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Designed to Deliver

Polymeric Multilayer Capsules in Drug Delivery

Liesbeth J. De Cock, Stefaan De Koker, Bruno G. De Geest, Johan Grooten, Chris Vervaet, Jean Paul Remon, Gleb B. Sukhorukov, and Maria N. Antipina*

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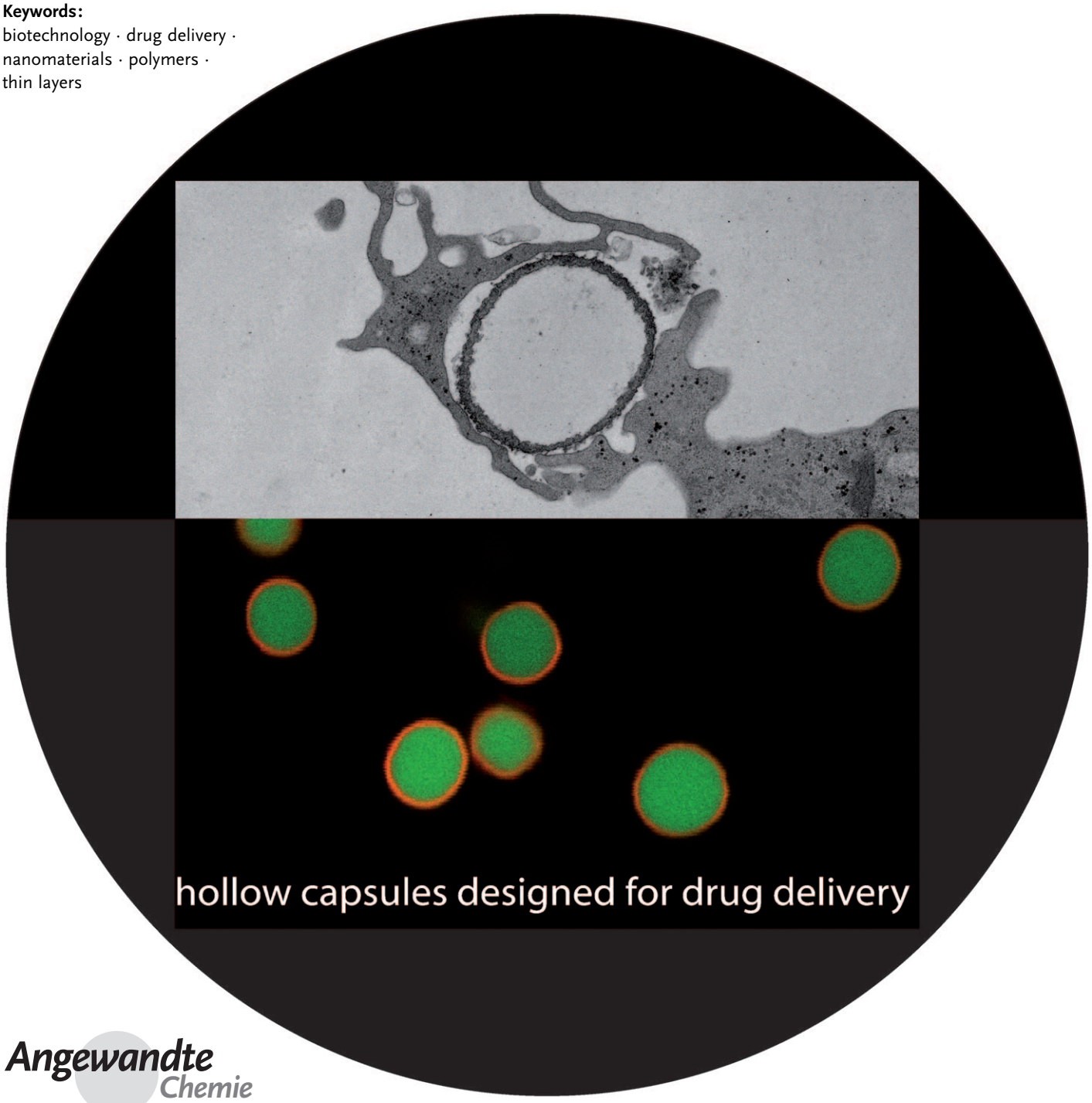

Recent advances in medicine and biotechnology have prompted the need to develop nanoengineered delivery systems that can encapsulate a wide variety of novel therapeutics such as proteins, chemotherapeutics, and nucleic acids. Moreover, these delivery systems should be "intelligent", such that they can deliver their payload at a well-defined time, place, or after a specific stimulus. Polymeric multilayer capsules, made by layer-by-layer (LbL) coating of a sacrificial template followed by dissolution of the template, allow the design of microcapsules in aqueous conditions by using simple building blocks and assembly procedures, and provide a previously unmet control over the functionality of the microcapsules. Polymeric multilayer capsules have recently received increased interest from the life science community, and many interesting systems have appeared in the literature with biodegradable components and biospecific functionalities. In this Review we give an overview of the recent breakthroughs in their application for drug delivery.

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1. Introduction

Drug-delivery science is driven by the need to develop systems that can deliver precise quantities of a therapeutic payload at a specific target site or tissue at a tailored release rate and/or after a specific trigger.[1,2] These requirements have resulted in a trend towards miniaturization, which has challenged scientists from multidisciplinary fields to engineer novel drug-delivery systems. Furthermore, several drug molecules cannot be formulated or administered by conventional techniques as they exhibit poor water solubility or suffer from limited stability in a complex environment such as the human body.

A beautiful example of a novel system that has recently emerged from cross-disciplinary scientific symbiosis is polymeric multilayer capsules (PMLCs).[3–5] These capsules are generated by sequential deposition of polymer layers from aqueous solutions onto a sacrificial template (Figure 1). Almost any type of interaction (for example, electrostatics, hydrogen bonding, covalent bonding, specific recognition) can be used as the driving force for the assembly of the multilayer shell. Dissolution of the sacrificial template then yields hollow capsules. PMLCs have been extensively explored for their physicochemical properties since their advent in the late 1990s,[6] and more recently they have attracted attention for drug-delivery applications.[7–12] PMLCs are now being engineered to encapsulate various classes of drug molecules, by using polymers that are biodegradable or that can respond and release their payload in response to well-defined stimuli.

The major benefit of PMLCs is without doubt their versatility. They can be fabricated using various templates, with sizes varying from a few nanometers to hundreds of micrometers, and their chemical and mechanical properties can be precisely tailored by modulating the thickness and constitution of the shell. In addition, the microcapsules can be modified with an almost unlimited number of compounds—ranging from polymers, to nanoparticles,[13] to biospecific motifs. In this Review we provide an overview of the important contributions in the development of PMLCs for drug-delivery purposes. We will also indicate those fields where PMLCs could offer distinct advantages compared to more-traditional systems.

2. Preparation of Polymeric Multilayered Capsules

2.1. Sacrificial Core Templates

PMLCs were initially templated on organic microparticles (typically 1–10 μm) such as polystyrene or melamine formaldehyde (MF).[3] These capsules required the use of organic solvents or acidic media to dissolve their core template, which hampered their applicability in a biomedical setting. A major step forward towards the biomedical application of PMLCs was the use of inorganic core templates that could be

[*] L. J. De Cock, Dr. B. G. De Geest,[+] Prof. Dr. C. Vervaet, Prof. Dr. J. P. Remon
Laboratory of Pharmaceutical Technology
Department of Pharmaceutics, Ghent University
Harelbekestraat 72, 9000 Ghent (Belgium)
E-mail: br.degeest@ugent.be
Dr. S. De Koker,[+] Prof. Dr. J. Grooten
Laboratory of Molecular Immunology
Ghent University (Belgium)
Prof. Dr. G. B. Sukhorukov
School of Engineering and Materials Sciences
Queen Mary University of London (UK)
Dr. M. N. Antipina
Institute of Materials Research and Engineering
A*STAR (Singapore)
[+] These authors contributed equally.

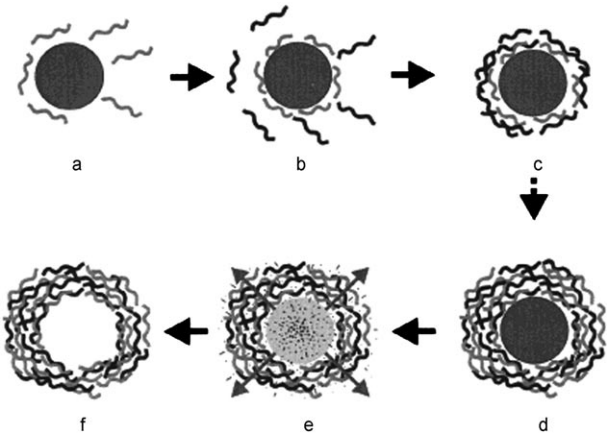
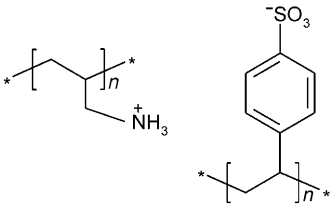


Figure 1. Schematic formation of PMLCs. a–d) The initial steps involve stepwise formation of the film by repeated exposure of the colloids to polymers with alternating interactions. The excess polymer is removed by cycles of centrifugation and washing before the next layer is deposited. e) After the desired number of polymer layers are deposited, the coated particles are exposed to conditions which cause the core template to dissolve. f) After further washing steps, a suspension of hollow PMLCs is obtained.[3]

decomposed under relatively mild conditions.[14–17] Amongst those, porous calcium carbonate (3–5 μm, and decomposed by aqueous EDTA) and (mesoporous) silica (0.5–5 μm, and decomposed in buffered dilute HF solutions)[18–20] have received most attention. The porous nature of these templates makes them well suited to absorb relatively large amounts of biomolecules that remain entrapped within the capsule void after layer-by-layer (LbL) coating and dissolution of the core.

2.2. Electrostatic Interactions

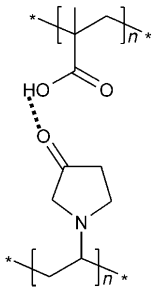
Based on the early pioneering work of Iler in 1966,[21] a LbL approach to coat charged surfaces was introduced by Decher et al. in the 1990s.[22–24] This approach was based on electrostatic attraction between alternating polyelectrolyte layers of opposite charge. The polyelectrolyte pair originally investigated most widely was sodium poly(styrene sulfonate)/poly(allylamine hydrochloride) (PSS/PAH; Scheme 1), which has been shown to yield stable capsules templated on a wide variety of cores. However, PSS/PAH capsules are nondegradable and irresponsive to physiological stimuli, thus limiting their potential in drug delivery. Inspired by the work of Picart et al.[25,26] and Lynn et al.,[27–29] who pioneered enzymatically and hydrolytically degradable planar electrostatic multilayer films, respectively, De Geest et al. developed degradable PMLCs with polypeptides or polysaccharides as enzymatically degradable components or charge-shifting polymers whose net charge balance changes on chemical hydrolysis of the polymer backbone.[30]



Scheme 1. Structures of PSS and PAH, which form a complex through electrostatic interactions.

2.3. Hydrogen Bonding

Pioneered by the Sukhishvili research group,[31] the use of hydrogen bonding has gained interest as it offers the possibility to fabricate multilayer capsules while avoiding the use of potentially toxic polycations. The most studied hydrogen-bonded system is poly(*N*-vinylpyrrolidone)/poly(methacrylic acid) (PVPON/PMA), in which PVPON acts as the hydrogen-bond acceptor and PMA the hydrogen-bond donor (Scheme 2), with the PMLCs from these polymers being generated at low pH values so that both polymers have a quasi-uncharged state. Under more physiological conditions (around pH 7.4), the carboxy groups of the PMA become deprotonated and thus charged, thus making these capsules unstable as a result of charge repulsion. Covalent stabilization of the capsule membrane is necessary to circumvent irreversible decomposition. The Sukhishvili research group coupled carbodiimide with the carboxy groups of the PMA by using ethylenediamine as a cross-linking agent.[32] The resulting cross-linked capsules were stable over the whole pH range, and released their PVPON fraction at alkaline pH values since it was no longer retained by the now deprotonated



Scheme 2. Structures of the polymers PMA and PVPON, which form a complex through hydrogen bonding.

Bruno De Geest graduated as a chemical engineer in 2003 from Ghent University in Belgium, where he obtained his PhD in 2006. Following two years of post-doctoral research at the University of Utrecht in The Netherlands he obtained a post-doctoral fellowship at the Laboratory of Pharmaceutical Technology at Ghent University. His main interests are the interfaces between chemistry, materials science, medicine, and biology.

PMA. The resulting single-component capsules exhibited interesting pH-dependent shrinking/swelling, with a steep reversible transition from a shrunken to a swollen state when the pH value increased above 6. This pH dependency is interesting in view of intracellular delivery mediated by PMLCs, since in endo/lysosomal vesicles—where PMLCs commonly succumb to phagocytosis—a slight acidic environment with a pH value around 5.4 is encountered.

The second major difference between the intracellular and extracellular medium, besides the acidic pH value, is the intracellular reductive environment arising from the presence of glutathione. Disulfide bonds are able to disassemble into single thiol moieties upon reduction, and have often been used as so-called bioresponsive linkages. The Caruso research group used this approach with PMLCs by grafting cysteamine (2-aminoethanethiol) moieties onto PMA, thereby providing the PMA backbone with pendant thiol moieties (PMA^{SH}).[33–36] PMLCs are fabricated by subsequent deposition of PMA^{SH} and PVPON onto silica microparticles, followed by cross-linking of the thiol moieties by oxidative treatment with hydrogen peroxide or chloramine T. Finally, hollow capsules are obtained by decomposition of the template in aqueous HF medium.

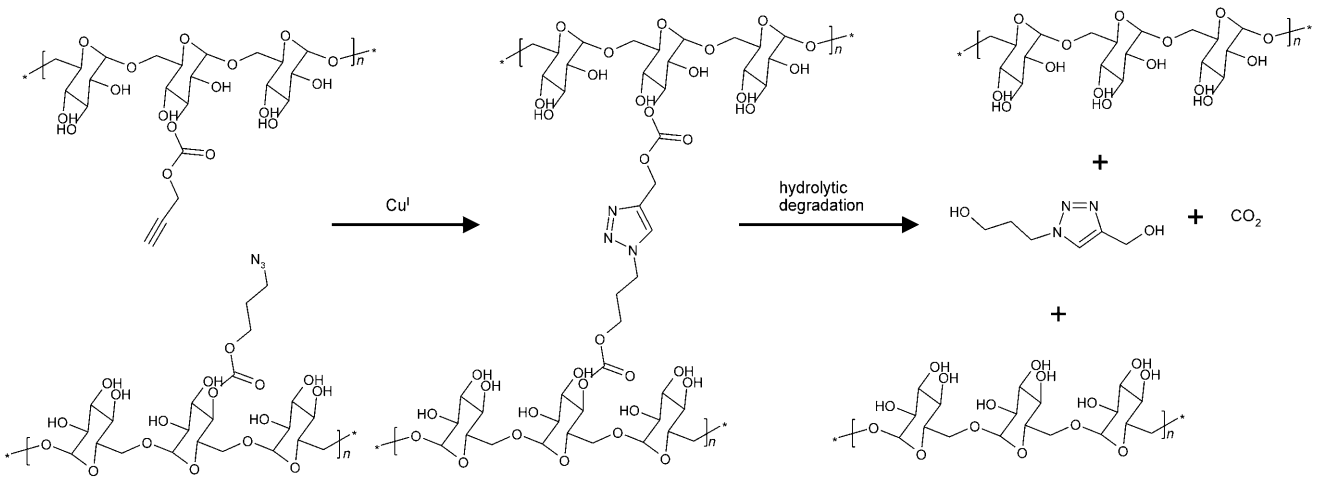
2.4. Covalent Reactions

Covalent reactions are a powerful method to prevent undesired disassembly of PMLCs (see Section 2.3). Besides providing merely stabilization, covalent reactions can also be exploited as a driving force for the build-up of multilayers. Several studies have reported on the use of the low-molecular-weight cross-linker glutaraldehyde to covalently link successive layers of polymers containing primary amines through the formation of imines.[37–39] Variations on this theme involving reactive polymers have also been reported: poly(dichlorophosphazene), which reacts readily with amines,[40] was used to fabricate PMLCs by reaction with hexamethylenediamine. Similarly, poly(glycidyl methacrylate) has been used in combination with PAH.[41]

A hot topic in the field of polymer science is click chemistry,[42,43] which has recently also been applied to the fabrication of PMLCs. According to the philosophy of Sharpless and co-workers,[44] the important characteristics of click reactions are their high selectivity and reactivity under mild conditions. This provides a versatile strategy for the fabrication of PMLCs without interfering with any functional groups on the encapsulated drug molecules, as is the case with, for example, amine-reactive chemistry. The most popular click reaction is the Huisgen reaction—the Cu^{I}-catalyzed 1,3-dipolar cycloaddition of azides and alkynes to form a stable triazole bond (see Scheme 3).[44] The Caruso research group has modified poly(acrylic acid) (PAA) with azide and alkyne groups to obtain "clicked" multilayers.[45,46] Silica particles were coated with alternate layers of PAA^{azide} and PAA^{alkyne} in the presence of $CuSO_4$ and sodium ascorbate (to reduce the Cu^{II} ions of the $CuSO_4$ to Cu^{I}) to allow formation of triazole bonds between the successive poly(acrylic acid) layers. Finally, after decomposition of the silica template, stable hollow "clicked" capsules were obtained which showed pH-responsive behavior because of the (de)protonation of the carboxy groups at different pH values. Temperature-sensitive PMLCs were synthesized by using a similar strategy with azide/alkyne-modified poly(*N*-isopropylacrylamide).[47]

However, the above-mentioned approaches only allow assembly of capsules without providing them with a mechanism to release their payload. Therefore, the introduction of degradability is required for them to enter the realm of drug delivery. Degradable or bioresponsive clicked capsules were reported by two research groups. Ochs et al. modified degradable polypeptides such as poly-L-glutamic acid and poly-L-lysine with azide and alkyne moieties, respectively.[48] Click reactions between like-charged modified PGAs or PLLs not only yielded PMLCs, but also allowed the authors to further functionalize the capsules with biomolecules such as biotin. This allowed further modification through ligation with streptavidin or with poly(ethylene glycol) (PEG), which significantly lowered the adsorption of albumin onto the surface of the capsules. A different approach involving degradable click cross-links was introduced by De Geest et al. (Scheme 3).[49,50] Dextran was modified with azide or alkyne moieties through activation of the azidopropanol and propargyl alcohol, respectively, with 1,1′-carbonyldiimidazole. This approach introduces degradable carbonate ester bonds between the dextran backbone and the pendant azide and alkyne moieties. The Cu^{I}-assisted cross-linking of $dextran^{alkyne}$ and $dextran^{azide}$ allowed the formation of both solid microgels (by using an emulsion technique) and hollow PMLCs when templating the click dextrans onto $CaCO_3$ microparticles, which were used as sacrificial templates. Carbonate esters can degrade under physiological conditions (that is, pH 7–4 and 37 °C) and, as such, drug release could be tailored from days to weeks by varying the degree of azide/alkyne substitution of the dextran backbone.

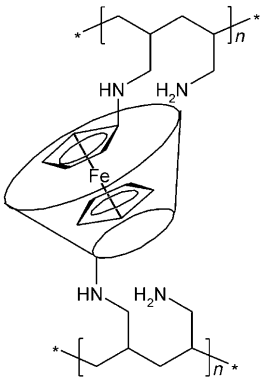
Caruso and co-workers used their experience on bioresponsive hydrogen-bonded capsules to combine click chemistry with bioreducible disulfide cross-linking.[51] PVPON was modified with alkyne groups, and click cross-linking with a bisazide was used to stabilize the hydrogen-bonded PMA/PVPON capsules. The bisazide cross-linker had a disulfide bond in the middle and thus bioreducible PMLCs based solely on PVPON (PMA is released at higher pH values) were obtained. Interestingly, the PVPON capsules tended to repel protein adsorption—an important property to allow circulation when administered into the bloodstream—while exhibiting low cytotoxicity. This approach was explored for drug-delivery applications by the same research group, who infiltrated PEG^{alkyne} into mesoporous silica microparticles and then cross-linked with a disulfide-bearing bisazide.[52] Prior to dissolution of the silica template, the anticancer drug doxorubucin (modified with a pendant azide moiety) was grafted onto residual alkyne moieties to yield drug-loaded capsules that could disassemble and release their doxorubicin payload under reductive intracellular conditions.



Scheme 3. Structures of alkyne- and azide-modified dextran, which form multilayers through formation of triazoles ("click" chemistry) in the presence of Cu^{I} ions. The multilayer structure degrades through hydrolysis of the carbonate esters which connect the dextran backbones to the triazole.

2.5. Specific Recognition

Although specific recognition has been elaborately exploited to prepare LbL films on planar surfaces, specific recognition has only rarely been reported for the fabrication of PMLCs. Host–guest interactions between β-cyclodextrin and ferrocene (Scheme 4) have been used by Wang et al. to generate PMLCs by alternate deposition of β-cyclodextrin- and ferrocene-modified PAH.[53] The inclusion complex formed by the two moieties allowed the formation of stable multilayers, and the resulting capsules exhibited interesting stimuli-responsive swelling and permeability properties in media of various pH values, ionic strength, and β-cyclodextrin concentration. Another approach to fabricate PMLCs by specific recognition through the formation of a stereocomplex between alternating layers of isotactic and syndiotactic poly(methyl methacrylate) layers was proposed by Akashi and co-workers.[54] The two above-mentioned strategies are certainly interesting and further development towards biodegradable or bioresponsive systems would be of interest for the field of drug delivery.



Scheme 4. Inclusion complex formed from a ferrocene- and cyclodextrin-containing polymer.

3. Encapsulation and Release

3.1. Pre- and Postloading

Molecules of interest, either low- or high-molecular-weight species, can be encapsulated within PMLCs by means of two different strategies: "preloading" and "postloading". In the "postloading" approach, already prefabricated capsules are loaded with the molecules of interest by altering the permeability of the capsule shell.[55–57] Figure 2 gives an overview of the different postloading possibilities. Under standard aqueous conditions, the capsule shell is permeable to low-molecular-weight compounds such as ions and small drug molecules (for example, ibuprofen), but impermeable to macromolecular components ($M_w >$ 5 kDa).[58] The loading of larger species is possible by altering either their own solubility or by altering the permeable PMLC shell. The former approach was used for the selective crystallization of various dyes by reversibly changing their solubility back and forth.[59]

Reversibly changing the permeability of the PMLC shell to macromolecules is achieved by changing the pH value, ionic strength, or solvent polarity, which leads to segregation of the polyelectrolyte network and defects in the shell.[55,57,60,61] After macromolecules have passed through the capsule wall, the PMLCs are transferred to their original medium, thereby entrapping the species in their hollow void. Ibarz et al. discovered the possibility of temperature-induced shrinkage of PMLCs.[56] This phenomenon was further explored by Köhler and Sukhorukov to encapsulate small (10 kDa) and large (70 kDa) molecules through the shrinking, membrane densification, and consequent decrease in the permeability of the PMLCs when subjected to heating above

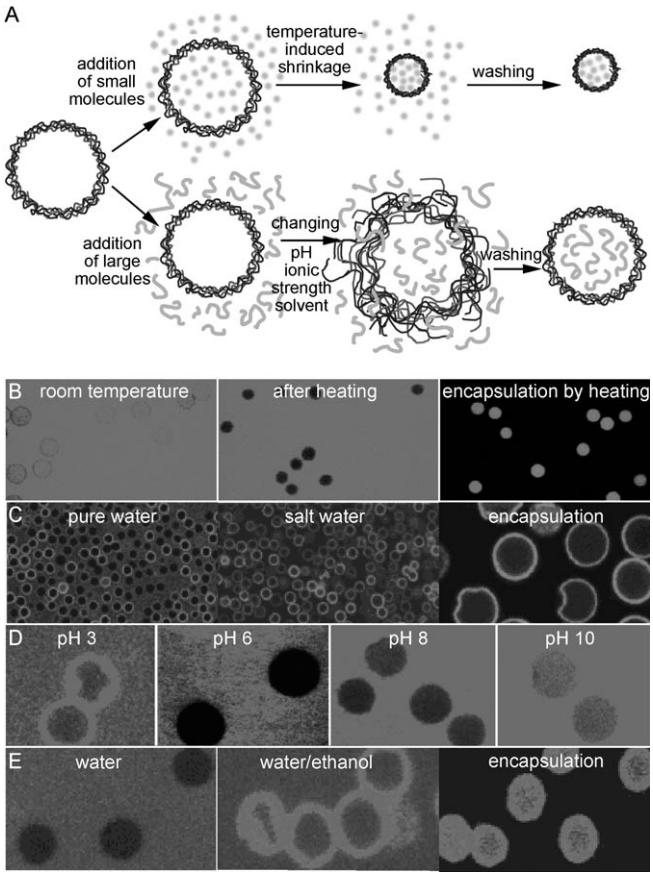


Figure 2. A) Schematic representation of the different strategies to generate postload PMLCs by changing their physicochemical environment. B)–E) Confocal micrographs: B) $(PDADMAC/PSS)_4$ capsules, whose permeability to FITC-dextran (10 kDa) changes upon heat treatment.[62] C) Loading of FITC-PAH (70 kDa) in $(PSS/PAH)_8$ capsules by reversibly changing the ionic strength of the medium.[55] D) $(TA/PAH)_5$ capsules incubated in FITC-dextran (2000 kDa) solutions at different pH values.[61] E) Loading of FITC-urease into $(PSS/PAH)_4$ capsules by changing the solvent polarity.[60]

the glass transition temperature T_g of the system.[62] Molecular diffusion is driven by the concentration gradient between the bulk phase and the interior of the capsule. Spontaneous accumulation occurs in the case of electrostatic interactions between the molecules of interest and an oppositely charged matrix inside the capsules, thus facilitating diffusion of water-soluble compounds from low to high concentrations. Such inner-charged networks can be created by incomplete dissolution of the organic core template (for example, MF) or by using charged gel templates such as calcium alginate beads.[63,64] A number of polymers, dyes, proteins, enzymes, and small drug molecules have been encapsulated in this way. In a special case of postloading, the species of interest is synthesized directly in the interior of the capsule or within the multilayer shell by polymerization or by enzymatic reaction.[65,66]

Although this method is applicable for a wide variety of molecules, it suffers from very low encapsulation efficiencies (that is, the amount of protein that becomes encapsulated within the capsules, relative to the amount of protein that was initially added), possible loss of bioactivity, and low integrity of therapeutic macromolecules because of the harsh conditions required to make the PMLC membrane permeable. Recently, $CaCO_3$ microparticles have emerged as a more "biofriendly" vessel for the encapsulation of proteins, enzymes, and nanoparticles.[15] The porous morphology of $CaCO_3$ generates a large surface area and offers the opportunity to capture macromolecules effectively. Species of interest can be preloaded into the $CaCO_3$ microparticles either by means of physical adsorption/pore diffusion or by co-precipitation during synthesis of the microparticles. Removal of the $CaCO_3$ template is performed after coating with a polyelectrolyte by treatment with EDTA, which has proved to be harmless for a wide range of biomolecules. Kreft et al. elaborated this method to generate PMLCs with two inner compartments for the spatially confined placement of different macromolecules (Figure 3 A,B).[67,68] These so-called shell-in-shell microcapsules were obtained by subjecting

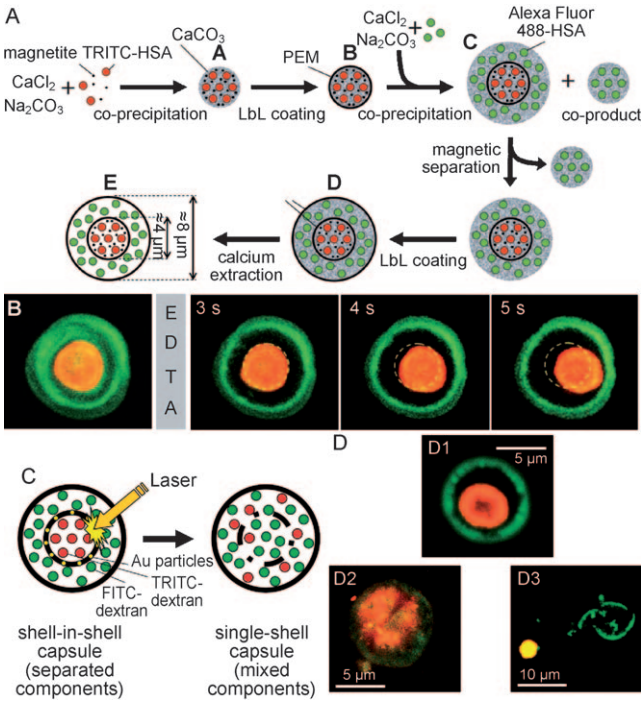


Figure 3. A) General route for the synthesis of shell-in-shell microcapsules. **A**: core; **B**: core–shell particle; **C**: ball-in-ball particle (type I); **D**: ball-in-ball particle (type II); **E**: shell-in-shell microcapsule.[67] B) CLSM imaging of **D** particles during extraction of the template material ($CaCO_3$). Individual $CaCO_3$ compartments are loaded with TRITC-HSA (orange, inner) and Alexa Fluor 488-HSA (green, outer). After conversion into shell-in-shell capsules by removal of the template, the two compounds remain separated because of the inner polyelectrolyte shell. The dashed circle indicates the original position of the inner capsule which moves to the outer shell upon extraction.[67] C) Schematic representation of the shell-in-shell polyelectrolyte multilayer capsule and laser-induced intercompartmentalized mixing. Enclosing and separating polyelectrolyte multilayers are represented by black circles.[68] D) CLSM images taken before (D1) and after (D2) laser illumination of the inner shell doped with gold particles. For some capsules, the rupture of the outer shell was observed accompanied by the release of the inner capsule as a side effect of the laser irradiation (D3).[68]

polyelectrolyte-coated and substrate-containing $CaCO_3$ microparticles to an additional $CaCO_3$ co-precipitation step, followed by coating with a secondary polyelectrolyte shell. The removal of the $CaCO_3$ template resulted in two microcapsules in which one was located inside the other (Figure 3C,D), thus allowing spatially confined reactions to be carried out.

The limitation of $CaCO_3$ as the PMLC template is in the encapsulation of pH-sensitive compounds as well as species containing di- or trivalent metal cations, since these can be decomposed by EDTA when dissolving the $CaCO_3$ core. Mesoporous silica similarly offers the advantage of a large surface area and has also been used as a porous core template for the encapsulation of macromolecules in PMLCs.[19,69] However, a much more careful handling is required as hydrofluoric acid (HF) is required to decompose the silica template. Balabushevitch et al. reported a preloading of enzymes into PMLCs by polyelectrolyte coating of salted-out aggregates of α-chymotrypsin that did not require the assistance of a template.[70] The concept of encapsulating lipid vesicles within polymeric multilayers[71] was applied as a preloading strategy for embedding macromolecule-loaded liposomes within the multilayers of the capsule shell. This procedure allows PMLCs to be obtained with a large number of subcompartments that can be destroyed by treatment with surfactants.[72–74]

3.2. Emulsion Templating

Oil-in-water emulsions are widely used in biomedicine as carriers for lipophilic bioactive compounds for controlled drug delivery and targeting.[75,76] For example, MF59 is an oil-in-water emulsion approved for influenza vaccines. A great demand for well-defined emulsions also exists in the food industry.[77] The encapsulation of droplets by polyelectrolyte multilayer coating has emerged as a method for the fabrication of smart delivery systems for oil-based drugs. A potential benefit of LbL coating is the possibility to tailor the physicochemical properties of the droplet surface, thereby resulting in more versatile drug-delivery systems.

Similar to water-soluble compounds, nonpolar species can be encapsulated by two means: postloading into prefabricated PMLCs or LbL coating of oil droplets dispersed in an aqueous phase.[78] The postloading approach was first demonstrated by Moya et al. Decane was successfully encapsulated within prefabricated PSS/PAH PMLCs by consecutively exchanging solvents from more polar to less polar five times. Sivakumar et al. reported the fabrication of PSS/PAH and PMA/PVPON PMLCs loaded with a range of oils, including silicon oil, paraffin oil, and thermotropic liquid crystals. Hollow PMLCs prepared by using a sacrificial template were first filled with the appropriate solvent and then brought into contact with oil. After the oil had infiltrated into the interior of the PMLCs, the solvent was exchanged with water again, thus resulting in stable multilayer-coated oil-in-water microemulsions.[79]

The main advantage of a postloading approach for the generation of oil-loaded carriers is the ability to obtain monodisperse emulsions, as this parameter is predetermined by the size and shape of the solid template used for the fabrication of the PMLCs. However, postloading of oils in PMLCs can be both material and time consuming, with encapsulation efficiencies always below 100%. Moreover, it results in restrictions regarding the composition of the PMLC shell, as the latter must be physically robust enough to withstand the solvent-exchange process and the multiple centrifugation steps.

LbL coating of previously stabilized oil droplets in an external aqueous phase appears to be a more universal and versatile approach. The easiest way to disperse an immiscible phase into a continuous phase is through the formation of droplets by high-speed shearing or sonication of the mixture. The resulting emulsions have a rather broad size distribution[81] and can be subjected to further homogenization.[82,83] More recently, microfluidic emulsification has emerged as the most straightforward method to generate monodisperse emulsions.[84] Stabilization of oil droplets is performed by adding an amphiphile to the oil/water emulsion. To facilitate further multilayer coating of the droplets the emulsifying surfactant should preferably have an ionic nature to provide the droplets with a sufficient surface charge. Proteins,[85] cationic,[81,86] and anionic lipids[82] as well as amphiphilic[86] polymers were reported as constituents of multilayer-coated emulsions. When choosing the emulsifier, its characteristics, such as its food-grade and cytotoxicity, should be taken into consideration.

After formation of a surfactant layer, the emulsion droplets are further subjected to multilayer coating in a similar fashion as solid-core templates. A continuous-flow process for multilayer-coating emulsion droplets was demonstrated by Priest et al. by using microfluidic emulsification of

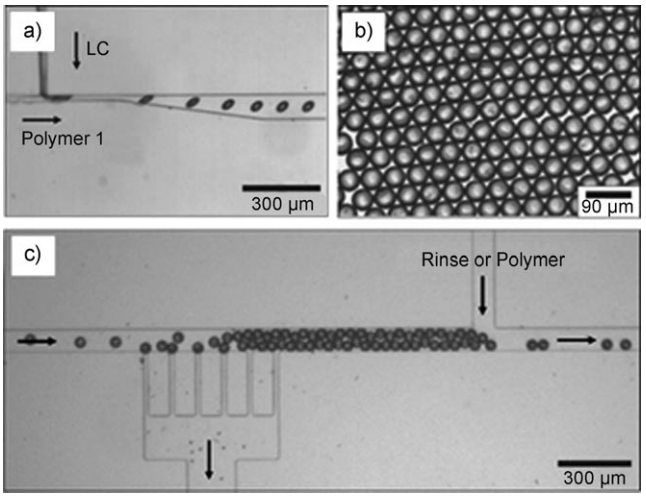


Figure 4. Synthesis of capsules in a microfluidic reactor. a) Emulsification of liquid crystals at a T junction, b) monodispersity is demonstrated by the hexagonal close packing of the polymer-coated particles prior to removal of the liquid-crystal core with ethanol, c) selective withdrawal of the continuous phase through the comb withdrawal channels and, downstream, infusion of subsequent polymer or rinse solution. The droplets are densely packed between the comb withdrawal and infusion channels because of the reduced volume fraction of the continuous phase.[80]

liquid-crystal droplets followed by multilayer coating within the same microfluidic device (Figure 4).[80] This approach is very attractive as it circumvents the multiple batch steps involved in common multilayer-coating methods. The potential of multilayer-coated emulsions has so far been exploited primarily to avoid coalescence of the droplets upon exposure to environmental stresses such as variations of the pH value, ionic strength, and temperature.[87] Polyelectrolyte multilayer coating has also been used as a protection barrier to prevent diffusion of Fe^{2+} ions, which accelerate lipid oxidation, into oil droplets.[88] Emulsions of polymeric multilayer-coated droplets for drug delivery were recently reported by the Caruso research group. Degradable multilayer-encapsulated microemulsions loaded with lipophilic anticancer drugs (doxorubicin or 5-fluorouracil) were able to induce a significant decrease in the viability of human colorectal cancer cells (LIM1215) in vitro.[89]

3.3. Stimuli-Responsive Release

One of the major aims in the field of drug delivery is to develop a carrier that would selectively release its payload either in response to an externally applied trigger or a trigger provided by the target tissue itself. The stimuli-responsive properties of PMLCs have been extensively reviewed previously.[11,12,90] In this section, we will focus mainly on those approaches that have reached the stage where biomedical applications are within sight.

In principle, most PMLCs can be considered stimulus-responsive. When the formation of the PMLCs is based on electrostatic interactions, changes in the pH value and/or ionic strength are evident triggers that can alter the interactions between the successive layers, and thus might be used to induce the release of encapsulated material.[91] These findings have been extended to hydrogen-bonded capsules containing a polyionic component.[31] Besides the pH value and ionic strength,[55] solvent polarity,[60] glucose,[92,93] temperature,[62] and oxidation[94] have also been reported to alter the permeability of PMLCs. However, these parameters are nonphysiological triggers, which generally impedes the use of these systems in vivo. Consequently, recent research has focused on developing PMLCs that are sensitive to more physiologically relevant stimuli, including enzymatic digestion,[95–99] or the reductive intracellular environment.[33–36,100,101] Enzymatically degradable PMLCs based on oppositely charged polypeptides and/or polysaccharides have now been generated by various research groups. De Geest et al. demonstrated that PMLCs consisting of dextran sulfate and poly-L-arginine could be degraded intracellularly by proteases upon phagocytosis by in vitro cultured cells.[96] Similar findings were reported later by different research groups, who used hyaluronidase and chitinase to decompose capsules containing hyaluronic acid or chitosan, respectively, as membrane components.[97–99,102]

The transition from an oxidative to a reductive environment has also been exploited to trigger the decomposition of PMLCs following cellular uptake. As mentioned in Section 2.3, disulfide bonds can act as bioresponsive cross-links that are cleaved to single thiols upon reduction. Haynie et al. were the first to stabilize PMLCs by disulfide bonds through the design of oppositely charged 32-mer peptides containing cysteine moieties.[100,101] At low pH values, these charged peptides can be assembled into PMLCs through electrostatic interactions; at a physiological pH value of 7.4, these PMLCs normally break down unless cross-linked through disulfide bonding of the cysteine moieties by oxidative treatment. The Caruso research group adopted this approach to develop hydrogen-bonded PMLCs by modifying poly(methacrylic acid) with cysteamine. The PMLCs were fabricated through sequential deposition of PVPON and PMA^{SH} onto sacrificial silica core templates followed by oxidative cross-linking of the thiol moieties and decomposition of the silica cores with HF.[34–36,103] The obtained PMLCs were broken down in vitro under reductive conditions, similar to those present in the intracellular space. This technology was applied by the same research group to encapsulate oligonucleotides, peptides, and low-molecular-weight anticancer drugs.[104]

In addition to physiological triggers, release from PMLCs might also be achieved by applying external stimuli to the capsules. This has mainly been accomplished by incorporating metal nanoparticles or light-responsive dyes into the walls of the capsules.[105] Several research groups have reported on such systems, including triggered release from a) dye-functionalized capsules by irradiation with light,[106] b) metal nanoparticle embedded metal nanoparticles by a magnetic field,[107] microwaves,[108] or ultrasound,[109–111] and c) noble metal (silver, gold) embedded capsules by irradiation with a focused laser beam.[106,112–117] The latter approach in particular has been explored extensively. Gold nanoparticles (Au^{NP}) exhibit a surface plasmon resonance signal in the visible spectrum around 530 nm.[114] As a result, Au^{NP} are locally heated when irradiated by laser light of this wavelength through conversion of photons into thermal energy. This heating leads to rupture of the capsules and release of the encapsulated material. Most interestingly, the surface plasmon resonance signal can be modulated by controlling the shape (namely, the aspect ratio) and aggregation state of the Au^{NP} on the surface of the PMLCs, with the signal shifted into the biologically friendly infrared region above 800 nm.[118] Moreover, further fine-tuning the composition of the PMLCs allows the PMLC membrane to be made reversibly permeable by IR irradiation, thereby releasing only discrete portions of encapsulated material, without destroying the whole capsule.[116] This principle was applied to drug delivery by Skirtach et al., who demonstrated that laser-triggered opening can be performed within living cells without impairing their viability.[115] In further studies it was shown that capsule breakage also resulted in the rupture of the phagosomal membrane surrounding the capsules, thus releasing the encapsulated material in the cellular cytoplasm.[119]

A third category of triggered-release capsules, which we will only briefly touch on in this Review, is the so-called self-degrading systems which are equipped with an internal trigger that causes the release of encapsulated species. This can be achieved by coencapsulating digestive enzymes into the hollow void of the PMLCs.[95] These enzymes either digest the PMLC membrane itself or process coencapsulated species

into smaller fragments that can be released through the PMLC membrane. Alternatively, "self-exploding capsules" have been generated by the LbL coating of a degradable microgel core which swells upon chemical hydrolysis at physiological pH values. When the swelling pressure exceeds the tensile strength of the PMLC membrane, the capsule ruptures and the encapsulated species are released.[120–127] Figure 5 shows a series of confocal microscopy images of

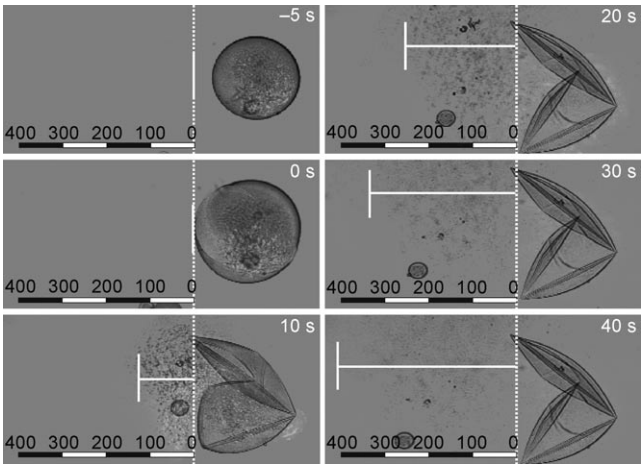


Figure 5. Confocal microscopy images taken at regular time intervals of $(PSS/DAR)_2$-coated microgels during degradation of the microgel core on addition of sodium hydroxide. The microgel contains fluorescent latex beads (50 nm), and during the dissolution the fluorescence and transmission channels are overlaid. The microcapsule explodes 10 s after addition of the sodium hydroxide. The edge of the propagating front of released nanoparticles is marked by the vertical white line. The scale bar is 400 μm long.[125]

such an exploding capsule that was loaded with fluorescent latex beads as a model. Remarkably, the released latex beads are able to travel relatively large distances within a short time frame, compared to purely Brownian motion.[125] These systems are more complex than conventional PMLCs and although promising, precise control of their release properties in regard to their potential benefit in drug delivery still has to be established.

4. Interactions with Living Cells and Tissues

4.1. In Vitro Interactions

4.1.1. Interaction between Polymeric Multilayers and Living Cells

Interactions between living cells and planar multilayers are under investigation for a wide range of biomedical applications and are beyond the scope of this Review, where we focus on colloidal systems. For the interested reader, excellent recent reviews can be found in Refs. [26, 128].

Human erythrocytes as well as fungal and bacterial cells have been used as sacrificial templates for PMLCs. The cellular template was removed after LbL coating by oxidative treatment.[129–131] The use of encapsulated living cells in polymeric multilayer shells could have many applications such as immune-isolation of non-autologous cells in the field of transplantation, targeted delivery, and tissue engineering. For such applications it is of utmost importance to have tight control over the pore size of the membrane as well as its chemical and mechanical stability and its biocompatibility with the host and graft tissue.[132,133] Diaspro et al. were the first to discover the ability of PSS/PAH multilayers to preserve the metabolic activity of *S. cerevisiae* yeast cells.[134] They later extended this approach to the coating of pancreatic islets, thereby protecting the cellular surface from antibody recognition, which is an important step towards shielding implanted cells from the host's immune system.[135] Successful in vivo transplantation of LbL-encapsulated pancreatic islets was shown by Wilson et al. by using coatings based on PLL/PEG and streptavidin.[136]

So far, numerous research groups have reported the polymeric multilayer coating of living cells. Recently, more exotic variations on this approach have also emerged. Swiston et al. demonstrated that instead of LbL coating the whole cellular surface, it is also possible to coat only part of it, thus equipping the cells with so-called multilayer patches (Figure 6A). The authors where able to show that cell function and mobility (as evaluated on T cells) where not hampered by

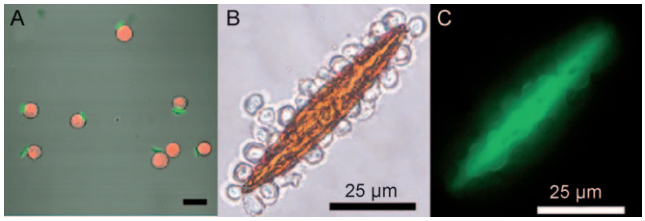


Figure 6. A) Red-fluorescent B cells coated with a multilayer patch (green fluorescence).[137] B), C) Optical and corresponding fluorescent microscopy images illustrating the viability of rodlike cellosomes treated with fluorescein diacetate which only stains living cells.[138]

these patches. This approach, therefore, offers great potential for delivery or sensing applications that exploit the natural cell behavior.[137] Another remarkable example is the use of cells themselves as constituents of a multilayer film. Such "cellosomes" were generated by Fakhrullin and Paunov by coating inorganic microcrystals with polyelectrolytes, magnetic nanoparticles, and polyelectrolyte-coated yeast cells followed by dissolution of the inorganic template.[138] Figure 6B and C shows micrographs of the obtained constructs which could, when developed further, find application as drug carriers, biological microreactors, or building blocks in tissue engineering.

4.1.2. Interaction between Polymeric Multilayer Capsules and Living Cells

Several drugs have an intracellular target but are poorly taken up when delivered in a soluble form or lack solubility. However, specific targeting by drug molecules is also often

desired to enhance the therapeutic efficiency or to avoid unwanted side effects. To evaluate the potential of PMLCs for drug delivery it is important to understand their interactions with living cells. One of the most important parameters is toxicity. Several research groups have assessed this topic by performing in vitro cell-viability assays such as the MTT test.[139,140] Generally, no acute toxicity was observed at moderate capsule concentrations, and an outermost polyanionic layer appeared to further decrease the toxicity (cationic PMLCs exhibit a pronounced tendency to adhere to the cellular surface).[141] Some toxicity was observed at elevated capsule concentrations; this is commonly attributed to sedimentation of the capsules on top of the cells as a result of competition for space between the capsules and cells. This effect hampers the metabolism of the cells, and thus affects their viability.[139,142,143]

Phagocyting cells such as most cancer cells and immune cells such as macrophages and dendritic cells are able to internalize PMLCs. This was first demonstrated by Sukhorukov et al. on a breast cancer cell line,[8] and the mechanisms of the uptake of capsules by living cells is under investigation by several research groups.[8,96,144,145] Studies on in vitro cancer cell lines by De Geest et al. and Parak and co-workers indicated that PMLCs end up in intracellular acidic vesicles upon cellular uptake. This was demonstrated by red-fluorescent lysosomal staining and observation of colocalization (that is, appearance of a yellow/orange signal; Figure 7B) with the green fluorescence of the capsules.[96,146] Further confirmation was provided by Kreft et al. by encapsulating SNARF-dextran, a pH-sensitive dye, in PMLCs (Figure 7A).[147] By calculating the ratio of the red and green emissions it was possible to determine that the pH value sensed by the capsules was 5.2, which corresponds to a endo/lysosomal environment. De Koker et al. inhibited different endocytotic pathways and actin polymerization and carried out a detailed confocal and transmission electron microscopy investigation of protrusions of the cellular membrane (Figure 7C). On the basis of their results they proposed macropinocytosis as the mechanism of uptake of capsules by bone-marrow-derived dendritic cells.[145]

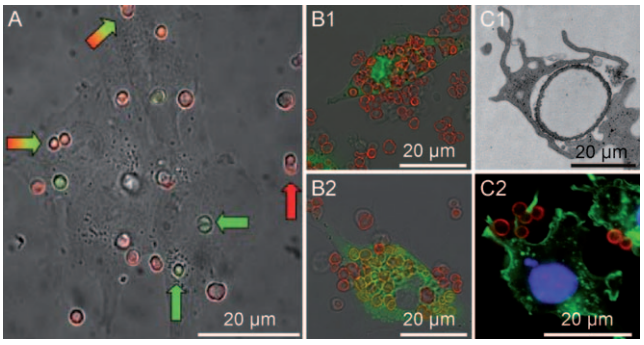


Figure 7. A) Confocal microscopy image of MDA-MB435S breast cancer cells incubated with PMLCs loaded with SNARF-dextran. The capsules outside the cells exhibit red fluorescence while the internalized capsules exhibit a shift towards green fluorescence (see arrows).[148] B) Confocal microscopy images of bone-marrow-derived dendritic cells incubated with red fluorescence PMLCs and with the cytoplasm (B1) and the endo/lysosomes (B2) stained with green fluorescence. Colocalization of the two channels yields a yellow/orange signal. C) Transmission electron (C1) and confocal (C2) microscopy images showing the engulfment of actin-rich (green fluorescent) protrusions on PMLCs.[145]

Several research groups have focused on the intracellular fate of the capsules, once internalized, and have observed a substantial deformation of the capsules, likely arising because of the pressure of the surrounding cytoplasm.[141,146] Incorporation of metal nanoparticles, which are known to enhance the mechanical strength of LbL films, rendered the capsule resilient to deformation upon cellular uptake.[149] Complete destruction and intracellular degradation was demonstrated by De Geest et al. by using degradable polycations such as poly-L-arginine and the hydrolysis-prone charge-shifting poly(HPMA-DMAE).[96] Co-incubation of VERO-1 cancer cells with such PMLCs resulted in internalization of the PMLCs and gradual disintegration of the capsules over a period of 60 h, after which no intact capsules could be observed.[96] Similar findings by the same research group were found when bone-marrow-derived dendritic cells were incubated with dextran sulfate/poly-L-arginine PMLCs.[139,145]

Biotechnological drug molecules, such as proteins and nucleic acids, or cancer therapeutics often have a specific target tissue, while delivery to other parts of the body is inefficient or even hazardous, as is the case, for example, for chemotherapeutics. For this purpose, functionalization of PMLCs with biological molecules plays an important role in shielding the capsules from unwanted uptake while also enhancing their uptake by the target cells.

The introduction of microparticulate matter in the body results in the opsonic proteins rapidly adsorbing onto the particle surface, thereby causing particle clearance by phagocyting cells. For prolonged circulation times and the targeted delivery of PMLCs, it would be beneficial to minimize the adsorption of proteins onto the capsule surface so as to avoid unspecific and undesirable phagocytosis.[150] Decreased protein interactions can be achieved by coating PMLCs with stealth polymers such as PEG. Coating PAH/PSS capsules with PLL-graft-PEG (namely, a polycation substituted with a protein-repellent "stealth" polymer) resulted in a drastic decrease in protein adsorption. This finding was attributed to the hydrophilicity of the PEG, which is fully hydrated in water, which likely prevents protein adsorption through hydrophobic interactions. Furthermore, the dense PEG brush layer shields electrostatic charges, thus minimizing electrostatic interactions between the capsules and proteins. Additional targeting capacity was introduced by using biotinylated PLL-graft-PEG. Such biotinylated stealth PMLCs were able to adsorb 40-fold more of the protein streptavidin than PMLCs with nonbiotinylated PLL-graft-PEG.[150] Further exploration of PEGylated PMLCs was performed by Wattendorf et al., who investigated the effect of PEGylation on cellular uptake. $(PAH/PSS)_4$ and $(PAH/PSS)_4$ PAH (4 = number of layers) PMLCs were coated with PLL-graft-PEG or PGA-graft-PEG and subsequently incubated with cultures of macrophages or dendritic cells. The PGA-graft-PEG coating did not show any significant effect on cellular uptake, probably because the PEG layer was not

dense enough. In contrast, PLL-graft-PEG could dramatically reduce the internalization of PMLCs.[151]

The targeted delivery of PMLCs involves functionalization of the capsule surface with monoclonal antibodies,[152,153] carbohydrates,[154–156] or magnetic particles.[157] Cortez et al. described the functionalization of PSS/PAH PMLCs with humanized A33 monoclonal antibodies (huA33 mAb), which bind to the human A33 antigen expressed by 95% of all human colorectal cancer cells. This functionalization resulted in a greatly enhanced uptake of PMLCs by cancer cells containing the A33 antigen compared to nonfunctionalized PMLCs.[152,153] Carbohydrates are often used as ligands for biospecific recognition. Galactose, for example, is specifically recognized by asiaglycoprotein receptors, which are exclusively expressed by liver parenchymal cells. Therefore, surface functionalization of PMLCs with galactose moieties could potentially enhance the binding of PMLCs to hepatocytes. Zhang et al. reported on the synthesis of a galactose-bearing polycation which was used to construct PMLCs in combination with PSS or hemoglobin as the polyanion.[154,155] These PMLCs showed a preferential binding to peanut agglutinin lectin rather then concanavalin A lectin, thus demonstrating the biospecificity of this type of PMLC. However, so far no studies on the in vitro or in vivo uptake of PMLCs by hepatocytes have been reported.

Magnetic targeting was established by Zebli et al. by using PSS/PAH capsules functionalized with magnetic metal nanoparticles. A flow-channel set-up combined with a localized magnetic field allowed the authors to demonstrate that PMLCs were preferably internalized by breast cancer cells growing in the proximity of the magnetic field.[157] Besides the use of antibodies, viruses have also been used to functionalize the surface of PMLCs to modulate the cellular uptake. Fischlechner et al. produced virus-modified PMLCs by incubating lipid-coated PMLCs with rubella-like particles or influenza A/PR8 viruses through lipid fusion of PMLCs and the viral surface. The key goals of these viral modifications were promoting the binding of PMLCs to the cellular surface, induction of endocytosis, and subsequent fusion with the late endosomal membrane. VERO cells showed an enhanced uptake of virus-coated PMLCs compared to lipid-coated PMLCs without virus particles.[158–161] Such virus-functionalized particles may find applications in diagnostics, vaccination, and gene delivery. For example, Toellner et al. described a bead assay based on virus-functionalized PMLCs for the simultaneous detection of viral antibodies in serum.[162]

4.2. In Vivo Interactions

Given their strongly charged nature, polyelectrolyte microcapsules may invoke significant tissue reactions when applied in vivo. De Koker et al. have examined the tissue reaction inflicted by subcutaneous injection of microcapsules composed of dextran sulfate/poly-L-arginine bilayers in mice. Injection resulted in a fast proinflammatory response, characterized by the recruitment of polymorphonuclear cells and monocytes.[139] The microcapsules behaved like a porous implant, with infiltration starting at the border and gradually moving towards the center of the injection volume (see the confocal microscopy images of tissue sections taken at different time points after injection in Figure 8). Phagocytic

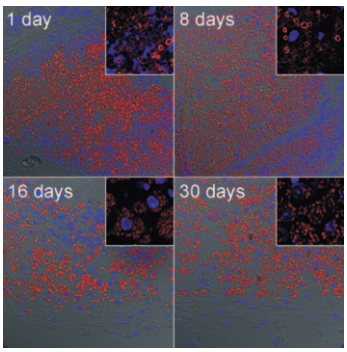


Figure 8. Confocal microscopy images of tissue sections taken at several times after subcutaneous injection of (dextran sulfate/poly-L-arginine)$_4$ capsules. The walls of the capsules were stained with rhodamine (red fluorescence) and the cell nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI; blue fluorescence). The insets in the top right corners show the cellular uptake and degradation at a higher magnification.[139]

mononuclear cells gradually replaced the polymorphonuclear cells. The inflammation remained confined to the injection site, which rapidly became surrounded by several layers of fibroblasts. Importantly, no tissue destruction or ulceration was observed. In this regard, polyelectrolyte microcapsules appear to elicit a similar degree of inflammation as other microparticles of the same size range, including the widely explored and Food and Drug Administration approved poly(lactic-*co*-glycolic acid). The same authors also further examined the in vivo fate of RITC-poly-L-arginine-labeled microcapsules following subcutaneous injection. The microcapsules were taken up by phagocytic cells, deformed, and subsequently degraded; microcapsules having a thicker shell (more bilayers) were more resilient to deformation and degradation. Taken together, these data have established the feasibility of using polyelectrolyte microcapsules in vivo. Moreover, given the tight association between inflammation and the induction of immune responses, and their capacity to target phagocytic cells in vivo, polyelectrolyte microcapsules may have interesting applications in the delivery of antigens (see Section 5.3).

5. Drug Delivery

5.1. Low-Molecular-Weight Drugs

Low-molecular-weight compounds are the predominantly used drugs and were amongst the first to be encapsulated in PMLCs. As the LbL coating is commonly performed in aqueous media, water-soluble drug compounds must be kept in a nonsoluble state, for example, by changing the pH value, to allow the LbL coating to be carried out. Numerous drug microcrystals including acyclovir, ibuprofen, dexamethasone,

ketoprofen, biotin, indomethacin, furosemide, and vitamin K_3 have been encapsulated in PMLCs in this way. Moreover, the ability of adsorbed polyelectrolytes to alter the dissolution profile of incorporated microcrystals towards more-prolonged drug release was observed.[156,163–168] Uncharged water-insoluble drug crystals can be encapsulated in a similar fashion by first providing the crystals with a surface charge through adsorption of an ionic surfactant, followed by LbL coating with polyelectrolytes.[169] Methods other than coating solid-drug microcrystals involve precipitation of the drug molecules in an organic phase followed by LbL coating with polyelectrolytes that are soluble in an organic solvent. This so-called reverse-phase layer-by-layer technique was reported by Beyer et al. for the encapsulation of glucose and vitamin C. Figure 9 shows the optical microscopy images of the encapsulation and release from such microcapsules.[170] An extension of this technique was demonstrated by the same research group by using hydrogel beads loaded with water-soluble compounds followed by stabilization of the beads through adsorption of colloids and reversed-phase LbL coating. This approach prevented premature release of the encapsulated water-soluble compounds and resulted in an encapsulation efficiency of nearly 100%.[171] Passive drug loading through electrostatic interactions and the use, as a layer component, of micelles loaded with a hydrophobic compound have also been demonstrated.[172,173]

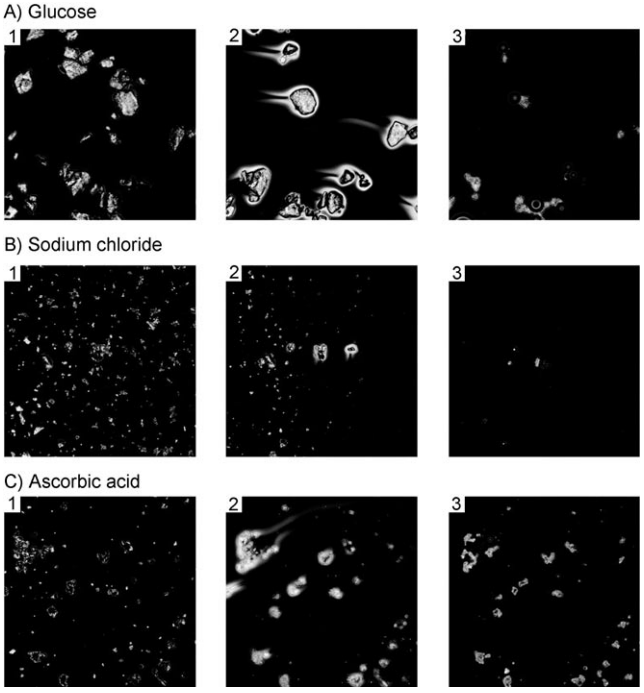


Figure 9. Microscopy images demonstrating the RP-LbL encapsulation of A) glucose, B) sodium chloride, and C) ascorbic acid. The images show 1) RP-LbL-encapsulated crystals in ethanol before the addition of water, 2) after addition of water, and 3) the capsule material after complete release.[170]

An "active postloading" approach was proposed by Radtchenko et al., who exploited the change in the solubility of (poorly water-soluble) drug molecules between the capsule interior and the external solution, thus allowing precipitation of the drug in the interior of the capsules.[174] A strongly hydrophilic polymer was encapsulated within the capsules, thereby providing a polarity gradient between the interior of the capsules and an external water/acetone solution. Drugs that were soluble in the water/acetone mixture experienced a higher partial water content within the capsules and thus precipitated. This process continues until the inner capsule volume is filled with precipitated drug. Molecular dynamics simulations and X-ray analysis revealed that the precipitated drug was in an amorphous state, while it is mostly in a crystalline state when suspended in water. This effect might favor drug release under physiological conditions.[175]

The findings of different research groups suggest that it is rather challenging to retain water-soluble substances within the PMLC wall when the capsule wall consists of highly water-soluble polyelectrolytes. The assembly of polyelectrolytes always results in small pores in the capsule wall, and small molecules can easily find their way through. The way to overcome this constraint might be to use more-hydrophobic compounds in the capsule shell or by rendering the capsules shell more hydrophobic after formation of the capsule. The use of a fluorinated polymer as a shell constituent followed by membrane densification by thermal shrinking of the capsules made the capsule impermeable to hydrophilic low-molecular-weight compounds.[62] A similar effect can be obtained by coating the capsule surface with a lipid bilayer.[176] Andreeva et al. performed a chemical modification by forming imide bonds between the successive polyelectrolyte layers.[177] In all of the above mentioned cases, drug release could only occur upon mechanical rupture of the capsules.

Anticancer drugs represent an important class of low-molecular-weight drugs. So far, chemotherapeutics such as doxorubicin, daunorubicin, 5-fluorouracil, and polyphenols have been successfully encapsulated in PMLCs.[143,178–181] Furthermore, a higher activity of the encapsulated chemotherapeutics compared to the free drugs was observed in the cases of doxorubicin- and daunorubicin-loaded capsules tested on in vitro H460, A549, and HepG2 cancer cell lines. A xenograft experiment in mice further demonstrated the effectiveness of encapsulated doxorubicin in reducing the size of tumors.[180,182] Electrostatic interactions are frequently used for encapsulating these drugs. Nevertheless, other techniques for encapsulating chemotherapeutics have also reported, such as the use of emulsion templating or hydrophobic association.[89,181] Emulsion-based encapsulation strategies investigated by Sivakumar et al. demonstrated the toxicity of encapsulated doxorubicin and 5-fluorouracil on a human colorectal cell line (LIM 1215).[89] Hypocrellin B was incorporated by Wang et al. in PMLCs by a solvent-displacement step with ethanol, in which the drug is soluble. The incubation of human breast cancer cells (MCF-7) with capsules loaded with hypocrellin B combined with light therapy resulted in a reduction of cell viability.[144] The above-described approaches comprise the encapsulation of the drug molecules themselves. An alternative approach involving the encapsulation of a chemotherapeutic pro-drug can also be followed. This was described by Wang et al. and Schneider et al., who encapsu-

lated a pro-drug of doxorubicin in multilayer capsules that released the active drug by enzymatic cleavage after endocytosis.[104,183]

5.2. Proteins

Proteins represent a growing and promising field of therapeutics with possible applications in the treatment and prevention of many metabolic and inflammatory diseases. Proteins are commonly administered through injection, as other routes often suffer from poor bioavailability. Most proteins, however, have limited stability when applied in vivo, thus often requiring multiple administrations through injection. For many applications, microencapsulation can be beneficial when it offers enhanced stability and/or targeting towards the site of action. In addition, protein-containing microcapsules might function as a depot that only releases its contents after a predetermined time or upon a specific stimulus.

Several requirements need to be fulfilled when incorporating proteins in PMLCs. The encapsulation process should not affect the biological activity of the encapsulated compound.[184] Many enzymes such as catalases, peroxidases, α-chymotrypsin, ureases, and glucose oxidases have been encapsulated in PMLCs through spontaneous loading into MF-templated PMLCs or by diffusion into porous silica or calcium carbonate core templates. It was demonstrated for all these systems that the encapsulated enzymes largely retained their biological activity, while keeping the enzymes within the confined geometry of the capsule shell, thus making them attractive candidates as microreactors.[15,16,60,185–189] The biological activity of PMLC-encapsulated insulin was evaluated in vivo in rats by Zheng et al. Insulin microparticles were coated with Fe^{3+}, dextran sulphate, and protamine and were shown to extend the tolerance to glucose from 2 to 12 h, and the glucose-lowering profile was prolonged and stable.[190]

Cytokines play an important role as mediators of inflammation and in tissue regeneration, where they control many cellular processes such as angiogenesis, proliferation, and differentiation. Cytokines greatly benefit from controlled release, to enhance their delivery in an intact form and at a sufficiently high concentration at their target sites, because of their fast diffusion and short half-life. Akashi and co-workers were the first to demonstrate the possibility of using PMLCs as cytokine carriers. Biodegradable PMLCs based on dextran sulfate and chitosan were loaded with basic fibroblast growth factor (bFGF) through pH-controlled switching of the capsule permeability. When placed in physiological media, competition with salts allowed a controlled release of the growth factor. Basic FGF-loaded PMLCs were able to prolong proliferation of L929 fibroblasts to 15 days, while soluble bFGF only supported L929 proliferation for 4 days.[98]

5.3. Delivery of Vaccines

Although vaccination schedules have dramatically reduced the incidence of many infectious diseases, no effective vaccines are today available for many insidious pathogens, such as HIV, malaria, and *Mycobacterium tuberculosis*. To successfully combat these pathogens vaccines are needed that mount the full armoury of the immune system, including CD4 T-helper responses and the induction of cytotoxic T lymphocytes (CTLs) that can recognize and kill infected cells.[191] Generating such CTL responses requires the antigen to be processed and presented by dendritic cells in the cleft of class I major histocompatibility (MHC) complexes.[192] This is barely possible with recombinant soluble antigens.[193] Indeed, MHCI presentation generally requires proteasomal processing of cytosolic antigens, while exogenous antigens are typically processed by lysosomal proteases and subsequently loaded onto MHCII molecules, thereby allowing the activation of CD4 T cells. Antigens derived from particulates such as whole bacteria and viruses can, in contrast, be efficiently presented by dendritic cells to both CD4 and CD8 T cells, thus stimulating the generation of much broader immune responses. The particulate nature of pathogens can be mimicked by encapsulating antigens in synthetic polymeric particles in the 0.1–10 μm range, which greatly enhances the targeting of dendritic cells and increases antigen presentation to both CD4 and CD8 T cells. Polymeric particles, such as poly(lactide-co-glycolide) microspheres and gel particles, currently being explored in antigen delivery, however, strongly suffer from practical drawbacks, including low antigen loading and antigen destruction as a result of the use of organic solvents and harsh reaction conditions, which largely limits their clinical application.[194–198]

LbL microcapsules might be interesting antigen-delivery systems because they can efficiently encapsulate proteins under nondenaturing conditions. PMLCs composed of the polyelectrolytes dextran sulfate and poly-L-arginine have been reported to be taken up highly efficiently by dendritic cells derived from mouse bone marrow, without exerting strong toxic effects.[139] These observations have been extended by De Rose and co-workers, who demonstrated the uptake of PMLCs composed of a variety of polymers by human antigen presenting cells (APCs). Both peptides and protein antigens have now been delivered to dendritic cells in vitro by using PMLCs. De Rose and co-workers used bioresponsive PMLCs composed of thiolated poly(methacrylic acid) (PMA^{SH}) to target a MHCI-restricted peptide epitope of the SIV (simian immunodeficiency virus) gag protein to macaque APCs.[199,200] PMA^{SH} microcapsules are stabilized by disulfide linkages at physiological pH values, but once internalized, the disulfide bridges are broken by the reductive environment, which results in decomposition of the microcapsules. These authors were able to couple the peptide to the capsules through a disulfide linkage by modification of the N terminus of the peptide with a cysteine group. Following uptake by APCs in the blood, the peptide was efficiently released from the microcapsules and presented to peptide-specific CD8 T cells, which resulted in their activation.

Recently, the effects of encapsulating protein antigens in LbL capsules on antigen presentation by dendritic cells have also been evaluated.[201] Protein antigens possess certain advantages over peptides. First, protein antigens are far more effective in inducing antibody responses compared to

peptides. Second, they often contain epitopes for both MHCI- and MHCII-mediated antigen presentation, thus allowing the induction of a broader immune response. These authors were able to show by using ovalbumin (OVA) as a model antigen that OVA encapsulated in dextran sulfate/poly-L-arginine microcapsules became readily accessible for proteolytic degradation following uptake of the microcapsules by the dendritic cells (Figure 10 A,B),[202] thus resulting in a strongly enhanced antigen presentation to both CD4 and CD8 T cells compared to soluble OVA (Figure 10 C).[145]

The real potential of PMLCs as antigen-delivery vehicles should, however, be in in vivo studies. As discussed earlier, dextran-sulfate/poly-L-arginine microcapsules are taken up and degraded by phagocytes following subcutaneous injection, which suggests their potential as antigen-delivery vehicles.[139] Whether these microcapsules, however, also target dendritic cells and promote T-cell responses in vivo is a topic of ongoing research. Recently, Selina et al. immunized mice with various PMLCs containing plasmid DNA encoding the E2 epitope of classical swine fever. Although no analysis of T-cell responses was performed in this study, antibody titers appeared elevated compared to immunization with naked plasmid DNA, thus indicating that PMLCs might also be beneficial for enhancing the efficiency of immunization protocols involving DNA.[203] Finally, an additional benefit of using PMLCs as antigen-delivery vehicles is the high versatility of the LbL technique itself, which allows one to modify the surface of the microcapsules with nearly any ligand of interest. Such an approach might be useful for enhancing specific cellular targeting by linking the microcapsules to antibodies, or to enhance the activation of dendritic cells by coating the microcapsules with immunopotentiators such as synthetic Toll-like receptor agonists.

5.4. Nucleic Acids

Nucleic acids are regarded as promising drugs for future therapies—curing genetic diseases as well as cancer.[204] Plasmid DNA (pDNA) and small interfering RNA (siRNA) have emerged as the primary candidates in gene therapy. To replace or suppress malfunctioning target genes these drugs have to be delivered in an intact form into the target cells, that is, the nucleus in the case of DNA and the cytoplasm in the case of siRNA. Nucleic acids are subjected to enzymatic degradation when administered unprotected to the body and are poorly taken up by cells. For this purpose, the key to gene therapy is the efficient formulation of nucleic acids into particles.[205]

DNA is polyanionic in nature and has been used as a constituent of electrostatic-bound multilayer films since the advent of the LbL technique.[207] Schüler and Caruso used DNA as wall components of PMLCs through complexation with spermidine.[208] The obtained capsules were destabilized at physiological salt concentrations, thereby providing them with a release mechanism, although also limiting their applicability. Shchukin et al. stabilized DNA/spermidine-coated colloids with additional polyelectrolyte multilayers to yield capsules with freely floating DNA in their hollow void upon dissolution of the core and destabilization of the DNA/spermidine complex.[209] A simple procedure to load DNA into human erythrocyte templated capsules was reported by the Möhwald research group, who applied an intermediate drying step which appeared to enhance the accumulation of DNA into the capsules.[131] The same research group reported a method for the release of encapsulated DNA by coencapsulation of enzymes that could digest the capsule wall.[95] Donath and co-workers incorporated plasmid DNA that encoded for enhanced green fluorescent protein (eGFP) and discosoma species red fluorescent protein (dsRED) within multilayers of dextran sulfate and protamine.[210] The resulting PMLCs could transfect cells, which internalized the capsules; this finding showed for the first time the successful delivery of functional DNA into cells mediated by PMLC capsules. Furthermore, DNA nanoparticles can be coated with polyelectrolytes to enhance their transfection.[211] So far these systems appear to be designed for paren-

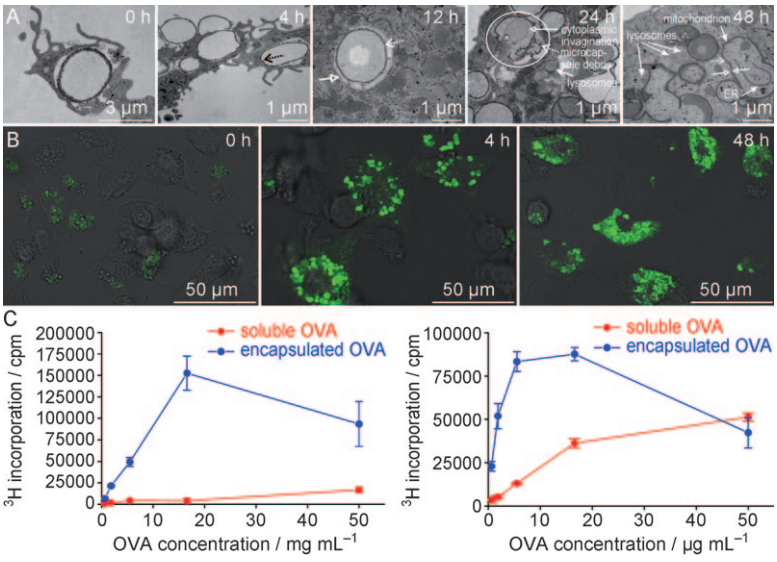


Figure 10. A) TEM images of bone-marrow-derived dendritic cells with internalized dextran-sulfate/poly-L-arginine microcapsules. Dotted arrows indicate the microcapsule shell; open arrows denote membranes surrounding the microcapsules. In the encircled area (after 24 h), microcapsule rupture and cytoplasmic invagination are clearly distinguishable. Lysosomes, endoplasmatic reticulum (ER), and a mitochondrion are indicated by the solid arrows. B) Processing of OVA encapsulated in dextran sulfate/poly-l-arginine microcapsules was analyzed using DQ-OVA (DQ-OVA is an ovalbumin oversaturated with BODIPY (boron-dipyrromethene) dyes). Confocal microscopy images of bone-marrow dendritic cells incubated with OVA-DQ microcapsules for 0, 4, and 48 h (overlay of green fluorescence and differential interference contrast). Upon proteolytic cleavage, quenching is relieved and green fluorescence appears. C) Presentation of antigens by bone-marrow dendritic cells after uptake of soluble and encapsulated OVA. The proliferation of cells of OT-I mice was used as a measure of MHC-I-mediated cross-presentation of OVA (left), and the proliferation of cells of OT-II mice as a measure of MHC-II-mediated presentation (right).[145]

teral administration. However, a recent report by Aouadi et al. showed very promising results after oral delivery of siRNA-loaded modified yeast cells to mice. These findings could pave the road for the oral application of PMLCs.[212]

Caruso and co-workers have extensively published on the use of short-length DNA and oligonucleotides for the fabrication of multilayer capsules.[206] Two distinct strategies were applied for this purpose: A first comprises the use of mesoporous silica microparticles which are modified with amino groups to facilitate diffusion and retention of oligonucleotides within the silica pores through charge interaction.[34,103] After coating the particles with a PMA^{SH}/PVPON multilayer followed by disulfide cross-linking and dissolution of the silica template, hollow capsules are obtained, which stably encapsulate oligonucleotides (Figure 11 A–D). This approach offers high versatility, since capsule disassembly can be performed in reductive media through cleavage of the disulfide bonds and nucleotides could be released by a triggered enzymatic reaction by using coencapsulated DNase I, which can be activated by the addition of Ca^{2+} or Mg^{2+} ions.[213] Moreover, the same research group has also reported on the application of DNA-loaded PMLCs for DNA biosensing. In this case they exploited the ability of single-stranded DNA (ssDNA) molecules to act as a molecular beacon which only becomes fluorescent in the presence of a specific DNA sequence.[214] This technique could be used, for example, for high-throughput screening applications, where multiple populations of PMLCs with different ssDNA beacons can be analyzed by flow cytometry.

A second methodology introduced by Caruso and co-workers is the use of DNA as a so-called programmable building block.[215] By taking advantage of base-pair recognition, one is able to construct multilayer films through hybridization of diblock oligonucleotides (shown schematically in Figure 11 E). Such diblock oligonucleotides must consist of one block (for example, polyA) which is complimentary to an underlaying layer (for example, polyT), thereby allowing hybridization of the two blocks through hydrogen bonding and π–π stacking of their aromatic base pairs. The second block (for example, polyG) should be able to hybridize a next block (polyC) of a subsequent diblock oligonucleotide (for example, poly(CT)). Hybridization of the base pairs provides a high degree of control over the obtained multilayer structures. Structural engineering of the capsule membrane was shown to be possible by engineering the sequences of the oligonucleotide blocks.[216] For example, shrinkage of the capsules upon dissolution of the core template could be controlled by the composition of the oligonucleotide diblocks: by exploiting the directional nature of DNA, which assembles through the formation of a double helix.[217] Such controlled shrinkage could be used to alter the properties of the capsule as well as to concentrate encapsulated compounds. Electrostatic repulsion between the anionic base pairs results in destabilization of the capsules occurring at low salt concentrations, where the negative charges of the nucleotides are no longer shielded. This issue could be circumvented by introducing triblock oligonucleotides, which offer additional cross-linking within the multilayer film, hence making them applicable under physiological conditions.[218] Finally, additional control over the destabilization and release properties of the capsules was achieved by incorporation of restriction-enzyme cut sites within the multilayer structure.[219] *Eco*RI is such an enzyme, which can specifically cleave the 5′-G | ATTC-3′ sequence. PMLCs were fabricated by DNA hybridization using triblock oligonucleotides having the 5′-G | ATTC-3′ sequence in their middle block. The addition of *Eco*RI resulted in the capsule starting to shrink and to continuously release the encapsulated bovine serum albumin used as a model drug. From a conceptual point of view, this is without doubt a beautiful and highly controllable system. It will, however, be a major challenge to prove cost efficiency. Specific niche applications might offer good opportunities, as production costs for such kind of capsules are relatively high.

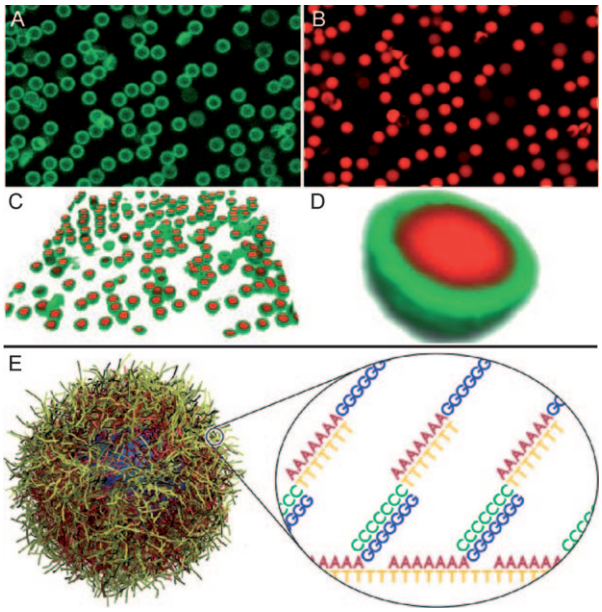


Figure 11. Confocal laser scanning microscopy images of 16-layer PMA^{SH}/PVPON capsules filled with poly($T_{15}C_{15}$). A) Fluorescence originating from the capsule walls as a consequence of the PMA^{SH} labeled with AF488 and B) fluorescence of TAMRA-poly($T_{15}C_{15}$). C, D) 3D cross-section images after reconstruction of the confocal data. The width of the images (A)–(C) is 30 μm. The capsule in (D) is 1.5 μm. E) Schematic representation of of an idealized orientation of oligonucleotides assembled from homopolymeric blocks of repeating nucleotides (poly(GC) and poly(AG)). A primer layer of polyT is also shown.[34,206]

6. Conclusions

In this Review we have attempted to give an overview of the recent developments in the use of polymeric multilayer capsules (PMLCs) for drug-delivery applications. The first section of this Review covered the different synthetic approaches for the synthesis of PMLCs. Initially, PMLCs were generated from synthetic nondegradable polyelectrolytes, but they, and especially the polycations, raise severe toxicity issues. More recently used polycations based on

polypeptides or polysaccharides can be degraded enzymatically, and PMLCs of these polycations show little or no toxicity. Whereas electrostatic interactions were initially used as the driving force for the assembly of multilayers, an increasing number of studies report on capsules held together by other types of interactions such as hydrogen bonding or covalent bonds. The main advantage of these methods is the avoidance of polycations, which are often toxic and could therefore hamper further clinical applications. On the other hand, the use of chemical synthesis to functionalize or cross-link polymers might also bring problems, as these materials most often do not have GRAS status. Moreover, all these approaches render the system more complex than when only electrostatic interactions are used. In this context, the major challenge is to identify those specific applications where the high degree of nanoengineering in the preparation of PMLCs offers advantages compared to using traditional, less-complex drug-carrier systems.

In the second section we reviewed the different approaches that can be used to load and unload molecules of interest into and out of PMLCs. The postloading approach, which involves a reversible change of the capsule permeability and was often applied during the initial years in the development of PMLC capsules, only offers low encapsulation efficiency. Preloading strategies, involving porous drug-loaded templates, as well as emulsion strategies are gaining increasing popularity in the field. Once encapsulated into PMLCs, the drug molecules also have to be released at their target site. While precisely controlling the release of small hydrophilic molecules is difficult with PMLCs, several successful strategies have been developed for the delivery of macromolecular drugs as well as hydrophobic compounds. For this purpose, PMLCs have been equipped with specific functionalities that allow them to be opened upon application of a specific trigger. So far, the most promising systems are those based on enzymatic degradation of the capsule shell or on reductive cleavage of a disulfide linkage, which leads to destabilization of the capsule shell. The recent findings of Skirtach et al.[115] on capsules activated by a laser beam also offer perspectives for controlled intracellular delivery of drugs. While PMLCs and other types of particles with similar sizes are commonly delivered to endo/lysosomal vesicles, this strategy allows escape from such vesicles and the delivery of drug molecules into the cellular cytosol. This process is important for the delivery of nucleic acids and peptides.

The synthesis of PMLCs is relatively time and cost consuming because of their multistep synthesis, and the essential monitoring to avoid particle aggregation during LbL coating and centrifugation/filtration steps. For this reason it can be excluded that PMLCs will emerge as a viable alternative to deliver traditional drug molecules which are already on the market in oral dosage forms. On the other hand, we are convinced that PMLCs would be advantageous for certain specific applications. So far this has been demonstrated by several research groups with the delivery of vaccines, where targeting cells of the immune system with PMLCs has shown to be a highly efficient process. The delivery of anticancer drugs undoubtedly also has potential. Several encapsulation methods have been shown to be successful in encapsulating anticancer drugs, and the functionalization of PMLCs by cell-targeting antibodies and stealth PEG coatings have been established. Moreover, the flexible nature of the PMLC membrane should allow facile passage through capillary veins compared to massive microspheres. The major challenge for the delivery of anticancer drugs as well as for nucleic acid and gene delivery, however, remains in getting the PMLCs to reach cancerous tissues in vivo. Thus, PMLCs should be able to circulate in the bloodstream without aggregating or prematurely releasing their payload. Moreover, their size also plays an important role: The well-known EPR (enhanced permeability and retention) effect of the blood vessels in cancerous tissues allows particles with a size of up to 200 nm to escape the blood vessel, but this is much smaller than the sizes of most PMLCs. Therefore, exciting future developments for intravenous administration of PMLCs will without doubt focus on the design of capsules with a size of less than 500 nm.

7. Abbreviations

CLSM	confocal laser scanning microscopy
DAR	diazoresin
EDTA	ethylenediaminetetraacetic acid
FITC	fluorescein isothiocyanate
HSA	human serum albumin
LbL	layer-by-layer
MF	melamine formaldehyde
MHC	major histocompatibility complex
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
PAH	poly(allylamine hydrochloride)
PDADMAC	poly(diallyldimethylammonium chloride)
PEG	poly(ethylene glycol)
PGA	poly-L-glutamic acid
PLL	poly-L-lysine
PMA	poly(methacrylic acid)
PMA^{SH}	thiolated polymethacrylic acid
PMLC	polymeric multilayer capsule
Poly(HPMA-DMAE)	poly(hydroxypropylmethacrylamide dimethylaminoethyl)
PSS	poly(styrene sulfonate)
PVPON	poly(*N*-vinylpyrrolidone)
RITC	rhodamine-B-isothiocyanate
RP-LbL	reversed-phase layer-by-layer
siRNA	small interfering RNA
SNARF	seminaphtharhodafluor
TA	tannic acid
TAMRA	carboxytetramethylrhodamine
TRITC	tetramethylrhodamine isothiocyanate

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