In Vivo Cellular Uptake, Degradation, and Biocompatibility of Polyelectrolyte Microcapsules**

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Polyelectrolyte microcapsules are made by layer-by-layer (LbL) coating of a sacrificial template, followed by decomposition of the template, to produce hollow microcapsules. In this paper, we report on the in vivo cellular uptake, degradation and biocompatibility of polyelectrolyte microcapsules produced from alternating dextran sulphate and poly-L-arginine layers on a template of calcium carbonate microparticles. We show that a moderate tissue reaction is observed after subcutaneous injection of polyelectrolyte microcapsules in mice. Within sixteen days after subcutaneous injection, most of the microcapsules are internalized by the cells and start to get degraded. The number of polyelectrolyte layers determines the stability of the microcapsules after cellular uptake.

1. Introduction

There has been a growing interest in the design and development of polymeric microspheres as controlled release systems for drugs and antigens. Different types of polymers have been used to design biodegradable polymeric microparticles.[1] Poly(lactic-co-glycolic) acid (PLGA) microspheres have been extensively investigated because of their biocompatibility and biodegradability.[2] PLGA has been approved by the FDA, and GMP grade PLGA is commercially available. However, preparation of PLGA microspheres requires the use of organic solvents and involves large shear forces, both of which can seriously affect protein stability.[3] Protein encapsulation efficiency and loading capacity of PLGA microspheres are often low.[3] Moreover, PLGA leads to acidification[4] of the injection site, because degradation of PLGA produces lactic acid, which can potentially denature encapsulated proteins. New types of injectable microspheres that can avoid these problems are needed.

A new type of biodegradable microcapsules[5–7] was recently developed using the layer-by-layer (LbL) technique.[8] This technique is based on the sequential adsorption of oppositely charged molecules, such as polyelectrolytes, onto a charged sacrificial template (Fig. 1). The template is then dissolved, resulting in hollow capsules with a wall thickness in the nanometer range. Degradable polyelectrolyte microcapsules made of polypeptides and/or polysaccharides were reported recently.[9,10] As polyelectrolyte multilayers on planar substrates start to emerge as multifunctional delivery platforms for implants, e.g., drug eluting stents or dental implants,[11–20] the potential of polyelectrolyte microcapsules as drug delivery systems could increase.[6,21] Polyelectrolyte microcapsules have the advantages of mild preparation conditions, multifunctionality, and the capacity to encapsulate large amounts of material.[22–24]
Polyelectrolyte microcapsules are produced at room temperature using simple bench-top equipment, without organic solvents or harsh reaction conditions. Due to the versatility of electrostatic interaction, the physicochemical properties of the microcapsules’ shells can be precisely tuned. Moreover, the capsule surface can be furnished with a wide variety of functionalities, such as lipids,[25] polyethylene glycol,[26] antibodies, or[27] viruses.[28]

In a previous paper we reported on the cellular uptake and degradation of polyelectrolyte microcapsules by an in vitro cultured cancer cell line.[9] In this paper we aim to assess the in vivo cellular uptake, degradation and tissue reaction of polyelectrolyte microcapsules. To assess in vivo biocompatibility, skin sections were made at different time points after subcutaneous injection of microcapsules in mice, and tissue reaction and cellular infiltration were examined. Furthermore, phagocytosis and in vivo degradation of fluorescently labeled microcapsules were evaluated. Because subcutaneously injected foreign particles in the 0.1–10 μm range are preferentially taken up by phagocytes, the in vitro cytotoxicity of the polyelectrolyte capsules was determined using bone marrow derived dendritic cells. Our data pave the way for further in vivo research on polyelectrolyte microcapsules.

2. Results and Discussion

2.1. Fabrication of Hollow Polyelectrolyte Capsules

CaCO₃ microparticles were LbL coated with several bilayers of dextran sulfate and poly-L-arginine.[24] The adsorption process was monitored by measuring electrophoretic mobility after deposition of each layer. Starting from a CaCO₃ microparticle with a zeta-potential of +12 mV, the zeta-potential alternated between –50 mV and 50 mV during the deposition of 8 polyelectrolyte layers (Fig. 2A).

Hollow particles were produced by dissolving the LbL coated CaCO₃ microparticles in a buffered EDTA solution (pH 5.4). The Ca²⁺ ions are complexed by the EDTA and can easily permeate the polyelectrolyte membrane. Polyelectrolyte microcapsules consisting of 1, 2, and 4 bilayers of dextran sulphate/poly-L-arginine were produced. Figure 2 shows confocal microscopy (2B) and scanning electron microscopy (2C) images of the capsules (see Supporting Information for DIC images of microcapsules consisting of 1, 2, and 4 bilayers). The microcapsules show a rather monodisperse size distribution with an average diameter of 3 μm. The capsules were fluorescently stained by using one layer of RITC labeled poly-L-arginine during the LbL coating process. The scanning electron microscopy image shows the microcapsules in a collapsed state because the sample was dried for imaging.

2.2. Uptake of Polyelectrolyte Microcapsules by Bone Marrow Derived Dendritic Cells

Uptake of polyelectrolyte microcapsules by cells was investigated by incubating cultured dendritic cells with fluorescently labeled (RITC) microcapsules, followed by confocal microscopy based on the procedures reported by Parak et al.[29–31] In Figure 3A, cells were stained with CellTrackerGreen, a fluorescent dye that accumulates in the cytoplasm of living cells. In...
Figure 3B, the cell surface was stained with CD11c, a marker for bone marrow derived dendritic cells. The fact that both the cellular membrane and the microcapsules are seen in the same confocal plane indicates that the microcapsules are effectively taken up by cells, and not merely sticking to the cell surface by electrostatic interactions. These observations are consistent with published data on cellular uptake of polyelectrolyte microcapsules by cancer cell lines. \[^{9,27,29,30,32,33,34}\] Figure 3 clearly shows that dendritic cells can take up very large amounts of capsules.

Microcapsules in the range of 0.1–10 μm can be easily phagocytosed by macrophages and dendritic cells. Moreover, rapidly degradable PLGA microspheres that deliver antigens intracellularly to dendritic cells have been shown to enhance antigen presentation and immune responses. As polyelectrolyte microcapsules can encapsulate large amounts of proteins,\[^{22,23}\] they can be useful for intracellular delivery of antigen to antigen presenting cells (APC), providing that they do not affect their viability.

### 2.3. In vitro Cytotoxicity

The *in vitro* cytotoxicity of the polyelectrolyte microcapsules was assessed by a MTT assay, because the uptake of large amounts of polyelectrolyte capsules by APC could be toxic. Indeed, it has been reported that polycationic polymers reduce cell viability.\[^{35–38}\] Fisher et al.\[^{38}\] showed that cytotoxicity of polyelectrolyte microcapsules consisting of four bilayers of dextran sulfate/poly-L-arginine. (see Supporting Information for enlarged and additional images) The top left image of each block shows an overview of the injection site, while R1, R2, and R3 are detailed images of the regions marked in the overview image. A PBS group was included as a negative control. As positive control groups for inflammation, Complete Freund’s adjuvant (CFA) and Al(OH)\(_3\), two frequently used adjuvants were used. CFA consists of a water-in-oil emulsion containing heat-killed mycobacteria or mycobacterial cell wall components and is known to cause extensive tissue inflammation, with possible side effects like skin ulceration, local abscess formation and tissue necrosis. Because it causes very intense inflammatory reactions, CFA use is restricted to research purposes in laboratory animals. Al(OH)\(_3\) on the other hand, causes only mild inflammatory reactions and is by large the most often used adjuvant in vaccines for human use. Table 1 gives an overview of the nature and extent of the tissue response at different time intervals after injection of the polyelectrolyte capsules, when compared to CFA and Al(OH)\(_3\).

One day after injection of the polyelectrolyte capsules (Fig. 5, day 1) inflammatory cells, mostly polymorphonuclear leukocytes (R3, \(\bullet\)), are recruited to the injection site. As the mass of injected microcapsules resembles a porous implant, there is a lag time between infiltration of the edge of the injection site, and infiltration of the centre. One day after injection only the edge of the injected site is infiltrated (R1). The microcapsules retain their spherical shape and appear as white discs (R1, R2, \(\bullet\)). Also lymphocytes (R1, \(\circ\)) and apoptotic cells (R1, \(\bullet\)), probably dying short-lived polymorphonuclear cells, are visible at the edge. No inflammation can be seen in the PBS control group (Fig. 6, day 1), while a massive inflammatory response is observed in the CFA group (Fig. 7, day 1). Injection of Al(OH)\(_3\) particles (Fig. 8, day 1, \(\bullet\)) results in a fast recruitment of polymorphonuclear cells to the injection site.
Figure 5. Bright field microscopy images of tissue sections of the injection spot at several time points after subcutaneous injection of mice with microcapsules consisting of four bilayers of dextran sulphate/poly-L-arginine. Tissue sections were assessed for the recruitment of inflammatory cells to the microcapsule mass. At early time points, only the edge of the microcapsule mass has been infiltrated, mostly by polymorphonuclear cells (△), predominantly eosinophils (▲). Individual microcapsules (●) can still be distinguished. Fibroblasts (★) start to surround the injection mass, even on day 4. After 18 days, macrophages (☆) appear to have phagocytosed the microspheres, which seem to have lost their spherical shape. On day 30 the entire injected mass has been infiltrated by cells, and encapsulated by 5–10 layers of fibroblasts, which can also be seen scattered throughout the injection mass. At the edge mainly macrophages are present, while at the centre the infiltrate consists of a mixture of macrophages and polymorphonuclear cells. Many new blood vessels (▴) are being formed.
Table 1. Overview of the inflammatory tissue response to polyelectrolyte microcapsules, PBS, CFA and Al(OH)₃ after subcutaneous injection. The response was rated according to the following scoring system: – = not present to +++ = extensive; n.a. = not assessed.

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Figure 6. Bright field microscopy images of tissue sections after injection of PBS. No inflammation can be observed.

As for the microparticles, infiltration starts at the edge and consists predominantly of polymorphonuclear cells.

Four days after injection (Fig. 5, day 4), inflammatory cells are still being recruited to the injection site. R1 shows leukocytes adhering and crossing the endothelial wall, and eosinophils (R1, Fig. 7) moving towards the microcapsule mass. At this time, fibroblasts start to surround the implant site (R2, Fig. 7). At the edge of the microcapsule mass, many eosinophils (R2, Fig. 7) and some macrophages (R2, Fig. 7) are seen. The microcapsules, which still appear spherical, have not been phagocytosed yet (R3, Fig. 7). At this early time point, the tissue response is clearly dominated by polymorphonuclear cells, which is characteristic of acute phases of inflammation.

Eight days after injection (Fig. 5, day 8), inflammatory cells have infiltrated a large part of the injected mass, but in its centre, few cells are seen and the microcapsules remain intact (R2, Fig. 7). Eosinophils (R2, Fig. 7) become scarcer, and many macrophages appear at the edge (R2, R3, Fig. 7) where microcapsules are becoming deformed and difficult to distinguish (R3). In the CFA control group (Fig. 7, day 8), a severe inflammatory response is observed, with the influx of predominantly polymorphonuclear cells, but also lymphocytes and fibroblasts. Macrophages start to appear at this stage. Infiltration is much less predominant in the Al(OH)₃ control group, as few cells are scattered between the particles. The injection site is surrounded by 5–10 layers of fibroblasts, and infiltrated by eosinophils, other polymorphonuclear cells and some macrophages (Fig. 8, day 8). In general, the tissue response to the large, inert Al(OH)₃ particles is less extensive than to the biodegradable, phagocytizable microcapsules.

Eighteen days after injection (Fig. 5, day 18) the whole injection site has become infiltrated by inflammatory cells and surrounded by a fibrous capsule consisting of approximately five layers of fibroblasts (R1, Fig. 8). The enlarged image R3 shows that the cytoplasm of the infiltrated macrophages (R3, Fig. 8) exhibits a remarkably granular structure that is most likely due to phagocytosed microcapsules (R3, Fig. 8). In the CFA control group, infiltration is much more widespread, and tissue necrosis is observed (Fig. 7, day 18, R1) Granulomatous structures are formed, consisting of a dense infiltrate of inflammatory cells (R2). Lots of macrophages and polymorphonuclear cells are present, as well as significant numbers of lymphocytes (R2, Fig. 8).

After one month (Fig. 5, day 30), most microcapsules have been phagocytosed, and debris of degraded/deformed microcapsules is observed in the cytoplasm of phagocytes (R3, Fig. 9). The injected mass is now entirely infiltrated by cells, and surrounded by five to ten layers of fibroblasts (R2, Fig. 9), which are also scattered throughout the injected volume.

Some lymphocytes are present too (R1, Fig. 9), but their numbers remain much lower than in the CFA group. The injection site is being vascularized, and many newly formed blood vessels (R1, R2, Fig. 9) are now visible throughout. At the edge of the implant site, the tissue response is dominated by macrophages and few polymorphonuclear cells can be seen, while at the centre they are still present in significant numbers. By this, the microsphere injection spot resembles a porous implant, with infiltration starting at the edge by polymorphonuclear cells followed by phagocytes and gradually proceeding to the centre of the microsphere mass over time. No giant cells could be observed at any time. In contrast to the CFA control group, inflammation remains locally and no necrosis can be seen. In the CFA group, the entire subcutaneous tissue is heavily inflamed, with formation of granulomatous structures, lots of macrophages, lymphocytes and polymorphonuclear cells (Fig. 7, day 30, R2). Inflammation remains mild in the Al(OH)₃ control group: besides macrophages, granulocytes, especially eosinophils (Fig. 8, day 30, R2), are still present throughout the injection side which is surrounded by fibroblasts.
2.5. In vivo Cellular Uptake and Structural Integrity

Confocal microscopy was used to investigate the fate of the polyelectrolyte capsules after subcutaneous injection. Figure 6 shows confocal microscopy images of the injection site at different time intervals after injection of capsules consisting of two bilayers dextran sulfate/poly-L-arginine, produced using RITC labeled poly-L-arginine to make them fluorescent red. The images show the overlay of the DIC image, the nuclear staining and the red fluorescence signal of the microcapsules. In line with the bright field images of Figure 5, cells infiltrate the edge of the injection spot within two days of injection, but it takes them longer to infiltrate the centre of the injection site (Fig. 9). Two days after injection, the microcapsules more or less retain their spherical shape and are scattered between the cells as seen in the DIC images (Fig. 9, third column, day 2). This indicates that the microcapsules have not yet been phagocytosed to an appreciable extent. Eight days after injection, most of the microcapsules are still scattered between the cells (Fig. 9, day 8), but at the edge of the injection site, microcapsules have been phagocytosed (Supporting Information, day 8, R2). These phagocytosed particles (day 8, R2) start to shrink and become deformed, while the extracellular capsules (day 8, R1) remain intact. The same observations can be made at day 16: particles that have been phagocytosed have lost their spherical shape and are much smaller (Fig. 9 & Supporting Information, day 16, R2), while those that have not been taken up remain intact. No particle debris can be observed outside the cells. This indicates that most of the capsule deformation and degradation occurs inside the cells. These observations are also in agreement with the bright field images in Figure 5: the granularity of the cytoplasm of the infiltrating cells observed from

Figure 7. Bright field microscopy images of tissue sections at different time intervals after subcutaneous injection of CFA. Injection of CFA results in a widespread inflammation, with the influx of large numbers of polymorphonuclear cells, lymphocytes and macrophages, formation of granulomatous structures and tissue necrosis.
day 18 is due to phagocytosed microcapsules. Thirty days after injection (Fig. 9) shows shapeless microcapsule debris in the cell cytoplasm, indicating severe capsule degradation, most likely due to a combination of enzymatic attack and mechanical deformation.

These results were obtained with microcapsules consisting of two bilayers of dextran sulfate/poly-L-arginine. As we were also interested in the influence of the number of polyelectrolyte layers on the fate of the microcapsules after in vivo cellular uptake, we injected mice subcutaneously with microcapsules consisting of 1, 2, and 4 bilayers of dextran sulfate/poly-L-arginine. Figure 10 shows confocal microscopy images of sections of the injection site 16 days after subcutaneous injection of the microcapsules. In all cases the infiltrated inflammatory cells have phagocytosed the microcapsules, but a clear influence of the number of polyelectrolyte layers on the microcapsules’ degradation and destruction is observed. Intact microcapsules consisting of one bilayer can no longer be distinguished within the cells, only debris of degraded microcapsules. This contrasts with microcapsules consisting of four bilayers, which can be distinguished individually, with some having retained their spherical shape after in vivo cellular uptake. For microcapsules consisting of two bilayers, an intermediate situation is observed, with most of the microcapsules reduced to unidentified debris, but some retaining an intact appearance. These observations lead us to conclude that incorporating more polyelectrolyte layers increases in vivo intracellular stability. Microcapsules consisting of too few layers have a lower mechanical strength and are degraded faster by enzymatic action.

3. Conclusions

We first studied tissue reaction following subcutaneous injection of microcapsules in mice. A moderate immune reaction was observed, with an acute phase characterized by recruitment of polymorphonuclear cells and a more chronic phase in which microspheres are phagocytosed by macrophages and the injection site is surrounded by fibroblasts. Similar tissue reactions have been reported for other types of biodegradable microspheres, such as PLGA,[2] methacrylated dextran,[39] and poly(ether-ester)[40] microspheres. Secondly, we demonstrated the in vivo uptake and intracellular degradation of the microcapsules. We also observed that the number of bilayers from which the microcapsules are constituted determines their stability after intracellular uptake. These combined observations indicate that polyelectrolyte microcapsules made from degradable polyelectrolytes are suitable for drug delivery. These polyelectrolyte capsules can be used to deliver[41] DNA,[42,43] proteins[44–46] nucleic acids[47] and other materials inside phi-
gocytic cells, which are key players in immunity and tolerance. Thus, they may be useful tools in the field of vaccination.

4. Experimental

Materials: Rhodamine isothiocyanate (RITC), FITC-dextran ($M_w \sim 2000$ kDa), dextran sulphate (DEXS; $M_w \sim 10$ kDa) and poly-L-arginine hydrochloride (pARG; $M_w > 70$ kDa) were purchased from Sigma–Aldrich–Fluka. Calcium chloride ($\text{CaCl}_2$) and sodium carbonate ($\text{Na}_2\text{CO}_3$) were purchased from Merck. RITC labeled pARG was synthesized by mixing 100 mg pARG and 4 mg RITC in 40 ml 0.1 M borate buffer, pH 8.5. After overnight reaction the mixture was dialyzed for several days against pure water using Spectra Por dialysis bags with a molecular weight cut off of 25 kDa to remove residual RITC.

Preparation of Polyelectrolyte Microcapsules: Polyelectrolyte microcapsules were made using calcium carbonate ($\text{CaCO}_3$) microparticles as a sacrificial template. $\text{CaCO}_3$ microparticles were synthesized according to Volodkin et al. [23,24] by mixing $\text{CaCl}_2$ and $\text{Na}_2\text{CO}_3$ solutions (0.33 M) with vigorous stirring for 30 s followed by extensive washing with pure water to remove unreacted reagents. Spherically shaped $\text{CaCO}_3$ microparticles with an average diameter of 3 $\mu$m were obtained.

The $\text{CaCO}_3$ particles were coated using the layer-by-layer (LbL) technique by dispersing them in 5 ml of a 2-mg-ml$^{-1}$ dextran sulphate solution containing 0.5 M NaCl. After 10 min shaking the microparticles were collected by centrifugation, and residual dextran sulphate was removed by washing twice with 10 ml of pure water. Thereafter the microparticles were suspended in 5 ml of a 1-mg-ml$^{-1}$ poly-L-arginine solution in 0.5 M NaCl and shaken for 10 min, followed by centrifugation and two washing steps. This procedure was repeated until the desired number of layers was deposited (2, 4, and 8 in this study).

Hollow capsules were obtained by removing the $\text{CaCO}_3$ core by incubating the coated microparticles for 10 min in 10 ml of 0.2 M EDTA solution (pH 5.2) to dissolve the $\text{CaCO}_3$. The dissolved ions were then removed by three centrifugation and washings steps. Finally the capsules were resuspended in 1 ml PBS. The capsule concentration was determined by haemocytometry.

Characterization of Polyelectrolyte Microcapsules: Electrophoretic Mobility: The electrophoretic mobility of the polyelectrolyte microcapsules was measured in deionized water at room temperature using a Malvern Zetasizer 2000 (Malvern Instruments). The zeta-potential ($\zeta$-potential) was calculated from the electrophoretic mobility ($\mu$) using the Smoluchowski function $\mu = \frac{g}{e}$ where $g$ and $e$ are the viscosity and permittivity of the solvent, respectively.

Electron Microscopy: A drop of capsule suspension was placed on a silicon wafer and dried under a nitrogen stream, and then coated with gold. SEM images were recorded with a Quanta 200 FEG FEI scanning electron microscope operated at 5 kV.

Optical Microscopy: The Olympus BX51 microscope, equipped with 4x, 10x, 20x, 40x, 60x, and 100x lenses and a Coolmap Color camera (from Roper Scientific – Tucson USA) were used for bright field inspection and recording.

Fluorescence Microscopy: The RITC labeled microspheres in the skin of the mice were imaged with a Leica SP5 AOBS confocal microscope (63x 1.4 oil objective, HCX PL APO 63.0 x 1.40 OIL UV), using the 543 nm HeNe laser line for the RITC-labelled microspheres. Nuclei were stained with DAPI included in the mounting medium Vectashield, and excited with the 405 nm line of a UV diode laser.

Generation of Bone-Marrow Derived Dendritic Cells: Dendritic cells were generated using a modified Inaba protocol [49]. Two to six months Balb/c mice were sacrificed and bone marrow was flushed from...
In vitro Cytotoxicity Assay: The cytotoxicity of dextran sulfate/poly-L-arginine microcapsules was evaluated on bone marrow derived dendritic cells using a classical MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Day 8 dendritic cells were plated in a 96-well plate at 2.5 x 10^3 cells/well and incubated with different concentrations of microspheres for 6 h. Afterwards, medium was refreshed and cells were cultured for another 48 h. Medium was removed and MTT, a water-soluble tetrazolium salt, was added. MTT is converted into an insoluble purple formazan dye by mitochondrial dehydrogenases of living cells. After 3 h of incubation at 37 °C, cells were lysed and formazan was dissolved in dimethylsulfoxide (DMSO). Measuring the absorbance at 570 nm assessed the relative viability of the cells.

Histological Examination: Dextran/pARG polyelectrolyte microcapsules were prepared under endotoxin-free and sterile conditions. Female C57/B6 mice (6–8 wks, Janvier) were housed under SPF conditions. After being anesthetized intraperitoneally with a mixture of ketamine/xylazine, mice were sacrificed and their femurs and tibias. After lysis of red blood cells with ACL lysis buffer (BioWhittaker, Walkersville, MD), granulocytes and B cells were depleted using Gr-1 (Pharmingen) and B220 (Pharmingen) antibodies respectively, and low-toxicity rabbit complement (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). Cells were seeded at a density of 2 x 10^6 cells mL^-1 in 175cm^2 Falcon tubes (Becton Dickinson) in DC medium (RPMI 1640 medium containing 5 % LPS-free FCS, 1 % phytohemagglutinin, 1 % β-mercaptoethanol) containing 10 ng mL^-1 IL-4 and 10 ng mL^-1 GM-CSF (both from Pepro-tech, Rock Hill, NJ). After two days and again after four days of culture, the non-adherent cells were centrifuged, resuspended in fresh medium and replated to the same falcons. On the sixth day, non-adherent cells were removed and fresh medium containing 10 ng ml^-1 GM-CSF and 5 ng ml^-1 IL-4 was added. On day 8 of culture, non-adherent cells were harvested and fresh medium containing 10 ng ml^-1 GM-CSF was added.

The integrity and cellular uptake of the poly-electrolyte capsules were evaluated using confocal microscopy. For each time interval, samples from three different mice were injected subcutaneously. Mice were sacrificed at different time intervals and the site of injection was disected. Tissue samples were fixed in 4 % paraformaldehyde, embedded in paraffin, and 5-μm sections were cut. After deparaffinisation, sections were mounted with DAPI-containing Vectashield mounting medium for fluorescence (Vector Laboratories, Inc.) to visualize nuclei. The integrity and cellular uptake of the poly-electrolyte capsules were evaluated using confocal microscopy.

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