Advances in Colloid and Interface Science 304 (2022) 102680

Contents lists available at ScienceDirect

Advances in Colloid and Interface Science

journal homepage: www.elsevier.com/locate/cis

Historical Perspective

Exploiting the layer-by-layer nanoarchitectonics for the fabrication of polymer capsules: A toolbox to provide multifunctional properties to target complex pathologies

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ARTICLE INFO

Keywords:
Polymer capsules
Layer-by-layer
Therapeutic applications
Functionalization
Multifunctional properties

ABSTRACT

Polymer capsules fabricated via the layer-by-layer (LbL) approach have attracted a great deal of attention for biomedical applications thanks to their tunable architecture. Compared to alternative methods, in which the precise control over the final properties of the systems is usually limited, the intrinsic versatility of the LbL approach allows the functionalization of all the constituents of the polymeric capsules following relatively simple protocols. In fact, the final properties of the capsules can be adjusted from the inner cavity to the outer layer through the polymeric shell, resulting in therapeutic, diagnostic, or theranostic (i.e., combination of therapeutic and diagnostic) agents that can be adapted to the particular characteristics of the patient and face the challenges encountered in complex pathologies. The biomedical industry demands novel biomaterials capable of targeting several mechanisms and/or cellular pathways simultaneously while being tracked by minimally invasive techniques, thus highlighting the need to shift from monofunctional to multifunctional polymer capsules. In the present review, those strategies that permit the advanced functionalization of polymer capsules are accordingly introduced. Each of the constituents of the capsule (i.e., cavity, multilayer membrane and outer layer) is thoroughly analyzed and a final overview of the combination of all the strategies toward the fabrication of multifunctional capsules is presented. Special emphasis is given to the potential biomedical applications of these multifunctional capsules, including particular examples of the performed *in vitro* and *in vivo* validation studies. Finally, the challenges in the fabrication process and the future perspective for their safe translation into the clinic are summarized.

1. Introduction

Biomaterials are in constant evolution to face the challenges of our current aging population. The definition of biomaterial has been accordingly adjusted to reflect this dynamic situation. According to a contextual dictionary published in 1999, a biomaterial can be considered as a "material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body" [1]. This definition was further reformulated by the same author to be in line with the new biomaterials paradigm, stating that "A biomaterial is a substance that has been engineered to take a form which, alone or as part of a complex system, is used to direct, by control of interactions with component of living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine" [2].

Within the frame of biomaterials for therapeutic and diagnostic applications, future biomaterials are expected to achieve several biological responses simultaneously, being multifunctional in nature and showing optimized performance to treat and track complex pathologies. These complex pathologies, led, among others, by cancer, cardiovascular diseases and central nervous system disorders, demand customized micro- and nanomaterials capable of crossing biological barriers and interacting with the peculiarities of the tissue microenvironment, thus opening new avenues in personalized medicine [3]. For example, central nervous system diseases, which show a rapidly increasing incidence, would be clearly benefited by the combination of multiple functionalities in a

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https://doi.org/10.1016/j.cis.2022.102680
Received in revised form 12 April 2022;
Available online 17 April 2022

single micro- or nanoplatform for their diagnosis and treatment. The ideal future formulations in this field should actively target the disease-associated markers to allow a more precise and efficient drug delivery, thus minimizing off-target side effects. At the same time, the formulation should be equipped with several contrast agents, favoring the multi-modality imaging for early detection. This combination will result in theranostic tools that will have to cross the blood-brain barrier in a noninvasive manner [4]. It is concluded from the previous example that, all in all, biomaterials displaying a single functionality will be clearly inappropriate to meet the requirements demanded by complex pathologies. Diagnosis and/or treatment of cancer represents another clear example where multifunctional biomaterials will be of vital importance. The combination of multiple therapy modalities (e.g., chemotherapy, photodynamic and photothermal therapy) in a nano- or micro-formulation capable of simultaneously acting as an imaging agent could overcome in the near future the limited clinical efficacy observed for monotherapy approaches [5,6].

In this review paper, the layer-by-layer (LbL) approach is presented as a potent tool to fabricate polymeric and hybrid micro- and nano-platforms displaying multifunctional properties, placing particular emphasis on their potential application as diagnostic and therapeutic tools (Fig. 1). Originally, the LbL approach was based on the alternate deposition of charged synthetic polyelectrolytes onto planar substrates to create self-assembled multilayer films through electrostatic interactions [7]. The versatility of the process was rapidly exploited to consider alternative building blocks or elementary units (e.g., natural polymers, inorganic nanoparticles, enzymes, growth factors, genetic material, drugs, etc.) to create multicomponent films not only in planar substrates but also in micro- and nano-colloidal (sacrificial) templates. As a result, polymer micro−/nanocapsules and micro−/nanoparticles of both scientific and technological interest were developed [8]. These systems are now regularly considered in a plethora of biomedical applications as drug/protein/gene delivery vehicles. Furthermore, the precise control over the permeability of the multilayer membrane, that results in a selective diffusion of reagents and byproducts through it, has allowed their use as micro- and nanoreactors where the reactions of interest are performed in a time- and spatial-controlled manner [9]. The shift from monofunctional to multifunctional platforms, together with validation experiments to test their suitability in real *in vivo* scenarios, could pave the way for polymer capsules towards representing a real alternative in the clinical practice.

2. Cavity

The fabrication of polymer micro- and nanocapsules relies on the alternate deposition of building blocks onto a (sacrificial) template. The choice of the template must be carefully considered accordingly because it will determine the shape and the size of the resulting capsules and their subsequent interaction with cells, tissues, and organs. The stability of the template must be preserved during the LbL process to ensure the proper deposition of the multiple layers. At the same time, the removal of the template should be carried out in mild conditions, avoiding any damage to the multilayer assembly and their sensitive components. A thorough consideration of the selected template is thus a must in any protocol intended to be used in the fabrication of polymer capsules for therapeutic and diagnostic applications.

2.1. *The core as the active agent*

The LbL method allows the deposition of nanometer-thick films on virtually any substrate through either physical or chemical interactions. Using drug crystals as a template for the LbL approach results in high loading capacities (i.e., usually in the range of 80–90%), thus representing an important advantage with respect to alternative carriers (e.g.,

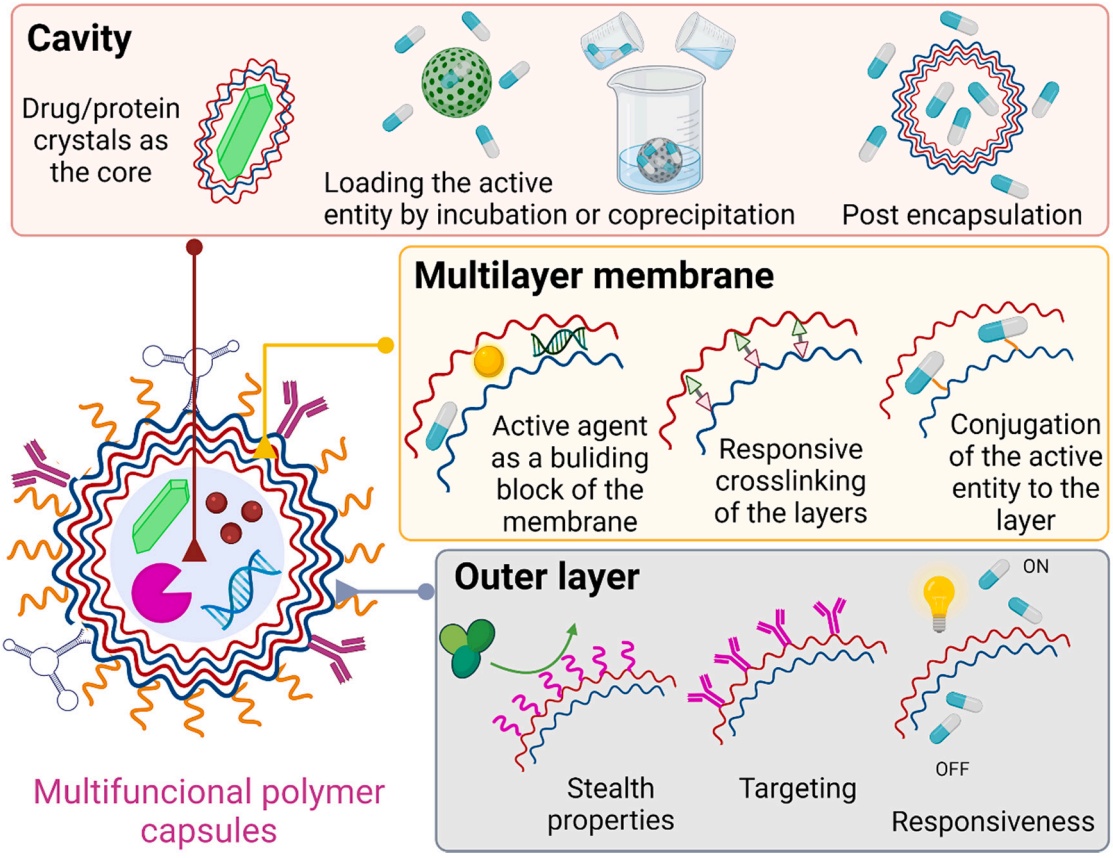

Fig. 1. Schematic representation of the strategies available for the fabrication of multifunctional/multicomponent polymer capsules via the LbL approach.

liposomes, micelles, polymer-drug conjugates), particularly for drugs showing poor solubility in water. The pioneering studies in this field were focused on the encapsulation of drug microcrystals and subsequent modulation of their release by controlling the permeability of the polymeric membrane [10–13]. Increasing the number of deposited layers (i.e., increasing the thickness of the membrane) [10], using polymers with a loopy conformation (e.g., gelatin) [10,13], or annealing the multilayer capsules [12] were some of the strategies that were explored to control the release kinetics. The shift from micron-sized to nano-sized drug crystals further expanded the applicability of these systems by developing novel nanoformulations that could be intravenously administered and boost their capacity to reach target areas passively (e.g., via enhanced permeability and retention (EPR) effect) or actively (e.g., via surface functionalization with antibodies [14], lectins [15], etc.). For the fabrication of nanocrystals, top-down approaches rely on particle size reduction, usually by ultrasonication and subsequent LbL coating, to stabilize the resulting suspension [16]. Contrarily, in bottom-up approaches, a sonication-assisted crystallization of the drug is favored by the progressive addition of a non-solvent to a drug solution (Fig. 2A) [17,18]. Alternative approaches for the synthesis of drug nanocrystals such as solvent evaporation emulsification method [19], nanoprecipitation [20] or spray drying [21] have also been explored. The modification of the outermost layer with poly(ethylene glycol) (PEG) (i.e., PEGylation) is regularly acquired as an effective strategy to improve the colloidal stability of the nanoparticles, reduce protein fouling and consequently increase the circulation time *in vivo* [22,23]. The therapeutic efficacy of this approach was, however, questioned when PEGylated nanoparticles were challenged in an *in vivo* context [24]. PEGylated nanoparticles were rapidly cleared from the blood circulation and did not reach the target tissue, probably due to the displacement of the last layer by serum components and subsequent adsorption of opsonins. This clearly demonstrates the need for more studies about the behavior of LbL particles in the complex environment of the body. The encapsulation of drug nanocrystals via the LbL approach has also been recently proposed to overcome the physiological and anatomical barriers of the gastrointestinal tract after oral administration [25]. In an experimental model of colitis in mice, the multilayer nanoparticles were able to reduce the leakage of the encapsulated cargo (i.e., curcumin) in the stomach (pH = 1.2) and small intestine (pH = 5–7). However, the nanoparticles were accumulated in the inflamed colon (pH > 7) thanks to the charge-reversal of the particles resulting from the dissolution of the negatively-charged outermost layer of cellulose acetate phthalate (CAP). Consequently, curcumin was released at the diseased tissue and reduced the symptoms of colitis (Fig. 2B).

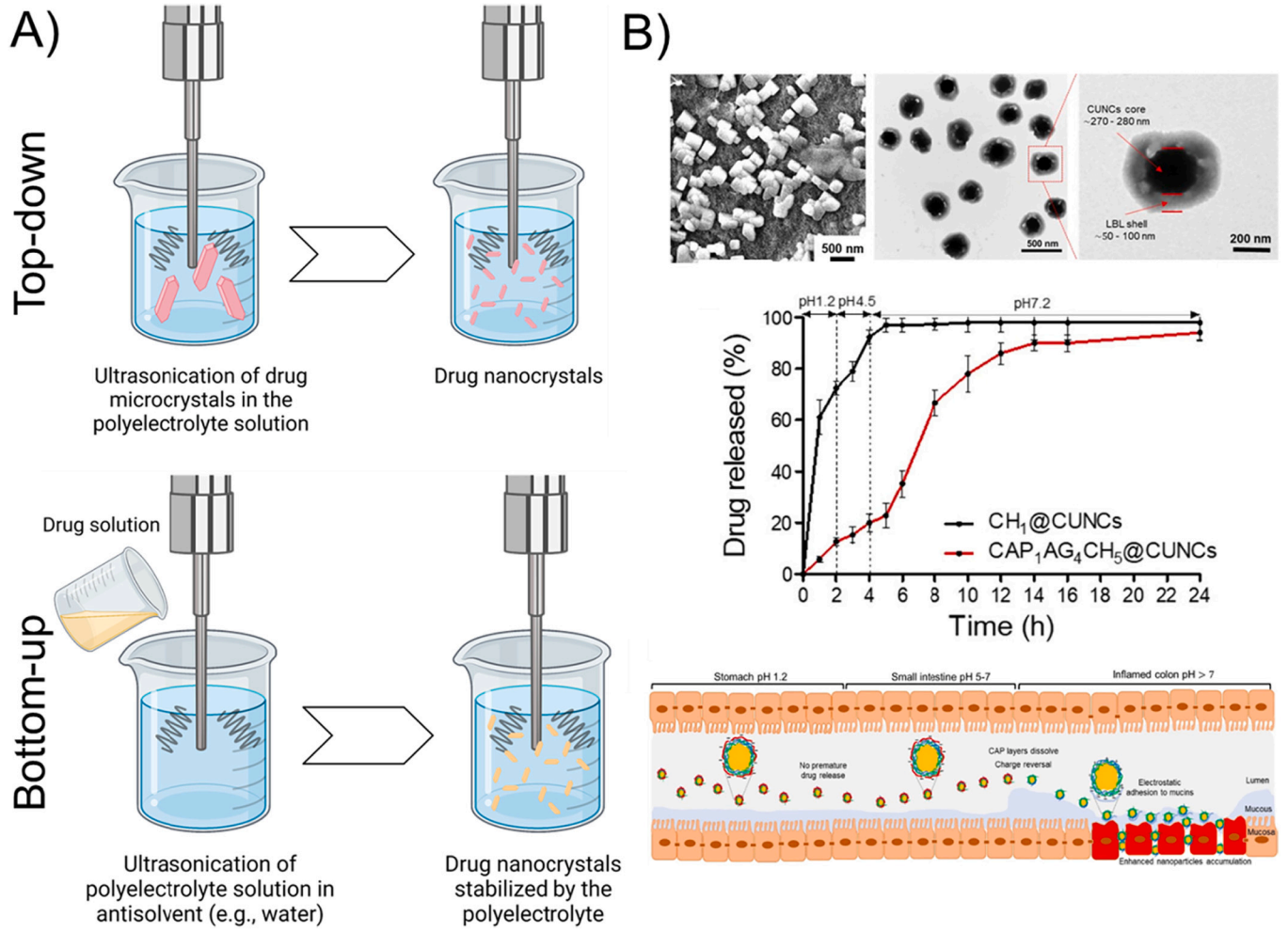

Fig. 2. A) Top-down vs. bottom-up approaches for the fabrication of drug nanocrystals. In this particular example, the figure below describes ultrasound-assisted antisolvent crystallization of the drug. B) Above, SEM and TEM micrographs of curcumin nanocrystals coated with chitosan and alginate and surface-coated with cellulose acetate phthalate (CAP). Increasing the number of multilayers limited the release of curcumin at pH 1.2 (stomach) and pH 5–7 (small intestine), whereas the release was accelerated at pH > 7 (inflamed colon). The dissolution of the outer layer (i.e., CAP) at pH > 7 exposed the positively charged chitosan and benefitted their electrostatic adhesion to mucins in the inflamed colon. Reprinted (adapted) with permission from Biomacromolecules 2020, 21, 9, 3571–3581. Copyright 2021 American Chemical Society.

The LbL approach has also been proposed for the encapsulation and delivery of (bio)macromolecules (e.g., enzymes, peptides), thus overcoming the limitations of these therapeutics such as poor permeability across biological barriers, short half-life in the bloodstream and susceptibility to undergo proteolytic degradation. The encapsulation and sustained delivery of insulin, which is an essential protein hormone for the treatment of diabetes, has attracted particular attention [26]. Insulin micro- and nano-aggregates can be synthesized via precipitation [27] or salting-out method and subsequently be used as the template for the LbL deposition of polymers [28]. The LbL approach opens the possibility to explore alternative delivery routes for insulin, including oral [29] or pulmonary [30] administration and could overcome the drawbacks associated with subcutaneous injections such as discomfort and poor compliance. The reported *in vivo* studies [27] showed that encapsulated insulin decreased blood glucose levels more efficiently and in a more sustained manner than the non-encapsulated counterpart after oral administration, thanks to a preserved stability of the insulin under acidic gastric conditions.

Encapsulation of active enzyme biocrystals allows the use of LbL micro- and nano-entities as micro- and nanoreactors. Contrarily to the aforementioned examples, where the encapsulated cargo is released by diffusion or triggered by a relevant stimulus, in micro- and nanoreactors the confined enzyme is protected from the outer environment while the substrates and reaction (by)products diffuse across the polymeric membrane [9,31]. The LbL deposition can be performed without causing any detrimental effect on the encapsulated enzyme and can further protect the activity of the enzyme against proteolysis [32]. In comparison to the conventional LbL deposition of enzymes in solution, the use of encapsulated enzymes results in films displaying up to 50 times higher biocatalytic activities [33]. This allows the development of more stable, robust and sensitive biosensors [34,35].

Recently, an alternative method that allows the encapsulation of virtually any active cargo at high loading capacity (> 80%) has been presented for the fabrication of capsules with controllable geometry [36,37]. This method represents a shift from traditional to reversed production processes, in which defined-shape open microchambers are first created by imprinting and subsequently covered with a polymeric film via the LbL approach. Then, the cargo is placed inside each microchamber through either powder deposition or crystallization from saturated solutions. Finally, the microchambers are sealed with the polymeric film and the template removed to yield a core-shell microstructure. Although this method presents important advantages with respect to more traditional approaches in terms of versatility in the choice of active substances and controllable geometry of the resulting microcapsules, the transition from micro- to nanoscale is currently a limitation.

2.2. Modification of the (sacrificial) template

Using drug crystals as a template in the LbL approach is a valid strategy to improve the stability and availability of drugs, particularly in the case of highly hydrophobic and poorly water-soluble compounds. However, controlling the shape, the polydispersity and the size at the nanometer scale of the resulting formulations still represents a limitation in this approach. The use of pre-synthesized templates as drug carriers and their subsequent coating via the LbL approach may overcome the aforementioned limitations (Fig. 3). The use of highly porous templates such as mesoporous silica or calcium carbonate nano- and microparticles are of great value in this context thanks to their high surface area and controllable pore size that results in high loading capacities. Dissolving the drug of interest in an adequate solvent (e.g., chloroform, ethanol) and incubating with the porous template has become a routine protocol to encapsulate high payloads of water-insoluble drugs within polymer capsules made by LbL [38]. The

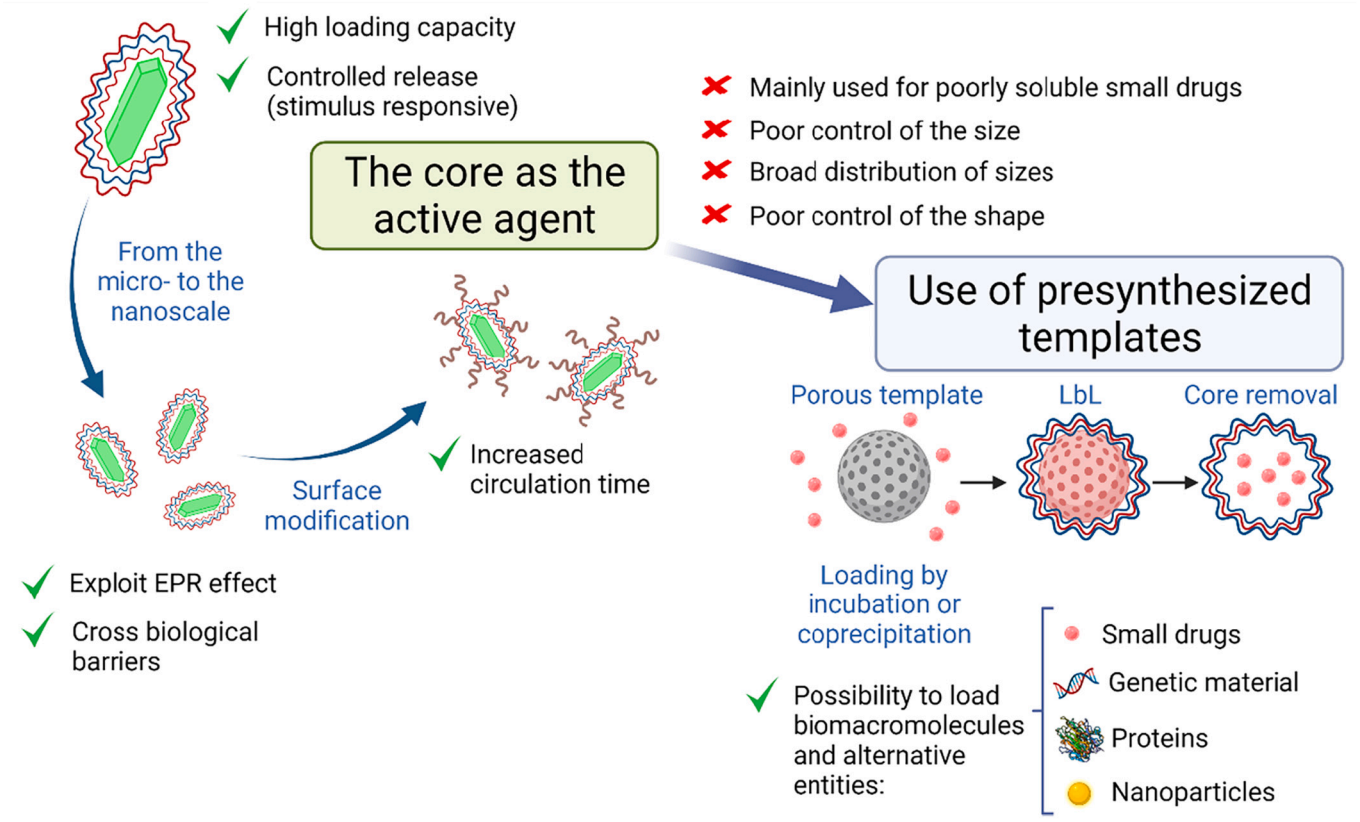

Fig. 3. Transition from drug crystals to presynthesized nano- and microparticles as templates for the fabrication of polymer capsules intended to be used in biomedical applications.

template can be finally eliminated or not, leaving behind polymer capsules or core-shell structures, respectively. This strategy has been used for the encapsulation of doxorubicin (DOX) within polymer capsules made out of tannic acid (TA) and poly(N-vinylpyrrolidone) (PVPON) that respond to ultrasound irradiation and allow a triggered release of the encapsulated cargo under both low-power and high-power irradiation [39]. Similarly, the co-incubation with porous templates can be further exploited to encapsulate alternative entities such as contrast agents, which are of high interest for imaging applications [40]. Incorporation of functional groups on the surface of the (sacrificial) template can be further considered to maximize the amount of incorporated agent (e.g., 1.96 wt% of indocyanine green was loaded into pristine mesoporous silica nanoparticles vs. 16.5 wt% for the amine-functionalized counterparts) and improve accordingly the photoacoustic signal intensity both *in vitro* and *in vivo*. Even if the use of porous templates has been the preferred method to encapsulate water-insoluble drugs into LbL structures, alternative approaches are also being explored. These include, among others, the use of zein nanoparticles as colloidal delivery systems for hydrophobic compounds with a reported loading capacity for curcumin loaded by antisolvent precipitation of 5.7% [41,42].

Apart from the incorporation of small molecules into solid templates, their modification with genetic material and subsequent coating with a multilayer membrane has been also widely reported [43–45]. The encapsulated material is protected from external insults (e.g., chemical degradation by nucleases) by the polymeric membrane and remains active after the encapsulation process. This approach requires fine control over the adsorbed DNA on the sacrificial template: a complete surface coverage of the template with DNA may hinder the formation of the capsules because of the poor interaction between the deposited polyelectrolyte and DNA [43]. Enzymes or nanoparticles are also prone to be incorporated in solid templates by simple co-incubation [46,47]. In the case of enzymes, a high loading capacity must be accompanied by the preservation of the enzyme activity to ensure the viability of the process. In this sense, the shape of vaterite (i.e., a polymorph of calcium carbonate) microparticles determined the activity of the adsorbed enzyme, being the star-like vaterite particles preferable over other shapes (e.g., spherical, elliptical and rhomboidal).

The coprecipitation process, which relies on the *in situ* incorporation of the active agent during the formation of calcium carbonate micro- and nanoparticles, has been widely reported as an alternative strategy to the coincubation process described above. This process allows the incorporation of a wide variety of theranostic agents (e.g., nanoparticles, growth factors, enzymes, DNA) in a simple way, displaying extraordinarily good encapsulation efficiencies. Besides, since the subsequent elimination of the template occurs in mild conditions via the incubation with a calcium-chelating agent, the process to obtain polymer capsules does not have any detrimental effect on the activity of the encapsulated entity. Following this approach, iron oxide nanocubes were incorporated into calcium carbonate particles that were used as a sacrificial template for the fabrication of polymer capsules [48]. In comparison to free nanocubes, which tend to agglomerate in the cellular microenvironment, encapsulated counterparts maintained higher specific absorption rate values and preserved the magnetic losses. This is of great importance for hyperthermia applications since the dynamic magnetic response is almost unaffected in a real biological environment. Following similar strategies, other inorganic nanoparticles (e.g., gold nanorods, superparamagnetic iron oxide nanoparticles (SPIONs)) have been coprecipitated within the calcium carbonate templates for the fabrication of polymer capsules displaying multifunctional properties (e.g., cancer chemo-photothermal therapy [49]), or for tracking stem cells after *in vivo* administration [50].

The encapsulation of proteins, including growth factors and enzymes, has clearly benefited from the coprecipitation process. Moreover, the coprecipitation process allows the incorporation of a cocktail of proteins in the calcium carbonate template, which may be essential to preserve the activity of the encapsulated entity [51,52]. For example, basic fibroblast growth factor (bFGF) preserved its activity during the encapsulation process when heparin and bovine serum albumin (BSA) were coprecipitated in vaterite microparticles, promoting the proliferation of L929 fibroblasts. Therapeutic enzymes, such as catalase (CAT) [53] and asparaginase [54], have also been successfully incorporated into calcium carbonate microparticles for the fabrication of polymer capsules with potential biomedical applications (Fig. 4A). Capsules loaded with CAT were able to scavenge hydrogen peroxide (Fig. 4B) in an inflammation model of nucleus pulposus cells and consequently attenuated the expression of major proteolytic enzymes (Fig. 4C). In the case of asparaginase, direct incubation with standard concentrations of the calcium chelating agent (i.e., 0.1 M) inhibited the activity of the enzyme. Therefore, alternative protocols were adopted to maintain its activity within the polymer capsules. The encapsulated asparaginase was accordingly protected from proteases by the polymeric membrane and showed potential application for the treatment of leukemia.

Finally, within the process of coprecipitation to functionalize the template, recent advances about the incorporation of genetic material must be highlighted. In a recently reported systematic study [55], plasmid DNA was co-precipitated in calcium carbonate submicron particles, together with organic additives (e.g., human serum albumin, dextran sulfate (Dex), glutathione (GSH), poly-L-arginine (Parg), protamine sulfate (Pro), TA, inhibitors of DNase II). The incorporation of organic additives, particularly the combination of Parg and DNase II inhibitor, significantly improved the transfection efficiency (from <6% in the absence of organic additives to 72%). Coprecipitation of messenger RNA (mRNA) and RNase inhibitors has also resulted beneficial in improving the transfection efficiency of the developed capsules [56].

The versatility and simplicity of the coprecipitation process in terms of the possibility to co-encapsulate a wide variety of entities within calcium carbonate particles could pave the way for the development of theranostic systems with multifunctional properties. Recently, positron emitters were incorporated in calcium carbonate core-shell particles via the coprecipitation approach, allowing to determine the biodistribution of the particles by both positron emission tomography (PET) and computerized tomography (CT) [57]. Among the studied strategies to incorporate the positron emitter into the core-shell particles, the coprecipitation technique was the most efficient in terms of radiolabeling stability. Alternatives to the most generally employed approaches for the modification of the template discussed herein (i.e., coincubation of the template with the active agent and coprecipitation) are regularly being reported. Freezing-induced loading appears as a promising strategy, considering that it can be used for the incorporation of proteins and inorganic nanoparticles into calcium carbonate nanoparticles and also due to the reported high loading efficiencies (four times larger encapsulated amount in comparison to coincubation and coprecipitation) [58,59]. However, the freezing/thawing cycles could have detrimental effects on the activity of more sensitive entities (e.g., enzymes) that could limit the universal application of this approach.

2.3. Post encapsulation

The controllable permeability of the polymeric shell allows the loading of therapeutic agents after the fabrication of the capsules. Although post encapsulation has also been applied for the loading of macromolecules, encapsulation of high-molecular weight entities requires the “opening” of the capsules, usually by reducing the interaction of its shell constituents. The conditions to promote the transition from the “closed” to the “opened” state should be carefully controlled to avoid the complete disassembly of the capsule and the degradation/denaturation of the encapsulated entity, which cannot always be guaranteed. Therefore, several authors studying the encapsulation of a library of entities have preferred the preloading process (described in the previous section) for molecular cargos with molecular weight above 10 kDa, while the post encapsulation process followed by a heat shrinking was

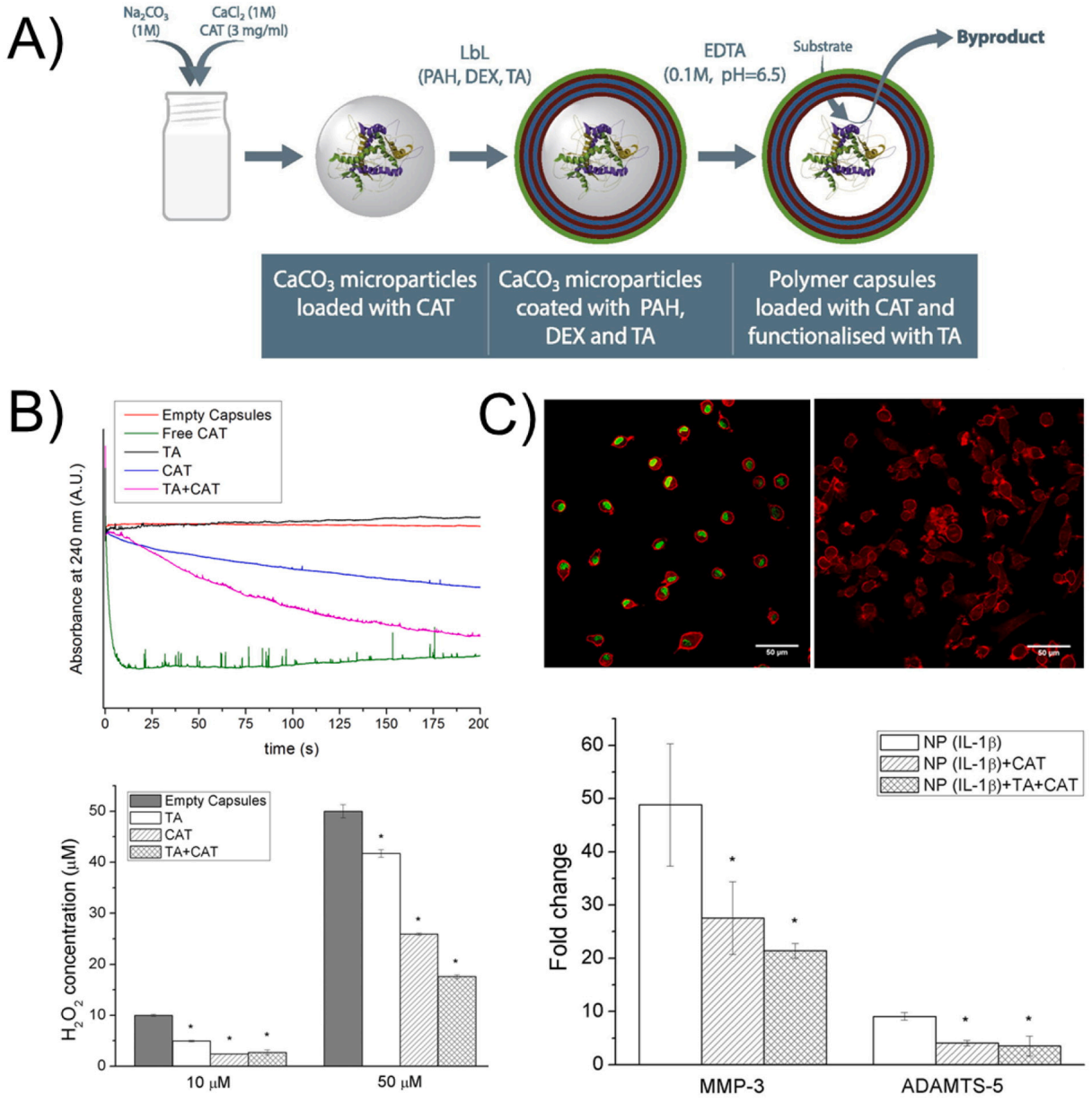

Fig. 4. A) Schematic representation of the fabrication of CAT-loaded capsules. The template is fabricated via the coprecipitation process in the presence of CAT. B) Stopped-flow (above) and hydrogen peroxide detection kit (below) measurements showing the capacity of CAT-loaded capsules (CAT) to scavenge H_2O_2 from solution. C) Cellular oxidative stress staining (green) of nucleus pulposus cells stimulated with a pro-inflammatory cytokine (IL-1β) (left) and further incubated with CAT-loaded capsules (right). Below, the expression of major proteolytic enzymes (i.e., MMP-3 and ADAMTS-5) by cells stimulated with IL-1β (NP (IL-1β)) and further treated with CAT-loaded capsules (NP (IL-1β) + CAT)). Reprinted from Acta Biomaterialia, 67, A. Larrañaga et al., Antioxidant functionalized polymer capsules to prevent oxidative stress, 21–31, 2018, with permission from Elsevier. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used for cargos of small-molecular weight [60].

The incubation of hollow polymer capsules with water-soluble drugs (e.g., DOX hydrochloride) in aqueous solutions is a simple method to load the drug of interest in the cavity of the capsules. However, since the process relies simply upon a diffusion mechanism, the release profile usually resembles the loading profile and results in a very rapid release rate. Crosslinking of the polymeric shell constituents [61] or temperature-induced shrinking of the capsules [62] are commonly followed to ensure a more controlled release of the encapsulated cargo. The latest was recently used for the encapsulation of small-molecular weight water-soluble drugs (i.e., gemcitabine and clodronate) into Parg/Dex capsules of ~300 nm that were preferentially accumulated in the lung tumors in comparison to healthy organs thanks to the EPR effect (Fig. 5) [63].

Although the incubation of water-soluble drugs with hollow polymer capsules may seem a trivial approach, there are several parameters (e.g., incubation time, solute concentration, pH, ionic strength) that need to be finely adjusted to achieve high loading efficiencies and capacities [64]. Drug molecules will not only fill the inner cavity of the capsule but will also be adsorbed by the constituents of the polymeric shell through molecular interactions. Considering the varying charge of the drugs and the polymeric constituents at different pHs and the influence of ionic strength on their conformation, small alterations in these parameters can result in dramatic changes in the loading efficiency and capacity. For the encapsulation of lipophilic drugs (e.g., DOX and 5-fluorouracil), incubation of capsules in a drug/oleic acid mixture allows the

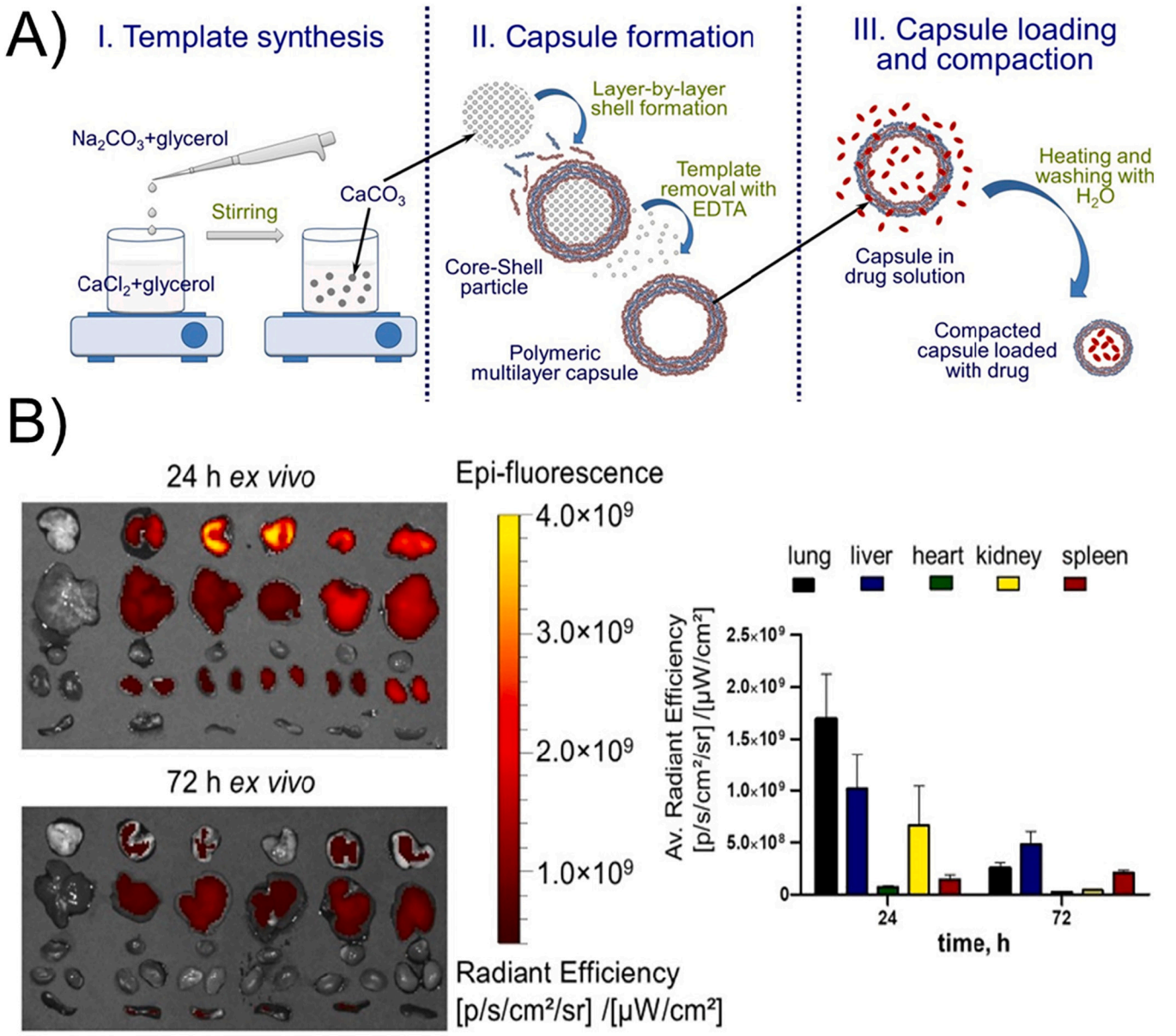

Fig. 5. A) Schematic representation of the capsule fabrication and drug loading by incubation and subsequent shrinking by heat treatment. B) Imaging of isolated organs after the administration of fluorescent capsules and its quantification, highlighting the preferential accumulation in lung and liver. Reprinted (adapted) with permission from ACS Appl. Mater. Interfaces 2020, 12, 5, 5610–5623. Copyright 2021 American Chemical Society.

infiltration of the drug and the filling of the cavity of the capsules [65].

The so-called spontaneous deposition has been widely reported as a strategy to load a plethora of active entities in the interior of polymer capsules under ordinary conditions (i.e., room temperature, neutral pH, in pure water) [66,67]. In this approach, a positively-charged drug can spontaneously diffuse from a low- (e.g., the bulk) to a high-concentration region (e.g., the interior of the capsule) thanks to the presence of negatively charged species in the interior of the capsule. This phenomenon was first reported for capsules fabricated with melamine formaldehyde (MF) as a core and poly(sodium 4-styrene sulfonate)/poly (allylamine hydrochloride) (PSS/PAH) as polyelectrolytes. During template removal at low pH, a complex between PSS and MF is formed, which remains inside the capsule due to its large size and provides the driving force to induce positively-charged molecules to penetrate through the capsule wall (Fig. 6). Biomacromolecules (e.g., enzymes) have similarly benefited from the spontaneous deposition [68]. By adjusting the pH of the enzymatic solution below its isoelectric point, the charge of the enzymes becomes positive, thus favouring the spontaneous diffusion towards the interior of the capsule. However, this process may not be valid for more sensitive enzymes, in which a slight change in the pH of the solution may result in conformational changes and a detrimental effect on their activity. Inspired by the spontaneous deposition first described for MF cores, other templates have been modified to facilitate the post encapsulation of therapeutic agents (Fig. 6), including PSS-doped $CaCO_3$ microparticles for the subsequent loading of daunorubicin [69], or the coprecipitation of heparin or cyclodexrin (CD) for the encapsulation of transforming growth factor (TGF-β1) [70] and hydrophobic entities (e.g., coumarin, Nile red) [71], respectively. The modification of nanoporous silica particles with poly-L-lysine (PLL) has been similarly used to post encapsulate small interfering RNA (siRNA) into poly(methacrylic acid) (PMA) polymer capsules [72]. By limiting the number of bilayers to five, siRNA could cross the polymeric membrane and be sequestered by PLL, forming a PLL-siRNA complex that is retained in the capsule interior. In this particular

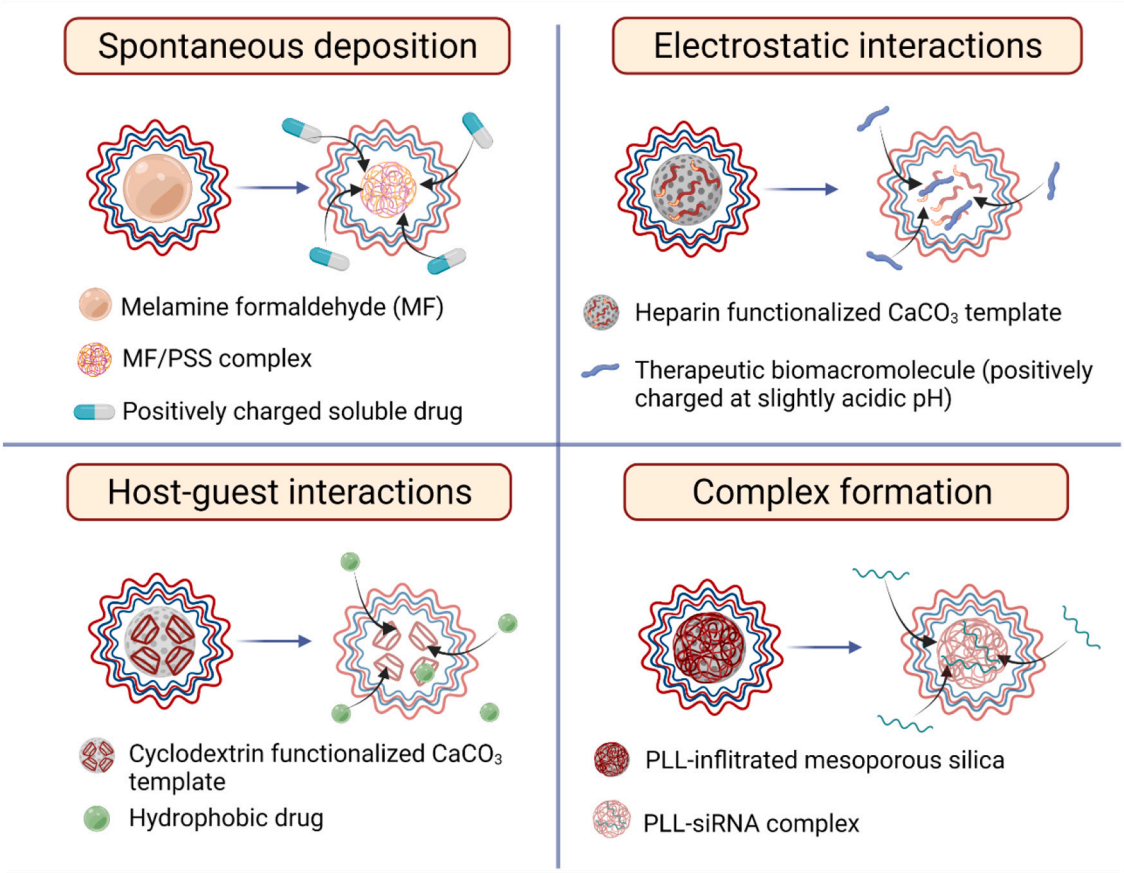

Fig. 6. Different strategies inspired by the original spontaneous deposition that allow the post encapsulation of active entities within the cavity of polymer capsules.

study, the expression of the target gene was downregulated even when scrambled siRNA or empty capsules were used, suggesting a capsule-dependent off-target effect due to a cellular macroautophagy response.

As mentioned at the beginning of this section, biomacromolecules (e.g., enzymes, growth factors) can also be post encapsulated in pre-fabricated microcapsules by reversibly switching the state of the capsules from a "closed" (impermeable) to an "opened" (permeable) state. The permeability of PAH/PSS multilayer microcapsules was reversibly switched when the NaCl concentration was varied between 0.5×10^{-2} and 2×10^{-2} M [73]. At increasing salt concentration, the ionic strength may weaken the electrostatic interaction between polyelectrolytes, thus favouring the swelling of the capsules and the corresponding diffusion of the macromolecules that were used as a control model. Raising the pH of the solution also resulted in increased permeability of chitosan (Chi)/Dex and Pro/Dex based capsules, presumably because of the electrostatic repulsion caused by the gradual deprotonation of the amine groups in the cationic polyelectrolyte [74,75]. This allowed the encapsulation of bFGF and peroxidase (POD), respectively, with reported loading capacities of 34 μg/mg of capsules in the case of bFGF and 2.2×10^8 molecules/capsule in the case of POD. Alternative approaches to reversibly switch the permeability of the capsule and allow the encapsulation of biomacromolecules, including the incubation of multilayer polymer capsules in water/ethanol mixtures, have also been reported [76]. Several aspects, such as multilayer shell composition [77] (e.g., the amount of encapsulated BSA in alginate (Alg)/Dex capsules was 2.5 times higher than in Dex/Parg counterparts), need to be carefully considered when trying to post encapsulate biomacromolecules in multilayer polymer capsules to ensure an efficient loading process and a sustained release. Above all, preserving the activity of pH-, salt-, solvent-sensitive biomacromolecules (i.e., enzymes) is of vital importance and this cannot always be guaranteed with the aforementioned strategies.

Table 1 contains several examples where the cavity of capsules has been functionalized and the potential of the resulting capsules in biomedical applications has been tested either *in vitro* or *in vivo*.

2.4. Conditions and implications for core removal

When hollow architectures are pursued (i.e., hollow capsules), the protocol to remove the core must ensure that neither the assembly/integrity of the multilayer membrane nor the activity of the active entity are compromised. Similarly, considering that the resulting capsules may find application in the biomedical field, the use of reagents that trigger a toxic and/or inflammatory response should always be avoided. There exist nowadays a plethora of commercially available colloidal formulations that can act as sacrificial templates for the LbL approach (Table 2). The synthesis of polymer based colloidal templates is well established and permits the incorporation of several chemical functionalities and molecules in nano- and microparticles showing a narrow size distribution. Polystyrene (PS) and poly(methyl methacrylate) (PMMA) with several functionalities (e.g., amine, carboxyl, streptavidin, antibodies) on their surface, fluorescently tagged and in a wide variety of size ranges (i.e., from 15 nm to 750 μm) are manufactured and supplied by several companies (e.g., CD Bioparticles). These nano−/microparticles, as well as similar alternatives based on bioresorbable polyesters (e.g., poly(lactic acid) (PLA), poly(lactic-*co*-glycolic acid) (PLGA)), require the use of organic solvents (e.g., tetrahydrofuran (THF), chloroform) [79,80] for template removal that may have a detrimental effect in terms of cytocompatibility if not totally eliminated. Similarly, within the commercially available polymeric particles, MF was first considered as a sacrificial template for the LbL approach. Despite the current availability of MF nano- and microparticles with controllable sizes (i.e., from 500 nm to 14 μm) and surface

Table 1
Strategies to incorporate functionalities into the cavity of LbL capsules intended to be used in biomedical applications.

Strategy	Encapsulated entity	Approach	Biomedical application	Outcomes	Ref.
Core as the active agent	Curcumin	Ultrasound-assisted antisolvent crystallization (core of c.a. 300 nm).	Ulcerative colitis (*in vitro* and *in vivo*)	• Preferential accumulation in inflamed colon. • Enhanced efficacy in controlling inflammation.	[25]
Modification of the (sacrificial) template	Paclitaxel	Solvent emulsification evaporation method (core of c.a. 100 nm)	Cancer (*in vitro* with Caco-2 cells)	• More targeted binding of the lectin-functionalized particles.	[15]
		Ultrasonication (core of c.a. 100 nm)	Cancer (*in vitro* with MCF-7 and BT-20 cells)	• Enhanced cytotoxic effect on antibody-functionalized nanoparticles. • Improved colloidal stability *in vitro* after PEGylation.	[14]
		Wet milling (core of c.a. 200 nm)	Cancer (*in vitro* and *in vivo*)	• Rapid clearance from the bloodstream *in vivo*.	[24]
		Nanoprecipitation (core of c.a. 150 nm)	Cancer (*in vitro* with MCF-7)	• The encapsulation of the drug did not weaken its mechanism of action.	[20]
	Campotothecin	Nanoprecipitation (core of c.a. 160 nm)	Cancer (*in vitro* with a glioblastoma cell line)	• Improved colloidal stability *in vitro* after PEGylation. • Prolonged activity of the drug.	[23]
	Ibuprofen	Ultrasonication (core of c.a. 100 nm)	Oral delivery of therapeutics (*in vitro* with Caco-2 cells)	• Good cytocompatibility of the capsules.	[16]
	Insulin	Core of c.a. 5–20 μm	Diabetes, pulmonary delivery (*in vivo*)	• Controlled delivery of insulin via intrapulmonary administration depending on the number of layers.	[30]
		Precipitation (core of c.a. 1 μm)	Diabetes, oral delivery (*in vivo*)	• Enhanced stability of the encapsulated insulin at acidic pH. • Sustained and dose-dependent reduction of blood glucose after oral administration.	[27]
		Salting out (core of c.a. 100–230 nm)	Diabetes (neither *in vitro*, nor *in vivo*)	• Low release of insulin at acidic pH but sustained release at neutral pH.	[28]
	Catalase	Catalase crystals (core of c.a. 10 μm)	Hydrogen peroxide biosensor	• Higher biocatalytic activities with respect to non-encapsulated counterpart.	[32–34]
	Glucose oxidase	Glucose oxidase crystals (core of c.a. 30 μm)	Glucose biosensor	• Faster response of the biosensor with respect to non-encapsulated counterpart.	[35]
	Paclitaxel	Coincubation with mesoporous silica nanoparticles (core of c.a. 400 nm)	Cancer (*in vitro* with LIM1899 colorectal cancer cells)	• Encapsulated drugs show similar efficacy to the free drugs.	[38]
	Doxorubicin	Coincubation with porous silica microparticles (core of c.a. 3–5 μm)	Cancer (*in vitro* with MCF-7)	• Ultrasound triggered release of the encapsulated cargo. • Ultrasound imaging contrast depend on capsule's rigidity, thickness and molecular weight.	[39]
	Indocyanine green	Coincubation with surface-functionalized mesoporous silica nanoparticles (core of c.a. 65 nm)	Photoacoustic imaging (*in vitro* and subcutaneously with mouse cadavers)	• Increasing photostability and photoacoustic signal for the LbL encapsulated formulations.	[40]
	Curcumin	Antisolvent precipitation with zein nanoparticles (core of c.a. 150 nm)	Oral delivery of therapeutics (neither *in vitro*, nor *in vivo*)	• Increased light, thermal and storage stability of the encapsulated drug. • Controlled release of the encapsulated drug.	[41,42]
	Iron oxide nanocubes	Coprecipitation in calcium carbonate particles (core of c.a. 1 μm)	Hyperthermia-Cancer (*in vitro* with SKOV-3 ovarian carcinoma cells)	• Higher specific absorption rate values for the encapsulated nanocubes. • More predictable heating dose inside biological matrices for encapsulated nanocubes.	[48]
	Gold nanorods	Coprecipitation in calcium carbonate particles (core of c.a. 4.5 μm)	Chemo-photothermal therapy-Cancer (*in vitro* with THP-1 cells)	• NIR triggered drug release. • Increased cell death under combination of drugs + NIR stimulation.	[49]
	Manganese dioxide nanoparticles	Coincubation with calcium carbonate particles (core of c.a. 2–3 μm)	Scavenging of reactive oxygen species (*in vitro* with HeLa cells)	• Surface charge of the capsules determine their interaction with cells. • Capsules prevent cell death in an oxidative stress *in vitro* model.	[47]
	Nerve growth factor	Coprecipitation in calcium carbonate particles (core of c.a. 2–3 μm)	Treatment of neuropathologies (*in vitro* with hippocampal neurons)	• The presence of capsules accelerate neurite growth and promote the development of primary and secondary-order branches.	[52]
	Catalase	Coprecipitation in calcium carbonate particles (core of c.a. 2–3 μm)	Intervertebral disc degeneration (*in vitro* with nucleus pulposus cells)	• Encapsulated catalase preserves its activity. • Capsules scavenge hydrogen peroxide from solution and attenuate oxidative stress in cells.	[53]
	Asparaginase	Coprecipitation in calcium carbonate particles (core of c.a. 1–2 μm)	Leukemia (*in vitro* with SD1 and MOLT-4 cancerous lymphocytes)	• Template removal under dialysis preserve the activity of the enzyme. • Encapsulated enzyme promotes cell death in the presence of proteases, which inhibited the activity of the free enzyme.	[54]

(*continued on next page*)

Table 1 (*continued*)

Strategy	Encapsulated entity	Approach	Biomedical application	Outcomes	Ref.
Post encapsulation	siRNA	Coprecipitation in calcium carbonate particles (core of c.a. 2–3 μm)	Treatment of influenza virus infection (*in vitro* with MDCK kidney and A549 epithelial cells)	• Reduction of viral nucleoprotein level and inhibition of influenza virus production in infected cells.	[78]
	Doxorubicin	Incubation with prefabricated capsules followed by crosslinking of the polyelectrolytes (core of c.a. 220 nm)	Combination cancer therapy (*in vitro* with HeLa cells)	• The release of the encapsulated cargo occurs too fast if the polymeric shell is not crosslinked.	[61]
		Incubation with prefabricated capsules followed by temperature induced shrinking (core of c.a. 490 nm)	Cancer (*in vitro* with MCF-7 and MCF7-ADR cells)	• Shrunken capsules allow a more sustained release of the encapsulated drug.	[62]
	Gemcitabine and clodronate	Incubation with prefabricated capsules followed by temperature induced shrinking (core of c.a. 500 nm)	Lung cancer (*in vitro* with mouse lung cancer spheroids and bone marrow-derived macrophages; *in vivo* in a lung cancer model with mice)	• Preferential accumulation of capsules in tumor lungs with respect to healthy tissue. • The tumor-promoting function of macrophages is suppressed after the pretreatment with capsules.	[63]
	5-fluorouracil	Filling hollow polymer capsules with an oil phase containing the drug (core of c.a. 500 nm)	Cancer (*in vitro* with LIM1215 human colorectal cancer cell line)	• Release of the encapsulated cargo under reducing conditions. • Encapsulated drugs are more effective in killing cancer cells than free drugs.	[65]
	Daunorubicin	Spontaneous deposition using melamine formaldehyde as a sacrificial core (core of c.a. 5 μm)	Leukemia (*in vitro* with HL-60 cells)	• Gradual cytotoxic effect was observed for encapsulated drugs in comparison to "just at one time" effect for free drugs.	[66]
	TGF-β1	Spontaneous deposition into heparin loaded prefabricated capsules (core of c.a. 5 μm)	Wound healing (*in vitro* with human dermal fibroblast)	• The growth factor remains active after the encapsulation process and is able to induce transdifferentiation to myofibroblast in a similar way to the growth factor in solution.	[70]
	bFGF	Loading the growth factor at high pH to increase the permeability of the polymeric shell (core of c.a. 3 μm)	Proof of concept work for the delivery of biomacromolecules (*in vitro* with L929 fibroblasts)	• Steady state growth of cells cultured with encapsulated growth factor thanks to the sustained release.	[74]
	siRNA	Complexation with preinfiltrated polylysine (core of c.a. 1 μm)	Prostate cancer (*in vitro* with PC-3 cells)	• Down-regulation of the target gene also occurs in the presence of empty capsules, suggesting a capsule-dependent off-target effect.	[72]

Table 2
Regularly considered sacrificial templates for the fabrication of polymer capsules and their commercial specifications.

Material	Commercially available sizes	Commercially available surface functionalities	Common solvent for elimination	Complete removal
Melamine formaldehyde	500 nm-14 μm	Limited: carboxyl, amine and methylol	HCl or organic solvents (DMF, DMSO)	Difficult
Thermoplastic polymers (PS, PMMS, PLA, PLGA)	15 nm-750 μm	Extense: amine, carboxyl, NHS, streptavidin, acrylate, antibodies, etc.	Organic solvents (THF, DCM)	Difficult
Silica	10 nm-50 μm	Extense: amine, carboxyl, NHS, streptavidin, acrylate, antibodies, etc.	hydrofluoric acid	Easy
Calcium carbonate	150 nm-5 μm[a]	N/A	EDTA	Easy
Hydrogels (calcium alginate and dextran)	N/A	N/A	EDTA/aqueous solution	Difficult

[a] Values obtained from several studies reporting the synthesis of vaterite particles via well-established protocols.

functionalities (e.g., carboxyl, amine, methylol), the difficulty to fully remove the sacrificial template, together with the need of extremely acidic pH (i.e., pH value <1.6) or the use of organic solvents (e.g., N,N-dimethylformamide (DMF) or dimethyl sulfoxide (DMSO)) [81,82], have gradually decreased the interest on the use of MF as a sacrificial template in favour of better alternatives.

Inorganic alternatives, such as silica and calcium carbonate nano- and microparticles, are gaining increasing attention for the fabrication of LbL polymer capsules thanks to their decomposition into low molecular weight ions that avoid osmotic stress during their removal. Silica particles are again commercially available in a wide range of sizes (i.e., from 10 nm to 50 μm), surface functionalities and fluorescent tags. Besides, their mesoporous counterpart with tuneable pore size and architecture allows high loading capacities due to their associated high surface area (i.e., 1500 m^2 g^{-1}). Moreover, the use of active drugs as a surfactant in the synthesis of silica nanoparticles results in much higher loading capacities with respect to the traditional incubation method (e.g., 34% of loading capacity vs. <1% for the traditional method) [83]. However, removal of silica-based sacrificial templates requires the use of hydrofluoric acid that may not always guarantee the integrity of the capsule and the protection of the encapsulated cargo [84]. The use of inorganic templates based on calcium carbonate is gradually gaining interest over the more traditionally considered silica particles thanks to their larger pore size, easy/inexpensive production and possibility to be removed under mild conditions (i.e., calcium chelating agent at neutral pH). The less thermodynamically stable polymorph of calcium carbonate (i.e., vaterite) is of particular interest for biomedical applications since it allows high loading of theranostic entities (e.g., small molecular

weight drugs, biomacromolecules, nanoparticles, etc.) by different routes (e.g., adsorption, infiltration, co-precipitation) within its porous structure [85]. Although achieving sub-micron vaterite particles represented an important limitation at the beginning, the use of ethylene glycol and other polyols (e.g., glycerol, erythritol [86]) has been regularly adopted as a universal protocol to achieve particles of around 400 nm [87,88]. Other additives, such as poly(vinylsulfonic acid) (PVSA) allows a further reduction of particle size up to 150 nm [89]. However, in comparison to the aforementioned polyols, alternative additives may precipitate in the calcium carbonate structure and alter the composition and toxicity of the final formulation. Development of protocols that allow a better size control and narrower size distribution, long-term stability avoiding the natural transition from vaterite to calcite (i.e., thermodynamically more stable polymorph), and commercial availability, will determine the ultimate establishment of vaterite micro- and nanoparticles as the gold standard template for future LbL formulations.

Alternative approaches to the most commonly used templates discussed above have been also reported in bibliography, including calcium alginate or dextran hydrogels [90,91]. However, considering all the benefits of calcium carbonate templates in terms of biocompatibility, porosity and available routes to incorporate a wide variety of active entities, the use of this template is foreseen as dominant in forthcoming studies and trials regarding the use of polymer capsules for biomedical applications.

3. Multilayer Membrane

A rational design of the polymeric membrane architecture and its subsequent assembly onto the colloidal template may affect both the mechanical integrity of the resulting capsule and its performance during a required application. Apart from shielding the encapsulated cargo from the surrounding harsh environment, the polymeric membrane endows the capsules with responsiveness towards relevant stimuli and structural stability to avoid their disassembly along their journey to the target site. The functionalization of the membrane can be achieved by using a wide variety of inorganic particles and/or therapeutic agents as building blocks in the assembly process. Alternatively, building blocks can be functionalized prior to or after their deposition on the template. This enables adaptation of the features and performance of the membrane to meet the requirements of a specific biomedical application.

3.1. The layer as the active agent

The use of an active agent as a building block of the polymeric membrane is one of the most employed strategies to provide functional properties to the multilayered capsules. The incorporation of inorganic nanoparticles between the layers endows polymeric capsules with functionalities such as the capability to be guided, activated by means of an external stimulus or monitored/tracked. Incorporation of iron oxide nanoparticles, especially magnetite (Fe_3O_4) nanoparticles, is a common

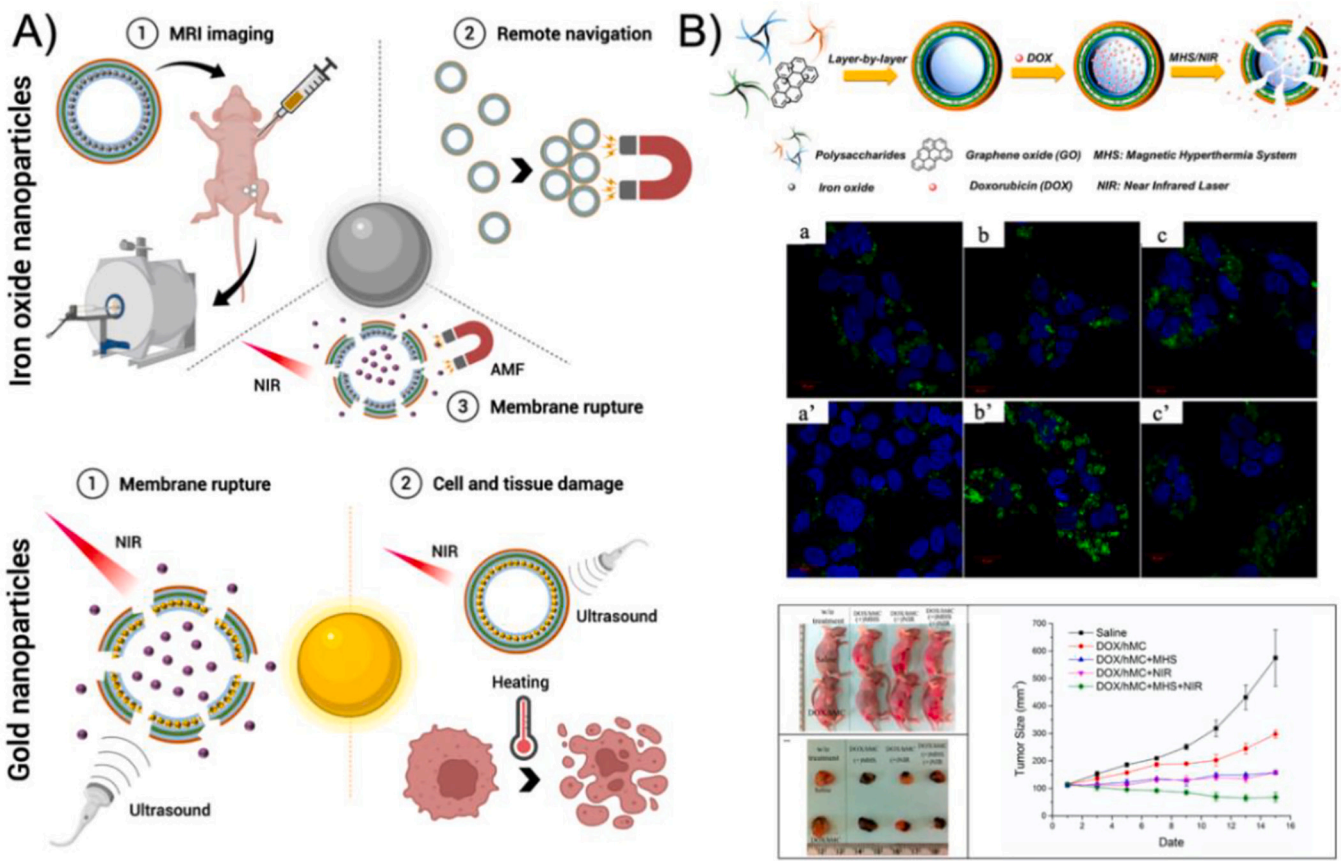

Fig. 7. A) Iron oxide vs. gold nanoparticle incorporation in LbL polymer membranes. The figure shows the characteristics and the possible applications of the incorporated nanoparticles. B) Above, schematic representation of the fabrication process of DOX loaded microcapsules. The iron oxide decorated graphene oxide (GO) nanosheets were incorporated between the polysaccharide layers used for the formation of the polymeric membrane. To enhance the targeting, an outer hyaluronic acid (HA) layer was added. The outer layer of HA, together with a low magnetic field (middle, b') or NIR light (middle, c') improved the internalization and the adhesion of the fabricated capsules comparing to the control (i.e., capsules without iron oxide, GO) (middle, b,c). Below, images of mice and extracted tumors after the administration of capsules and the quantification of the tumor size, highlighting that the synergistic effect of magnetic hyperthermia and NIR light promoted tumor reduction. Reprinted (adapted) with permission from ACS Appl. Mat. Interfaces 2016, 8, 11, 6859–6868. Copyright 2021 American Chemical Society. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

approach in those scenarios in which an external *in vivo* monitoring and visualization of the capsules is needed [50,92–94], or when the guiding and controlled release are a must to obtain an efficient treatment (Fig. 7A) [95–100]. In stem cell regenerative medicine therapies, the monitoring and tracking of the implanted cells is a prerequisite to determine the safety and efficacy of the treatment [50]. In drug release applications, the possibility to track the *in vivo* fate of the administered particles could provide useful information about the biodistribution, which is directly associated with the release of the bioactive substance and its effect on the treatment [92–94]. Introducing magnetite, especially SPIONs as contrast agents, allows monitoring the polymer capsules via magnetic resonance imaging (MRI) analysis, which shows high resolution and penetration with a high safety level [50,93,94]. The obtained contrast depends on size, shape and aggregation level of the nanoparticles [94]. Another benefit of using magnetite nanoparticles as an element of the polymeric membrane is the possibility to fabricate LbL capsules that can be remotely navigated to the target site and release their cargo in response to an external trigger (e.g., alternating magnetic field (AMF) or near-infrared light (NIR)) [95,97–99]. In cancer treatment, the triggered-release of a chemoterapeutic drug, in combination with a magnetic hyperthermia system (MHS), results in a synergistic effect that allows an efficient tumor reduction (Fig. 7B) [100]. Another way to induce the loosening/rupture of the polymeric membrane is the use of high-intensity focused ultrasound (HIFU) [96,101]. These individual harmless beams focused on a small area can create a collective energy that will lead to the rupture of the carrier walls in the presence of magnetite nanoparticles. The position of the layer including the magnetic nanoparticles is a key parameter in this case since it will affect the stability of the capsule before and during the sonication process [96].

Remote control on the fabricated capsules and controlled release can also be achieved by using gold nanoparticles and nanorods (Fig. 7A) [102–106]. In the presence of NIR light, gold nanoparticles exhibit strong surface plasmon resonance (SPR) and, consequently, are capable of converting this NIR into heat, which triggers the drug release [102]. This strategy is helpful in cancer treatments, where a specific and controlled release of the encapsulated cargo is required only at the site of action, with the aim of reducing side effects associated with the cytotoxic drugs and avoiding cellular drug resistance [107,108]. Furthermore, these nanoparticles hinder the leakage of the encapsulated agent and mitigate the so common initial burst release observed on similar release platforms [104]. By using ultrasound instead of NIR, increased permeability of the polymeric membrane can also be achieved, with an increase in the release efficiency by up to 4-fold [105]. The local increase of the temperature associated with the response of gold nanoparticles to NIR or high-frequency ultrasound can also be used to damage the surrounding tissues and cells (Fig. 7A) [105,106]. Accordingly, a plethora of works has focused on the use of free gold nanoparticles to cancer cell ablation, obtaining significant tumor volume reductions [109–112]. However, these free nanoparticles usually need their functionalization to promote their accumulation in the tumor and avoid the aggregation and systemic clearance [110–112]. Within this context, the incorporation of gold nanoparticles in the membrane of LbL capsules could be a useful tool to simultaneously protect them and provide additional functionalities, improving the outcomes of the treatment accordingly.

Less common alternatives to the use of iron- or gold-based nanoparticles mentioned above are titania (TiO_2) [113], silica (SiO_2) [114], quantum dots [115], cerium oxide (CeO_2) [116,117] nanoparticles and graphene derivatives [118–120]. These nanoparticles are useful for imaging and as protective barriers of the encapsulated active entity, thus maintaining its activity during their *in vitro* or *in vivo* application. In the specific case of cerium oxide nanoparticles, their remarkable redox potential inactivates a wide variety of reactive oxygen species (ROS) and protects the activity of the encapsulated agents (e.g., enzymes) from surrounding insults [116,117]. As an example, Popov and co-workers demonstrated that adding CeO_2 nanoparticles in the shell of the fabricated capsules protected the activity of the encapsulated enzyme in the presence of H_2O_2 [116]. Thanks to their unique properties, graphene derivaties have gained increased interest as constituents of the multilayer membranes as structural elements and also for the remote-controlled release triggered by low doses and low laser power [118–120].

The incorporation of enzymes like glucose oxidase (GOx) [121–125], CAT [124,125], POD [122,126], or urease [127,128] in the membrane of polymer capsules fabricated via the LbL approach is a facile way to obtain micro- and nanoreactors. In the case of some enzymes, their immobilization between the membrane layers enhances their stability against fluctuations in temperature and pH and also prolongs their storage time [122,127]. For example, taking advantage of the stability obtained by trapping GOx between layers, these polymer capsules can be potentially used as glucose sensors and as micro-platforms for diabetes treatment [121,123–125]. The by-products resulting from the reaction between GOx and glucose can react with a fluorescence substrate in the presence of a catalyzer, thus resulting in an increase in the fluorescence intensity, which enables their use as glucose sensors [121,123]. For the treatment of diabetes, the main requisite is to have a system that is switched on when the glucose level is above the established value and switched off when it drops, thus mimicking normal physiological processes [125]. As an example, Xu *et al.* fabricated multilayer capsules that were capable of being repeatedly activated during four cycles obtaining a total insulin release of 30% under normal glucose levels and of 70% release under hyperglycemia conditions [125]. This phenomenon was achieved thanks to the increase in the permeability of the layers as a consequence of pH drop resulting from the reaction of GOx with glucose [123,124].

siRNA is a promising tool to improve the therapeutic efficacy of some treatments towards more personalized medicine, particularly for cancer [129,130]. siRNA is capable of targeting oncogenes and genes implicated in cancer cell growth and metabolism [129,131]. However, the efficiency of free siRNA has limitations such as immunogenicity, nuclease degradation, low cellular uptake, fragile stability and systemic side effects as a consequence of uncontrolled distribution and limited transfection. Consequently, encapsulation or protection of siRNA is a must to ensure safe use of this technology [132–135]. Incorporation of siRNA in the multilayer membrane prolongs its circulation time in the bloodstream, ensures a high siRNA loading and minimizes its potential toxicity [129–131,133,136–138]. As reported by Choi *et al.*, the LbL nanoparticles containing siRNA between their layers led to the down-regulation and silencing of B-cell lymphoma 2 (BCL-2) expression, causing the apoptosis of blood cancer cells in culture and also in animal models (Fig. 8) [131]. One issue to take into account in these capsules is their internalization by cells. The most important thing is not only the number of internalized capsules, but also the number of active molecules delivered, which depends on the loading capacity of the multilayer capsule [139–141]. Hence, several works are focused on the optimization and enhancement of the nucleic acid adsorption modifying the salt concentration of the incubating solution [140,141].

The versatility of the LbL approach also allows the deposition of small molecules (e.g., drugs) into the polymeric membrane [142], as well as the incorporation of growth factors (GFs) [143–145]. The use of GFs is a useful tool to enhance or accelerate cell differentiation to the desired tissue. Some works have accordingly developed LbL polymer capsules comprising within their membrane osteogenic factors such as recombinant human bone morphogenetic protein-2 (BMP-2) or osteogenic growth factors (OGF) to enhance bone tissue formation [143,144].

The incorporation of the above-mentioned active agents usually relies on the electrostatic interaction between the building blocks. Consequently, the deposited active agent could be released before reaching the target site, thus losing its therapeutic potential and causing side effects associated with off-target accumulation. As an alternative to physical interactions, chemical conjugation techniques can be considered to improve the interaction between the constituents of the

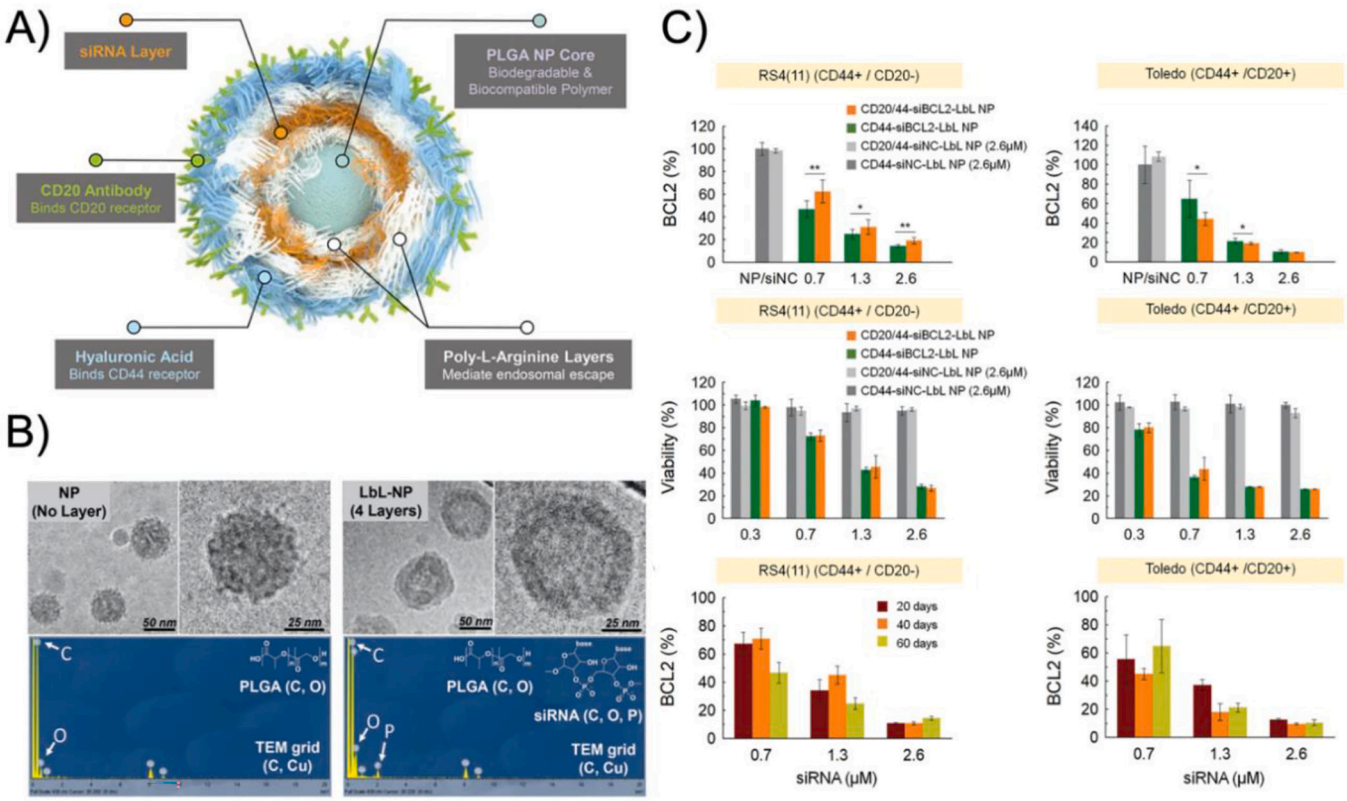

Fig. 8. A) Schematic representation of dual-targeted (i.e., CD20 and CD44) nanoparticles for siRNA release for the treatment of hematological cancers presenting an up-regulation of antiapoptotic BCL-2 protein. B) Cryogenic TEM images and energy dispersive X-ray (EDX) spectra of PLGA core nanoparticles (left) and 4-layer LbL nanoparticles containing siRNA (right). The particles show a uniform nanolayer coating in TEM images and the incorporation of siRNA was demonstrated by the presence of phosphate atoms in the EDX spectra. C) *In vitro* gene silencing effect of siRNA (i.e., siBCL2) containing nanoparticles. The presence of siBCL2 between the nanoparticle layers suppresses BCL2 gene expression (above) and, as a consequence, a decrease in the viability of the cells was observed (middle). The storage stability of the LbL nanoparticles was further analyzed and the results show a gene silencing capacity after 60 days (below). Reprinted (adapted) with permission from Adv. Funct. Mater. 2019, 29, 20, 1,900,018. Copyright 2021 WILEY-VCH GmbH.

polymeric membrane.

3.2. Crosslinking and functionalization of the building blocks

Crosslinking of the building blocks constituting the multilayer membrane is regularly adopted to impart mechanical integrity and/or responsiveness to the polymeric capsules. Using thiol-modified polyelectrolytes for the LbL deposition and its subsequent crosslinking to form disulfide bonds results in redox responsive vehicles capable of releasing an encapsulated agent in response to the GSH levels encountered in the cellular microenvironment [146–150]. Thus, the fabricated capsules are stable at physiological pH but are deconstructed under reducing conditions allowing the targeted release of the cargo intracellularly [147,148]. This strategy is useful in vaccine formulations since traditional strategies have been unsuccessful in immunizing against many chronic diseases such as HIV [148,150]. The capsules are subjected to phagocytosis by antigen-presenting cells (APC) and to micropinocytosis by dendritic cells (DC), both being of utmost importance in the immune response coordination [148]. Within this field, Sexton and co-workers fabricated LbL capsules for vaccination, formed by a polymeric membrane of thiolated poly(methacrylic acid) (PMASH) and PVPON capable of encapsulating ovalbumin (OVA) peptide. They observed that these LbL capsules containing the peptide within their core were capable of efficiently stimulating T immune cells in a lower dose than the free protein. Instead of using disulfide crosslinked polymeric membranes to protect and control the release of the encapsulated cargo, other authors employ polyelectrolytes that are sensitive to an external or internal trigger, such as UV light or pH change [151,152]. By covalently assembling poly(2-vinyl-4,4-dimethylazlactone) (PVDMA) with polyethylenimine (PEI), the degradation of the LbL occurred only at acidic pH values, allowing the release of the encapsulated cargo under these conditions [151]. Using poly(diallyldimethyl ammonium) chloride (PDADMAC) and poly[1-[4-(3-carboxy-4-hydroxyphenylazo) benzenesulfonamido]-1,2-ethanediyl, sodium salt] (PAZO) as the constituents of the polymeric membrane, the release of the cargo was controlled using UV light thanks to the formation of azobenzene (AZO) aggregates which induce a phase separation on the polymeric membrane [152].

The conjugation of the active agent to a polyelectrolyte is a useful alternative to avoid the aforementioned limitations associated with the weak electrostatic interactions between the constituents of the capsules. The incorporation and encapsulation of small hydrophobic molecules is usually a challenge in the fabrication of LbL micro- and nanocapsules. Many works have described the conjugation of the drug or prodrug to one of the polyelectrolytes to improve its solubility, stability and transport of the therapeutic hydrophobic molecule [153–160]. Thanks to the conjugation, a better control over the release rate and an improvement in the targeting are achieved. This is very useful in pathologies like cancer, in which the control over the dosage and efficient targeting of the anticancer drug determines the success of the adopted approach [159,160]. Anticancer drugs like DOX or paclitaxel (PTX) have been successfully conjugated to a wide variety of polyelectrolytes [154,157]. Within this context, several strategies have been followed for the conjugation of the drug, including amine bond formation for the conjugation of DOX [154,158] or the use of CD to host various guest molecules into their hydrophobic cavity (Fig. 9A) [157]. For example,

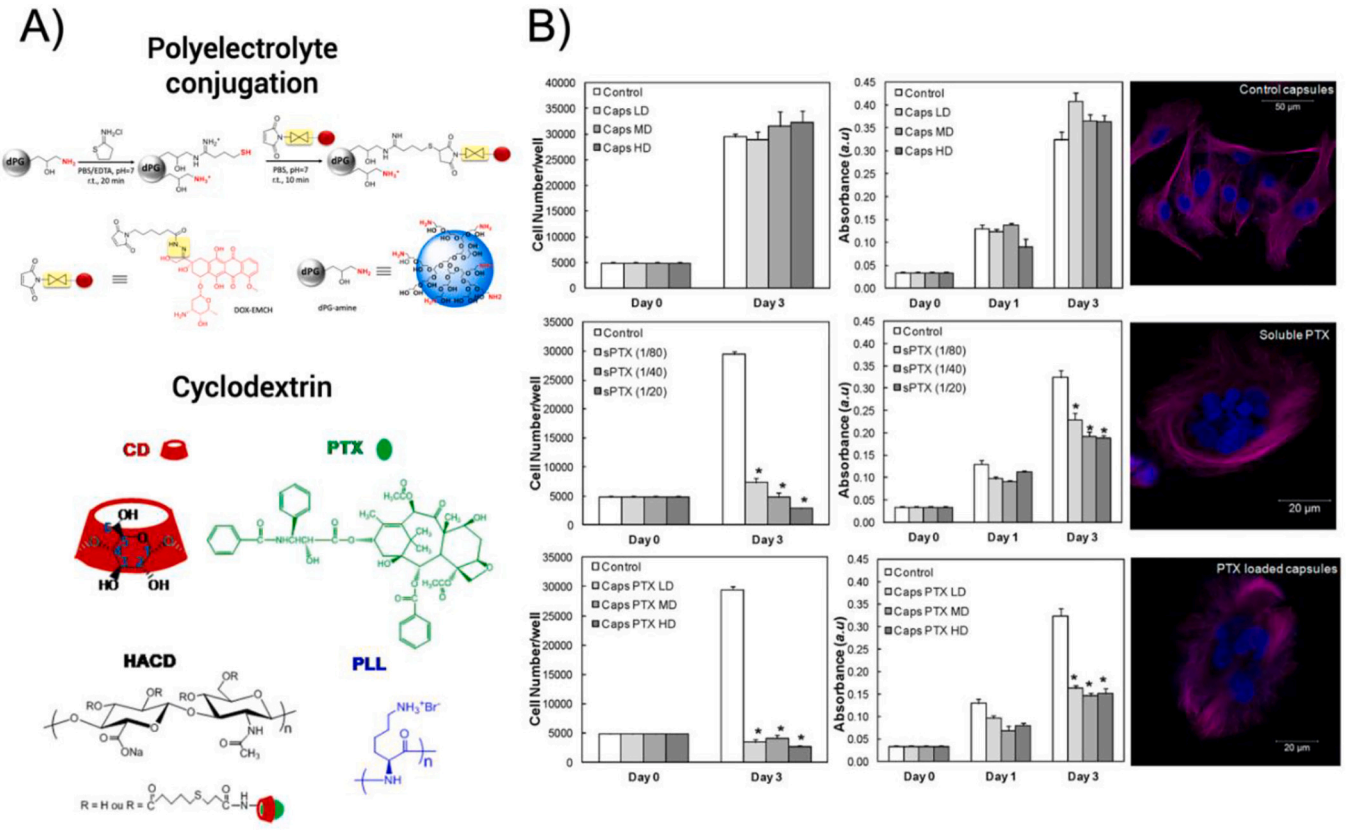

Fig. 9. A) Chemical conjugation (above) vs. cyclodextrin complex formation (below) for the incorporation of poorly soluble molecules. The image above has been reprinted (adapted) with permission from ACS Appl. Mat. Interfaces 2021, 13, 16, 18,511–18,524, and the image below has been reprinted (adapted) with permission from Chem. Mater. 2013, 25, 19, 3867–3873. Copyright 2021 American Chemical Society B) Viability results and fluorescence micrographs of MDA-MB-231 cells in response to unloaded capsules (upper row), paclitaxel (PTX) in solution (middle row) and PTX-loaded capsules using cyclodextrin complex formation (row below). Capsules with the cyclodextrin-PTX complex induce a higher cell toxicity obtaining thus an efficient anticancer treatment. Reprinted (adapted) with permission from Chem. Mater. 2013, 25, 19, 3867–3873. Copyright 2021 American Chemical Society.

Jing and collaborators fabricated LbL capsules using a hyaluronic acid (HA)-CD conjugate as a building block, which was subsequently loaded with PTX. This system exhibited a controlled release and high effectiveness in reducing the viability of breast cancer cells (Fig. 9B). In cancer treatment, another strategy is the use of prodrugs conjugated to the polymer membrane layers. The use of Pt(II)-based drugs has been extensively reported since they are capable of inhibiting the growth of cancer cells interfering in transcription and other DNA-mediated cellular functions [159]. However, limitations in terms of poor solubility, lack of functional groups and associated side effects have limited their clinical use. The use of its prodrug (i.e., Pt(IV)) represents a promising alternative because it shows better pharmacokinetics and reduced side effects [159,160]. Several works have conjugated the Pt(IV) prodrug to a polypeptide or a polysaccharide to exploit them as part of the polymeric membrane [159,160]. In this way, higher toxicity than free Pt(II) was observed thanks to a higher intracellular accumulation of Pt [159]. As reported by Zhang and co-workers, capsules fabricated via the LbL approach combining in their membrane Pt(IV)-Chi conjugates and complementary anticancer drugs (e.g., gemcitabine (GEM)-conjugated to HA) were capable of releasing the active agents in a sustained manner, obtaining a synergistic effect that reduced lung cancer tumor while avoiding toxic effects in off-target organs and tissues [160].

Table 3 includes several examples were the multilayer membrane of polymer capsules has been functionalized, together with *in vitro* and *in vivo* studies to validate their potential in biomedical applications.

4. Outer layer

It is evident from the previous sections that the LbL methodology offers numerous advantages including the colloidal stability, degradability, drug release and biocompatibility, among others [161]. These advantages derive through the careful design of the LbL micro/nanocapsules, that include the selection of the appropriate components that act as the core, as well as the building blocks that are used as the coating layers and their functionalization. Although both the core and the multilayer membrane affect the *in vitro* and *in vivo* behavior of the capsules, the most important component is the outer layer. The outer layer is the one that comes first into contact with the biological microenvironment defining the *in vitro/in vivo* fate of the capsules. Thus, a suitable functionalization can improve characteristics like circulation time in the blood and cell uptake or provide responsiveness to a physical, chemical or biological stimulus (Fig. 10).

4.1. Colloidal stability and circulation time

Colloidal stability inside a biological environment is of crucial importance since it strongly affects the fate of the administered capsules. One of the most common techniques for improving the colloidal stability and circulation times (through opsonization inhibition) of nano- and microcapsules, is their surface-functionalization with PEG [99,162–168]. The ability of PEG to create brushes that inhibit the attachment of proteins present in the blood, and the steric hindrances that promote the individual stability (through aggregation inhibition) of each capsule, make PEG one of the best stability enhancement

Table 3
Strategies to incorporate functionalities in the multilayer membrane of LbL capsules intended to be used in biomedical applications.

Strategy	Active agent in the membrane	Encapsulated entity	Layer functionalization approach	Biomedical application	Outcomes	Ref.
Layer as the active agent	Iron oxide	N/A	Electrostatic interaction	Imaging, fate and biodistribution (*in vivo* and *in vitro* studies in MSC cells)	• Two-fold higher Fe uptake when encapsulated. • After intravenous administration, distribution of capsules was determined by MRI *in vivo*.	[50]
		N/A	Electrostatic interaction	Imaging, fate and biodistribution	• Caspules were accumulated in liver, as determined by MRI.	[93]
		N/A	Electrostatic interaction	Imaging, fate and biodistribution (degradation analysis)	• The signal intensity change did not exceed 25% with high Fe_3O_4 concentration. • The signal intensity change was 100% with low Fe_3O_4 concentration. • MRI contrast increase upon degradation.	[94]
		Doxycycline	Electrostatic interaction	Drug release	• The release of the drug is promoted by AMF. • Increasing the cell to capsule ratio increased the expression of endothelial growth factor receptor.	[97]
		RITC-BSA	Electrostatic interaction	Remote navigation (Localization in blood flow)	• Thanks to AMF guiding endothelium penetration is achieved in 30–40 min. • Accumulation of capsule accumulation did not block blood flow.	[98]
		N/A	Electrostatic interaction	Cancer	• AMF induced morphological change and disruption of the capsules.	[99]
		Doxorubicin	Electrostatic interaction	Imaging, guiding and drug release (studies in cancer cells)	• AMF guidance to the target site. • HIFU promoted the release of the drug.	[101]
	Iron oxide and graphene oxide	Doxorubicin	Electrostatic interaction	Cancer (*in vitro* studies in HeLa cells and *in vivo* in HeLa bearing BALC/nude mice)	• HA and low magnet irradiation enhanced cell targeting. • On demand therapy. • Efficient tumor reduction.	[100]
	Gold	Doxorubicin	Electrostatic interaction	Cancer (*in vivo* and *in vitro* studies in MCF-7 cells)	• NIR promoted the release of the drug. • Reduced tumor volume *in vivo*.	[103]
		Dextran	Electrostatic interaction	Drug release in cancer (*in vitro* studies in MDA-MB-435 cells)	• Laser activated release into the cytosol.	[106]
	Cerium oxide	Luciferase	Electrostatic interaction	Oxidative stress (reactive oxygen species) attenuation	• Cell protection against 1.5 mM H_2O_2. • Enzyme activity retention.	[116]
		N/A	Electrostatic interaction	Oxidative stress (reactive oxygen species) attenuation	• B50 rat neuronal cell protection from high H_2O_2 doses. • Preservation of cell viability.	[117]
	Graphene oxide	DOX	Hydrophobic interaction	Drug release	• After NIR irradiation nearly 80% of drug release.	[120]
	Glucose oxidase and catalase	Insulin	Electrostatic interaction	Diabetes	• In presence of glucose, capsules were capable to release nearly 55% of insulin.	[124]
		Insulin	Electrostatic interaction	Diabetes	• Release activity activated when glucose level increase. • 70% of insulin release in hyperglycemia.	[125]
	siRNA	Doxorubicin and Indocyanine green dye	Electrostatic interactions	Cancer (*in vivo* and *in vitro* studies in A549 cells)	• Synergistic effect of thermal/gene/chemotherapies. • High tumor volume reduction. • High gene transfection.	[129]
		Doxorubicin and MRP1	Electrostatic interaction	Cancer (*in vivo* and *in vitro* studies in MDA-MB-468 cells)	• 4–8-fold tumor volume reduction.	[130]
		N/A	Electrostatic interaction	Cancer (Hematological BCL-2 protein silencing)	• BCL-2 protein reduction induced blood cancer cell apoptosis.	[131]
		N/A	Electrostatic interaction	Cancer (*in vivo* and *in vitro* studies in OVACAR8 cells)	• 54% knockdown of the target gene.	[136]
		Cisplatin	Electrostatic interaction	Cancer (Lung tumor targeting)	• Increased cytotoxicity *in vitro*.	[137]

(*continued on next page*)

Table 3 (*continued*)

Strategy	Active agent in the membrane	Encapsulated entity	Layer functionalization approach	Biomedical application	Outcomes	Ref.
Crosslinking and functionalization of the building blocks	Growth factor	N/A	Electrostatic interaction	Gene transfection	• RNAs and cisplatin combination enhanced tumor volume reduction. • Enhanced transfection efficiency than commercial liposome-based transfection kits.	[138]
		N/A	Electrostatic interaction	Bone regeneration	• Using BMP-2 and TGFβ1 bone regeneration was favoured in the absence of a cartilage template.	[143]
		Osteogenic peptide	Electrostatic interactions	Osteogenic differentiation	• Increased cell elongation/ stretching. • Upregulation of bone related proteins.	[144]
	Disulfide link	Ovalbumon	PMA functionalized with thiol groups and subsequent crosslinking	Vaccine (Chronic infections)	• Preferential degradation of the disulfide link intracellularly. • Ovalbumin stimulate T cell immunity.	[148]
	Doxorubicin	N/A	Click-chemistry	Cancer (*in vitro* studies in LIM1899 cells)	• Decreased cell viability (32%) and proliferation.	[154]
		CAT	pH-cleavable link to dendritic polyglycerol	Oxidative stress (reactive oxygen species) attenuation and drug release	• Higher drug releases at lower pH values. • Cell protection against H_2O_2 insults.	[158]
	KP9 peptide	N/A	Conjugation via disulfide link to PMA	Vaccine (Chronic infections)	• Stimulate lymphocyte immune response.	[156]
	Paclitaxel	N/A	Host-guest interaction with cyclodextrin	Cancer (*in vitro* studies in MDA-MB-231)	• Limited cell proliferation and metabolic activity.	[157]
	Pt (IV) prodrug	N/A	Covalent conjugation via amination to PLL	Cancer (*in vitro* studies in CT-26 cells)	• Higher intracellular accumulation of Pt. • Enhanced cytotoxicity.	[159]
		Gemcitabine	Covalent conjugation via amination to chitosan	Cancer (*in vitro* studies in NCl-H460 cells)	• Sustained release of the drugs. • Synergistic effect capable to reduce lung cancer and avoid toxicity in off-target organs and tissues.	[160]

approaches [99] and avoid the unspecific clearance, via phagocytosis, of immune cells like macrophages [163,165,166]. As an example, Wattendorf and collaborators functionalized the outer layer of the fabricated microcapsules with PEG-grafted PLL (PLL-g-PEG) and polyglutamic acid (PGA) (PGA-g-PEG) to analyse their stealth effect in human monocyte derived DC and macrophages [166]. As they observed, PLL-g-PEG coated capsules were capable to resist the internalization by DC and macrophages in comparison to the non-PEGylated counterparts. The capsules remained freely in the cellular microenvironment, thus reducing the uptake rate by about two-thirds and a half obtaining a drop in the phagocytosis mechanism by up to 85–90%.

However, this conventionally used PEG coatings present several limitations including, its degradation by oxidation, hypersensitivity, poor bioavailability, immunogenicity and non-biodegradability [169]. As an alternative, other brush-like structures have been studied, including poly(2-oxazoline)s (POxs). These POxs present excellent biocompatibility, a great stealth behavior and protein repellence, and an ease of fabrication, thus increasing the circulation time and avoiding the clearance via phagocytic pathways [169–172]. In their work, Kempe *et al.* observed that capsules fabricated with these brush-like structures and incubated in model protein serums like BSA and lysozyme, were capable to reduce in 40% the association of proteins in comparison to its linear counterparts [169]. The better hydration and subsequent increased resistance to protein adhesion was the main reason behind the observed behavior. In a similar work, it was observed that capsules fabricated with poly(2-ethyl-2-oxazoline) (PEtOx) and incubated in human serum presented a three-fold reduction in protein fouling with respect to the control [171].

4.2. Targeting and selective internalization/interaction

Surface functionalization using proteins or sugars on the surface of the capsules can improve cell internalization while enhancing the selective uptake by a specific cell type. Additionally, a surface protein or sugar can be used as a recognition molecule rendering the capsules able to act as biosensors or biomolecule separators. In an interesting work [173], fibrinogen was used to functionalize the surface of Dex/PLL/PGA multilayer capsules aiming at attracting the circulating platelets. Due to fibrinogen, the platelets adhered to the microcapsules via the αIIbβ3 integrin, forming a hybrid platelet-microcapsule system. The system was able to travel to the clot area and release the encapsulated anti-clotting factor VIII in an *in vitro* model of Hemophilia A. Notably, the release of the pro-clotting factor was achieved through the rupture of the capsules upon activated platelet contraction. On a different approach, Dex/Parg LbL capsules were coated with a variety of liposomal formulations aiming at the activation of DC and a specific immune response [174]. The data of this study showed that the lipids acted as immunopotentiators enhancing DC activation with the combination of a lipid A derivative with 1,2-Dioleolyl-3-trimethylammonium-propane chloride salt (DOTAP)/1,2-dioleoyl-sn-glycero-3-phospho-ethanol-mine (DOPE), giving the strongest activation.

The use of lectin as a target is another approach that has been reported in the literature [175]. The authors of the study fabricated an Alg/Chi microcapsule coated with a lipid bilayer. This bilayer allowed the functionalization with a laboratory-synthesized glycolipid that shows high affinity to lectins. To further improve the targeting and selective uptake of the system, the authors used an additional lipid-based

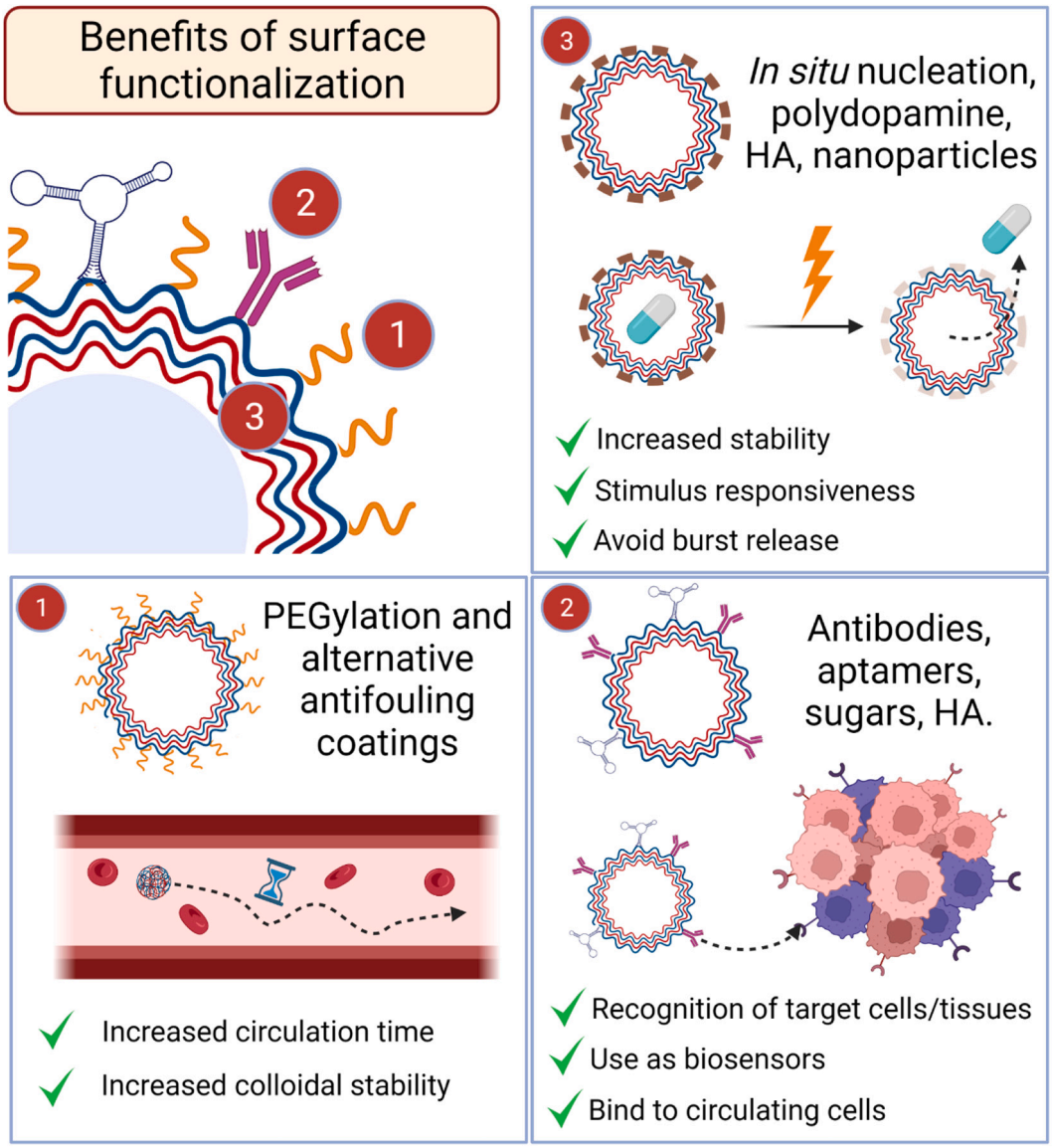

Fig. 10. Some of the benefits of decorating the surface of LbL capsules with several functionalities (e.g., PEG, antibodies, aptamers, nanoparticles, etc.).

folate group (DSPE-PEG_{2000}-folate) that has the ability to target the MCF-7 cancer cells. A different system that also made use of lectin as a targeting group was reported by Zhang and coworkers [176]. In their work, the authors made a polyelectrolyte microcapsule constituted of poly(vinyl galactose esterco-methacryloxyethyl trimethylammonium chloride)s (PGEDMC) and PSS. PGEDMC contained galactose branches able to recognize membrane-bound galactose receptors (ASGPR). The *in vitro* studies revealed a peanut agglutinin (PNA) lectin recognition that allows adhesion to the HepG2 receptor, suggesting the potential of the system for hepatic targeting.

Decorating the surface of nano- and microcapsules with antibodies allow their exclusive binding to specific targets, opening the possibility to use these systems as bimolecular recognition platforms [177,178]. In a particular example [179], PAH/polyacrylic acid (PAA) microcapsules were functionalized with antibodies (Y3, 5D3 and W6/32) against the major histocompatibility complex I (MHC I) (Fig. 11A). The results of this study showed that the targeting towards this complex is selective and allotype specific (Fig. 11B and C). Additionally, when the microcapsules were coated with the *staphylococcus aureus* protein A before the antibody functionalization, then the targeting efficiency increased by 40–50% compared to the direct antibody functionalization (Fig. 11C) thanks to the specific orientation of the antibodies deriving from the optimized immobilization protocol.

Enhanced targeting can be also achieved using antibodies specific to receptors that are overexpressed in various cells, like the endothelial growth factor receptor (EGFR) that is overexpressed, among others, in breast cancer. In a particular study, the authors fabricated quantum dots that were subsequently coated with PAA/PAH and functionalized with cetuximab, a monoclonal antibody against the EGFR [180]. The *in vitro* studies that were carried out in the MDA-MB-468 and MCF-7 human breast adenocarcinoma cell lines showed that the antibody-conjugated capsules interacted only with the MDA-MB-46 (EGFR positive), and not the MCF-7 (EGFR negative) cells. Other commonly overexpressed marker is A33, which is present in 95% of human colorectal tumor cells [181]. Functionalization of polymer capsules with the humanized A33 (HuA33) antibody allowed their binding to corresponding receptors overexpressed in the LIM1215 colorectal cancer cell line. This binding was the first step towards an efficient internalization, and thanks to this, nearly all cells were capable of containing one particle, demonstrating an efficient targeting to the tumor site.

Aside from proteins and antibodies, oligonucleotides like aptamers have also been used for surface functionalization [182,183]. In one of

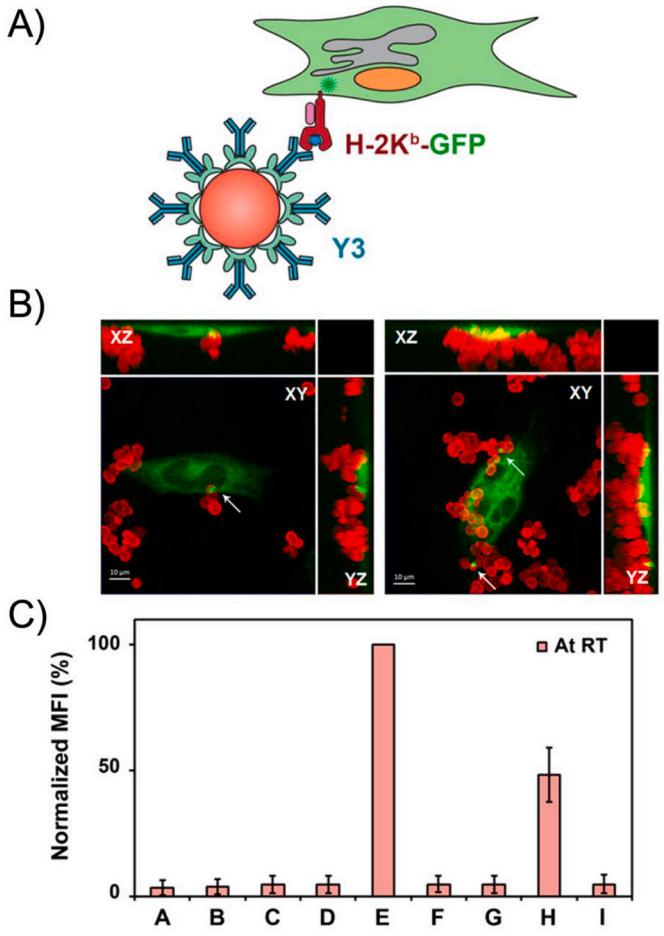

Fig. 11. A) Schematic representation of $(PAH/PAA)_2$ microcapsules coated with protein A and post-functionalized with the murine Y3 antibody capable to specifically recognize specific class I molecules at plasma membrane of STF1 cells. B) Confocal microscopy images showing the cell, antibody and allotype specific targeting of the capsules. In the particular case of these images, capsules post-funcionalized with Y3 were attached after 3 h to STF1 cells. White arrows highlight the specific contact point between the cells and the capsules. C) Flow cytometry results summarizing the interaction study between the capsules functionalized with different antibodies and T1 cells. The used capsules were: (A) non functionalized, (B) coated with protein A, (C) functionalized with the Y3 antibody in optimized orientation, (D) functionalized with the 5D3 antibody in optimized orientation, (E) functionalized with the W6/32 antibody in optimized orientation. (F) Y3 antibody, (G) 5D3 antibody, (H) W6/32 antibodies were randomly oriented and (I) BSA coated capsules. The results showed the allotype and cell specific tendency in this specific case using T1 cells. Optimizing the orientation of the antibodies, the targeting efficiency increased by 40–50%. Reprinted (adapted) with permission from ACS Appl. Mater. Interfaces 2017, 9, 13, 11,506–11,517. Copyright 2021 American Chemical Society.

the works presented by Liao *et al.*, aptamer-functionalized microcapsules offered selective targeting and responsiveness to overexpressed cancer cell biomarkers like the vascular endothelial growth factor (VEGF) and adenosine triphosphate (ATP) [182]. When the microcapsules were incubated with the cancer cells (MDA-MB-231), the VEGF on the surface of the cells or the intracellular ATP were bound to the anti-VEGF/anti-ATP on the surface of the capsules, leading to their unlocking (higher effectiveness with ATP functionalization) and releasing of the encapsulated drug DOX.

Other alternatives for the functionalization of the outer layer and the subsequent improvement of their targeting rely on the use of polysaccharides like HA. HA presents great affinity to CD44, a cell surface receptor present in many cancer types, that plays a pivotal role in cancer progression and metastasis (Fig. 12A) [184,185]. Within this context, many works have focused on the fabrication of capsules with a HA outer layer to enhance the targeting and also the internalization of the capsules (Fig. 12B) [100,131,184–186]. Choi and collaborators fabricated capsules encapsulating siRNA to disable the BCL-2 present in hematological cancers [131]. These capsules were functionalized with an outer HA layer conjugated to a specific antibody (CD20), accordingly improving their internalization by cancer cells thanks to their binding to two different surface receptors.

4.3. *Providing stability and stimulus-responsive release*

In the aforementioned sections we have already described how nanostructures like nanoparticles, in the intermediate layers of the LbL microcapsules, can provide advanced functionalities. However, nanoparticles and nanorods have also been used in the outer layer of various microcapsules aiming at improving the release properties by rendering the capsule responsive to a stimulus. As an example, silica [187–191] and titania [192] nanoparticles have been formed through *in situ* nucleation on the surface of microcapsules resulting respectively in an ultrasound and UV/ultrasound-dependent release of the encapsulated cargo. As an illustration, those microcapsules containing an outer silica layer were capable to hinder the release of the encapsulated DOX, whereas it was rapidly released under ultrasound treatment obtaining 87% of drug release after 120 s [189]. In another example, gold nanorods were used to decorate hydroxyapatite (HAP)/Chi/HA microcapsules aiming at the creation of a pH- (i.e., due to presence of Chi, which shows an isoelectric point around physiological values) and NIR-responsive (i.e., thanks to the presence of gold nanorods) system [193]. The microcapsules showed a significant increase in the release of DOX (~72%) when low pH (4.5) and NIR irradiation were combined.

Another way to obtain stimuli-responsive capsules by functionalizing the outer layer of the polymeric membrane relies on the use of polysaccharides such as Chi and HA. One LbL system that made use of the pH-sensitivity of Chi was presented by Verma *et al.* [194]. In their Chi/Alg system, the surface was functionalized with Vitamin 12 aiming at its uptake in the intestine and through the intrinsic factor receptor-mediated endocytosis. The system was able to safely deliver the encapsulated insulin both *in vitro* and *in vivo* presenting a 4.3-fold increase in its absorption when loaded in the nano-capsules (< 250 nm), compared to the free insulin. HA is also capable to be hydrolyzed by hyaluronidase (Hyal) which usually is overexpressed under bacterial infections and cancer environments (Fig. 12A) [195,196]. Using this strategy, Zheng and co-workers developed a capsule with an outer layer of HA which was degraded in the presence of Hyal, thus promoting the release of the encapsulated protein cytochrome C (Cyt) [195]. In this way, capsules in the presence of Hyal were capable of releasing 98% of the encapsulated protein after 96 h (Fig. 12C).

Apart from the above-described properties that the LbL coatings offer, there are still a few attributes that are worth mentioning. As an example, improved stability, but this time in terms of degradation time of the polyelectrolyte multilayer, was also studied after the coating of PAH/PSS capsules with bacterial self-assembled proteins isolated from *Bacillus thuringiensis* [197]. These proteins, called S-layers [198], are composed of glycoprotein subunits that cover the outer layer of gram (+), gram (−) bacteria and archaea and have great potential to be used as biomimetic coatings.

Polydopamine has also been reported as an additional functionalization of the outer layer to improve the mechanical stability [199]. This stability could be attributed to the creation of covalent bonds between the repeated monomeric units that interconnect through the polyelectrolyte layers enhancing the mechanical strength of the polyelectrolyte system.

Table 4 summarizes those studies where the outer layer of polymer capsules has been functionalized, together with *in vitro* and *in vivo* studies to validate their potential in biomedical applications.

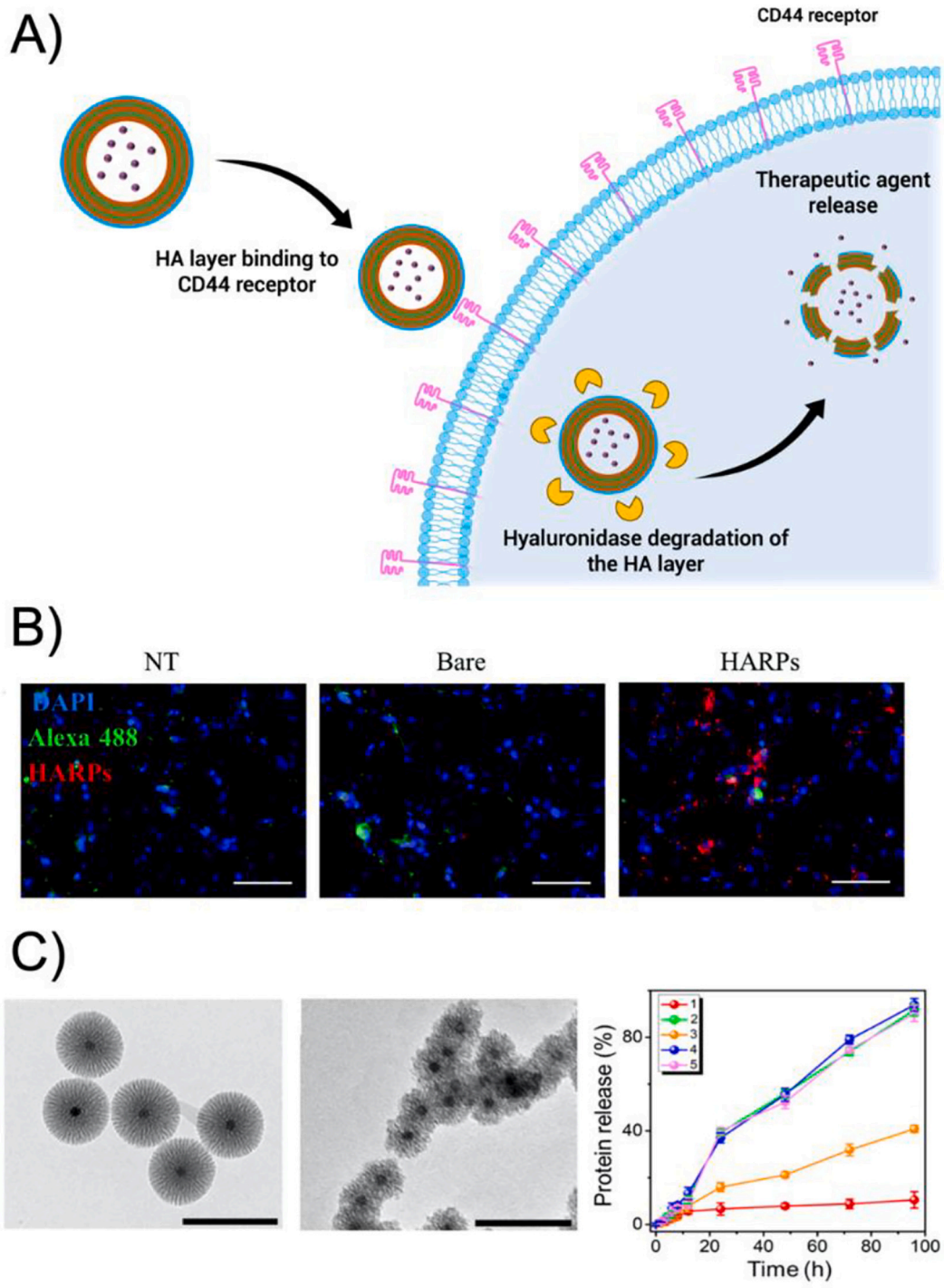

Fig. 12. A) Schematic representation of the capability of HA to specifically bind to the CD44 receptor and subsequent Hyal degradation allowing the release of the encapsulated therapeutic agent. B) Fluorescence micrographs showing the interaction between non treated capsules (NT), bare carboxylate-modified (CML) polystyrene latex beads (Bare) and HA functionalized capsules (HARPs). Nuclei of the cells were stained with DAPI, cell body structure with AlexaFluor 488 and capsules are shown in red. Replublished with permission of Royal Society of Chemistry, from Enhancing chemoradiation of colorectal cancer through targeted delivery of raltitrexed by hyaluronic acid coated nanoparticles, Rosh J. *et al.*, 11, 29, 2019; permission conveyed through Copyright Clearance Center, Inc. C) TEM images of HA coated nanocapsules degradation by Hyal after 0 h (left) and 96 h (middle). Encapsulated Cyt protein release percentage of the nanocapsules (right). The analyzed capsule conformations were: (1) capsules coated with 163 mg/g HA, (2) capsules without HA coating, (3) capsules coated with 80 mg/g HA and (4) and (5) capsules coated with the considered amounts of HA in the presence of Hyal. Reprinted (adapted) with permission from ACS Nano 2019, 13, 11, 12,577–12,590. Copyright 2021 American Chemical Society. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5. Towards multifunctional capsules

The LbL fabrication method allows the precise functionalization of all the constituents that form the polymeric capsule, from the inner cavity/core to the outermost layer through the polymeric membrane. Since capsules displaying a single functionality are inappropriate to overcome the challenges involved in complex pathologies, many works have developed capsules combining multiple functionalities within their

Table 4
Strategies to incorporate functionalities on the outer layer of LbL capsules intended to be used in biomedical applications.

Strategy	Encapsulated entity	Surface functionalization	Surface functionalization approach	Biomedical application	Outcomes	Ref.
Colloidal stability and circulation time	Iron oxide	PEG	PEG grafted on the PGA electrolyte	Cancer (neither *in vitro*, nor *in vivo*)	• AMF-induced hyperthermia resulting to morphological alterations.	[99]
	$LaVO_4:Tb^{3+}$		PEGylation	Bioimaging (*in vitro*/ *in vivo* toxicity)	• Fluorescence signal of capsules within cells and tissues.	[162]
	N/A		PEG grafted to PSS polyelectrolyte	Cancer (*in vitro* with LIM1215 and LIM2405 A33+ cells)	• Decreased non-specific binding. • HuA33 functionalization increase cell binding.	[165]
	N/A		PEG grafted to PLL and PGA polyelectrolytes	Shielding	• Capsules freely distributed and not adsorbed by macrophages and dendritic cells.	[166]
	N/A		PEG grafted to PLL polyelectrolyte	Shielding	• Reduced protein adsorption.	[167]
	N/A		Electrostatic interaction	Shielding	• Reduced adhesion to biological cells. • Reduced human serum albumin adsorption.	[168]
	Urokinase plasminogen activator	Poly(2-ethyl-2-oxazoline)	Electrostatic interactions	Thrombolysis	• High platelet affinity. • Thrombin responsiveness and thrombus dissolution.	[172]
	N/A	Poly (2-oxazoline)	Electrostatic interactions	Antifouling	• 40% lower protein association in brush like POx than in linear.	[169]
	N/A	Poly(2-ethyl-2-oxazoline) copolymerized with methacylic acid	Electrostatic interactions	Antifouling	• Preferential uptake of dendritic cells instead of macrophages.	[170]
	N/A	Poly(2-ethyl-2-oxazoline) copolymerized with methacylic acid	Electrostatic interactions	Antifouling	• Three-fold reduction in human serum protein adsorption with poly(2-ethyl-2-oxazoline) copolymerized with methacylic acid systems.	[171]
	Factor VIII	Fibrinogen	Electrostatic interactions	Hemophilia A (*in vitro* with activated platelets)	• Delivery system exploiting platelets' contractile forces. • Faster hemostasis.	[173]
	Daunorubicin	• Lab-synthesized glycolipid • Commercial DSPE-PEG2000-folate	Hydrophobic interactions	Breast cancer (*in vitro* with MCF-7 cells)	• High affinity towards lectin concavalin A and MCF-7 cells through the overexpressed folate receptor.	[175]
	Acyclovir	Galactose branches	Electrostatic interactions	Herpes & Hepatitis B	• Number of layers affects the release.	[176]
	Iron oxide nanoparticles	Anti-Horseradish peroxidase antibody	Covalent bonding	Biomolecular recognition (neither *in vitro*, nor *in vivo*)	• Recognition, extraction and detection of HRP.	[177]
	N/A	PEG + Collagen type IV antibody	Biotin-streptavidin reaction	(neither *in vitro*, nor *in vivo*)	• Enhanced binding affinity to collagen type IV.	[178]
	N/A	Y3, 5D3 and W6/32 antibodies	Covalent bonding	MHC I class receptors (*in vitro* with RMA, STF1 & T1 cells)	• Optimized antibody orientation through protein A coating resulting in enhanced targeting.	[179]
Targeting and selective internalization	QDs	Anti- endothelial growth factor receptor	Covalent bonding	Breast cancer (*in vitro* with MDA-MB-468 and MCF-7)	• Enhanced uptake by the MDA-MB-468 (EGFR expression) compared to the MCF-7 (no EGFR expression) cells.	[180]
	Doxorubicin	DNA crosslinked with anti-vascular endothelial growth factor or anti-adenosine triphospate	Electrostatic interactions	Cancer (*in vitro* with MDA-MB-231 and MCF-10A)	• Selective uptake by cancer cells. • ATP-crosslinked capsules show higher toxicity on cancer cells compared to normal cells. No toxicity in all the cell lines when VEGF is used. • ATP overexpression results to DOX-D release.	[182]
	• TMR-D • CdSe/ZnS QDs • MP-11	DNA crosslinked with anti-ATP	Electrostatic interactions	Cancer (*in vitro* with MDA-MB-231) – not extensive study	• Multiple cargo loaded. • ATP-responsive release.	[183]
	N/A	HuA33	Physical adsorption	Cancer (*in vitro* in LIM1215 cells)	• Cell binding and rapid internalization. • Nearly all cells contain one particle within their cytosol.	[181]
	Raltitrexed	Hyaluronic acid with raltitrexed	Electrostatic interactions	Cancer (*in vitro* in CT26 and *in vivo*)	• Higher directed uptake of HA containing capsules.	[184]

(*continued on next page*)

Table 4 (*continued*)

Strategy	Encapsulated entity	Surface functionalization	Surface functionalization approach	Biomedical application	Outcomes	Ref.
Loading stability and stimulus-responsive release	siRNA	Hyaluronic acid and CD20 marker	• HA: Electrostatic interactions • CD20: click chemistry	Cancer (*in vitro* and *in vivo* hematological BCL-2 protein silencing)	• Radiation and capsules combination increase tumor inhibition. • Successful directed internalization. • BCL-2 downregulation and cell apoptosis induction.	[131]
	Docetaxel and an anionic porphyrin ($TPPS_4$)	Hyaluronic acid	Electrostatic interactions	Cancer (*in vitro* in MD4-MB-231 and MCF-7 cells lines)	• Higher capsule adsorption in MDA-MB-231 cells (higher CD44 marker expression). • Both drug combination enables improvement in cell killing.	[186]
	Upconversion nanoparticles sensitive to UV and TiO_2	Hyaluronic acid	Electrostatic interactions	Cancer (*in vivo* and *in vitro* studies in HeLa cell line)	• Cancer cell apoptosis triggered by 808 nm light irradiation.	[185]
	Rhodamine-B	Silica	*In situ* nucleation based on the hydrolysis of TEOS	Controlled drug delivery (neither *in vitro*, nor *in vivo*)	• Ultrasound-dependent Rhodamine-B release.	[187]
	Dextran	Titania	*In situ* nucleation based on the hydrolysis of TIBO	Controlled drug delivery (neither *in vitro*, nor *in vivo*)	• UV and Ultrasound-dependent release of TRITC-Dextran.	[192]
	Doxorubicin	Au nanorods	Electrostatic interactions	Cancer (*in vitro* with MCF-7 cells)	• Controlled release. • Dual responsiveness.	[193]
	Insulin	Vitamin B12	Vitamin B12 grafted on the chitosan electrolyte	Diabetes, oral delivery (*in vitro* with Caco-2 cells and *ex vivo/in vivo* in male Wistar rats)	• The VitB12 capsules enhance 4.3 folds the absorption of insulin. • Sustained hypoglycemic effect for 12 h.	[194]
	Upconversion nanoparticles senstitive to NIR and therapeutic protein cytochrome c	Hyaluronic acid	Electrostatic interactions	Cancer (*in vitro* studies in HeLa and NIH3T3 cell lines)	• Tracking and delivery of capsules via NIR. • In presence of Hyal, capsules release 96% of the cargo in 96 h.	[195]
	Tirapazamine and $TPPS_4$	Hyaluronic acid	Electrostatic interactions	Cancer (*in vitro* studies in SCC-7, MCF-7 AND COS 7 cell lines)	• NIR/MR imaging and guiding capability. • In presence of Hyal 50–60% cargo release in 12 h. • Specific uptake obtained thanks to CD44 overexpression.	[196]
	Doxorubicin	Silica	*In situ* nucleation based on the hydrolysis of TEOS and TESPT	Cancer (*in vitro* studies in HeLa cells)	• Negligible drug release without any stimuli (silica shell protection). • Ultrasound stimuli: 87% release in 120 s. • GSH presence: 71% release in 48 h.	[189]

architecture. This resulted in multifunctional micro- and nanoplatforms capable of exploiting a synergistic treatment/diagnosis adapted to the particular characteristics of the pathology. Considering the huge socio-economic impact in our current society, most of the studies reporting the use of multifunctional LbL capsules are focused on the development of improved therapeutic, diagnostic and theranostic agents for cancer disease. The most commonly used chemotherapies are usually unable to ensure a complete tumor elimination on its own, partially due to the primary and secondary chemoresistance developed by tumor cells. Combinatorial approaches based on chemical, photothermal, photodynamic and radionuclide therapies have shown enhanced therapeutic outcomes compared to single therapy [200]. For example, Gaio et al. developed polymeric LbL nanoparticles loaded with the chemotherapeutic drug docetaxel (DTX) and a photosensitizer to exploit the photodynamic therapy [186,201]. The nanoparticles were further decorated with an external layer of HA to promote their internalization by CD44 expressing tumor cells. Interestingly, as concluded from the *in vitro* studies using DTX-sensitive and DTX-resistant cells, a clear synergistic effect was observed when both drugs were assembled in one single nanoparticle with respect to the administration of free drugs or drugs in separate nanoparticles. In a different example [129], a LbL microplatform combining photothermal (due to the incorporation of indocyanine green), chemical (thanks to the incorporation of DOX) and gene therapy (due to the incorporation of siRNA) was developed and its therapeutic efficacy was tested *in vivo*. Tumor-bearing mice were intravenously administered with this LbL formulation as well as with free DOX, which was used as a control. The tumor volume was reduced and the survival rate was enhanced in those mice treated with the LbL formulation under NIR laser irradiation in comparison to the administration of free DOX. Moreover, the mice had a stable body weight (i.e., similar to saline) when the LbL formulation was administered. In contrast, DOX administration resulted in a significant body weight decrease, indicating the

systemic toxicity of the free drug in comparison to the encapsulated counterpart.

Polymer and hybrid LbL capsules combining multimodal imaging and drug release also result in multifunctional entities enabling simultaneous diagnostic, monitoring and therapy, which may find application as theranostic agents for cancer and other diseases [101,200]. Capsules made out of TA/PVPON multilayers allowed high imaging contrast and a controlled DOX release in response to ultrasound irradiation [39]. The imaging contrast of these capsules was achieved without the need to incorporate nanoparticles (e.g., metal and metal oxide nanoparticles) into their architecture and could be tuned by adjusting the rigidity of the polymeric membrane. These nanoparticle-free capsules displayed improved stability (i.e., no significant ultrasound contrast intensity change was observed in solution for 6 months) and a better ultrasound contrast than commercially available alternatives. Accordingly, they represent a promising approach for the efficient tracking and delivery of therapeutics in tumor tissues. The same authors further improved their formulation by incorporating iron oxide nanoparticles in the polymeric membrane, thus combining the diagnostic potential of MRI with ultrasound-triggered drug release [202]. As a result, the LbL capsules allowed an imaging-guided safe and precise delivery of the encapsulated cargo (i.e., DOX) at the target site while avoiding off-target accumulation and associated side effects in a mouse model of breast cancer. Following a similar strategy, the same authors also formulated radionuclide-functionalized vehicles that were imaged via PET, allowing to monitor the *in vivo* fate of the capsules with high sensitivity and stability up to 7 days post-injection [203]. The use of iron oxide nanobeads as the core in the LbL approach can similarly result in multifunctional stimuli-responsive entities capable of combining several therapies and imaging modes by applying an external magnetic field [204]. However, the aforementioned systems only provide visual information about the bulk tumor, while ignoring important molecular and cellular insights that may be vital for a better patient prognosis. Hence, the group of P. Hammond went a step further and fabricated nanoparticles capable of obtaining information about precise pathological changes, thus obtaining more dynamic systems not only focused on the local image information provided by the aforementioned counterparts [136]. The developed nanoparticles, which included an urinary biosensing peptide on their outer layer, were activated by matrix metalloproteinase-9 (MMP-9), which is overexpressed in tumors. As observed in pancreatic, colorectal and ovarian cancer models in mice, the peptide reporter concentration was significantly higher in the urine of tumor-bearing mice, proving the potential of the nanoparticles as a non-invasive diagnostic tool. Simultaneously, the nanoparticles were capable of releasing encapsulated siRNA, obtaining a prolonged gene silencing, endowing multifunctionality (i.e., diagnosis + treatment) to the system.

Other pathologies in which the multifunctional LbL capsules may find application are the cerebrovascular and cardiovascular disorders [173,205]. Polymer LbL capsules functionalized with iron oxide nanoparticles to confer MRI capabilities and loaded with recombinant tissue plasminogen activator (rtPA), delivered via ultrasound stimulation, may serve as thrombolytic agents for ischemic stroke [205]. In a different example, by modifying the surface of the capsules with fibrinogen and filling their cavity with a pro-clotting agent via coprecipitation, the resulting LbL capsules were bound to the circulating platelets, who are the "first responders" in the blot clot formation [173]. Then, by elegantly adjusting the mechanical properties of the multilayer membrane, the encapsulated pro-clotting agent was delivered through platelet contractile forces, allowing a faster clot formation.

The intensive research around novel nanoparticles, including quantum dots, nanodiamonds and upconverting nanoparticles, will open the possibility to develop LbL capsules with additional/improved functionalities and stimulus-responsiveness (e.g., response to microwave, temperature nanosensors) [206,207]. However, these formulations are still under optimization and further *in vitro* and *in vivo* validation studies will determine their real potential for biomedical applications. In other cases, although strictly speaking, these cannot be considered multifunctional capsules, the combination of several types of capsules in a device can render the resulting construct multifunctional [208]. For example, by decorating the surface of electrospun mats with LbL capsules separately loaded with antibiotic and osteogenic molecules, a polymeric device that shows two functionalities can be fabricated. Besides, since the LbL capsules are engineered to respond to different stimuli, the release of each factor is prone to be controlled by either physiological or remote stimulus. In summary, considering the versatility of the LbL approach, together with recent advances in (bio) chemistry, biology, genetics, etc., further multifunctional systems displaying advanced functionalities are expected for the near future. For example, an interesting multifunctional approach was demonstrated by encapsulating DNA-encoding sensing elements (SE) in the interior of silk fibroin capsules and their further functionalization with gold nanoparticles and the IgG antibody [209]. The fabricated capsules acted as microreactors allowing the transcription and translation of the immobilized SE theophylline riboswitch and a broccoli aptamer, resulting in the production of the GFP1a reporter protein. Although at a proof of concept level, using these capsules with various DNA plasmids in their interior or in between the different surface layers could result in the expression of various therapeutics and/or diagnostic proteins. Additionally, the surface functionalization demonstrated the ability of these capsules for tissue-specific targeting.

6. Current challenges and future perspective

As discussed along this review paper, the LbL methodology offers huge versatility for the fabrication of micro- and nanocapsules with tunable characteristics, which makes it highly attractive for the development of multifunctional entities with potential use to treat and diagnose several pathologies. However, this approach presents some limitations which are hindering their rapid translation to the clinic (Fig. 13).

The production of these capsules remains at an artisanal level. In fact, the most commonly used fabrication protocols rely on the sequential immersion of a sacrificial template in different polyelectrolyte solutions, followed by repetitive washing steps [210]. This time-consuming method implies the continuous manual intervention leading to a lack of homogeneity in the capsule size distribution and reproducibility, together with low efficiency of the process associated with the loss of a great amount of capsules during the washing steps. Thus, making a robust and reproducible production still represents a challenging objective [210–213]. Production methods that enable the automatization and up-scaling of the fabrication of these capsules are consequently of significant need to move forward in the implantation of these systems in clinical use. Within this context, several works have focused on the optimization of the fabrication of LbL capsules suggesting methods based on microfluidics [212], tangential flow filtration (TFF) [210,213], semi-continuous membrane filtration [214], immobilization-based techniques [215] or continuous flow tubular reactors [211]. Although many of them present some limitations, including difficulties in adapting to the huge variety of available templates and building blocks, these fabrication systems are a promising tool for the up-scaling of the LbL approach.

Depending on the selected building blocks, the fabrication cost of LbL capsules can range from very low to very high. For example, the use of synthetic polyelectrolytes like PAH and PSS can result in low-cost LbL capsules (estimated cost: ~40 €/g – Sigma Aldrich), where the use of other materials like Parg can significantly increase the cost by 100 times. Notably, these costs are similar or, in certain cases, lower compared to commercial therapeutics like the BioNTech/Pfizer lipid-based Covid-19 vaccine (estimated cost: ~4000 €/g – Sigma Aldrich) or the anticancer drug Doxil (estimated cost: 20000 €/g – Sigma Aldrich). Although the above-mentioned estimated costs are based on prices from laboratory

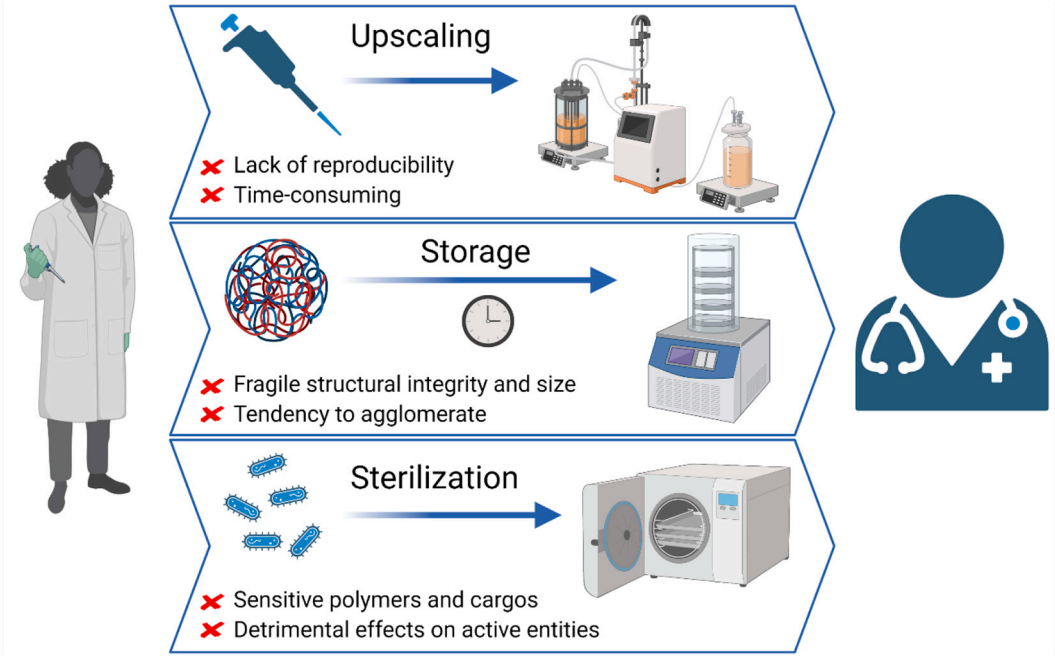

Fig. 13. Main challenges encountered by LbL capsules that need to be addressed prior to their safe translation into the clinical practice.

suppliers and do not represent industrial prices for raw materials, it can be assumed that the cost for capsule fabrication is not one of the limitations for their clinical translation and/or commercialization.

The long-term storage is another issue that needs to be considered to guarantee the safe translation of these capsules into the clinic. As in real clinical scenarios the fabrication of fresh polymer capsules is utopic, adequate storage conditions (e.g., temperature, pH) and states (e.g., in solution, dried, lyophilized) need to be further explored with the aim of preserving the integrity of the systems until their use. Several works have analysed the long-term storage of polymer capsules subjecting them to different temperatures (e.g., 4 °C or 37 °C) [41,42,54,131,216], storage solutions (e.g., NaCl [54,217], phosphate buffers or distilled water [131]) or even to dehydration-hydration cycles [218]. The results derived from these studies were promising in terms of size and charge preservation, as well as activity retention of the active agents. Nevertheless, these are individual examples and although promising, a general evaluation specifying the proper storage conditions for each polyelectrolyte type and each encapsulated or functionalized entity remains absent.

The sterilization of the fabricated capsules is also a key factor that has received little attention in bibliography. Sterilization is a fundamental step in the manufacturing of biomedical devices and it should guarantee the preservation of physical, chemical, mechanical and biocompatibility properties of the fabricated systems [219,220]. The most commonly used methods include autoclaving, dry heating, chemical treatments (e.g., ethylene oxide and hydrogen peroxide), UV radiation or ionizing radiations (e.g., gamma and beta radiation) [219,220]. However, it should be taken into account that some of these methods could irreversibly alter the original properties of the polymer used and also have a detrimental effect on the encapsulated active entity. For example, capsules based on thermal- or hydrolytic-sensitive biomaterials should avoid the use of autoclaving. Sterilization by chemical treatments, specifically the use of ethylene oxide, result in the formation of toxic by-products and a softening of the polymers [220]. For this reason, proper optimization and study should be done in the case of multilayer capsules and encapsulated therapeutic agents in order to ensure the preservation of the properties when translating to bigger production scales.

Despite all these challenges, polymer capsules fabricated via the LbL approach represent promising systems for accurate and personalized treatments. With the optimization of the above mentioned up-scaling issues and with the help of emerging technologies like artificial intelligence, which allows the pre-simulation of human functions to predict the actuation of the biomedical systems [221,222], the translation to the clinical use of these capsules may be much closer.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are thankful for funds from the Basque Government, Department of Education (IT-927-16 and PIBA_2021_1_0048).

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