POLYMERIC MULTILAYER CAPSULES DELIVERING BIOTHERAPEUTICS

Stefaan De Koker,[1,2] Liesbeth J. De Cock,[1] Pilar Rivera Gil,[3] Wolfgang J. Parak,[3] Rachel Auzély Velty,[4] Chris Vervaet,[1] Jean Paul Remon,[1] Johan Grooten,[2§] and Bruno G. De Geest[1*§]

1 Laboratory of Pharmaceutical Technology, Department of Pharmaceutics, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium

2 Laboratory of Molecular Immunology, Department of Molecular Biomedical Biology, Ghent University, Technologiepark 927, 9052 Zwijnaarde, Belgium

3 Biophotonic Group, Phillips University Marburg, Renthof 6, D-35032 Marburg, Germany

4 Centre de Recherches sur les Macromolécules Végétales (CERMAV-CNRS), BP53, 38041 Grenoble cedex 9, France

§ These authors equally supervised this work

* Corresponding author: Dr. Bruno De Geest

Laboratory of Pharmaceutical Technology
Department of Pharmaceutics
Ghent University
Harelbekestraat 72
9000 Ghent
Belgium
br.degeest@ugent.be

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Abstract

Polymeric multilayer capsules have emerged as a novel drug delivery platform. These capsules are fabricated through layer-by-layer sequential deposition of polymers onto a sacrificial core template followed by the decomposition of this core yielding hollow capsules. The resulting nanometer thin membrane is permselective, allowing diffusion of water and ions but excluding larger molecules. Moreover, the sequential fabrication procedure allows a precise fine-tuning of the capsules' physicochemical and biological properties. These properties have put polymeric multilayer capsules under major attention in the field of drug delivery. In this review we focus on polymeric multilayer capsule mediated delivery of biotechnological macromolecular drugs such as peptides, proteins and nucleic acids.

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1. Introduction

Recent advances in molecular biology and biotechnology have lead to the discovery of an entirely new gamma of biomolecular therapeutics such as peptides, proteins and nucleic acids. [1] These biotherapeutic drugs require special handling as they are often prone to degradation or denaturation upon oral intake and often do not easily reach their target after mere parenteral injection.[2, 3] In order to be protected before reaching their target and/or to assist them reaching their specific target location, advanced drug formulation is of paramount importance.

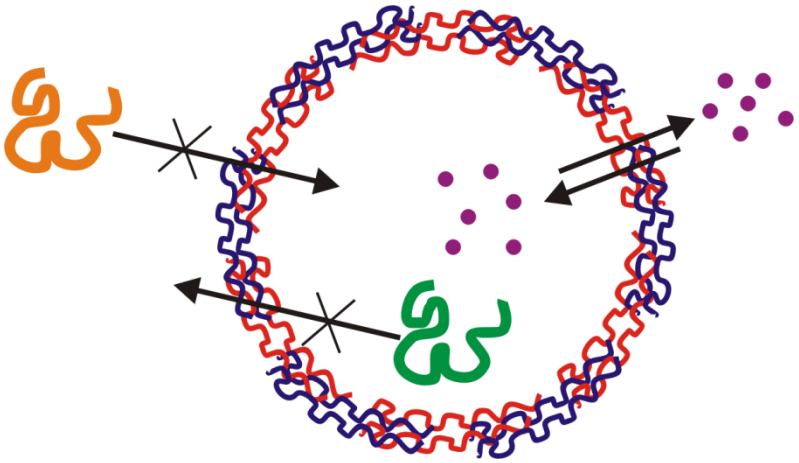

Figure 1. Schematic representation of the permselective nature of polymeric multilayer capsules. The polymeric multilayer membrane is fully permeable to water molecules and the inner compartment of the capsules is fully aqueous. Low molecular weight compounds can freely diffuse in and out of the capsules while larger species are excluded or remain encapsulated.

For several reasons, microparticulate encapsulation often offers a solution to the formulation issues of biotherapeutics: (1) Microparticles can be easily administered both by injection, topical administration as well as oral intake. (2) Microparticles offer protection of the therapeutics of interest against enzymatic degradation on a microscopic scale. (3) Microparticles can be internalized by many different cell types while tailoring the size and surface chemistry of the particles can alter their tager cell population. (4) Microparticles can be engineered in such a way that they release their payload in a sustained fashion of only after a specific physic-chemical stimulus when reaching their target site. [2, 3]

Polymeric multilayer capsules are fabricated through step-wise adsorption of polymers using electrostatics, H-bonding, covalent chemistry, etc… as driving force, followed by the dissolution of the core template. [4, 5] One of their most striking properties is their permselectivity in aqueous medium.[6] While the polymeric shell is fully permeable to low molecular weight compounds such as ions and small drug molecules, they are impermeable

to larger molecules. This property, schematically illustrated in Figure 1, renders polymeric multilayer capsules ideally suited for the encapsulation of biotherapeutics such as proteins, peptides, and nucleic acids like DNA, siRNA etc… [7-9] While the aqueous void of the capsules should provide physico- and bio-chemical stability to the molecules of interest, the capsule surface could be engineered in order to (1) target specific cell populations, (2) activate certain cell functions upon binding of the capsules to the cell surface or upon intracellular uptake, (3) release the capsule content at the required moment when reaching the target site or (4) upon a well-defined stimulus.

In this paper we review the recent literature data on the encapsulation and delivery of high molecular weight compounds for biomedical applications.

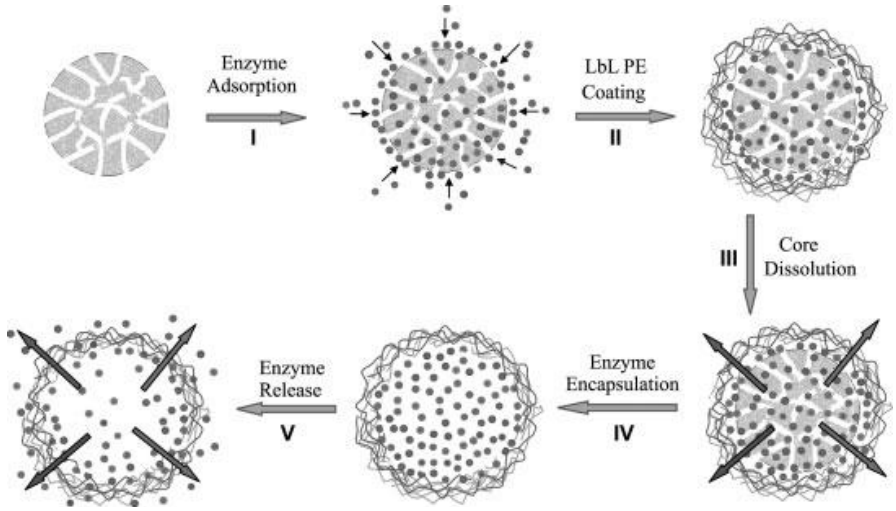

Figure 2. Schematic representation of the procedure for encapsulating biotherapeutics in polyelectrolyte microcapsules using porous microparticles as templates: (I) biotherapeutic immobilization in mesoporous spheres; (II) LbL assembly of oppositely charged polyelectrolytes (PE); (III) dissolution of the microspheres template; (IV) biotherapeutic encapsulated in a polyelectrolyte microcapsules; and (V) biotherapeutics release via altering the shell permeability.

2. Encapsulating biotherapeutics

2.1 Pre-loaded templates

Pre-loading procedures make use of sacrificial templates that already contain the molecules of interest. For this purpose, emulsions [10, 11] are used to encapsulate hydrophobic compounds – these are dissolved into an organic phase which is subsequently coated with a multilayer film – while porous inorganic templates can adsorb both hydrophilic as well as hydrophobic molecules in their pores. Mesoporous silica (SiO_2) [12-14] and calcium carbonate ($CaCO_3$) [15-18] microparticles have been used for this purpose. Mesoporous silica (Figure 2)

benefits from the advantage that such templates can be obtained in a monodisperse state and that they are stable over the whole pH range which allows the use of polyelectrolyte solutions with acidic pH. This is e.g. of importance for the construction of hydrogen bonded multilayers [19, 20] based on the use of polycarboxylic acids as electron donor, since the solution pH has to be lowered in order to keep the carboxylic acid moieties protonated. An important drawback of silica based sacrificial templates however, is the requirement of hydrofluoric acid (HF) to dissolve the silica cores. As HF is extremely toxic, extreme caution has to be exercised during subsequent washing steps in order to remove all residual HF. So far urease, [21] catalase [13] and DNA [22] haven been loaded into mesoporous silica templated PMLC. A number of other components have been encapsulated into non-porous silica templated capsule by using them as first layer in the LbL assembly or through covalent coupling to on the polyelectrolytes. [23]

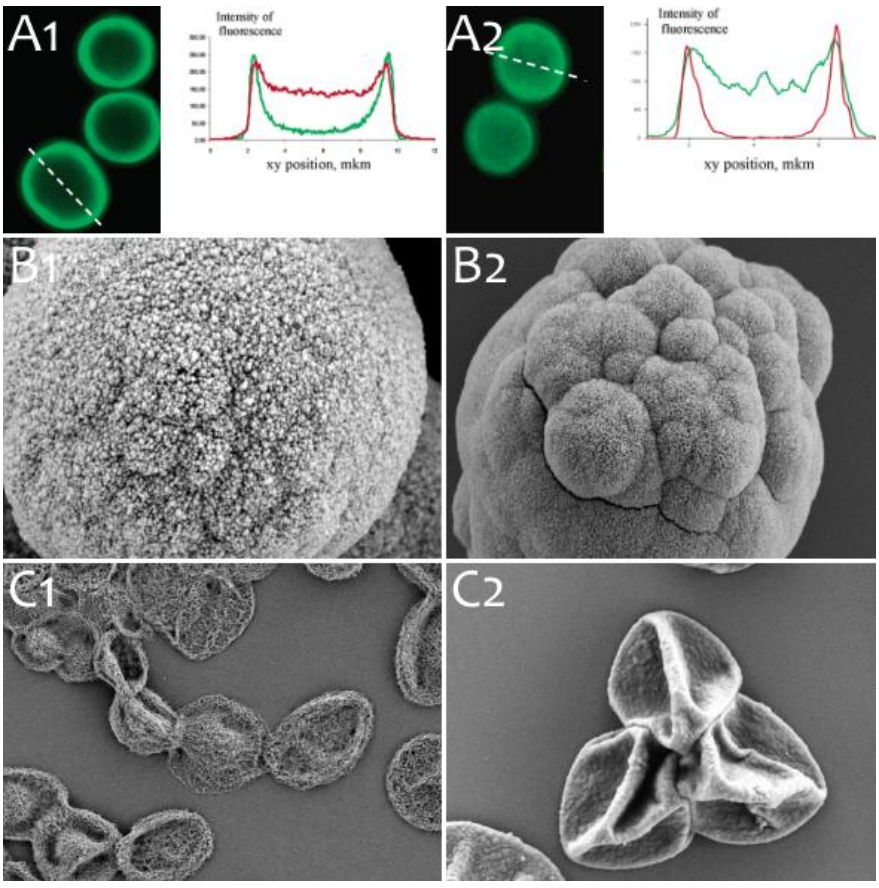

Figure 3. (A) Confocal microscopy image of $(PAH/PSS)_5$ polyelectrolyte capsules loaded with FITC-BSA through (A1) physical adsorption on preformed $CaCO_3$ templates and (A2) co-precipitation during $CaCO_3$ synthesis. (B) Scanning electron microscopy images of (B1) bare $CaCO_3$ templates and (B2) BSA loaded $CaCO_3$ through co-precipitation. (C) Scanning electron microscopy images of (C1) empty and (C2) BSA loaded $(PAH/PSS)_5$ capsules.

An alternative to HF are calcium carbonate microparticles ($CaC0_3$) [16] which are formed through mixing of calcium chloride and sodium carbonate. After LbL coating the

$CaCO_3$ particles are easily dissolved in an aqueous EDTA solution. This technique is especially well suited for the in situ encapsulation of proteins by means of physical adsorption of through a co-precipitation reaction. $CaCO_3$ microparticles exhibit a high porosity (SEM image in Figure 3B1) and can therefore adsorb large amounts of biotherapeutics. A limitation in this process is the molecular weight of the macromolecules, it has been reported that FITC-dextran with a molecular weight of 4 kDa can diffuse through the whole interior of $CaCO_3$ particles while 40 kDa FITC-dextran can only diffuse into the periphery of the particles. This is demonstrated in Figure 3A1 where FITC-BSA was loaded into $CaCO_3$ particles followed by LbL coating and dissolution of the $CaCO_3$ template with EDTA. As can be derived from the distribution of the green fluorescence the protein is only found in the capsule periphery. To encapsulate higher amounts of protein with a more homogeneous intra-capsule protein distribution, a co-precipitation approach is more interesting. [15] In this method the precipitation reaction of $CaCl_2$ and Na_2CO_3 is carried out in the presence of the protein of interest. In first instance $CaCO_3$ nanoparticles are formed onto which the protein adsorbs followed by aggregation of the nanoparticles into microparticles. (SEM image in figure B2). After LbL coating and dissolution of the $CaCO_3$ template a much more homogeneous protein distribution throughout the capsule volume is observed (Figure 3A2). Moreover, a co-precipitation procedure allows to encapsulate much higher amounts of protein compared to physical adsorption. In Figure 3C SEM images are shown of hollow capsules that are respectively empty and loaded with BSA. The loaded capsules exhibit a pronounced higher density compared to the empty ones. So far the following biotherapeutics have been encapsulated in $CaCO_3$ templated PMLC: dextran, a-lactalbumin, lysosyme, horseradish peroxidise, glucose oxidase, catalase, ovalbumin, bovie serum albumin, a-chymotrypsin, insulin DNA and pronase. [15, 17, 18, 24-28]

A third category of template particles that have gathered considerable attention in literature are hydrogel beads. Hydrogels are strongly hydrated 3D networks which, due to their aqueous environment offer good preservation of the biotherapeutics' bioactivity upon encapsulation. The first report of such approach was done by the McShane group who used calcium-alginate beads to load oppositely charged proteins through electrostatic interaction with the anionic alginate followed by the deposition of an LbL coating onto the calcium alginate bead surface. [29] Encapsulation of glucose oxidase with high yields and excellent preservation of enzymatic activity was subsequently demonstrated. [30] De Geest *et al.* elaborated on the use of degradable dextran microgels employing methacrylated dextran whose methacrylate groups were connected to the dextran backbone through a hydrolysable carbonate ester link. As dextran phase separates from poly(ethylene glycol) in aqueous medium at elevated concentrations, microgels could be fabricated in all aqueous conditions

through a water-in-water emulsion approach and the preferential distribution of many proteins into a dextran phase over a poly(ethylene glycol) favours protein encapsulation into the dextran microgels. By engineering the LbL coating of the microgels it was possible to equip the capsules with different release properties. [31-33] For example, a strong coating yielded stable capsules upon dissolution of the microgel core while a loosely bound coating allowed sustained release through pores in the capsule wall. A tightly optimized coating composition yielded so-called self-exploding capsules that retained their payload during degradation of the microgel core. [34, 35] However, upon total degradation, when the osmotic pressure of the microgel core exceeds the tensile strength of the capsule membrane, the capsule explodes and released its payload in a pulsatile fashion. [36]

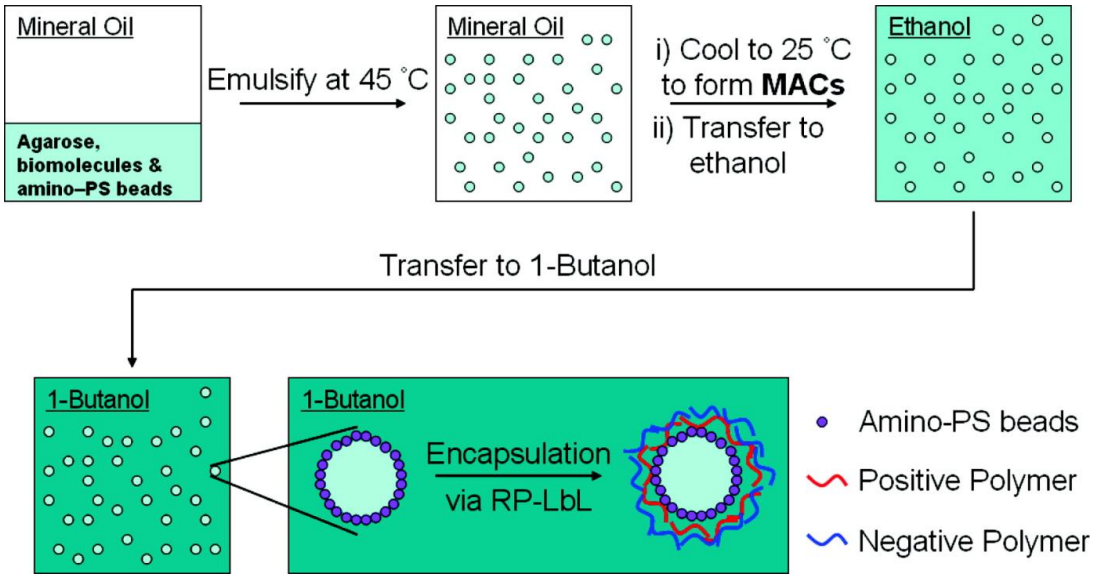

Figure 4. Schematic diagram illustrating the matrix-assisted colloidosome reverse-phase layer-by-layer (MAC RP-LbL) microcapsules fabrication process.

Recently the Trau group reported on a novel so-called reverse-phase LbL encapsulation technique. [37] This approach is especially suited for very hydrophilic molecules and involves non-ionized polyelectrolytes that are dissolved in organic medium (Figure 4). Contrary to the traditional aqueous phase electrostatic LbL build-up where coulomb interactions are the driving force for polyelectrolyte assembly, the authors hypnotized that in reverse-phase LbL a solid phase acid-base reaction occurs between the template surface and the non-ionized polyelectrolyte, yielding a thin layer of ionized polyelectrolyte, which provoques particle stabilisation through repulsion and also creates a concentration gradient between the particle surface and the solution which allows further polyelectrolyte transport through diffusion. Initially, bovine serum albumin (BSA) microparticles in ethanol were used to demonstrate reverse-phase LbL with non-ionized poly(methactylic acid) and non-ionized poly(diallyl dimethyl ammonium chloride). The approach was in subsequent publications

further elaborated for the coating of protein loaded agarose hydrogel beads allowing a quasi 100% encapsulation efficiency of hydrophilic compounds. [38] As schematically depicted in Figure 4, a first step comprised the formation of protein loaded agarose hydrogel beads formed through an emulsification process involving amino functionalized polystyrene beads as colloidal stabilizing agent. Subsequent reverse-phase LbL yields stable microcapsules that can be transferred into aqueous medium without losing their integrity nor losing their protein payload. Furthermore they demonstrated successful encapsulation of the bi-enzyme system based on glucose oxidase (GOx) and horseradish peroxidase (HRP) where GOx consumes D-glucose into D-gluconolactone and hydrogen peroxide which is then consumed by HRP to oxidise Ampliflu Red into a UV-active product that can be measured by spectrophotometry.

2.2 Post-loading strategies

In a post-loading approach, the molecules of interest are loaded into pre-fabricated capsules. This can be done in two ways: (a) by reversibly changing the permeability of the capsule shell or (b) by creating a driving force for certain molecules to accumulate inside the capsules.

Due to their permselectivity, polymer multilayer capsules are permeable to low molecular weight species while they exclude high molecular weight ones. [6] Taken this into consideration, there is however no rule of thumb nor a theoretical model which allows to predict the molecular weight cut-off of the capsule membrane. This is due to several reasons: (a) different polyelectrolytes can results in different structures of the multilayer films with very large differences in permeability, (b) a high surface roughness of the template increases the amounts of adsorbed polyelectrolytes, and thus a thicker and less permeable membrane and (c) due to their ionic nature, polyelectrolytes and their complexes are highly sensitive to variations in pH and ionic strength of the surrounding aqueous medium.

This last property is exploited to reversibly change the permeability of the capsule wall, ‘opening’ the capsules during which the capsules are loaded with molecules of interest followed by ‘closing’ of the capsules resulting in the entrapment of the molecules of interest. [39-41] This is shown in Figure 5 where a combination of adding salt and heating is applied to encapsulate 70 kDa FITC-dextran into $(PDADMAC/PSS)_4$ microcapsules. [42] Figure 5a demonstrates that under conditions of low ionic strength the FITC-dextran cannot diffuse through the polyelectrolyte shell. However, in the presence of 50 mM salt (NaCl; Figure 5b) the polyelectrolyte shell becomes permeable and the capsules fill with FITC-dextran. In order to re-seal the capsule shell, the capsules are heated above the glass transition temperature

of polyelectrolytes. This leads to a shrinkage and by consequence thickening and densification of the polyelectrolyte shell, providing a stable encapsulation of FITC-dextran into the polyelectrolyte capsules. Figure 5c illustrates the importance of the addition of salt to allow encapsulation of high molecular weight species into PMLC through heat treatment. The microscopy images in Figure 6, obtained with various techniques (i.e. confocal laser scanning microscopy, scanning electron microscopy and transmission X-ray microscopy), further illustrates what happens with the polyelectrolyte shell during this heat treatment. [43-48] All three microscopy techniques show a shrinkage of the capsules with higher temperatures of the heat treatment. Note that the same trend can also be obtained at a fixed temperature with variation of the treatment time. The scanning electron microscopy images demonstrate the transition from an inflated structure of the hollow capsules upon drying of the sample, to a hardened structure which can maintain its spherical shape upon drying. The transmission X-ray microscopy images give final evidence of the increase in shell thickness as a function of temperature. These images also point out the powerfulness of this technique to characterise objects in water with a resolution higher than conventional confocal microscopes, avoiding the use of fluorophores and without the extensive sample preparation that is required for electron microscopy methods.

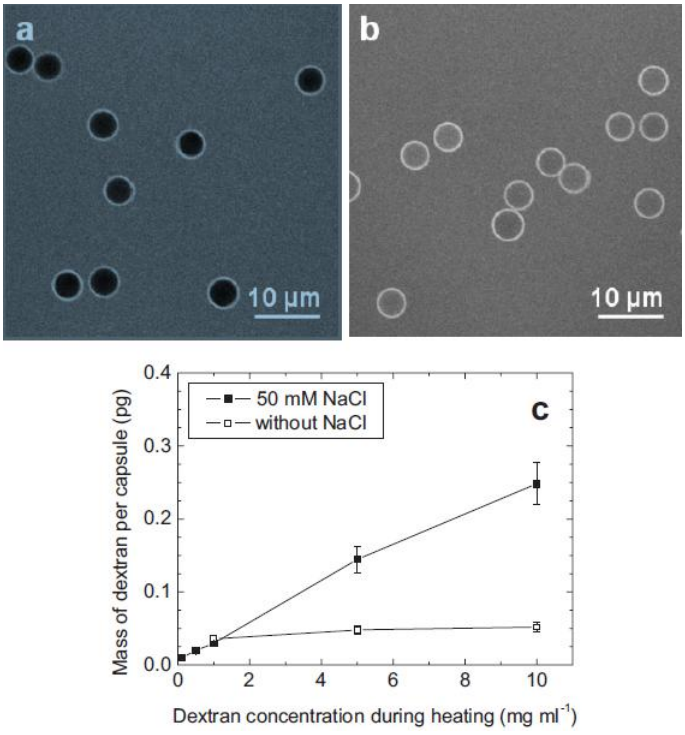

Figure 5. Confocal micrographs of $(PDADMAC/PSS)_4$ capsules incubated for one hour in 1 mg/mL FITC-dextran 70 kDa (a) without NaCl and (b) with 50 mM NaCl. (c) Comparison of the encapsulated amount of FITCd-extran 70 kDa per $(PDADMAC/PSS)_4$ capsule as a function of dextran concentration during heating in 0 and 50 mM NaCl.

Heat shrinkage is definitely an elegant method to encapsulate a wide variety of molecules that is applicable to both small (i.e. 1 to10 kDa) as well as larger molecules. Note that smaller ones do not need additional salt to further permeabilize the capsule shell. However, one does have to take into account that many biotherapeutics are sensitive to heat and therefore extreme caution should be exercised to avoid denaturation. Moreover, encapsulation efficiencies in post-loading methods are inherently very low as the majority of the material to be encapsulated will be outside the capsule shell.

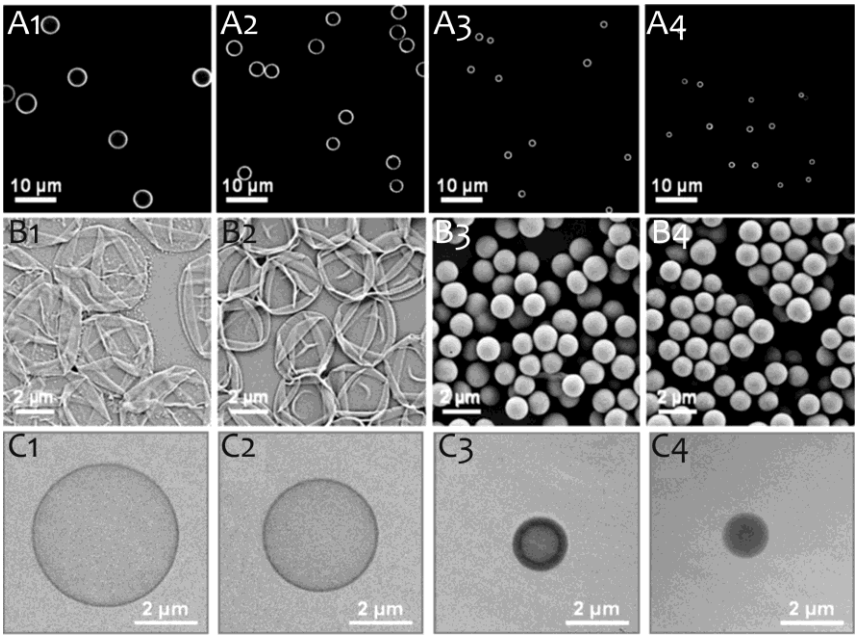

Figure 6. (A) Confocal, (B) scanning electron an (C) transmission X-ray microscopy images $(PDADMAC/PSS)_4$ capsules: non-heated (left), heated at 50 °C, heated at 70 °C, and heated at 90 °C (right) for 20 min.

3. Engineering the capsules to release biotherapeutics

In order to play a role as drug delivery system, it is evident that polymeric multilayer capsules should not only be able to encapsulate and transport drug molecules but they should also be able to release their payload, preferably in a desired fashion. [8] Therefore, numerous release mechanism have been incorporated within these capsules. Inherent to their polyionic nature, polyelectrolyte capsules are prone to ionic strength and pH. The presence of ions in the surrounding medium shields the attractive forces between the successive polyelectrolyte layers, loosening their structure allowing outwards diffusion of encapsulated payload through the capsule membrane. Similarly, when changing the pH of the medium the charge density of the individual polyelectrolytes is also altered. By changing the charge balance between attractive and repulsive forces in the successive polyelectrolytes a rearrangement of the membrane structure is induced, often leading to increased or

decreased permeability towards macromolecules. [49] However alterations in pH (e.g. the shift from extracellular pH of 7.4 to intracellular ph of 5.2 in phagosomes) and ionic strength are very common in living tissue, they are most often too weak to alter major changes in the structure of polyelectrolyte capsules in order to induce release of encapsulated compounds.

The presence of metabolites such as glucose could also be used as trigger, potentially for insulin release in the treatment of diabetes mellitus patients. Several research groups have reported on polymeric multilayer capsules containing glucose responsive components. The first one was demonstrated by De Geest *et al.* using a copolymer containing phenylboronic acid moieties that undergo a glucose dependent shift in charge density. [50] Several other groups also reported on phenylboronic acid based systems, however, a major drawback of this approach was the restriction to work above physiological pH values. [51] Alternatively, protein based systems employing glucose oxidase or Concanavalin A have been reported as well but all suffered from low sensitivity and robustness under physiological conditions. [52-54]

Two major breakthroughs in putting polyelectrolyte capsules en route towards biomedical applications were reported by Zelikin *et al.* and De Geest *et al.*, taking advantage of the of the change in oxidative state, respectively enzymatic activity when crossing the cellular membrane. Zelikin *et al.* designed capsules based on thiolated poly(methacrylic acid) as hydrogen bond donor in conjunction with poly(N-vinylpyrrolidone) as hydrogen acceptor for the formation of H-bond based multilayer capsules. [55, 56] Cross-linking of the successive thiolated poly(methacrylic acid) layers is performed by chloramine T mediated oxidation of the pending thiol moieties to form disulfide linkages. Due to the reductive environment (gluthathione) upon intracellular uptake in phagosomal vesicles, these disulfides become reduced to thiols leading to disassembly of the capsules and release of the encapsulated payload. Based on this principle the Caruso group has reported several modifications, further engineering the physcio-chemistry of the capsules onto the nano-scale. [55, 57-60]

Alternatively to exploiting glutathione mediated reduction, De Geest *et al.* took advantage of the proteolytic activity within intracellular acidic vesicles to trigger capsule disassembly. [24] First they showed that capsules composed of oppositely charged polysaccharides and polypeptides, such as dextran sulfate and poly-L-arginine, decomposed gradually when incubated with a mixture of proteases. Secondly, these capsules were found to degraded intracellularly upon incubation with a cancer cell line (VERO – African green monkey kidney cells), whereas by contrast non-degradable PSS/PAH capsules stayed intact intracellularly for several days.

Both strategies paved the road towards applying polymeric multilayer capsules for intracellular delivery of therapeutic molecules as they could retain their payload stably encapsulated in the extracellular environment, but readily allow release of their payload upon cellular entry. Besides using triggers offered by nature itself also several systems are under investigation where external physical sources such as laser light, [61] magnetic field [62] or ultrasound [63] have been used to trigger release from polyelectrolyte capsules. Intracellular laser triggered release has shown to be possible within living *in vitro* cultured cells but so far no therapeutic applications have been demonstrated.

4. Targeting specific cell populations with polymeric multilayer capsules

Since many biotechnological molecules have a specific target tissue, biospecific interactions between PMLC and cells are important when PMLC are applicated *in vivo*. Functionalization of drug loaded PMLC with cell-targeting structures allows delivery of the cargo to a cell type of interest avoiding unwanted uptake. In this way, high drug concentration can be obtained in the target tissue using a minimum amount of drug loaded carriers and undesirable effects in other tissues or cells are avoided. To target PMLC to a specific cell type, carriers have been functionalized with monoclonal antibodies, [64, 65] carbohydrates [66, 67] or magnetic particles [68] in the past.

Monoclonal antibodies have a remarkable specificity which makes them extremely promising as cell recognition tool. When envisioning anti-cancer therapy, it would be beneficial to target chemotherapeutic loaded PMLC to tumour tissue using antibodies recognizing the cancer cells. Targeting PMLC to human colorectal tumour cells was performed by Cortez *et al.* by functionalizing the carriers through non-covalent (adsorption) or covalent (click-chemistry) interaction with humanized A33 monoclonal antibody which binds to the human A33 antigen, expressed by 95 % of the targeted cell type. [64, 65, 69] A preferential binding of the functionalized particles compared to non-functionalized particles to colorectal cancer cells was demonstrated. Zebli *et al.* demonstrated magnetic targeting of PSS/PAH capsules coated with magnetic metal nanoparticles using a flow-channel set-up. PMLC were trapped by a magnetic field resulting in higher uptake of the carriers by breast cancer cells growing in the proximity of this magnetic field. [68] Since liver parenchymal cells contain asiaglycoproteinreceptors which are recognized by galactose, Zhang *et al.* fabricated PMLC composed of PSS and a galactose-bearing cationic polymer in the perspective of targeting hepatocytes. [66, 67] Specific interaction of the PMLC with peanut agglutinin lectin

rather then concanavalin A lectin suggested the potential of the PMLC to interact specifically with hepatocytes.

Administration of PMLC in the body results in adsorption of opsonic proteins on the surface of the carriers followed by uptake by phagocytic cells. This unspecific clearance is undesirable and it would be beneficial to reduce adsorption of proteins to obtain a prolonged circulation time. Therefore, PMLC were equipped with a protein-resistant coating composed of polyethylene glycol (PEG). Several factors contribute to reduced adsorption of proteins to PEG coated PMLC. The hydrophilic character of PEG avoids hydrophobic interactions with proteins. Additionally, PEG shields charges of the PMLC thereby minimizing potential electrostatic interactions with proteins and flexible PEG chains provide steric repulsion. [70] Wattendorf *et al.* investigated the effect of PEG grafted polymers incorporated within PMLC on the internalization by phagocyting cells. [71] PAH/PSS PMLC were functionalized with PGA-g-PEG or PLL-g-PEG. Their findings demonstrated that PGA-g-PEG did not have a significant effect on cellular uptake which could be caused by an insufficient PEG density. In contrast, PLL-g-PEG could block internalization of the carriers by phagocyting cells. To combine both specific interaction and reduction of protein adsorption Heuberger *et al.* produced PAH/PSS capsules and functionalized them with biotinylated PLL-g-PEG. [70] The functionalized PMLC exhibited a severe reduction of protein adsorption compared to PAH/PSS carriers in addition to an interaction with streptavidin which was 40-fold higher compared to PMLC coated with non-functionalized PLL-g-PEG.

5. Intracellular fate of polymeric multilayered capsules

5.1. In vitro behaviour of LbL capsules and their cargo

Despite their relatively big size, LbL capsules are readily internalized not only by professional phagocytes (DCs [25, 72, 73] and macrophages[74]), but also by numerous other cell types including breast cancer cells, hepatoma cells, fibroblasts, [75] epithelial kidney cells (VERO), human embryonic kidney cells… How particles are internalized by cells strongly impacts their intracellular trafficking and hence the fate of the encapsulated cargo. Although the exact mechanism of cellular uptake of LbL capsules still remains largely unexplored, several papers have recently begun to address this issue. In general, microcapsule internalization appears not only to be dependent on microcapsule intrinsic factors (size, composition, charge…) but also on the cell type taking up the particles. Dendritic cells (DCs) are specialized in the continuous sampling of antigens and pathogens by macropinocytosis, phagocytosis and receptor mediated endocytosis. Using TEM and confocal microscopy (Figure 7A-B), De Koker *et al.* demonstrated that DCs form large

cytoplasmatic extrusions to engulf polyelectrolyte microcapsules composed of dextran-sulfate/poly-L-arginine (DS/pARG) bilayers. Uptake of these microcapsules by DCs was largely reduced by cytochalasin D, indicating particle internalization involves an actin dependent process. Furthermore, particle uptake was reduced by incubation with rottlerin, suggesting macropinocytosis as the predominant route of uptake in DCs. [25] To a lesser extent, membrane ruffling and the formation of plasmamembrane protrusions were also observed following incubation of colorectal LIM1899 cells with disulfide stabilized poly(methacrylic acid) capsules (PMA), consistent with particle uptake by phagocytosis or macropinocytosis. Nevertheless, while uptake of DS/pARG microcapsules by DCs [25] but also by VERO cells appears to be lipid raft mediated, [24] the uptake of the PMA capsules by various epithelial cell lines in contrast is clathrin dependent, pinpointing to important differences that might be both cell and particle dependent. [76] Zelikin *et al*. suggested that these discrepancies in internalization route might be due to the hydrogel nature and inherent softness of the PMA particles as the uptake of rigid PMA particles with a SiO_2 core in contrast was totally clathrin independent. Intriguingly, uptake of the hydrogel particles by RAMOS B cells was also not dependent on clathrin, stressing the fact that different cell types might use different pathways for internalizing and processing the particles. [76]

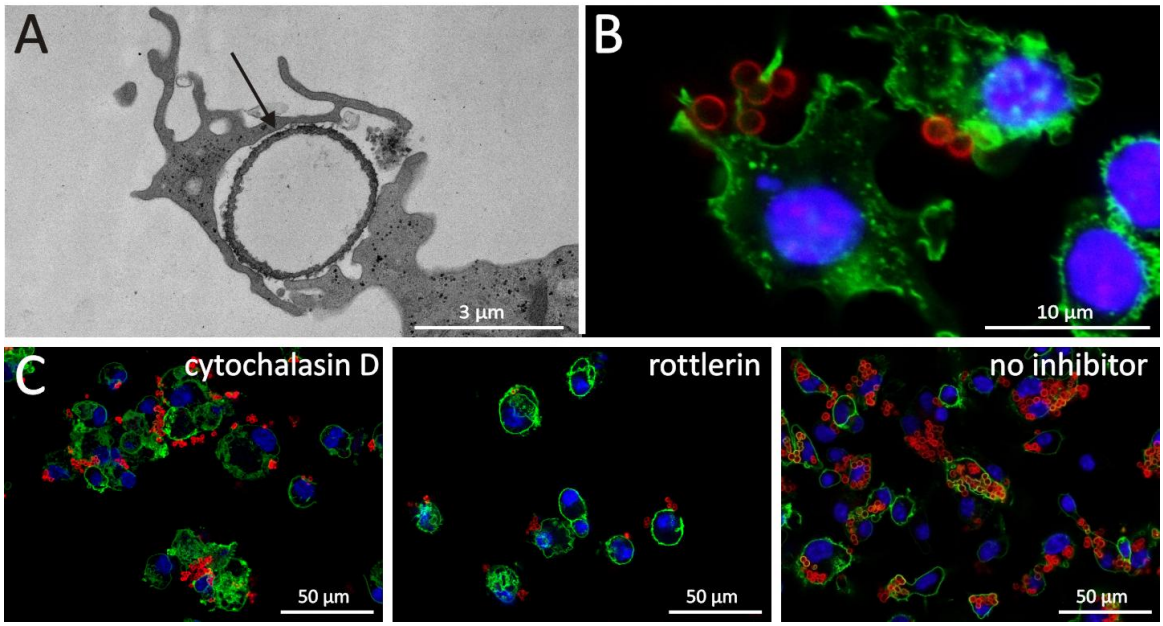

Figure 7. Images of BM-DCs taking up dextran sulfate/poly-l-arginine microcapsules. The TEM image **(A)** shows a BM-DC forming cytoplasmic protrusions to engulf the microcapsules (black arrow), which are visible as hollow disks with a dark, electron dense wall. Scale bar: 3 mm. **(B)** Confocal microscopy images showing the formation of actin-rich protrusions. The actin cytoskelet was stained with alexa 488 phalloidin (green). Microcapsules were labelled red by incorporation of RITC–poly-L-arginine. Nuclei were stained blue with Hoechst 33258. Scale bar: 10 mm. **(C)** Confocal images of the effects of cytochalasin D and rottlerin on microcapsule uptake by BM-DCs.

Once internalized, most authors now agree that LbL capsules end up in acidic compartments, although partial escape to the cytosol has been occasionally reported. Co-localization of fluorescently labelled microcapsules with lysotracker, a dye that selectively stains acidic vesicles, has been described for DS/pARG capsules internalized by DCs and VERO cells and for capsules composed of poly-lysine (PLL) and hyaluronic acid (HA) in RAW macrophages. [74, 77] Most strikingly, PLL/HA capsules promptly deform and rupture upon cellular internalisation (Figure 8) while this process appears to be much slower for other types of capsules. Encapsulation of the dextran labelled with the pH sensitive fluorophore SNARF-1 constitutes an elegant approach to visualize trafficking of LbL capsules to acidic compartments. Following excitation at 488 nm, the fluorescence intensities of SNARF-1 at 580 nm and 640 nm are dependent on the local pH environment, with the 580 nm intensity decreasing while the 640 nm intensity increasing as the pH increases. Using this approach, Parak *et al*. were able to demonstrate the localisation of PEM inside acidic vesicles. [77, 78] Several groups have investigated the intracellular fate of LbL capsules following cellular uptake by immunostaining for the early endosome antigen 1 (EEA1) and the lysosome-associated membrane protein 1 (LAMP-1). Partial or total co-localisation with the lysosomal marker LAMP1 was observed, further demonstrating the phago-lysosomal fate of LbL capsules following uptake . None of these studies was however able to demonstrate co-localisation with the early endosomal marker EAA1, a feature which may however also be due to the late time intervals following particle uptake generally assessed in these studies. Using TEM (Figure 11A), a detailed picture of the fate and structural integrity of DS/pARG LbL capsules following uptake by DCs was recently provided by De Koker *et al*. Following uptake, the microcapsules could be distinguished as hollow particles with an electron dense shell which was clearly surrounded by a membrane. After a 24 hours incubation period, the microcapsules' shell ruptured, possibly as a consequence of enzymatic degradation, and cytoplasmatic content invaginated the microcapsules' hollow core, which however remained separated from the cytoplasm at all times by a membrane. [25]

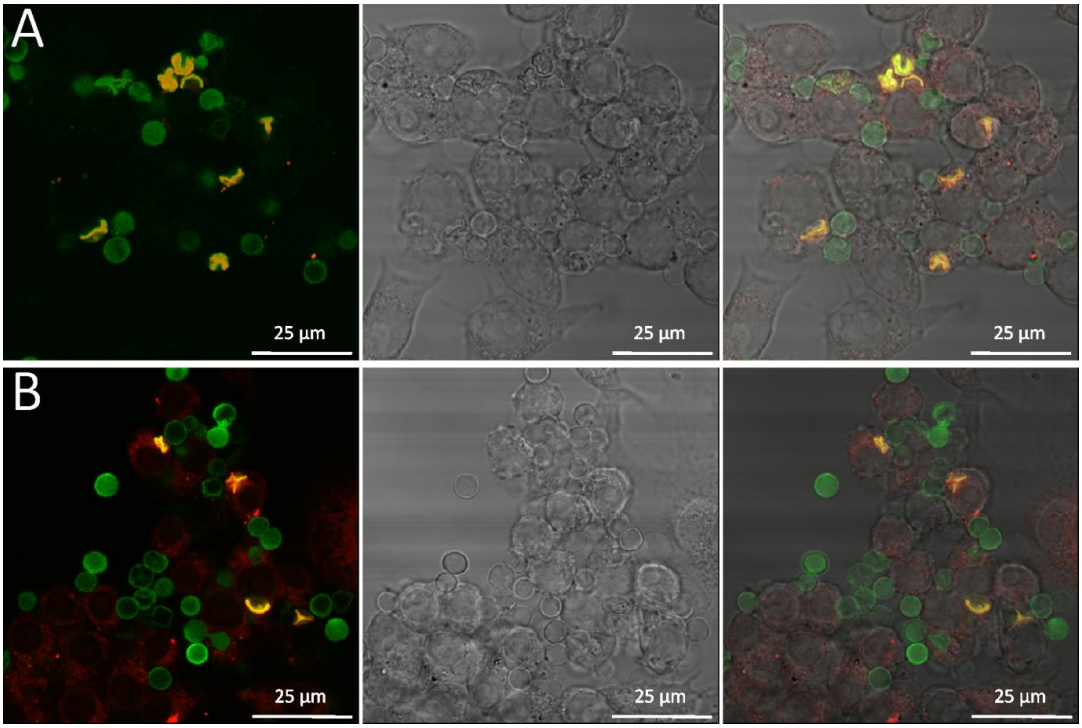

Figure 8. Confocal microscopy images of (A) cross-linked HA/PAH and (B) cross-linked HA/PLL capsules after 2 h co-incubation with RAW mouse macrophages. Capsules are stained green fluorescent using HAFITC, while the cellular lysosomes are stained using LysoTracker Red.The left pane gives the overlay of the green and red channel, the middle pane is the DIC channel and the right pane is the overlay of green, red and DIC. Co-localization between the green and red channel is observed as a yellow/orange colour. When capsules are taken up by the RAW cells – as visual from the yellow/orange co-localization signal between green capsule fluorescence and red lysosomal fluorescence – they are readily deformed.

The most important question when using LbL capsules for the intracellular delivery of macromolecules obviously remains the fate of the capsules' cargo itself. Recently, the Parak and De Geest group jointly compared the accessibility of encapsulated proteins for proteases between non-degradable PSS/PAH and biodegradable DS/pARG capsules (Figure 9). Accessibility to proteases is of paramount importance when using capsules to deliver inactive pro-drugs that need to be enzymatically cleaved in order to become active. In addition, proteolytic cleavage is also crucial when delivering antigens for immunisation, as will be discussed later on. To address these issues, Parak *et al*. used DQ-ovalbumin (OVA), which is composed of OVA extensively labelled with bodipy dyes. Due to the close proximity of the dyes, their fluorescence becomes self-quenched. When OVA gets degraded into small peptides by enzymatic digestion, the quenching is relieved and a bright green fluorescence is emitted. After uptake by NIH/3T3 fibroblasts, OVA-DQ encapsulated inside biodegradable DS/pARG LbL capsules got readily degraded, while the protein remained intact when encapsulated in non-degradable PLL/PAH particles, thereby clearly showing the importance

of using degradable polymers which can be eroded and cleaved by intracellular proteases. [79]

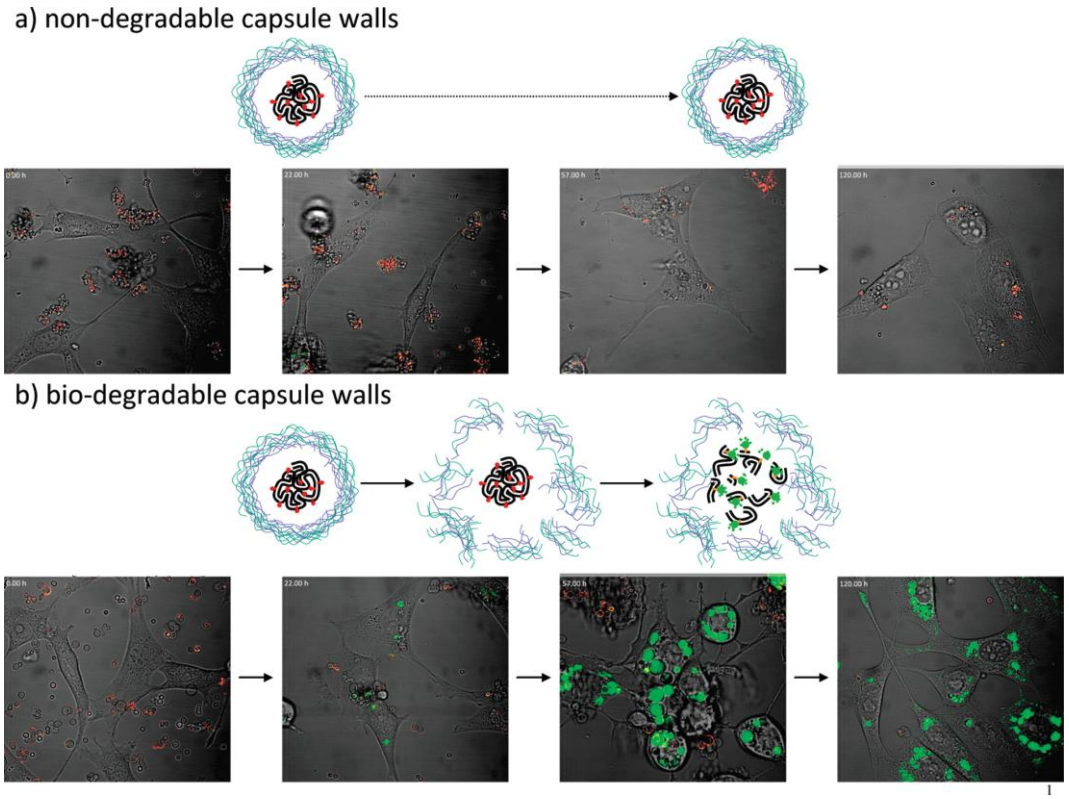

Figure 9. Enzymatic cleavage of protein cargo. Embryonic NIH/3T3 fibroblasts were incubated with (a) non-degradable PSS/PAH or (b) degradable DEXS/pARG capsules filled with the fluorogenic protein cargo, DQ-OVA. Images were taken immediately after addition of the capsules (*t*) 0 h)over time up to 120 h with a confocal microscope in different channels, green, red, and transmission. An overlay of the different channels is presented in the figures.

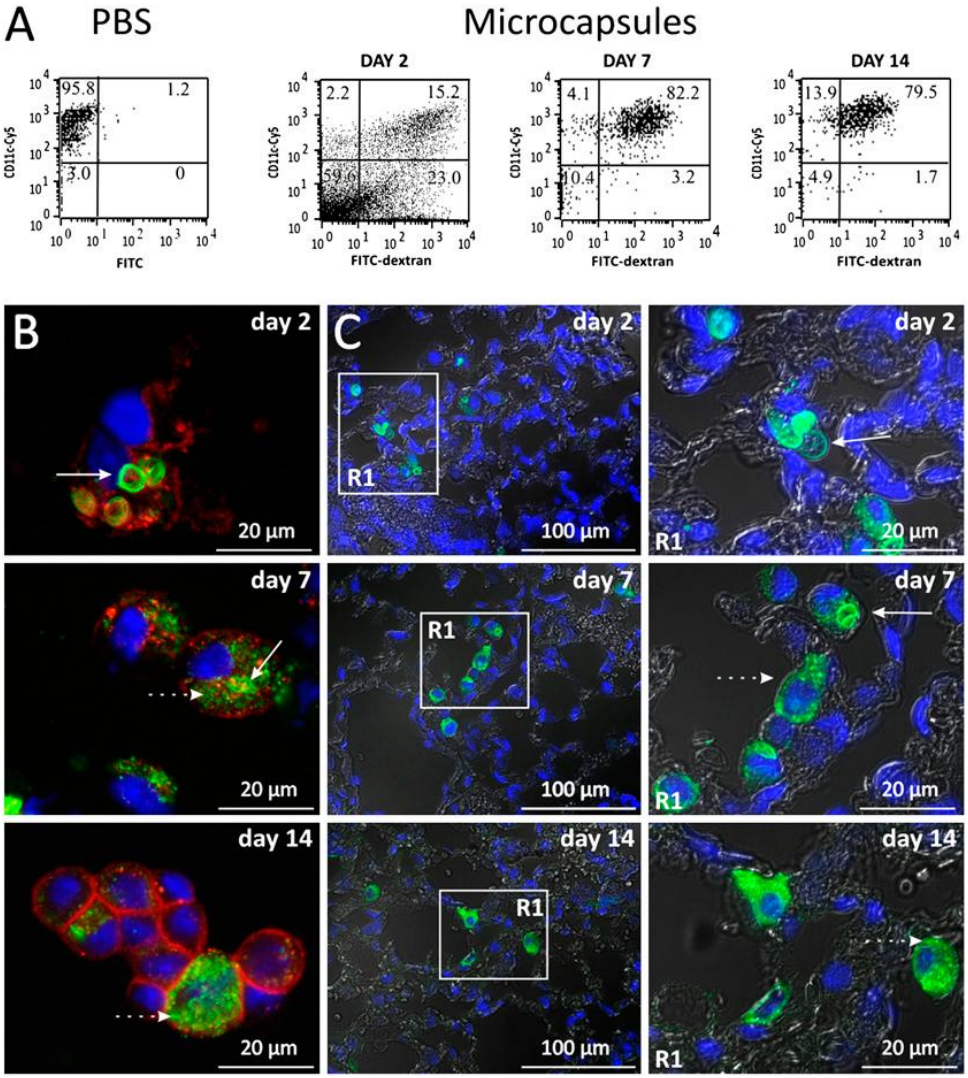

Figure 10. (A) Flowcytometric analysis of the uptake of FITC-dextran containing PEMs by BAL cells at different time intervals post instillation. Alveolar macrophages/ DCs were stained with CD11c-PE-Cy5. The respective percentages of cells are depicted in each quadrant. **(B)** Confocal images of BAL cells at different time intervals post instillation of FITC-dextran (green) containing microcapsules. Cells were stained with the macrophage/DC marker CD11c-PE-Cy5 (red). Nuclei were stained with DAPI (blue). C, Confocal images of lung sections at different time intervals after instillation of FITC-dextran (green) containing PEMs. Nuclei were stained with DAPI (blue). Figures show an overlay of differential interphase contrast, green and blue fluorescence, with the right panel being an enlarged picture of the R1 region of the left panel. Microcapsule structures are indicated by full arrows, whereas leaked FITC-dextran is indicated by dotted arrows.

5.2 In vivo fate and biocompatibility of LbL capsules

Obviously, the first prerequisite for LbL capsules to be applied *in vivo* is their biocompatibility. In this respect, capsules composed of biodegradable components such as polypeptides and polysaccharides have the best potential to reach *in vivo* application, whether it may be for the delivery of anti-cancer drugs or as antigen carriers in vaccination.

Up to date, relatively few papers have explored the *in vivo* behaviour of LbL capsules. Following subcutaneous injection in mice, DS/pARG microcapsules evoked a moderate and localised inflammatory reaction, characterized by the fast recruitment of granulocytes and monocytes which gradually infiltrated the microcapsule mass. Over time, microcapsules were taken up by mononuclear cells, most likely macrophages. Importantly, the DS/pARG capsules retained their structural integrity before cellular uptake, but got rapidly degraded after internalization. [80] DS/pARG capsules were also explored as antigen delivery vehicles for pulmonary immunisation, [72] which recently has draw a lot of attention as it allows a non-invasive route of delivery and might harbour the benefit of inducing local mucosal immune responses in addition to systemic immunity. Pulmonary delivery of DS/pARG capsules induced a transient inflammatory response with recruitment of mainly monocytes and granulocytes. Particles were rapidly taken up by alveolar macrophages, but also by DCs that transported them to the draining mediastinal lymph nodes. By encapsulating high molecular weight FITC-dextran, the capsules' fate was followed over time. As depicted in Figure 10, intact capsules were visible inside alveolar macrophages two days following instillation. One week after instillation however, most of the capsules had leaked their content although some heavily deformed capsule debris could still be distinguished. Two weeks after delivery, no capsules were visible anymore and the FITC-dextran appeared spread throughout the cells.

6. Delivery of biotherapeutics

6.1 Antigens

Recently, nano- and microparticles have gained a lot of interest as antigen delivery vehicles to selectively target antigens towards professional antigen presenting cells. [81] Most importantly, particulate antigen delivery has the capacity to elicit CD8 cytotoxic T cell (CTL) responses, a feature hardly achievable when using soluble antigens. Induction of such CTL responses is crucial to kill virally infected cells or even tumour cells. As they combine an efficient antigen encapsulation with an efficient uptake by DCs and monocytes, LbL capsules appear appealing antigen delivery systems for vaccination. Sexton *et al.* have nicely demonstrated that encapsulation of OVA in disulfide stabilized PMA capsules indeed is capable of stimulating antigen presentation by DCs in vitro. PMA particles however mainly increased antigen presentation to CD4 T cells (5.7- 42 fold), while CD8 T cell proliferation was enhanced by merely a factor 3.8 to 7.9, which is at best moderate compared to other particles such as PLGA or acid degradable particles. These observations were extended *in vivo*, with OVA loaded PMA capsules predominantly stimulating CD4 T cell responses (70-fold increase) and to a lesser extent also CD8 T cell proliferation (6-fold). [82]

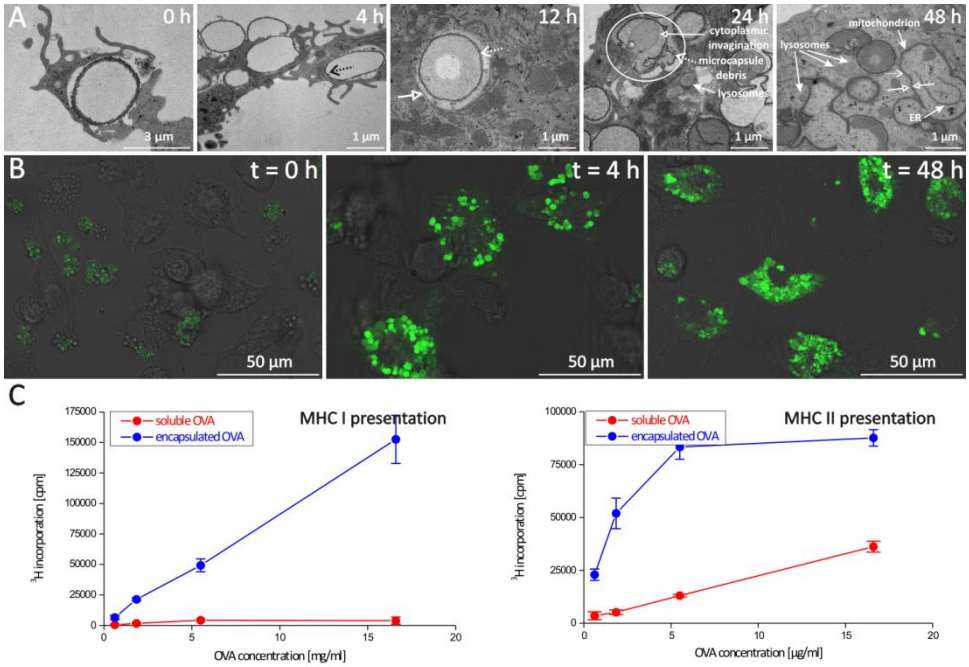

Figure 11. (A) TEM images of BM-DCs that have internalized dextran sulfate/poly-L-arginine microcapsules at the indicated time intervals. Microcapsule shell: dotted arrows; membranes surrounding the microcapsules: open arrows. In the encircled area, microcapsule rupture and cytoplasmic invagination are clearly distinguishable. Lysosomes, endoplasmatic reticulum (ER), and a mitochondrion are indicated by the solid arrows. (B) Processing of dextran sulfate/poly-larginine microcapsule encapsulated OVA was analyzed using DQ-OVA. Confocal microscopy images of BM-DCs incubated with OVA-DQ microcapsules for 0, 4 and 48 h (overlay of green fluorescence and DIC). (DQ-OVA is ovalbumin oversaturated with BODIPY dyes. Upon proteolytic cleavage, quenching is relieved and green fluorescence appears. (C) Antigen presentation by BM-DCs after uptake of soluble and encapsulated OVA. Proliferation of OT-I cells was used as a measure for MHC-I-mediated cross-presentation of OVA (left graph), proliferation of OT-II cells as a measure for MHC-II mediated presentation (right).

Similarly, DS/pARG capsules have been exploited to deliver OVA to DCs. Using DQ-OVA (Figure 11B), De Koker *et al*. have demonstrated that antigens inside these particles become readily available for enzymatic processing, even before visual rupturing of the particles' shell as seen by TEM. In contrast to the PMA particles however, DS/pARG particles appeared to mainly promote CD8 T cell proliferation *in vitro*, although there was also a clear increase in CD4 T cell proliferation (Figure 11C).

OVA loaded DS/pARG capsules were also used to further characterize the immune response following pulmonary delivery. [72] Instillation of these particles supported a strong humoral and cellular immune response. Strikingly, capsules with encapsulated OVA were much more potent in eliciting immune responses compared to mixtures of empty capsules and soluble antigens, stressing the fact that antigen encapsulation is crucial to obtain better targeting of DCs. On the level of the CD4 T cell response, a strong polarization towards IL-17

secreting Th17 cells was observed. Given the recent insights in the role of Th17 responses in combating extracellular bacteria such as *Pseudomonas aeruginosa* but also against the intracellular pathogen *Mycobacterium tuberculosis* it would be of great interest to examine the potential of DS/pARG capsules as antigen carriers to evoke protective immune responses against these insidious pathogens. Whether these particles also elicit Th17 responses following more conventional routes of immunization (intramuscular or subcutaneous) remains to be established. Finally, considering the high versatility of the LbL technique, modifying the capsules with immunopotentiators that activate DCs might be an interesting approach not only to further increase the strength of the induced response but also to modulate the type of response generated.

6.2 Peptides

In an attempt to restore tissue structure and/or function, the implantation of tissue engineered constructs developed by seeding cells onto a biodegradable synthetic scaffold was proposed as a promising approach. [83] To fabricate those constructs signalling molecules such as growth factors to regulate cellular activities i.e. proliferation, differentiation and migration are pivotal elements. [84] Successful administration of these proteins involves their delivery to the target site while maintaining biological activity and retention of the proteins in the tissue during a period in which their activity can be exerted. [83, 85]

Bolus injection of growth factors to the target site is generally not effective due to the short half-life of the growth factors and their fast diffusion from the injection site with concomitant toxicity in other tissues caused by non-specific distribution. [86] As a consequence controlling the release of the growth factor in terms of concentration and time span is an important issue. [87] Therefore, administration of growth factor loaded centres releasing their cargo in a controlled fashion at the target site has attracted attention.

PMLC have been loaded with growth factors and the *in vitro* and *in vivo* activity of the cargo were investigated. Itoh *et al.* established a mitogenic stimulation of mouse fibroblasts during a prolonged period by basic fibroblast growth factor (bFGF) released in a controlled fashion from PMLC compared to bFGF in solution. [88] Caruso *et al.* embedded the growth factors bone morphogenic protein 2 and transforming growth factor beta 1 in the multilayer film of PMLC composed of poly-L-lysine and poly-L-glutamic acid. After having demonstrated *in vitro* successful induction of bone formation from stem cells incubated with active capsules, osteogenic differentiation of embryoid bodies (EB) was shown in mice after subcutaneously implanting a mixture of an alginate gel containing growth factor loaded capsules and EBs. PMLC protected the growth factors against degradation and acted as

growth factor reservoir, since *in vivo* experiments with free growth factor resulted in absent mineralization. [89] These results demonstrated the potential of PMLC as growth factor delivery systems in the process of tissue engineering.

Administration of hormones through injection suffers from low protein stability *in vivo* requiring multiple administrations. This limitation can be overcome by encapsulating the hormones in PMLC which act as a protein reservoir and release their cargo in a controlled fashion. Benkirane-Jessel *et al.* loaded poly-L-lysine replica particles with alpha-melanocyte-stimulating-hormone through adsorption and demonstrated successful stimulation of melanin production from murine melanoma cells after incubation with the functionalized particles compared to untreated cells. [90]

6.3 Nucleic acids

Intracellular delivery of DNA, siRNA, oligonucleotides etc… is the primal goal for gene therapy, aiming to introduce new genes or replace defect genes. [91] Due to its polyionic nature, DNA has frequently been used in the past as building block in LbL films. Through condensation with the small cationic molecule spermidine, Schuler *et al.* demonstrated the possibility to construct multilayer capsules, however with limited stability under physiological salt concentrations. [92] Shchukin *et al.* condensed DNA with spermidine onto the surface of sacrificial manganese carbonate microparticles followed by polyelectrolyte coating and dissolution of the core templates leading to the liberation of freely floating DNA in the microcapsule cavity. [93] Direct incorporation of DNA by co-precipitation into calcium carbonate ($CaCO_3$) microparticles followed by LbL coating and dissolution of the $CaCO_3$ templates by EDTA has been shown by the Sukhorukov group. [28]

Alternatively to the use of electrostatic interactions, the Caruso group has elaborated on the use of hydrogen bonding to incorporate oligonucleotides either in the cavity or in the wall of polyelectrolyte capsules. Amine modified mesoporous silica microspheres could accumulate oligonucleotides through electrostatic interactions into their pores and multilayer build-up of hydrogen bonded poly(methacrylic acid) and poly(vinylpyrrolidone) followed by etching of the silica core templates yielded hollow capsules with oligonucleotides stably encapsulated within their cavity. [23, 60] Also DNA hybridization based on hydrogen bonding between homopolymeric oligonucleotide blocks has been explored to construct multilayered capsules. [94-96]

Although the feasibility to incorporate nucleic acid based macromolecules into polyelectrolyte capsules or to use them as wall constituent, there are only few reports of functional biological experiments with polyelectrolyte capsule mediated gene delivery. The

Donath group has reported on the incorporation of pDNA encoding for green fluorescent protein into the membrane of LbL coated silica colloids followed by transfection onto an *in vitro* cancer cell line. [97, 98] Selina *et al.* reported on the in vivo use on a swine fever DNA vaccine by incorporating plasmid DNA into $CaCO_3$ based degradable bio-polyelectrolyte (dextran sulfate / carragenan) capsules. [99] Recently Zhang *et al.* reported on single-polyelectrolyte capsules based on mesoporous silica particles infused with poly-L-lysine (PLL) followed by cross-linking of the PLL's amine groups with a reduction prone disulfide cross-linker. [90] These so-called PLL replica particles could accumulate plasmid DNA through electrostatic interaction and successful transfection experiments were conducted by transfecting an *in vitro* melanoma cell line with SPT7pTL plasmid DNA encoding for a nuclear transcription factor.

Despite these early reports, applications of polyelectrolyte capsule mediated gene therapy are still in a very early stage. An important drawback of polyelectrolyte capsule so far, is the fact that they end up upon cellular internalization in acid phagosomal vesicles and do not escape into the cellular cytoplasm. Although, for gene therapy phagosomal escape is essential and for plasmid DNA delivery, even the cell nucleus needs to be reached. Therefore, further developments in the field of LbL capsules with respect to gene therapy should focus on the incorporation of mechanisms to concur the intracellular phagosomal barrier.

7. Conclusions

In conclusion, we have reviewed in this paper the recent advances in encapsulation and delivery of biotherapeutics mediated by LbL capsules. Polymeric multilayers are an emerging field with application opportunities in numerous disciplines. Whereas in the nineties, mostly physicochemical characterization was conducted in order to gain understanding into the fundamental aspects of multilayer formation, during the first decade of the 21st century, many applications including those in drug delivery started to emerge. However, so far polyelectrolyte capsules did not reach a clinical stage yet. This is due to several reasons:

(1) Polyelectrolyte capsules contain polycations as a building block while these are often considered as toxic. However, several studies have pointed out that both planar films as well as capsules consisting of polyelectrolyte complexes do not exhibit significant toxicity. [7, 100, 101] Therefore, a thorough debate should be performed in order to assess in which conditions polycation related toxicity might be an issue or not. On the other hand, there are several research groups switching from the use of electrostatic

interactions to the use of hydrogen bonding as driving for multilayer assembly, thus avoiding the use of polycations. [19, 102, 103]

(2) Nano- and micro-technology are omnipresent in the contemporary drug delivery literature and many nano/micro-scale drug delivery systems have been or are being developed. [2, 3]Therefore, it is a major challenge for scientists active in the field of LbL-films to define those specific areas where polyelectrolyte capsules are beneficial compared to other drug delivery systems.

(3) Due to the inherent sequential manufacturing procedure, involving many batch operation steps, polyelectrolyte capsules are laborious and time-consuming to fabricate. This fact further emphasizes the necessity, as mentioned above, to focus to those cases where LbL technology offers sufficient added value. On the other hand, several recent studies from pioneering groups in the LbL field report on novel strategies that dramatically reduce the amount of batch operations while till aiming to mimic as much as possible the highly versatile concept of LbL technology. [104-106]

Taking into account the above mentioned considerations we are convinced that there is a bright future for polymeric multilayer capsules, providing that several important issues are being addressed the coming decade.

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