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Review

Polymer Capsules for Enzymatic Catalysis in Confined Environments

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Abstract: Catalysis is at the base of a series of biological and technological application processes. In recent years, the tendency has developed to carry out catalyzed reactions within confined structures, thus forming systems called micro or nanoreactors. Compartmentalized structures are cavities delimited by a wall where specific functions are introduced with a defined concentration and in the desired sites. These containers are generally referred to as nano or microcapsules, assuming the function of reactors in the presence of chemical reactions. Among the various types of existing structures, one of the most interesting is represented by systems made with polymers. This review aims to highlight some of the current advances in the use of functionalized structures that are useful for catalysis reactions, paying particular attention to polymer capsules and enzymes. The built-up methods used for the production of polymer capsules, as well as the aspects that influence membrane permeability and reactivity to environmental conditions, are discussed. Recent advances on biocatalysis confined in polymeric capsules are illustrated, and the strengths and weaknesses of the principal nanoreactors are considered.

Keywords: polymers; capsules; catalysis; enzymes; nanoreactors

1. Introduction

In recent years, the design of the self-assembled chemical ensemble is becoming the basis for the development of large-scale processes in a wide range of research fields. Relevant outcomes have been achieved in the assembly of micro/nano compartments, like hollow spheres having an internal core surrounded by a surface structure. Catalysis performed in these confined environments fits with the concept of micro/nanoreactor systems. The confinement of molecules in small spaces has large implications in chemistry since it can help in stabilizing the molecule structure and in adapting their reactivity [1].

Confined microenvironments can be constructed using a large variety of molecules like lipids, amphiphilic diblock and triblock copolymers [2,3], metal oxides [4], polymers, etc. This category of vessels, generally referred to as nano or microcapsules, is a multifunctional material that, among other uses, can find application as a reactor. Reactors within size-confined environments are of tremendous interest due to their promising applications in catalysis, biomedicine, materials, energy, electronics, separation technology, food, and environmental science. The design of nanoreactors, regardless of the material used to fulfill the requirements of a nanoscale or microscale space, should guarantee specific features, like the efficient encapsulation of a catalyst within the capsules, and the ability to properly respond to environmental changes like pH, temperature, and ionic strength variations [5–7]. Among the various typologies of existing structures, one of the most intriguing examples is the category of polymeric vessel systems [8]. The main properties of polymeric capsules are related with

suitable functionality strongly connected with high flexibility and stability. In the defined structures, it is possible to perform cascade reactions by introducing suitable catalytic functionalities localized in the shells or inside the internal core. The great variety of micro/nanocontainers, the fabrication routes, and the high potential of such aggregates in a wide range of areas have been recently reviewed [9–11]. The different methods used to build such containers and their functionality, as well as the molecular diffusion through the polymeric membrane and the responsiveness to environmental conditions, were discussed in detail. Through the previous bibliography, it was deduced that various terms were used to identify a space confined by a polymer, e.g., polymerosomes, capsosomes or capsules. The present review mainly focuses on the ability of such capsules to be used as nanoreactors, emphasizing the biocatalytic events without neglecting other aspects related with the fabrications routes, the responsiveness, and the mass transfer phenomena.

2. Compartmentalized Spaces

Compartmentalization plays a central role in the biological cell where several reactions and transport processes are accomplished in tandem. Compartmentalized structures are characterized by hollow spaces where catalysts can be inserted at controllable densities and desired sites [12]. One of the simplest examples is represented by lipid and surfactant assemblies, namely liposomes and micelles, self-assembled colloidal systems that are able to solubilize macromolecules for various applications [13–18]. Liposomes were especially developed for application areas ranging from drug delivery and pharmaceuticals to nanotechnology. Such aggregates can enclose either hydrophilic or hydrophobic compounds at high concentrations, and are currently presented as promising systems to be used as nanocarriers and artificial cells or organelles. Capsosomes are polymer capsules containing liposomal subcompartments that are valuable for the sustained delivery of cargos [19]. Amphiphilic block copolymers in water, like natural phospholipids, can self-assemble into various ordered mesophases, known as polymersomes (polymer-based liposomes) [3]. Over the past years, polymersomes have been developed and used as structural analogues of liposomes in order to overcome the chemical limitations and the instability of lipid vesicles [20]. Polymer-based vesicles are obtained from the controlled self-assembly of amphiphilic block copolymers through a number of ways. The synthetic nature of block copolymers makes them highly versatile materials with tunable structural and mechanical properties [21]. By varying the copolymer molecular weight, it is possible to tune the membrane thickness of the polymersomes, and thus, to control membrane permeability. Multishell capsules represent another interesting class of compartmentalized carriers, formed by the alternate adsorption of oppositely charged polyelectrolyte multilayer films. Various synthetic and natural polyelectrolytes, biopolymers (proteins and nucleic acids), lipids, and inorganic particles have been used to produce multilayer films [22,23].

2.1. Hollow Polymer Capsules Preparation Routes

Several procedures are recognized for producing polymer capsules, whose characteristics and properties depend on the fabrication route employed. Among the procedures are the copolymer self-assembly, the emulsion polymerization, the flow focusing method, and the layer-by-layer (LbL) assembly [22]. The general aspects of these assembly techniques are briefly described. A representative illustration of the main routes of fabrication is schematized in Figure 1.

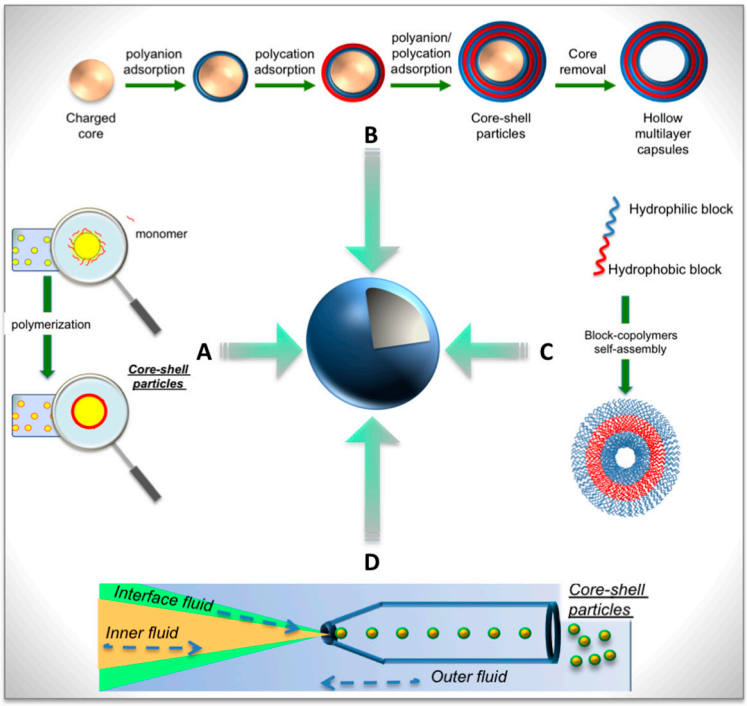

Figure 1. The representative illustration of polymer capsule main routes of fabrication; (**A**) emulsion polymerization; (**B**) layer-by-layer (LbL) assembly; (**C**) block copolymer self-assembly; (**D**) flow focusing method.

The production of polymer capsules via emulsion polymerization (A) starts at the interface of the emulsion droplets (oil in water or water in oil). Here, generally, condensation reactions occur where monomer units react to give the polyelectrolyte. The synthesized polymer forms a layer at the droplet interface, thus making a core-shell structure. More than one polymerization cycle can be carried out based on the desired shell thickness [24]. An improvement of the emulsion polymerization method is given by the Pickering emulsion polymerization, which makes use of solid particles at the liquid-liquid interface of the emulsion as polymerization vessels to fabricate hybrid polymer capsules [25,26]. LbL assembly (B) is an appealing and simple technique that is suitable for preparing multishell capsules. The assembly process is based on the ordered and alternated deposition of oppositely charged polyelectrolytes onto appropriate templates. Finally, template material is dissolved and a free-standing multilayer is realized. The type and number of polyelectrolytes adsorbed and the template dissolution method will influence the multishell capsule properties. Besides the electrostatic interaction, hydrogen [27] or covalent bonding [28], base-pairing interactions (if polyelectrolytes are nucleic acids) [29], and van der Waals interactions [30] are also brought up to keep the polymer shells connected together. Size and shape are well controlled when using the LbL method and wall thickness can be finely tuned by varying the multishell compositions. Polymerosomes manufacturing (C) is generally based on the self-assembly of amphiphilic block copolymers that are produced through synthetic procedures, usually two or three sequential polymerizations [31]. Block copolymers, whose physicochemical characteristics are inspired by amphiphilic molecules like lipids and surfactants, consist of polymer chains covalently linked, forming two or more "blocks". These molecules in the bulk polymer phase, in absence of organic solvents, arrange themselves in ordered domain morphologies, including the lamellar phase (dry lamellae). Their hydration results in a stable dispersion of amphiphilic block copolymer aggregates, i.e., the polymerosomes [32]. The hydrodynamic flow focusing method (D) generates a double emulsion where a single droplet (inner fluid) is covered with a layer of a second fluid (interface fluid) that is in turn dispersed into another liquid (outer fluid). The polymer is provided with the second fluid and forms the wall of a core-shell structure. The device dedicated to the flow focusing technique forces two or more fluids

to move co-axially through an orifice. The flow rate of the outer fluid is higher than the others and generates a pressure drop on the inner phase, which is induced to break up into droplets once it has passed the orifice [33].

2.2. Molecular Diffusion Through Polymeric Capsules

One of the most important aspects to consider when a compartmentalized micro/nano reactor is designed is the molecular diffusion of substrates or products through the border wall. Conventionally, to exploit polymer capsules as reaction vessels, the polymer membrane separating the inner aqueous lumen from the outer compartment should allow the passage of molecules between the outside and the inside of polymer capsules [2]. The study of the molecular release takes account of the relationship between the structure and the function of the device and the possible interactions between the capsule wall and the drug molecules [34,35]. The most used models for quantitative studies of drug release profiles from particles are the Higuchi model, the Ritger-Peppas model, the Weibull function, and the Hixson-Crowell model. These models describe the transport behavior as strictly related to the Fick's law of diffusion and suggest that the description of solute transport from polymeric matrices can be divided into two categories: Fickian and non-Fickian behavior. However, considering the introduced types of vessels above, the permeability of the polymerosomes membrane is strongly reduced compared to that of liposomes or of polyelectrolyte multishells, for example [36]. Some polymerosome membranes, like those that have the PDMS poly(dimethylsiloxane) block in the copolymer composition, are practically impermeable to water, while those made from biodegradable copolymer PEG-PLA (polyethylene glycol-polylactic acid) or PEG-PCL (poly(ethylene glycol)-b-poly(ε-caprolactone)) are semipermeable [37]. One of the strategies used to enhance the membrane permeability is the enclosure of channel proteins (outer membrane protein F, OmpF) added to the copolymer mixture [38].

Considering the polymerosomes structure and the polymer multishell capsules, it is evident that polymerosomes have a hydrophobic zone generated by the hydrophobic portion of the constituent block copolymer that makes their membranes less permeable compared to a polyelectrolyte multishell membrane.

Although less selective, multishell polymer capsules enable the tuning of membrane permeability according to the application request. The versatility of polymer multishell capsules is found, in particular, in varying the number of polymer layers, consequently modulating the membrane cut off and decreasing the molecular permeability, or by choosing different kinds of polymers, like those sensitive to pH, temperature, or light, thus controlling the release triggering.

Parekh et al. used LbL to produce nanocapsules that contained camptothecin in the core and had a multishell formed by heparin, poly-l-lysine, and polyethylene glycol as its layers. [39]. The authors demonstrated that the modification of the nanocapsules' outer surface with additional mPEG 5 kDa tails decreased the initial burst release of camptothecin and the prolonged release time in biological media. Thomas et al. [40] investigated the release of doxorubicin that was encapsulated in hollow biocompatible nanocapsules made of chitosan/heparin assembled onto SiO_2 nanoparticles. From the release studies, they found a strong dependence on a medium pH, a parameter that is able to affect the multilayer association and integrity. Yan et al. [41] demonstrated the loading and sustained release of 5-fluorouracil, a hydrophilic molecule, from microcapsules made of poly (L-glutamate)/chitosan. In that investigation, the drug was encapsulated into the capsule core after template (melamine formaldehyde particles) dissolution. The release profiles were studied at different pH levels and after multishell cross-linking. The latter reduced the release rate because cross-linking limits the mobility of the polyelectrolyte chain, slowing down the drug diffusion through the microcapsule shell. Recently, the permeability of multishell capsules was investigated according to the different molecular weight of the loaded molecules and according to variation in the wall thickness.

Cuomo and coworkers reported that low or high molecular weight molecules were released with a non-Fickian type of transport from polyelectrolyte capsules made of alginate and chitosan. The release rate decreased, while the molecular weight and the multishell thickness increased [42,43] (Figure 2).

On the same line, Kaoui et al developed a novel in silico model for computing the drug release from multi-layer capsules [44]. Considering the numerous release studies from polymer capsules, it can be deduced that polymer capsules are very different from one another and that every single system, each loaded with different molecules, has its own peculiar release behavior depending on the size, the assembly conditions, and the multishell composition.

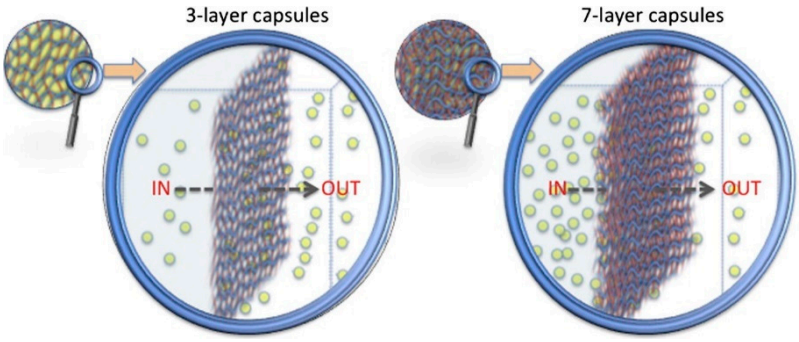

Figure 2. A schematic representation of the effect of the multishell thickness on the molecule release (reproduced with permission from [42], Copyright 2015, Elsevier).

From the information reported above, it is evident that confinement in polymer capsules offers a wide range of opportunities for the encapsulation and release of molecules that can also meet the requirements of biocatalysis application in confined spaces. According to the type of enzyme, one would need to select the access inside the capsules of very small chemical species or bigger ones. In the case of more complex enzymatic cascade reactions, like those mimicking the cell functioning, the coupling of polymerosomes and multishell capsules could be considered.

3. Polymer Capsules as Reactors—General Outlook

Cargo containing polymer capsules are characterized by an aqueous lumen that is separated from the external aqueous environment via a polymer film in which both hydrophilic and hydrophobic molecules can be encapsulated. Based on their characteristics, cargo molecules can be loaded in the internal core or in the polymer membrane. As reported in 2.1 and further highlighted by the examples below, different building strategies are used to produce catalytic carriers. The enormous advantages provided by the possibility of finding different kinds of environments in a unique carrier give attractive opportunities for different research areas. Additional advantages exist because of the more complex systems that make use of polymers for fabrication of hollow inorganic spheres [45] with catalytic activity [46,47].

3.1. Hollow Capsules for Inorganic Catalysts

Even though this review is mainly centered on biocatalysts containing reactors, it also takes a quick look at how inorganic catalysts react in such confined environments. They deal with interesting applications, like the development of efficient, environmentally friendly, and health friendly approaches for energy conversion and storage. This is one of the pivotal challenges in the development and application of modern photodynamic therapy, the production of solar fuels via CO_2 reduction, water splitting, degradation of dyes, etc. [48–50]. Traditionally, the most important inorganic metal oxides are the semiconductors titanium dioxide and zinc oxide [51–53]. Titanium dioxide is a widely used photocatalyst because of its high photocatalytic activity, stability, non-toxicity, and low cost [54]. The catalytic activity of semiconductors is strongly connected with the transport of electrons and electron holes within the solid catalyst. Thus, depending on the specific application, inorganic metal oxide reactors are designed while taking into account important parameters like size and morphology, light harvesting aptitude, and the electronic structure related with the visible light absorption efficiency.

Examples of capsule core/shell or yolk/shell systems as organized multi-scale assemblies that provide photocatalytic units on hollow scaffolds have recently been reviewed [55]. Among them was one of the first attempts to design polymer microreactors with light responsive capability given by the insertion of TiO_2 in the polymeric wall, as proposed by Shchukin and coworkers [56]. The authors designed photocatalytic microreactors for microheterogeneous photoreduction of metal ions from aqueous solutions. Later, Galeano and co-workers synthesized gold yolk shell materials that were used as model catalysts for the study of support effects in CO oxidation [57]. Through nanocasting, two different nanoporous-core/mesoporous-shell exotemplates were synthesized: $Au/SiO_2/ZrO_2$ and $Au/SiO_2/m\text{-}SiO_2$. In this investigation, the highly positive effect of metal oxide as the support material to gold activity was demonstrated. These kinds of reactors were made of hollow micron-sized PSS/PAH polyelectrolyte capsules templated onto $MnCO_3$ with photoactive TiO_2 nanoparticles incorporated in the walls. Another important example of TiO_2 involvement was reported by Tu et al. In that study, by means of an LbL deposition, hollow spheres consisting of molecular-scale alternating titania nanosheets and graphene were prepared [58]. The authors proved that the ultrathin nature of titania nanosheets allowed the charge carriers to move rapidly onto the surface to participate in the photoreduction reaction. Additionally, the photogenerated electron transferred quickly from the titanium nanosheets to the graphene in order to enhance the lifetime of the charge carriers. Tseng and coworkers designed silver/titania (Ag/TiO_2) core/shell composite microcapsules. The TiO_2 shell protected the encapsulated, movable Ag nanoparticles from breaking away under moderate loading, whereas the mesoporous shell served as a conduit for Ag ions that were released from the caged Ag nanoparticles to kill *Escherichia coli* in aqueous solutions under dark conditions [59]. Han et al. proposed yolk–shell nanocatalysts made of Au–polymer hollow hybrids having a single Au nanoparticle encapsulated in each porous polymer shell with great catalytic efficiency [60]. With this approach, by means of the polymer POMA (poly(o-methoxyaniline), Au-core-shell hybrids were prepared. In those carriers, active catalytic cores encapsulated in permeable shells were easily reached by the reagents, thus enhancing the catalytic efficiency.

3.2. Polymer Capsules for Biocatalysts

Enzymes confined in small environments have very advantageous results that make it easier to isolate them from the reaction products, protect their structure from denaturation, and increase their stability. In polymer capsules, enzymes can be entrapped in the polymer shell or within the internal core. These kinds of assemblies have proven to be promising candidates for biomedicine, biomaterials, and biosensing applications. Some examples of enzyme confinement are illustrated and discussed in this section. For the sake of clarity and shortness, the enzymes discussed in this manuscript are listed in Table 1. The membrane compositions, made by either copolymers or membrane multilayers, are reported in Table 2, recalling the names of the various types of polymers and their acronyms, respectively.

3.2.1. Polymerosomes

Among the various compartmentalization categories, polymerosomes represent the simplest structures of synthetic block copolymers characterized by high stability. One of the most studied assemblies for bio-catalytic conversion was first proposed one decade ago by Vriezma et al. [61]. The polymerosomes they proposed were made of a diblock copolymer that, after the self-assembly, formed a structure with an internal aqueous core separated by a hydrophobic ring from the external aqueous environment. The diblock copolymer, named PS-PIAT, was a rod-coil type copolymer in which the polystyrene unit constituted the flexible part (coil) and the polyisocyanide was the rigid part (rod) (Table 2). The polymeric vesicles of PS-PIAT provided a selective membrane that was only permeable to low molecular weight molecules. This carrier was first used to carry out a cascade reaction involving three enzymes, i.e., lipase B from *Candida antartica* (CALB), horseradish peroxidase (HRP), and glucose oxidase (GOX). The cascade reaction started with CALB activity, localized in the

external bulk, that catalyzed the production of glucose from 1,2,3,4-Tetra-O-acetyl-β-D-glucopyranose. Glucose molecules diffused through the polymer membrane became a substrate of GOX that was located inside the polymerosome core and oxidized hydrogen peroxide into its lactone. The latter substrate was finally used by HRP, still inside the same polymerosome, to convert 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ABTS (added in the external bulk), in $ABTS^{\bullet+}$.

In a successive study, the same polymerosomes [62] were used to separately load HPR and GOX into different polymerosome cores and to carry out the enzyme cascade reaction due to the substrate/product diffusion through the porous membrane provided by PS-PIAT polymerosomes. Successively, the PS-PIAT polymerosome composition was enriched with a molecule with anchoring functions (acetylated terminal functionalization), which was able to anchor enzymes on the polymerosomes' surface [63]. This new architecture allowed enzyme positioning in different levels of the capsule—CALB in the membrane, GOX in the lumen, and HRP anchored to the external surface. The cascade reaction was again successfully realized. A further modification of the PS-PIAT-tat polymerosomes recipe was made to promote their cellular uptake [64]. The cell penetrating peptide, protruding from the polymerosome surface, was provided by the PS-PEG-tat copolymer mixed with PS-PIAT. Three cell lines, i.e. HeLa, Jurkat, and HEK 293 cells, effectively internalized the assembled nanoreactors loaded with HRP inside. The HRP activity in the cells had a half life of 15 days. More recently, PS-PIAT nanoreactors have been used for the assembly of multi-compartmentalized polymerosomes [65].

In the systems resembling the cell organization, enzyme filled PS-PIAT nanoreactors were encapsulated with other free-enzymes (cytosolic) and different substrates in PB-PEO polymerosomes. The suggested compartmentalization was suitable for the coexistence of two enzymes that could not be compatible in a polymerosome if they were not insulated.

A well studied and characterized polymerosomes composition is composed by the triblock copolymer PMOXA-PDMS-PMOXA, and is able to encapsulate Cu,Zn-SOD [66]. The main focus of this enzyme is the possibility of controlling the oxidative stress due to an imbalance in reactive oxygen species, such as superoxide radicals. The chosen block copolymers with the PMOXA units as hydrophilic domains showed good levels of biocompatibility [67]. The polymer membrane of these polymerosomes was stable and only permeable to small ions, such as the superoxide anion ($O_2^{\bullet-}$) [36]. The catalytic activity of Cu,Zn-SOD loaded inside the capsule lumen confirmed that the polymerosome membrane was permeable to superoxide anion and that a valuable antioxidant nanoreactor was assembled. Additional studies examined the antioxidant activity of SOD encapsulated in amphiphilic PMOXA-PDMS-PMOXA block copolymer polymerosomes. The studies also analyzed the enzyme encapsulation efficiency and the membrane permeability as functions of the copolymer composition, like changing the length of the different polymer block [68]. Here, it was demonstrated that the encapsulation efficiency is mainly dependent on the vesicle dimensions, and that the length of the hydrophobic PDMS middle block mainly affects the membrane permeability. For very long PDMS chains, superoxide anion transport across the membranes was too slow to be detected. In line with these studies, Tanner et al. [38] obtained a different permeability for capsules made of PMOXA-PDMS-PMOXA by adding a channel protein membrane (OmpF) to the triblock copolymer membrane, allowing the passive diffusion of small molecules. The enzymatic cascade reactions, realized by loading Cu,Zn-SOD and lactoperoxidase (LPO), inside the polymeric nanocontainers were tested to control superoxide radicals. The cascade reactions involved a superoxide anion that was converted in hydrogen peroxide by SOD and hydrogen peroxide used by LPO to convert amplex red reagent in resorufin. The passage of substrates and products through the membrane proved that OmpF changed the capsule permeability. Polymerosomes modified in this way were also used by Langowska et al. [69] to encapsulate the enzyme penicillin acylase for producing the antibiotic penicillin. In this example, the nanoreactor was successfully employed to convert a non-active compound into an active molecule. Lately, PMOXA-PDMS-PMOXA with enhanced permeability were used for mimicking the natural organelles,

peroxisomes [70]. The system, based on two enzymes (SOD and LPO or CAT) working in tandem and encapsulated in polymer vesicles, was used as an artificial antioxidant nanoreactor on a HeLa cell. The cell uptake, the absence of toxicity, and the in situ activity in cells that were exposed to oxidative stress demonstrated that the artificial peroxisomes detoxify superoxide radicals and hydrogen peroxide after endosomal escape. In order to modify the permeability of the PMOXA-PDMS-PMOXA membrane, a further strategy was applied. For this purpose, three different amphiphilic block copolymers based on α,ω-hydroxy-end-capped PMOXA-PDMS-PMOXA, α,ω-acrylate-end-capped PMOXA-PDMS-PMOXA, and PEO-PB were considered [71]. The three systems were reacted with a photoreactive compound, 2-hydroxy-4′-2-(hydroxyethoxy)-2-methylpropiophenone, under UV-irradiation. The light treatment resulted in a substantial increase in permeability for organic compounds without affecting the size and shape of the vesicles. Permeability was estimated by encapsulating HRP into vesicles and measuring the accessibility of substrates to the enzyme. The irradiated nanoreactors retained their ability to protect encapsulated biocatalysts from degradation by proteases.

Another significant example of compartmentalization, polymerosomes in polymerosomes, was provided by Siti et al. [72] by encapsulating a PMOXA-PDMS-PMOXA/OmpF nanoreactor containing HRP into a PS-PIAT polymerosome loaded with GOX. The functionality of this multicompartment architecture was demonstrated by a cascade reaction between enzymes that were segregated in separate compartments. The cascade reaction did not occur in the absence of the membrane protein channel. Finally, very recently, [73] the suitability of PMOXA-PDMS-PMOXA/OmpF was tested as an efficient solution for detoxifying the harmful effects of uric acid and for preventing the accumulation of the derived hydrogen peroxide by using the combined activity of fungal uricase and HRP. The presence of uric acid and hydrogen peroxide are associated, for example, with gout and oxidative stress. The two enzymes were separately encapsulated within nanocompartments that were equipped with channel porins as gates to allow passage of substrates and products from each step of the reaction (Figure 3).

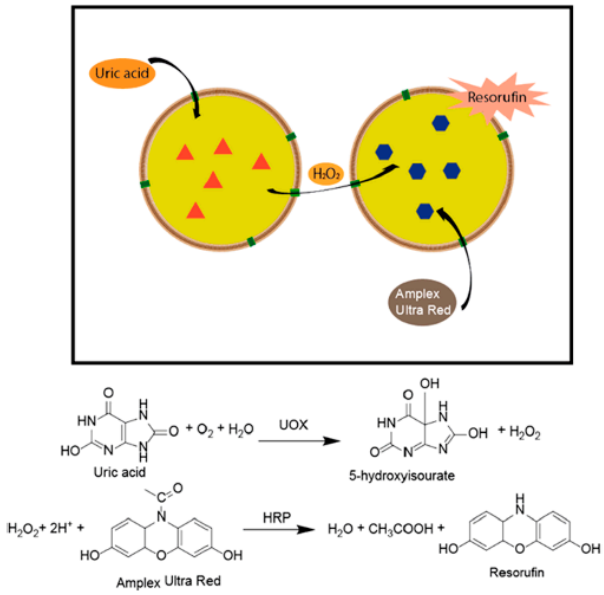

Figure 3. The schematic representation of catalytic nanocompartments [outer membrane protein F (OmpF), green rectangle] working in tandem, and a detailed cascade reaction mediated by a combination of uricase (UOX, red triangles) and horseradish peroxidase (HRP, blue hexagons). The oxidation of uric acid results in the formation of 5-hydroxyisourate and hydrogen peroxide. The latter is a co-substrate for HRP in the presence of substrate Amplex Ultra Red, AR. The final product, resorufin, can be monitored by fluorescence spectroscopy (reproduced with permission from [73], Copyright 2018, American Chemical Society).

Enzyme encapsulation in UV cross-linked polymerosomes was proposed in 2012 by Gaitzsch et al. [74]. These authors put forward polymerosomes made of the triblock copolymer PEG-PDEAEM-PDMIBM that were UV cross-linked within 30 seconds. Myglobin was the model enzyme encapsulated in the capsule lumen. Myoglobin catalyzed, among others, the oxidative reactions of guaiacol with hydrogen peroxide. The central copolymer block, PDEAEM, was sensitive to pH and became fully hydrophobic at pH 8, where it unprotonated, a condition that did not allow the substrate to reach the polymerosome lumen. The enzyme activity could not be detected. Differently, at pH 6, the molecule trafficking through the capsule membrane occurred and the enzyme activity could be measured. The pH-tunable properties of PEG-PDEAEM-PDMIBM polymerosomes have also been used to study their use in cascade enzymatic reactions (GOX and myoglobin). Enzymes were loaded in one common polymerosome or in two different containers [75]. It was demonstrated that substrates and products moved or did not move across the polymerosomes membran according to the swelling-deswelling states of the enzyme compartments, which coincided with pH 6 and 8, respectively.

Furthermore, polymerosomes made of the PNVP-b-PDMS-b-PNVP triblock copolymers were used for producing a nanoreactor protecting the biocatalyst laccases (LAC), an oxidizing enzyme with a broad range of applications [76]. Oxygen (O_2) was used to catalyze the production of the reactive oxygen species (ROS). The polymersomes membrane, permeable to oxygen, allowed the substrate to encounter the LAC inside the polymerosome. It also allowed the ROS to diffuse out of the polymersomes and to oxidize ABTS outside the membrane. Overall, the PNVP-b-PDMS-b-PNVP nanoreactors protected the LAC from enzymatic degradation by proteinase K and against the inhibition by sodium azide.

A slightly different system of compartmentalization was formed through non-cross-linked polyion complexes (PIC) forming the so called PICsomes. In aqueous media, the assembly of the PICsomes was mediated by the electrostatic interaction of oppositely charged block- and homoionomers, PEG-PAsp and homocatiomer Homo-P(Asp-C8) [77]. The activity of the encapsulated enzyme β-galactosidase (β-GAL) that catalyzed the cleavage of terminal β-linked galactose residues from various substrates was maintained, even with the presence of trypsin in the exterior of the PICsome. More recently, β-GAL was cross-linked to the PICsomes and also maintained its activity after linking the enzyme [78]. It was seen that after intravenous injection, β-GAL cross-linked PICsomes selectively accumulated in the tumor tissue of mice. The enzyme activity at the tumor site continued four days after administration.

3.2.2. Layer by Layer Assemblies

Considering the polymer multishell compartmentalization, the LbL assembly represents one of the more simple and versatile approaches. Almost 20 years ago, soon after LbL was introduced, the enzyme encapsulation was proposed based on the use of enzyme crystals as templates [5]. Catalase crystals were indeed used, and the alternate depositions of PSS and PAH were carried out on crystals to obtain a mulishell capsule. By modulating the medium pH, the enzyme was solubilized and its activity was studied. The enzyme retained its activity completely after incubation for 100 min with protease. On the contrary, the activity of uncoated catalase rapidly decreased after exposure to proteases.

Although there are few examples of LbL capsules hosting enzymes compared to polymerosomes, some meaningful examples are illustrated below. An interesting and challenging application of enzyme encapsulation in LbL capsules was the set up of a glucose-sensitive multilayer shell as a carrier for the encapsulation and controlled release of insulin [79]. Insulin particles were used as templates and glucose oxidase (GOX) and catalase (CAT) were used as polymers for the LbL assembly. The two enzymes were cross-linked via glutaraldehyde. The catalysis interaction of CAT/GOX shells to glucose introduced externally lead to the H^+ production and to the pH decrease. This caused a membrane modification (bonds rupture) and an increase in permeability that allowed insulin to be released from the multishell capsule.

A successive development on the insulin release was recently published [80]. A similar strategy of the LbL coating was applied on silica vesicles (SV) loaded with insulin. The multishell was made of GOX and CAT enzymes and a layer of PEI was interposed, functioning as a buffer for the pH variations. This new architecture was effective for controlling the membrane permeability, i.e., the insulin release threshold was tuned to the hyperglycemia range (Figure 4). In vivo tests on mice with diabetes type I demonstrated that the nanoreactor carrier was effective for regulating the glycemia levels to normal values for more than 80 hours with a single administration.

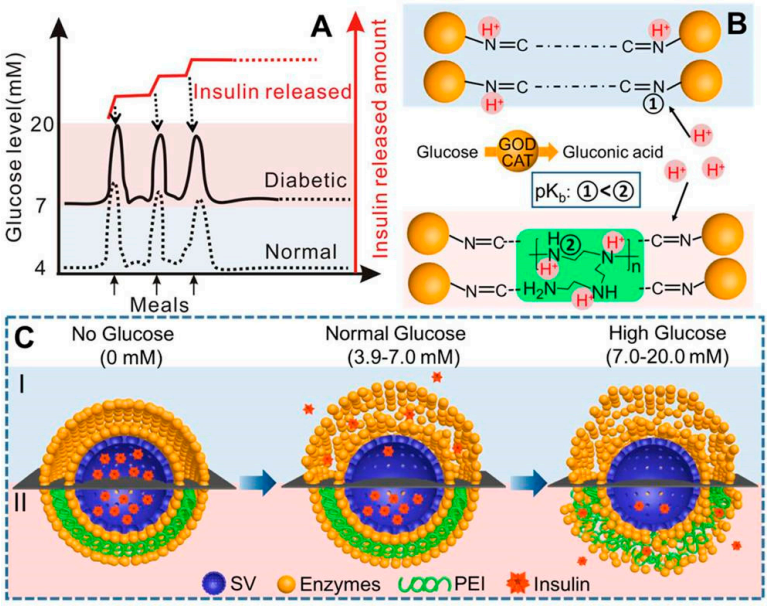

Figure 4. (**A**) Ideal insulin-release behavior under different blood glucose levels. The black dashed and solid curves correspond to the blood glucose level fluctuation in one day in healthy nondiabetic and diabetic humans, respectively; (**B**) the mechanism of glucose-responsive insulin release in the enzyme system (up, blue area) and enzyme−PEI system (bottom, pink area); (**C**) comparison between (I) traditional glucose-responsive insulin release systems where insulin would be released under normal glucose levels, and (II) physiological glucose-responsive systems, which release insulin only under "diabetic" glucose levels (reproduced with permission from [80], Copyright 2017, American Chemical Society).

Another recent application was developed for potential uses for biomedical applications [81]. PLGA-enzyme particles were produced by immobilizing the enzymes acid phosphatase (AP) or β-galactosidase (β-GAL) between the PSS and PAH layers via glutaraldehyde cross-linking. The β-GAL assembled LbL capsules were injected into the blood circulation of zebrafish, demonstrating enzymatic activity in a biological environment and thus confirming the potential adaptability of that system to meaningful technological applications.

Following the LbL principles, in 2009, Chandrawati et al. [19] assembled polymer capsules containing multiple liposomes as subcompartments. The hierarchical structures assembled were called capsosomes and were particularly interesting as microreactors. Capsosomes were fabricated on silica template particles that were first covered with a precursor layer (PLLc). This layer had a cholesterol functionalization for better interacting with the successive layer of liposomes, encapsulating enzymes that were deposited between the PLLc and the PMAc capping layer. The PMAc was also modified with cholesterol moiety. Subsequently, PVP and PMA_{SH} were assembled onto the pre-adsorbed layer of liposomes. The assembled capsosomes were still intact after silica template dissolution, and the encapsulated enzymes (luciferase or β-lactamase) were not affected by acidic or physiological pHs. The enzymatic cargo was well retained by the liposomal sub-compartments for at least 14 days. On the same kind of capsosomes it was demonstrated that, after the addition of TritonX-100, which promotes

the liposome disassembly, the enzyme β-lactamase was released and available for catalyzing the hydrolysis of the nitrocefin substrate [82].

An example of a capsosome carrier with antioxidant activity and with the ability to release molecules was also designed with some modifications compared to the previous examples [83]. Capsosomes templated on silica particle were coated with the following sequence of layers: PVPc precursor, zwitterionic liposomes loaded with glutathione reductase, PVPc capping layer, PMA and PVP, PMA-KP9 polymer-peptide conjugates, and three other bilayers of PVP and PMA_{SH}. The liposomes assembled in the capsosomes had a transition temperature close to 37 °C (Figure 5). In correspondence to this temperature, the enzyme substrate, glutathione disulfide (GSSG), was free to penetrate the multishell and was converted in the sulfhydrilic form (GSH). GSH cleaved the sulfide bonds of the polymer-peptide conjugates, thus allowing the release of the KP9 peptide. With this approach, it was demonstrated that the enzyme activity was temperature triggered and that, as a cascade, the enzyme reaction also activated the peptide release.

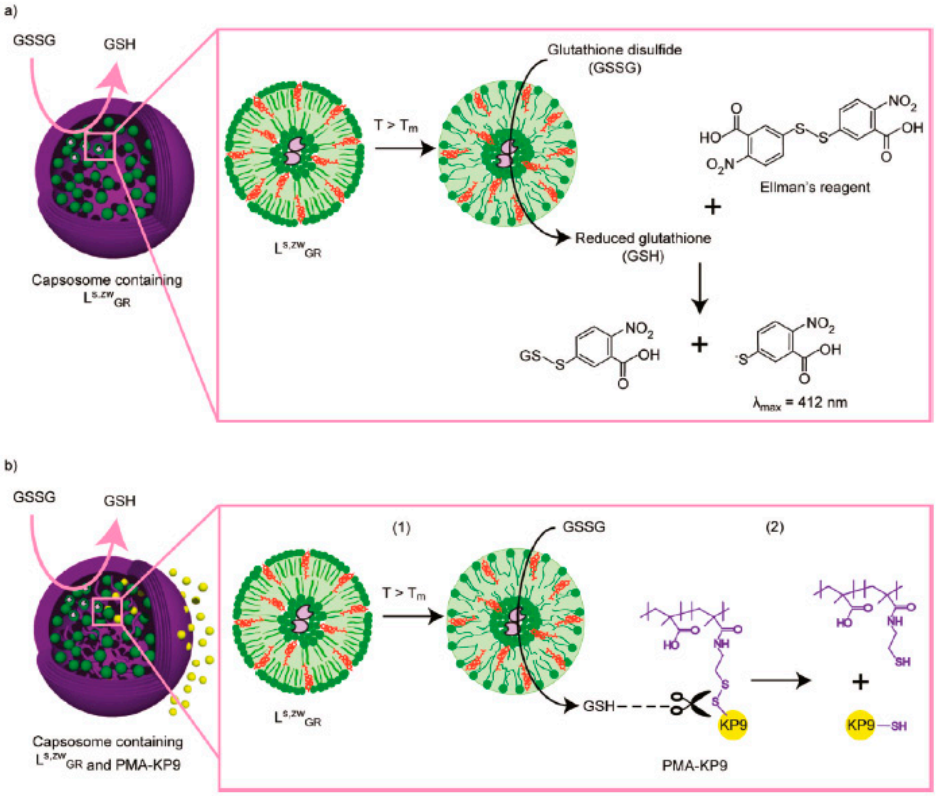

Figure 5. (**a**) Temperature-triggered catalysis of encapsulated glutathione reductase in the "free-floating" liposomal compartments of capsosomes, which reduces glutathione disulfide (GSSG) to its sulfhydryl form (GSH); (**b**) the release of encapsulated oligopeptides triggered by the catalytic activity of glutathione reductase in capsosomes. The production of GSH is measured using Ellman's reagent as an indicator. The reduction of GSSG to GSH by the activity of encapsulated glutathione reductase in the liposomal subcompartments (1) facilitates the release of the encapsulated peptide due to the cleavage of disulfide bonds linking the polymer carrier (PMA) and the peptide (KP9) (2) (reproduced with permission from [83], Copyright 2011, American Chemical Society).

A very recent study proposed the capsosome architecture to design artificial cell organelles that can be internalized in cells to replace malfunctioning organelles [84]. A bi-enzymatic model cascade reaction catalyzed by HRP and GOX, loaded in separate liposome subcompartments, was proposed to evaluate the capsosomes intracellular activity. Capsosomes were internalized in macrophages, preserving their integrity inside the cell, and the enzymatic activity was triggered in macrophages at 37 °C (the transition temperature of liposomes). The authors demonstrated that enzymes confined in

separate capsosome compartments preserved their activity intracellularly, allowing for the control of enzymatic cascade reactions within a host cell.

3.2.3. Pickering Emulsions

When hydrophilic enzymes have hydrophobic molecules as substrates, the best solution to adequately accommodating both the catalyst and the educts is an emulsion system—in particular, a Pickering emulsion that is characterized by a higher stability compared to classical surfactant stabilized emulsions. Pickering emulsion for enzyme immobilization was proposed by Wu et al. in 2011 [85]. SiO_2 nanoparticles were used to stabilize droplets of an aqueous solution containing enzymes in heptane (CALB, CALA, or benzaldehyde lyase). The aqueous core of the emulsion was jellified with agarose. The catalytic activity of the enzymes was higher in the Pickering emulsion than in the simple biphasic system. Wang et al. stabilized a Pickering emulsion with polymerosomes made of the block copolymer PEG-P(S-co-TMI) with a isocyanate group for cross-linking [86]. Polymerosomes hosted CALB at the oil/water interface (inside the polymerosome lumen) or in the aqueous emulsion droplet. The enzyme performance was better when CALB was inside the polymerosomes.

Liu et al. [87] stabilized the Pickering emulsion with nanocages of mesoporous silicas FDU-12 (cubic, Fm3m) (Figure 6).

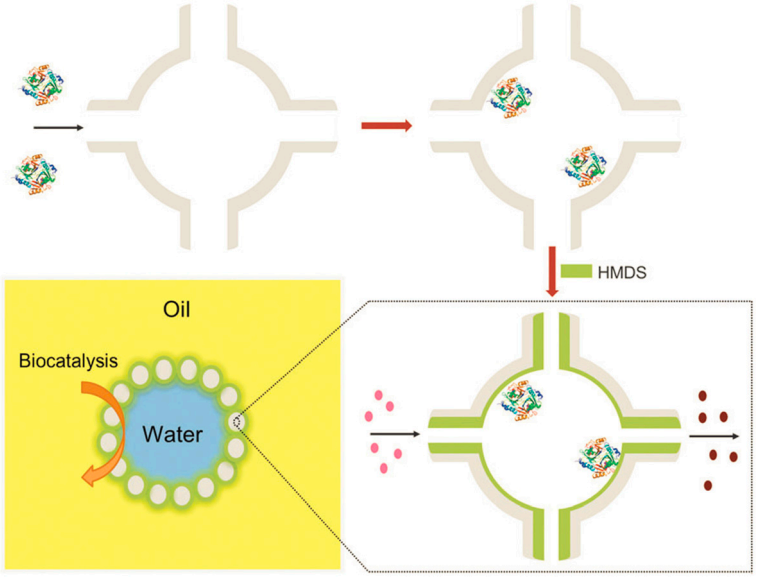

Figure 6. The schematic illustration of the strategy to encapsulate enzymes into the nanocage of FDU-12 using hexamethyldisilazane (HMDS) as the silylation reagent, and the Pickering emulsion stabilized by modified FDU-12 particles with enzymes confined in the nanocages to perform biphasic enzymatic reactions (reproduced with permission from [87], Copyright 2013, Royal Society of Chemistry).

By applying a silylation procedure using hexamethyldisilazane (HMDS), they optimized the encapsulation of PCL (lipase from *Pseudomonas cepacia*) in the FDU-12 nanocages and the loaded particles were used to stabilize the Pickering emulsions. In comparison with the biphasic system, catalytic performance of PCL, measured through the hydrolysis of triacetin, was enhanced in the Pickering emulsion, possibly due to the effects of increasing the oil/water interface.

3.2.4. Flow Focusing

Finally, very recently, Wang et al. projected an enzyme cascade reaction system in microcapsules obtained through microfluidic electrospray [88]. Enzymes were encapsulated in inverse opal particles (three dimensionally ordered mesoporous structures with very high surface areas) with different structural colors, choosing an individual color for each enzyme. All of these structures were protected

with alginate hydrogel shells that contributed to further stabilizing and protecting the enzymes. Among others, the enzymes alcohol oxidase (AOX) and CAT were encapsulated and the effectiveness of the cascade reaction was successfully tested to reduce alcohol levels. The designed multienzyme system showed a great potential against alcohol intoxication.

Table 1. List of enzymes encapsulated in polymer capsules.

Catalysts	Reference
Acid phosphatase (AP)	[81]
Alcohol oxidase (AOX)	[88]
Benzaldehyde lyase (BL)	[86]
Catalase (CAT)	[5,70,79,80,88]
β-galactosidase (β-GAL)	[77,78]
Glucose oxidase (GOX)	[61–63,72,75,79, 80]
Glutathione reductase (GR)	[83]
Horseradish peroxidase (HRP)	[61–64,71–73,75]
Laccase (LAC)	[76]
β-lactamase (BL)	[19,82]
Lactoperoxidase (LPO)	[38,70]
Lipase A (CALA)	[86]
Lipase B (CALB)	[61,63,65,86]
Lipase (from Pseudomonas cepacia) (PCL)	[87]
Luciferase (LUC)	[19]
Myoglobin (MB)	[74,75]
Penicilln acylase (PA)	[69]
Superoxide Dismutase (SOD)	[38,66,68,70]
Uricase (UOX)	[73]

Table 2. List of polymers and membrane domains of polymer capsules.

Membrane Composition		Reference
Polymer Name	**Acronym**	
Poly(o-methoxy aniline)	POMA	[60]
Polystyrene-polyisocyanoalanine (2-thiophen-3-yl-ethyl)amide)	Copolymer PS–PIAT	[61–65]
Poly(2-methyloxazoline)-poly(dimethylsiloxane)-poly(2-methyloxazoline)	Copolymer PMOXA-PDMS-PMOXA	[36,38,66–73]
Poly(ethylene glycol)-poly(diethylaminoethyl methacrylate)-poly(3,4-dimethyl maleic imidobutyl methacrylate)	Copolymer PEG-PDEAEM-PDMIBM	[74]
Poly(ethylene glycol)-poly-(styrene-co-3-isopropenyl dimethylbenzylisocyanate)	Copolymer PEG-P(S-co-TMI)$_n$	[86]
Poly(N-vinylpyrrolidone)-poly(dimethylsiloxane)-poly(N-vinylpyrrolidone)	Copolymer PNVP-b-PDMS-b-PNVP	[76]
Poly(ethylene glycol)-b-poly(aspartic acid):poly([8-aminoctyl]-α,β-aspartamide)	Copolymer PEG-PArg:Homo-P(Asp-C8)	[77,78]
Poly(ethylene glycol)-block-poly(ε-caprolactone)	Copolymer PEG-PCL	[34]
Cholesterol-modified poly(L-lysine)/liposomes layer/poly(methacrylic acid)-co-(cholesteryl methacrylate)/poly(N-vinyl pyrrolidone)/thiol-functionalized poly(methacrylic acid)	Multilayer film PLL$_c$/liposomes/PMAc/PVP/PMA$_{SH}$	[19,82]
Alginate/Chitosan	Multilayer film ALG/CHI	[7,22,42,43]
Poly(sodium-styrene sulfonate)/poly(allylamine) hydrochloride	Multilayer film PSS/PAH	[5]
Glucose oxidase/Catalase	Multilayer film GOX/CAT	[79]
Glucose oxidase/polyethylenimine/Catalase	Multilayer film GOX/PEI/CAT	[80]

4. Conclusions and Perspectives

When important challenges are faced, such as the design of nanoreactors for applications in biological and technological fields, the main goal can be achieved starting from less complex steps. The present review gives an overview of some important outcomes on the biocatalysis in polymer capsules, illustrating some achievements at different levels of complexity that can be taken in consideration for the set-up of micro and nanoreactors. Through the years, polymer science has offered a great variety of molecules to meet the different requests in terms of chemical composition, molecular weight, and alternation of hydrophilic and hydrophobic blocks. It has also characterized polyelectrolytes, including proteins, in depth. Each of those polymers self-assemble in a unique way according to their own characteristics that are generated by confined volumes. Some important aspects of the capsules production methods and the relative implications on the final applications were highlighted in this study. Also addressed was the significant progress in the development of nanoreactors using emulsion polymerization, LbL assembly, and polymerosomes hydrodynamic flow focusing methods.

The entrapment and localization of the enzymes for cascade reactions in confined volumes, as well as their accessibility to the substrates, can be adequately controlled, as demonstrated by the in vitro studies here recalled.

If we consider the applications discussed, polymerosomes are the most studied nanoreactors because they are characterized by higher stability and selectivity to permeation when compared to the multishell capsules. One of the few aspects that could limit the interest toward polymerosomes is their too-tight cut off of their membrane for many reaction substrates, a property that has been modified with the inclusion of protein channels within the membrane. Polymer capsules assembled through LbL, although more versatile in the composition of their multilayer membrane, risk being damaged in their integrity when removing the internal core. This is a drawback that can be overcome by using non-sacrificial cores like lipid vesicles as capsule templates. Moreover, in the majority of cases, the trafficking of molecules from the inside to the outside of the capsule (and vice-versa) is governed by the sensitivity of the membrane to variations of pH, temperature, ionic strength, etc., and in some circumstances, this can cause enzyme denaturation or damage.

Overall, polymer capsules are promising and attractive tools for different kinds of application. Even though some seeds have been thrown for mimicking trafficking in biological cells, there is still room for researchers to fully understand the design strategies for making the polymer capsules valuable candidates for the treatment of a number of diseases or technological applications.

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