) emulsions [224]. To achieve an optimal blood circulation half live, the size of the final polymer should be in range of 50-200 nm [120]. As most of the capsules obtained using microfluidics are in the micro-sized range, more progress is required to achieve capsules with a diameter in the nanoscale. By employing a 3-channel (one main middle and two side channels) microfluidic setup, a PEG-*b*-PS diblock copolymer solubilized in THF/dioxane was injected into the main middle channel, while water was injected into both side channels [225]. Upon mixing the two different phases, a solution gradient flow was generated initiating the block copolymer self-assembly. Interestingly, variation in initial polymer concentration (1-10 mg/mL), the mode of collection, and the fluid flow rate between the main and side channels dictated the formation of polymer stomatocyte-like vesicles with sizes of 120-200 nm, polydispersity indexes of 0.092 – 0.220, with loading capacity for drugs (e.g., DOX) making them feasible for biomedical applications.

Microfluidics has also been proved as a useful technique for the development of very attractive microreactor systems with increased complexity for biomedical applications such as biosensors for implantable device-based detection [226] (Figure 9). Firstly, glucose oxidase was conjugated either on Quantum Dots (QD-GOx) or on gold nanorods (Gold NR-GOx) to act as glucose nanosensors, while NRs were also conjugated with amine groups (NRs-NH_2) to sense heparin, following their encapsulation in PEG-based microreactors, to protect and preserve the sensor activity. Secondly, the shell of the microcapsules prevented leakage from the nanosensors but allowed permeation of the biomolecules of interest. Permeability of the shell to glucose (MW 180 Da) was tested via exposing the microreactor to a 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-Amino)-2-deoxyglucose (2-NBDDG, MW 342) fluorescent analogue as visualized by confocal microscopy and quantified with fluorescence recovery after photobleaching (FRAP).. Larger molecules, such as heparin (MW 10 kDa) permeated in approximately one hour through the shell, while streptavidin-FITC (MW > 60 kDa) took one week until a fluorescence signal was observed within the inner core of the microcapsule. Glucose was oxidized by GOx leading to the production of gluconic acid and H_2O_2. Both products induced a decrease in the zeta-potential of encapsulated NR-GOx and their aggregation, while H_2O_2 quenched the fluorescence of encapsulated CdSe/ZnS QD. Similarly, heparin induced the aggregation of encapsulated NR-NH_2. These exciting results clearly demonstrate the biosensing ability and the promising versatility of microfluidics for the preparation of micro reactors for therapeutic applications.

5. Current challenges and future directions

The use of polymer capsules as micro-/nanoreactors relies on, but is not limited to, an efficient encapsulation of active biomacromolecules (including enzymes and enzyme mimics), the preservation of their biological activity, spatial control over the encapsulated species [227] size, morphology, flexibility (such as the ability of the capsule to contract) and capsule stability. These are all critical factors which should be considered when designing these systems regardless of their preparation strategy (absence or presence of a sacrificial template) for therapeutic applications. Considering the complexity of biological entities in terms of pH-, temperature-, ionic strength- and solvent-dependence, the development of a universal protocol to encapsulate bio-active catalysts in polymer capsules remains a challenging task. In the case of polymer capsules prepared employing a sacrificial template, those methods based on extreme changes of conditions to allow the post-loading of particular catalysts may not be valid for other catalysts. Thus, in our opinion, those templates that allow the preloading of catalysts are more promising for the fabrication of polymer capsules acting as micro-/nanoreactors; however, even in these cases, the loading efficiency is limited by the size and capacity of the template to capture the species to be encapsulated. In the case of polymer capsules prepared without a sacrificial template such as polymersomes, encapsulation efficiency is still an issue [228]. Moreover, the concentration of the catalyst should be balanced, as too high of an initial concentration of the enzyme impacts the self-assembly of the polymer capsules [229], as is the case with PMOXA-*b*-PDMS-*b*-PMOXA polymersomes, prepared without the need of a sacrificial template [13].

Kinetic Monte Carlo simulations have demonstrated that activity of cascade reactions under diffusion- and reaction-limited conditions was greatest when enzymes were arranged randomly at high densities [230]. Even though this was shown by random immobilization of enzymes in two-dimensional scaffolds, this could be extended to polymer capsules where the achievement of optimal activity based on dense packing and close arrangement of the catalytic sites is desirable.

Size, morphology or flexibility will impact upon the fate of capsules, which is not yet fully understood, within a living organism while these parameters will influence the capsules ability to escape from the reticuloendothelial system's uptake. Small capsules are more difficult to properly control and more challenging to characterize (especially for very dilute or nanometre scaled objects). Additionally, micrometer sized capsules are challenging for the secretory system, due to the release of cargo in higher amounts which imposes hydrostatic pressure and thus instability on nearby systems [231, 232].

For the intended use of polymer capsules as injectable micro-/nanodevices, their surface characteristics need to be finely tuned to avoid non-specific interactions with biomolecules and to improve their blood circulation time [233, 234]. Polymers such as PEG, PVPON or thiol-containing poly(2-ethyl-2-oxazoline) ($PEtOxMA_{SH}$) exhibit improved non-fouling properties due to their lack of electrostatic charge. Besides, specific targeting properties can be conferred to polymer capsules by the incorporation of antibodies, peptides, aptamers, on their surface. Even though promising results have been reported in this regard, it is only those strategies that result in low signal-to-background ratios and which are simple to construct that will find real life applications for the development of polymer capsules with selective targeting of cell surface receptors [235].

The key challenge in the field remains the need to develop synthetic systems which mimic biomimetic approaches to facilitate the translation of robust synthetic systems for therapeutic applications. Even though considerable progress has been made in the fabrication of polymer capsules acting as micro-/nanoreactors, these systems still lack the complexity of biological cells, where multiple bio-chemical reactions are simultaneously executed in response to the microenvironment. To overcome this problem, the development of multicompartment systems via liposomes-in-liposomes (vesosomes), polymersomes-in-polymersomes, capsules-in-capsules, polymersomes-in-liposomes, polymersomes-in-capsules, liposomes-in-polymersomes (capsosomes), are being extensively explored [236]. Most of the enzymatic cascade reactions reported in the literature to date are not therapeutically relevant and represent theoretical proof of concept research studies without a clear pathway to clinical translation for specific applications. Therefore, to promote the clinical translation of polymer capsules as therapeutic micro-/nanoreactors, significant efforts are required in the near future to validate the performance of polymer capsules in biological systems, both *in vitro* and *in vivo* [13, 47, 141, 144, 237].

6. Conclusions

Recent progress in polymer synthesis allows the development of (co)polymers with controlled properties (chemical composition, molecular weight, or hydrophilic-to-hydrophobic block length ratio). This opens the possibility of preparing polymer capsules to act as micro-/nanoreactors or artificial organelles. Inside the inner cavity of these polymer capsules, active biomolecules (e.g. enzymes) are protected and act *in situ*, due to a continuous transfer of biologically relevant entities (substrates/products) through a shell/membrane that can be rendered selectively permeable via the several strategies

elucidated within this review. These micro-/nanocapsules function without any compromise in their mechanical integrity.

Polymer capsules prepared with the aid of a sacrificial template generally undergo complete dissociation/rupture during their application, limiting their potential use as micro-/nanoreactors. Additionally, the proposed strategies to post-encapsulate active compounds (mainly enzymes) are usually based in pH or solvent changes, both adversely affecting the activity of the enzyme because of possible denaturation. To overcome this, pre-encapsulation of the active compound via the co-precipitation method in calcium carbonate template might be a promising alternative in terms of increased encapsulation efficiency and preserved enzymatic activity. In any case, examples of polymer capsules fabricated with the aid of a sacrificial template as micro-/nanoreactors for therapeutic applications are scarce with efforts still ongoing.

The use of polymer vesicles (polymersomes), without the aid of a sacrificial template and based on the self-assembly of block copolymers in aqueous solutions, allow instead mild conditions for the preparation of micro-/nanoreactors, which is a prerequisite for the preservation of the activity of biomolecules. Although enormous progress has been achieved in recent years, challenges still exist in terms of controlling membrane permeability, increasing the encapsulation efficiency of active cargo, and especially towards the development of more complex biomimetic compartments with multiple functionalities.

The design of micro-/nanoreactors, scaling-up, optimization and their implementation in dysfunctional cells as artificial organelles may in the near future represent an effective strategy to deal with several pathologies, though still more exciting progress in this field is

anticipated, as now the first *in vivo* reports of active and stable nanoreactors are starting to appear.

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Appendix: List of abbreviations

7-ADCA	7-aminodesacetoxycephalosporanic acid
ABTS	2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
ADH	Alcohol dehydrogenase
AEC	3-amino-9-ethyl carbazole
Alg	Alginate
ANG-2	Asante natrium green-2
APG-2	Asante potassium green-2
Apy	4-aminopyridine
ATRP	Atom transfer radical polymerization
BSA	Bovine serum albumin
BLA	β-lactamase
BP	Benzophenone
CAT	Catalase
CAL B	Candida Antarctica Lipase B
Chi	Chitosan
CMA	Coumarin
CPO	Caldariomyces fumago
CRL	Candida Rugosa Lipase
DAPN	2,3-diaminophenazine
DAR	Diazoresin
DEX	Dextran
DiFMU	6,8-difluoro-4-methylumbelliferyl
DIP	Dipyridamole
DLS	Dynamic light scattering
DMIBM	3,4-dimethyl maleic imidobutyl methacrylate
DMIEM	3,4-dimethyl maleic imidoethyl methacrylate
DOX	Doxorubicin
EDC	1-ethyl-3-(3-dimethylaminopropil)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron paramagnetic resonance
FCS	Fluorescence correlation spectroscopy

FGF	Fibroblast growth factor
GA	Glutaraldehyde
GdnHCl	Guanidine hydrochloride
GOx	Glucose oxidase
HA	Hyaluronic acid
Hb	Hemoglobin
HEBIB	2-hydroxyethyl-2-bromoisobutyrate
HRP	Horseradish peroxidase
IAA	Isocyano-L-alanine-L-alanine
IAH	Isocyano-L-alanine-L-histidine
LbL	Layer-by-layer
LPO	Lactoperoxidase
MF	Melamine formaldehyde
MNGIE	Mitochondrial neurogastrointestinal encephalomyopathy
MRI	Magnetic resonance imaging
Myo	Myoglobin
NEV	Nuclear envelope-like vesicles
NHS	N-hydroxysulfosuccinimide
NR	Nanoreactor
OPD	o-phenylenediamine
PAA	Poly(acrylic acid)
PAD	Poly(N-amidino)dodecyl acrylamide
PAH	Poly(allylamine hydrochloride)
PAMO	Phenylaceton monooxygenase
PAr	Polyarginine
P(Asp)	Poly(α,β-aspartic acid)
P(Asp)-AP	Poly(2-aminopentyl-α,β-aspartamide)
PB	Poly(butadiene)
PBLA	Poly(β-benzyl-L-aspartate)
PCEMA	Poly(2-cinnamoylethyl methacrylate)
PDADMAC	Poly(diallyldimethylammonium) chloride
PDDA	Poly(diallyldimethylammonium chloride)
PDEAEM	Poly(diethylaminoethylmethacrylate)
PDMAEMA	Poly(dimethylaminoethylmethacrylate)

PDMS	Poly(dimethylsiloxane)
PEG	Poly(ethylene glycol)
PEGA	Poly(ethylene glycol) methyl ether acrylate
PEI	Poly(ethyleneimine)
PEO	Poly(ethylene oxide)
PEtOz	Poly(2-ethyl-2-oxazoline)
PGK	Phosphoglycerate kinase
PGME	Phenylglycine methyl ester
PIAT	Isocyano-L-alanyl-amino-ethyl-thiopene
PIB	Poly(isobutylene)
PLL	Poly(lysine)
PMA	Poly(methacrlylic acid)
PMETAI	Poly[2-(methacryloyloxy)ethyl]trimethylammonium iodide
PMOXA	Poly(2-methyl-2-oxazoline)
PNBOC	Poly(2-nitrobenzyloxycarbonyl aminoethyl methacrylate)
PNIPAm	Poly(N-isopropylacrylamide)
PNVP	Poly(N-vinylpyrrolidone)
PPDSM	Pyridyldisulfideethylmethacrylate
PP-OH	2-hydroxy-4´-2-(hydroxyethoxy)-2-methylpropiophenone
Pro	Protamine
PS	Poly(styrene)
PSBA	Poly(styrene boronic acid)
PSS	Poly(styrene sulphonate)
PUA	Poly(urethane-amine)
PVPON	Poly(vinyl pyrrolidone)
RAFT	Reversible addition-fragmentation chain transfer
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TMB	3,3´,5,5´-tetramethylbenzidine
TMSPMA	3-[(trimethoxysilyl)propyl methacrylate]
TP	Thymidine phosphorylase
TvNH	Trypanosoma vivax
WST-1	(2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-(2H-tetrazolium)

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Vitae

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Aitor Larrañaga received his PhD degree in Engineering in Advanced Materials from the University of the Basque Country (Spain) in 2014. Since then, he joined the Centre for Research in Medical Devices (CÚRAM) in Galway (Ireland) as a Postdoctoral Researcher under the supervision of Prof. Abhay Pandit. His research work is focused on the development of polymer microreactors with theranostic properties for the treatment of several degenerative diseases.

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Mihai Lomora studied Environmental Engineering at the Transilvania University of Brasov, Romania, and received his MSc degree in 2011. He obtained a PhD in Chemistry under the supervision of Prof. Dr. Cornelia G. Palivan, at the Department of Physical Chemistry in Basel. Currently, he is working on the development of novel strategies in tissue engineering for the treatment of ischemic diseases under the supervision of Prof. Abhay Pandit at the Centre for Research in Medical Devices (CÚRAM) in Galway (Ireland).

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Cornelia Palivan
Cornelia G. Palivan is Professor in Physical Chemistry at the University of Basel. She received several awards for her research. Her domain of interest is based on development of hybrid materials (nanoreactors, active surfaces, artificial organelles, nanodevices, functional membranes) by combining biomolecules or mimics with synthetic supramolecular assemblies for medical, environmental, technological, and food science oriented applications.

Abhay Pandit
Prof Abhay Pandit is the Science Director of the SFI Centre for Research in Medical Devices (CÚRAM), a multi-disciplinary academic-industry-clinician translational research centre. His research interests' focus on the development of next generation targeted hollow controlled-drug-release reservoir delivery vehicles which facilitate spatiotemporal localised sustained delivery of biomolecules to target injury mechanisms at the molecular and cellular levels. These macromolecular complexes form functional interfaces between implanted devices and biological systems to endow the former with bio-responsiveness and/or biological function. He is a Fellow of the Tissue Engineering and Regenerative International Society and an International Fellow in Biomaterials Science and Engineering.

Figure Captions

Figure 1: Schematic representation of the fabrication of polymer capsules with the aid of a sacrificial template.

Figure 2: Schematic representation of those strategies that allow the control of permeability of polymer capsules fabricated with the aid of a sacrificial template.

Figure 3: Concept of a nanoreactor and artificial organelles for promising therapeutic applications.

Figure 4: Polymer nanoreactors for "*in situ*" production of reactive oxygen species "on demand" (A) when up-taken by cells (B) and irradiated with the appropriate wavelength. HeLa cells treated with nanoreactors before (C) and after irradiation (D). White arrows indicate formation of blebs, as a sign for apoptosis (D). Scale bar: 20 μm. Adapted by permission of The Royal Society of Chemistry from [156].

Figure 5: Schematic design of β-galactosidase-loaded PICsome based nanoreactors and their corresponding TEM showing 100 nm vesicular structures (A); *In vivo* imaging (top) and *ex vivo* imaging of the excised tumor and organs (bottom) depicting accumulation of NRs in C26 tumor mouse; β-galactosidase-loaded NRs (blue) and β-galactosidase activity based on the time-dependent change in relative fluorescence intensity in the tumors of treated mice. Adapted with permission from ref. [15]. Copyright (2016) John Wiley and Sons.

Figure 6: Smart hybrid membranes with induced stimuli-responsiveness through insertion of ion channels (in this case, an amphiphilic helical one), ion carriers, or membrane proteins to induce selective permeability to ions or biomolecules (A). The membrane of the polymer vesicle is able to compress (B) and an increase in thickness induces a reduction in the relative, effective diffusion coefficient, D_{MP}/D_{eff} (C), as experimentally observed with specific model ion channels/membrane proteins (KcsA, AqpZ and OmpF). Adapted with permission from ref. [168]. Copyright (2016) American Chemical Society.

Figure 7: Simultaneous crosslinking and permeabilization of polymersomes based on PEO-*b*-PNBOC block copolymers, triggered by UV irradiation (left). Encapsulation of ALP enzymes inside polymersomes (a) and enzyme activity monitored by the formation of a fluorescent product (b and c) before (black curve) and after (red curve) UV-induced permeabilization. Reprinted with permission from ref. [197]. Copyright (2016) John Wiley and Sons.

Figure 8: Half-sectioned model of a synaptic vesicle containing space-filling models of all macromolecules at near atomic resolution. Reprinted from ref. [238], with permission from Elsevier.

Figure 9. Schematic depicting the principle of microcapsules with encapsulated QD-GOx and their sensing ability for glucose: Glucose permeates the shell of the microcapsule, and it is converted by GOx to gluconic acid and H_2O_2. H_2O_2 quenches the intrinsic fluorescence of encapsulated QD-GOx (A). Proof of principle for the glucose biosensor: Quenching of fluorescence intensity of QD-GOx in solution (B) and QD-GOx inside microcapsules (C) without (blue curve) and in the presence of 10 mM glucose (red curve) via fluorescence spectroscopy. Similar effect as in (C), but visualized in real time over 30 min via confocal microscopy. Scale bar: 500 μm (D). Schematics depicting the principle of microcapsules with encapsulated NR-GOx and their sensing ability for glucose (E) and with encapsulated NR-NH_2 and their sensing ability for heparine (F). 6 hrs exposure of NR-GOx in solution (G, left) and NR-GOx encapsulated inside microcapsules (G, right) to 100 mM glucose (G, red band) induced a significant change in the surface plasmon resonance (SPR) band as compared with lack of glucose (G, blue band). 3 hrs exposure of NR-NH_2 in solution (H, left) and NR-NH_2 encapsulated inside microcapsules (H, right) to 0.1 mg/mL heparin (H, red band) induced a significant change in the SPR band as compared to lack of heparin (H, blue band). Reprinted with permission from ref. [231]. Copyright (2017) American Chemical Society.

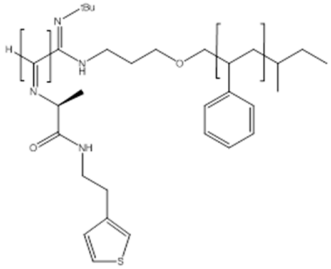
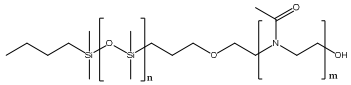
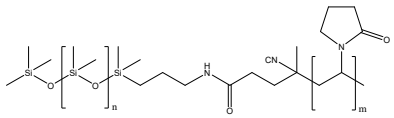
Table 1: Polymer capsules prepared with the aid of a sacrificial template that find potential application as micro-/nanoreactors for therapeutic applications

Polymer(s)	Encapsulated entity	Encapsulation strategy	Variable parameter	Proposed application	Outcome
PSS/PAH	α-chymotrypsin [57]	Electrostatic interaction with PSS/MF core+opening polymeric shell at low pH	• Effect of pH on encapsulation efficiency.	Microcontainers for bioreactions	No *in vitro/in vivo* results are reported.
	Urease [52, 103]	Opening polymeric shell in the presence of ethanol		Biomineralization processes	
PMA/PVPON	DNase I [25]	Infiltration in mesoporous silica template	• Effect of divalent ions (Mg^{2+}, Ca^{2+}) on the enzymatic activity of DNase I.	Mimicry of cell processes	No *in vitro/in vivo* results are reported.
PSS/Pro	CAT [82]	Coprecipitation in $CaCO_3$ template	• Effect of number of layers and external silica layer on enzyme leakage.	H_2O_2 scavenging microreactors	No *in vitro/in vivo* results are reported.
DEX/Pro	HRP [56]	Electrostatic interaction with PSS/MF core+swelling polymeric shell at high pH	• Effect of pH and number of layers in enzyme leakage.	Analytical systems and bioseparation	No *in vitro/in vivo* results are reported.
DEX/PArg	L-asparginase [47]	Coprecipitation in $CaCO_3$ template	• Thermal and proteolytic resistance of enzyme upon encapsulation.	Treatment of blood cancer	*In vitro*, leukemic cells are driven to apoptosis in the presence of polymer capsules loaded with L-asparginase.
PSS/PDADMAC	HRP [55]	Electrostatic interaction with PSS/MF core	• Effect of temperature on the activity of free *vs*. encapsulated enzyme.	Microreactors for biocatalysis	No *in vitro/in vivo* results are reported.
PSS/DAR	GOx [84]	Entrapment after UV crosslinking		Enzyme biosensor	No *in vitro/in vivo* results are reported.
PDEAEM-co-PPDSM	Myo [24]	Opening polymeric shell at low pH	• Effect of pH and crosslinker on permeability of the polymer shell.	Microcontainer of oxidative bioreactions	No *in vitro/in vivo* results are reported.
Polydopamine	CAT [87],[88],[89] CAT+GOx Candida Rugosa Lipase	Coprecipitation in $CaCO_3$ template	• Effect of pH and temperature on the activity of free vs. encapsulated enzyme. • Effect of dopamine concentration on shell thickness and thermal stability of the capsules.	Microcontainers for bioreactions	No *in vitro/in vivo* results are reported.

Table 2. Commonly employed sacrificial templates for the fabrication of polymer capsules

Sacrificial template	Advantages	Disadvantages
MF	• Commercially available in a wide range of sizes (500 nm-12 µm) and low polydispersities.	• Requires HCl or organic solvents (DMF, DMSO) to remove the template. • Osmotic pressure during template removal may results in the decomposition of the membrane. • Difficult to completely remove the template. • Inability to preload the active component.
PS/PMMA	• Commercially available in a wide range of sizes (100 nm-300 µm for PS/100 nm-200 µm for PMMA) and low polydispersities. • Tuneable surface charge. • Available with several surface functionalities (carboxyl, hydroxyl, sulfate or amine groups).	• Requires organic solvents (THF, DCM) to remove the template. • Osmotic pressure during template removal. • Difficult to completely remove the template. • Inability to preload the active component.
Mesoporous silica particles	• Commercially available in a wide range of sizes (50 nm-15 µm) and controlled pore size/structure. • High surface area (1500 $m^2 g^{-1}$)/loading capacity. • Possibility to preload the active component. • Complete removal of the template.	• Requires hydrofluoric acid to remove the template.
Calcium carbonate particles	• High surface area (1500 $m^2 g^{-1}$)/loading capacity. • Possibility to preload the active component. • Mild conditions to remove the template (EDTA solution). • Complete removal of the template.	• Poor control over the particle size and distribution. • Difficult to obtain nanosized particles.
Hydrogel microspheres (calcium alginate and dextran)	• Possibility to preload the active component. • Mild conditions required to remove the template (EDTA solution for calcium alginate/aqueous solution for dextran).	• Poor control over the particle size and distribution due to the fabrication process (emulsion). • Osmotic pressure during template removal may result in the decomposition of the membrane.. • Difficult to completely remove the template.

Table 3: Amphiphilic block copolymer showing intrinsic permeability, or by means of a chemical modification, and their potential applications as NRs.

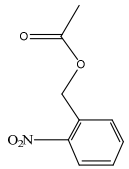
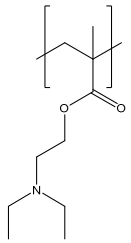
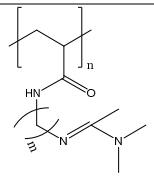
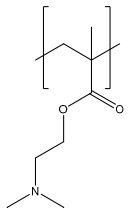
Block copolymer	Chemical structure	Encapsulated entities	Permeable to	Variable parameter	Proposed application	Outcome
PS-*b*-PIAT [148-154, 239]		CAL B, GOx, HRP, ADH, CPO, SOD, CAT	DiFMU octanoate, lactones, glucose, H_2O_2, primary alcohols, ester compounds, $O_2^{\cdot-}$, pyrogalloll, thioanisole, ABTS	• Location of encapsulated enzyme (aqueous cavity vs. bilayer). • Effect of pH in enzymatic activity (HRP-GOx) • Effect of hydrophilic block (PIAT vs. PEG) on the diffusion of $O_2^{\cdot-}$ and H_2O_2	Ring-opening polymerization, cell mimicry, enantioselective epoxidation, ROS scavenging	No *in vitro/in vivo* results are reported.
PMOXA-*b*-PDMS-*b*-PMOXA [156, 157, 160, 161, 240],[162, 163]*,		SOD, PGK, BLA, HRP, RB-BSA	$O_2^{\cdot-}$, GdnHCl, HEBIB (initiator)*, PEGA (monomer)*, ABTS*, pyrogallol*, TMB*, AEC*, H_2O_2*	• Effect of the length of the hydrophobic block on permeability. • Effect of photoreaction on the permeability of the membrane.	ROS scavenging, PDT, protein-folding studies, enzyme replacement therapy, ATRP, controlled release	No *in vivo* results are reported
PNVP-*b*-PDMS-*b*-PNVP [155, 157, 241]		Ceria nanoparticles, Lac, RB-BSA	$HO^{\cdot}$, $O_2^{\cdot-}$, O_2	• Effect of hydrophilic to hydrophobic ratio on ROS scavenging capacity and cellular uptake.	ROS scavenging, ROS production	Viability of HeLa cells exposed to oxidative stress via paraquat was higher after treatment with antioxidant polymersomes. ROS production was less effective than with NRs based on PMOXA-*b*-PDMS-*b*-PMOXA.
PEG-*b*-PAsp and Homo-P(Asp-AP) [15]		β-galactosidase	HMDER- β Gal, HMDER		enzyme prodrug therapy	Selective accumulation and activity for 4 days after administration in C26 tumors subcutaneously inoculated into mice

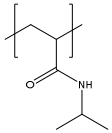
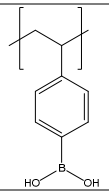
*if the membrane is chemically modified by photoreaction with PP-OH

Table 4: Protein channels and biopores incorporated in the membrane of amphiphilic block copolymers and their potential application as nanoreactors.

Protein channel	Block copolymer	Encapsulated entities	Permeable to	Variable parameter	Proposed application	Outcome
OmpF [13, 175, 177-181, 183-185, 242]	PMOXA-*b*-PDMS-*b*-PMOXA	Acid phosphatase, TvNH, SOD, LPO, Hb, CAT, Penicillin acylase, β-lactamase	Phosphatase substrate, inosine, adenosine, guanosine, 2-fluoroadenosine, $O_2^{\cdot-}$, peroxynitrite, acridine orange, 7-ADCA, PGME, ELF 97, ampicillin	• Effect of protein channel to polymer ratio on the permeability of the membrane. • Effect of incorporating protein channels on the permeability of the membrane.	Drug delivery, ROS scavenging, oxygen transport, development of active surfaces	• *In vitro*, SOD-LPO loaded polymersomes detect intracellular ROS in THP-1 cells via resofurin formation. • Viability of HeLa cells exposed to oxidative stress via paraquat was higher after treatment with antioxidant polymersomes. • Polymersomes uptaken by HeLa cells stimulated with pyocyanin were able to detoxify ROS. • Surfaces functionalized with polymersomes inhibit bacterial growth *in vitro*.
AqpZ [165, 187, 188]	PMOXA-*b*-PDMS-*b*-PMOXA PB-*b*-PEO		H_2O	• Effect of protein channel to polymer ratio on aggregate morphology. • Effect of crosslinking on membrane permeability.	H_2O purification	No *in vitro/in vivo* results are reported.
Tsx [179, 189]	PMOXA-*b*-PDMS-*b*-PMOXA	TvNH	inosine, adenosine, guanosine, 2-fluoroadenosine, thymidine, deoxyuridine	• Effect of protein channel (OmpF *vs.* Tsx) on membrane permeability.	Drug delivery, MNGIE	*In vitro*, polymersomes are not cytotoxic to hepatocytes and do not induce macrophage-induced inflammatory response neither *in vitro* nor *ex vivo*.
LamB [190]	PMOXA-*b*-PDMS-*b*-PMOXA				DNA translocation	*In vitro* λ phage DNA can be translocated across the membrane of the polymersome.
FhuA [174]	PMOXA-*b*-PDMS-*b*-PMOXA	Polylysine, HRP	Sulforhodamine B, TMB		Electrostatic trap	No *in vitro/in vivo* results are reported.
gA [171]	PMOXA-*b*-PDMS-*b*-PMOXA	Pyranine, ANG-2, APG-2	Na^+, K^+, H^+	• Effect of membrane thickness on protein channel incorporation.	Cell mimicry	No *in vitro/in vivo* results are reported.

Table 5: “Smart” amphiphilic block-copolymers specifically designed to change their properties in the presence of a stimulus and their potential use as nanoreactors.

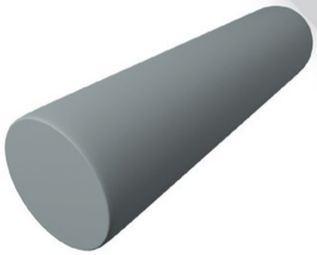
Block copolymer	Stimulus	Chemical structure (responsive unit)	Encapsulated entities	Permeable to	Variable parameter	Proposed application	Outcome
PEO-*b*-PNBOC [197]	UV		Nile red, Dox·HCl, dye labeled dextran, ALP	Nile red, Dox·HCl, phosphate-caged fluorescein, fluorescein	• Location of encapsulated entity (aqueous cavity vs. bilayer). • Effect of UV on the crosslinking and permeability of the membrane.	controlled triggered release, light-switchable biocatalysis of enzyme entrapped vesicle nanoreactors	No *in vitro/in vivo* results are reported.
PEG-*b*-PDEAEM-*stat*-PDMIEM/ PEG-*b*-PDEAEM-*stat*-PDMIBM/ PEG-*b*-PDEAEM-*stat*-BMA/ PEG-*b*-PDEAEM-*stat*-TMSPMA/ PEO-*b*-PS-*b*-PDEAEM/ PEO-*b*-PDEAEM-*stat*-CMA/ PAE-g-PEGLA/ PEG-P(Aps): PEG-P(Asp)-AP/ PEG-P(Aps):PEG-P(Asp)-AE [2, 198-207, 243-245]	pH		Myo, Rhodamine B, doxorubicin, GOx, HRP, Hb, O_2	Guaiacol, H_2O_2, glucose, ABTS	• Effect of pH on membrane structure and corresponding permeability. • Effect of hydrophilic to hydrophobic ratio on aggregate morphology. • Effect of pH on hydrodynamic radius and drug release kinetics. • Effect of crosslinking on membrane permeability. • Effect of crosslinking in cell viability.	Drug delivery, nuclear envelope-like vesicles, oxygen carrier, bioreactors	Developed polymersomes are not cytotoxic for MCF-7 cells *in vitro*.
PEG-*b*-PAD [208]	CO_2		Myo	GSH, O_2, Trypsin, PEI	-	Drug delivery, biochemical reactions	No *in vitro/in vivo* results are reported.
220P-star-PCL-*b*-PDEAEMA-*b*-PEG	pH/CO_2		RB	RB, other molecules expected (to be tested)	• Effect of CO_2 and N_2 on the level of morphological transition of the self-assembled nanoarchitecture • PCL (Tg~−60 °C) expe□ted to induce new morphologies	Nanocarrier, controlled release, mimicking nucleus pore complex and breathing process	No *in vitro/in vivo* results are reported.

Block copolymer	Stimulus	Chemical structure (responsive unit)	Encapsulated entities	Permeable to	Variable parameter	Proposed application	Outcome
PCEMA-*b*-PNIPAm/ PEO-*b*-PAA-*b*-PNIPAm/ PEO-*b*-PNIPAm [246-249]	Temp.		Apy, Dextran		• Effect of temperature on hydrodynamic radius and drug release kinetics. • Effect of molecular weight of PNIPAm on polymersome size and distribution • Effect of crosslinking on reversibility of structural changes.	Drug delivery	No *in vitro/in vivo* results are reported.
PEG-*b*-PS:PEG-*b*-PSBA [211]	Glucose		CAL B	DiFMU octanoate	• Effect of the ratio of the two block copolymers on the permeability of the membrane.	Bioreactors	No *in vitro/in vivo* results are reported.

SACRIFICIAL TEMPLATE

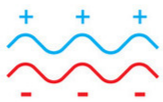
Determines size, shape, encapsulation method.

Organic	MF PS PLGA
Inorganic	$CaCO_3$ $MnCO_3$ SiO_2

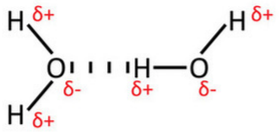


LbL DEPOSITION

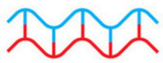
Electrostatic



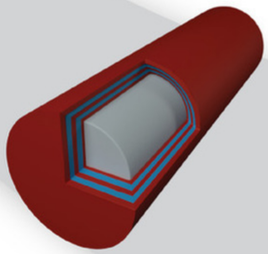
H-bonding

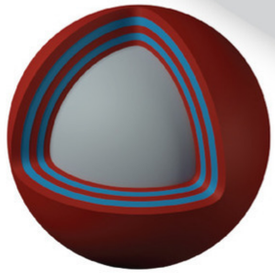


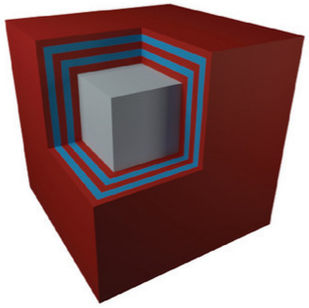
Covalent bonding



Single polymer assembly

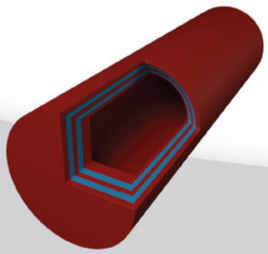


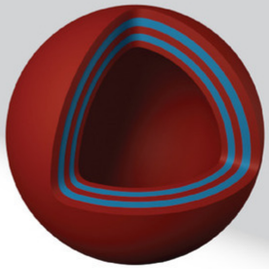


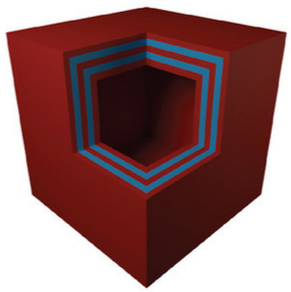


TEMPLATE REMOVAL

pH (MF), organic solvents (PS, PLGA), HF (SiO_2), EDTA ($CaCO_3$)

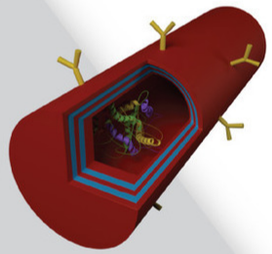


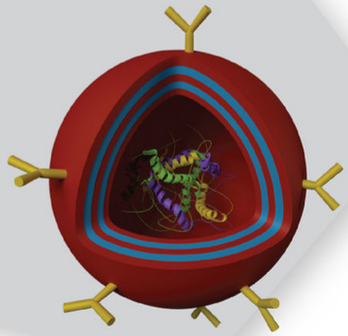


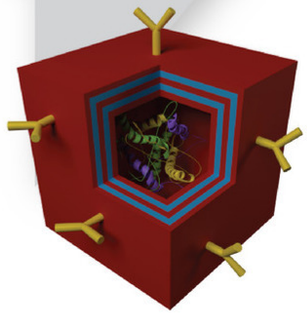


ENCAPSULATION & FUNCTIONALISATION

Nanoparticles, proteins, enzymes, antibodies

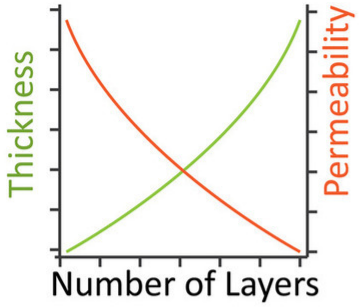




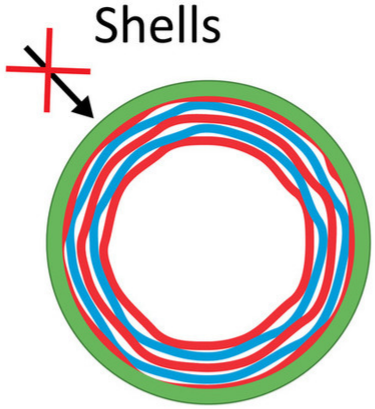


LIMITING THE PERMEABILITY

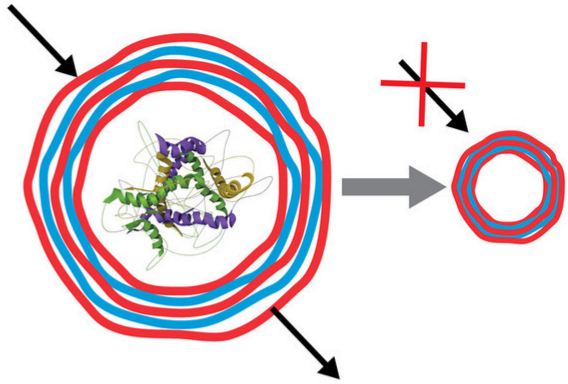
Number of Layers

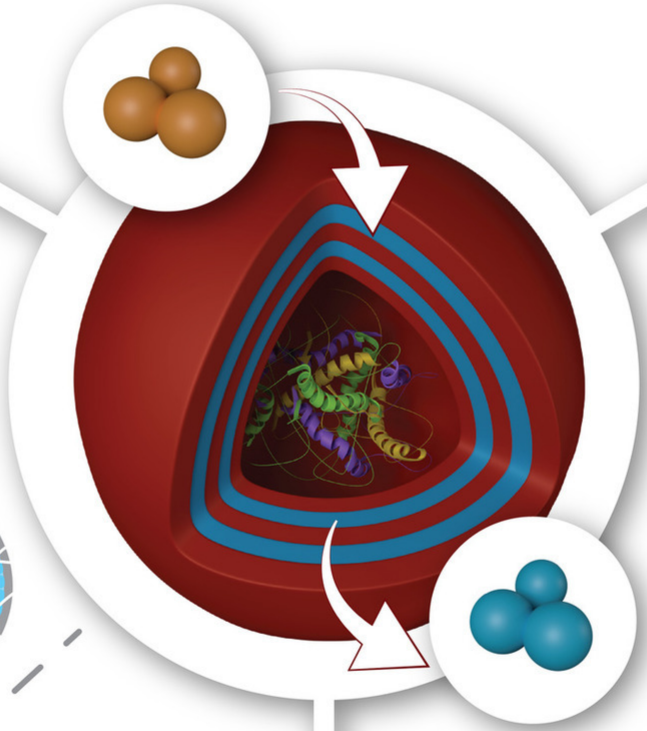


Composite Shells



Crosslinking

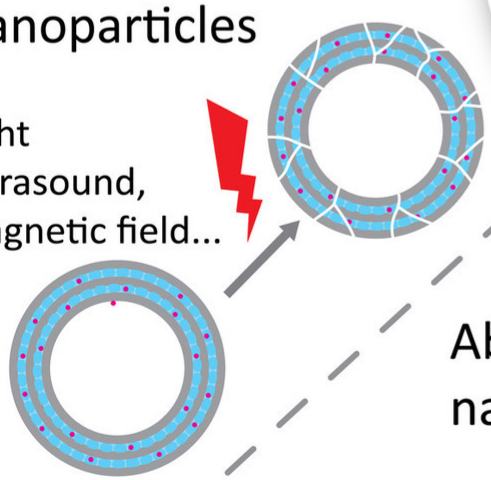




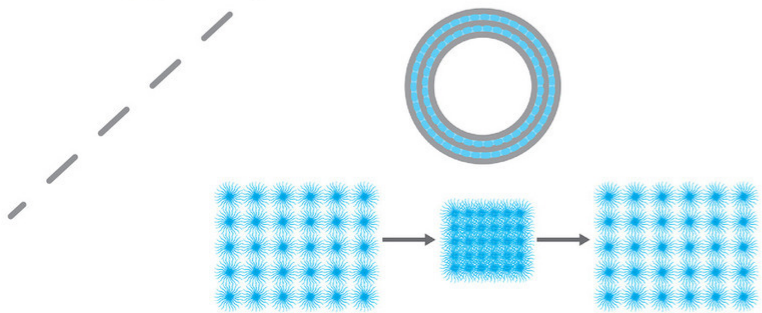
REMOTE CONTROL OF PERMEABILITY

Incorporation of Nanoparticles

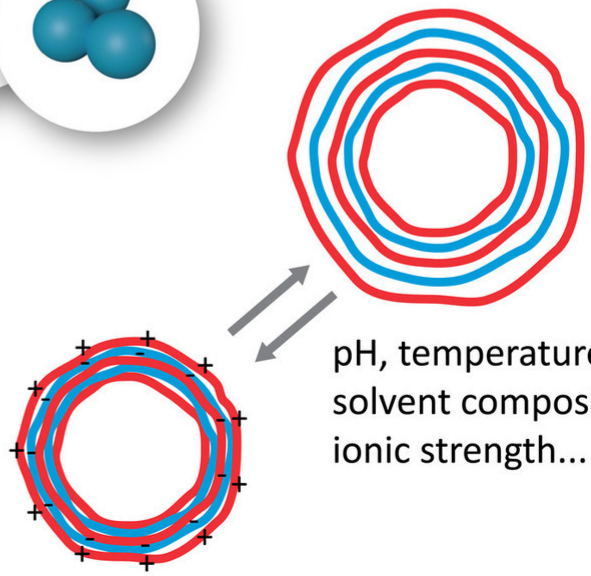
Light
Ultrasound,
Magnetic field...



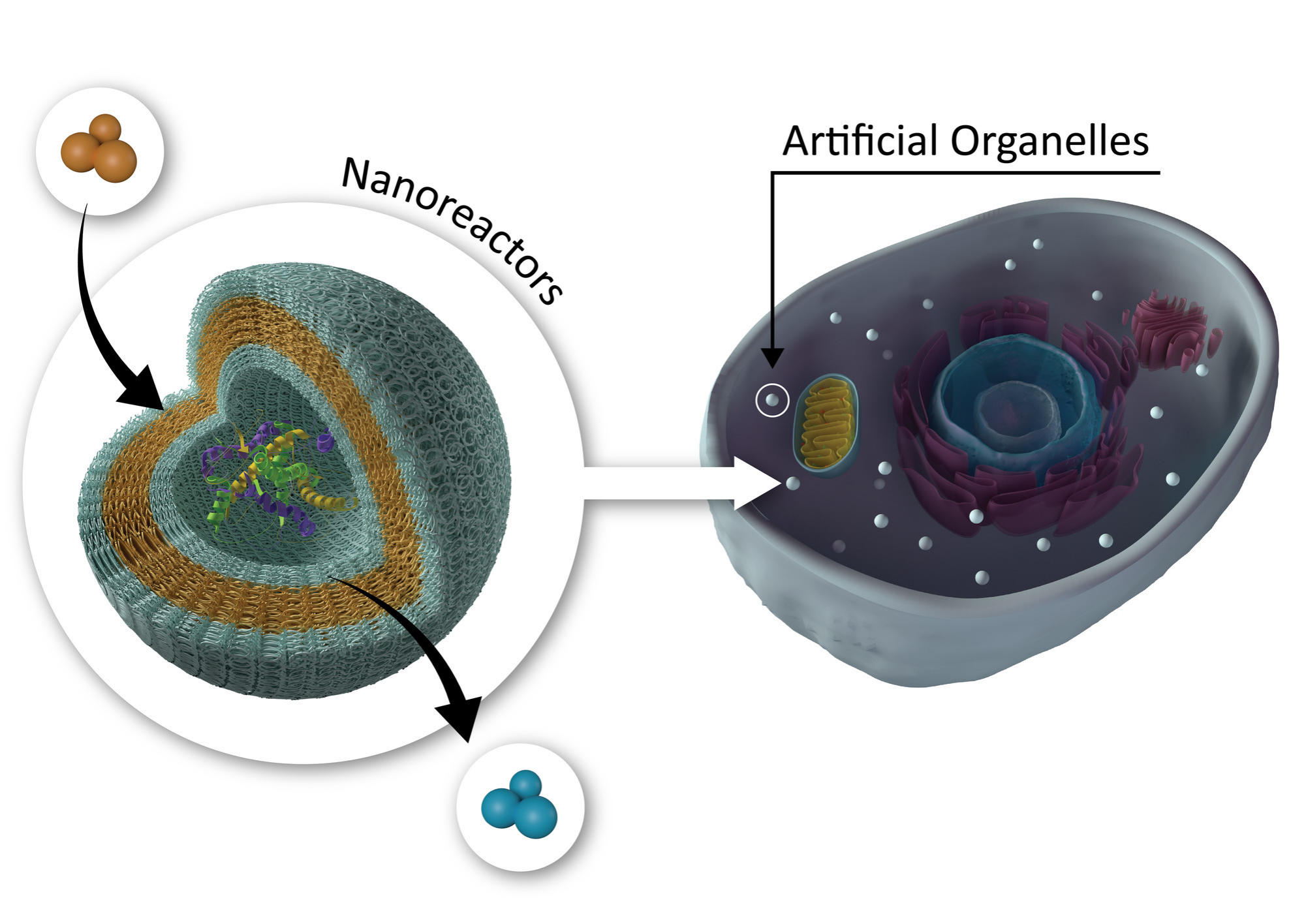
Absence of nanoparticles

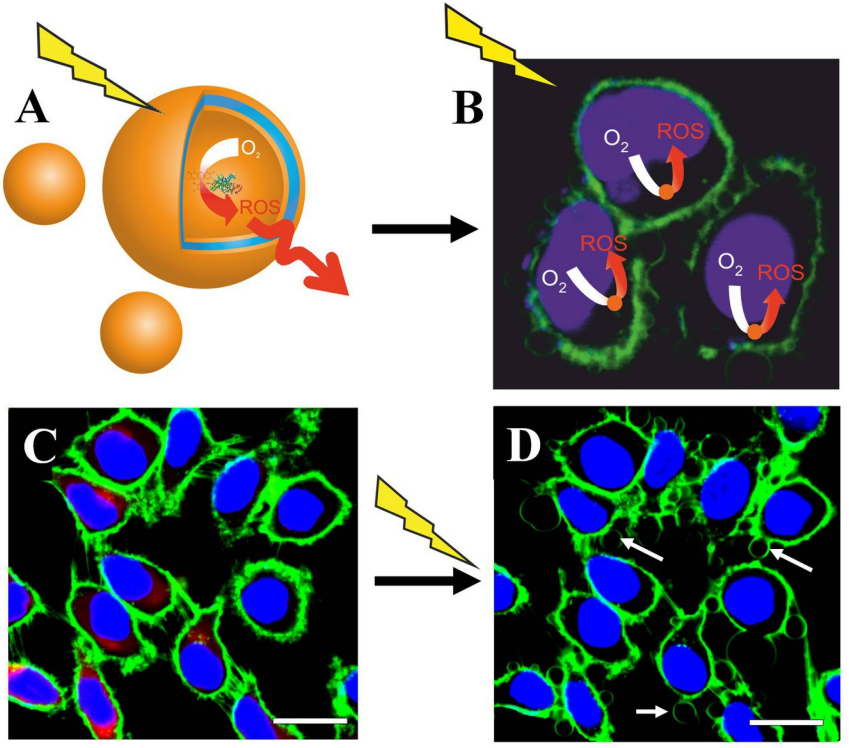


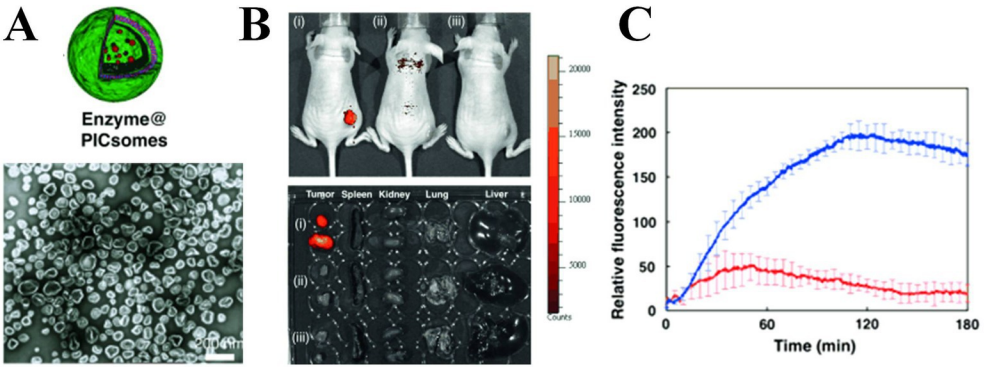
LOCAL STIMULI DRIVEN PERMEABILITY

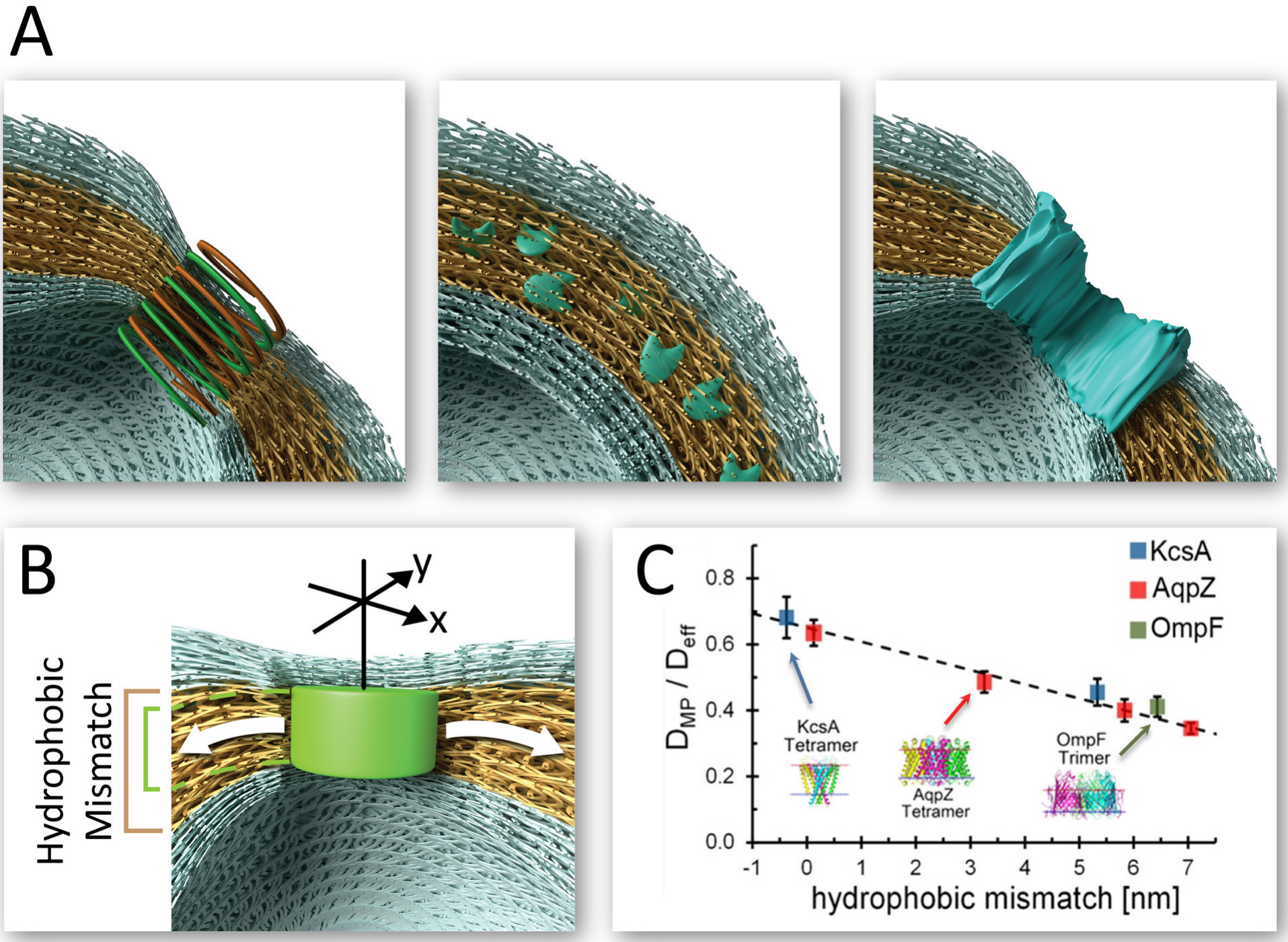


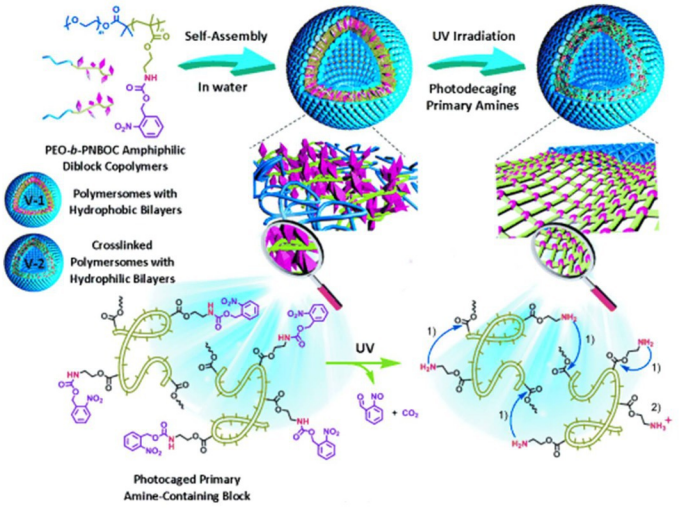
pH, temperature,
solvent composition,
ionic strength...

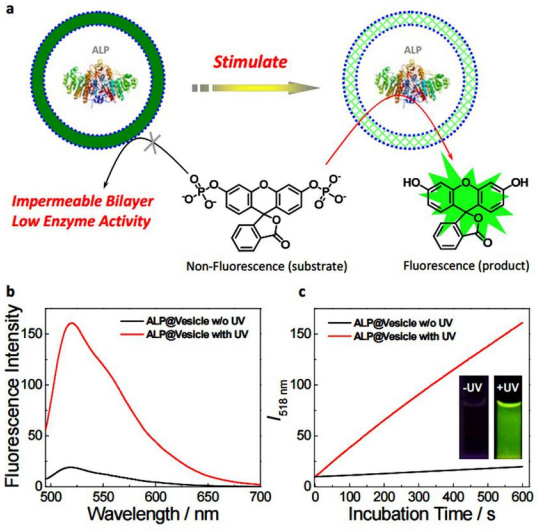

Nanoreactors
Artificial Organelles

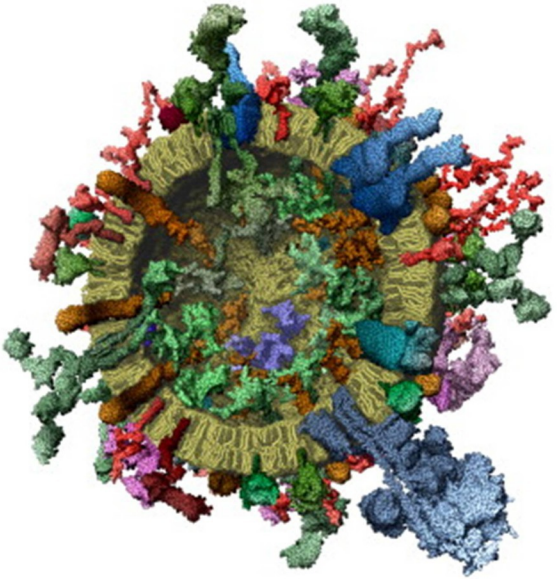

A
O2
ROS
B
O2
ROS
ROS
O2
O2
ROS
C
D

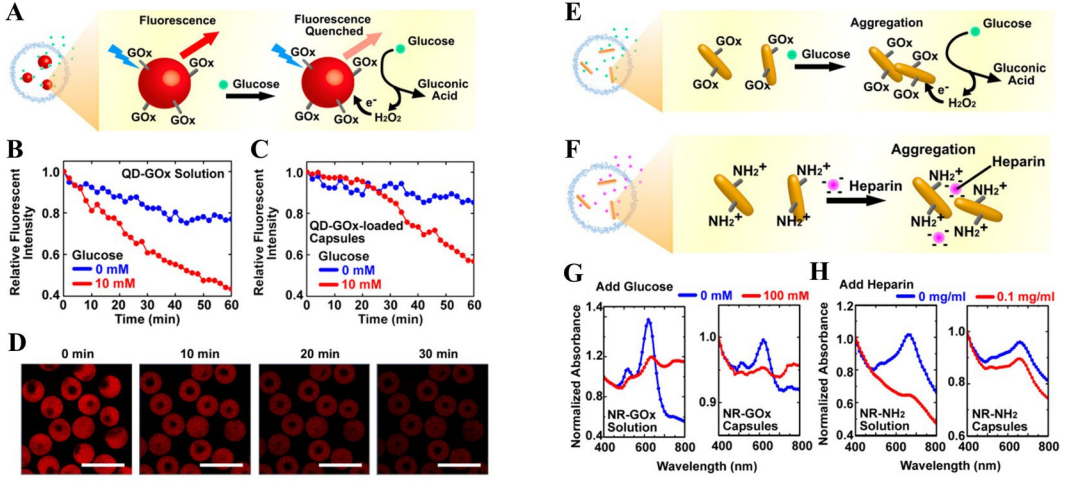

A
Enzyme@
PICsomes
B
(i)
(ii)
(iii)
Tumor
Spleen
Kidney
Lung
Liver
20000
15000
10000
5000
Counts
C
Relative fluorescence intensity
250
200
150
100
50
0
0
60
120
180
Time (min)


A
B
Hydrophobic
Mismatch
y
x
C
KcsA
AqpZ
OmpF
D_{MP} / D_{eff}
0.0
0.2
0.4
0.6
0.8
-1
0
1
2
3
4
5
6
7
hydrophobic mismatch [nm]
KcsA
Tetramer
AqpZ
Tetramer
OmpF
Trimer


Self-Assembly
In water
UV Irradiation
Photodecaging
Primary Amines
PEO-b-PNBOC Amphiphilic
Diblock Copolymers
V-1
Polymersomes with
Hydrophobic Bilayers
V-2
Crosslinked
Polymersomes with
Hydrophilic Bilayers
UV
Photocaged Primary
Amine-Containing Block


a
ALP
Stimulate
Impermeable Bilayer
Low Enzyme Activity
Non-Fluorescence (substrate)
Fluorescence (product)
b
ALP@Vesicle w/o UV
ALP@Vesicle with UV
Fluorescence Intensity
Wavelength / nm
c
ALP@Vesicle w/o UV
ALP@Vesicle with UV
-UV
+UV
Incubation Time / s




A
Fluorescence
Fluorescence Quenched
GOx
Glucose
Gluconic Acid
e-
H2O2
B
QD-GOx Solution
Relative Fluorescent Intensity
Glucose
0 mM
10 mM
Time (min)
C
QD-GOx-loaded Capsules
D
0 min
10 min
20 min
30 min
E
Aggregation
F
Heparin
G
Add Glucose
0 mM
100 mM
NR-GOx Solution
NR-GOx Capsules
Normalized Absorbance
Wavelength (nm)
H
Add Heparin
0 mg/ml
0.1 mg/ml
NR-NH2 Solution
NR-NH2 Capsules