Journal of Controlled Release 162 (2012) 599–605

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

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

NANOMEDICINE

Controlled enzyme-catalyzed degradation of polymeric capsules templated on $CaCO_3$: Influence of the number of LbL layers, conditions of degradation, and disassembly of multicompartments

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ARTICLE INFO

Article history:
Received 27 March 2012
Accepted 4 August 2012
Available online 11 August 2012

Keywords:
Microcapsules
Layer-by-layer
Drug delivery
Calcium carbonate
Controlled release

ABSTRACT

Enzyme-catalyzed degradation of $CaCO_3$-templated capsules is presented. We investigate a) biodegradable, b) mixed biodegradable/synthetic, and c) multicompartment polyelectrolyte multilayer capsules with different numbers of polymer layers. Using confocal laser scanning microscopy we observed the kinetics of the non-specific protease Pronase-induced degradation of capsules is slowed down on the order of hours by either increasing the number of layers in the wall of biodegradable capsules, or by inserting synthetic polyelectrolyte multilayers into the shell comprised of biodegradable polymers. The degradation rate increases with the concentration of Pronase. Controlled detachment of subcompartments of multicompartment capsules, with potential for intracellular delivery or in-vivo applications, is also shown.

1. Introduction

Polyelectrolyte multilayer polymeric capsules serve as prominent candidates for delivery of biologically active molecules [1–3]. A broad range of materials and stimuli [4] can be used for construction of carriers, while different stimuli can be used for encapsulation and release of cargo from capsules [5] and films [3,6–8]. Efficiency of encapsulation [9–12], and its mechanisms [13,14] play an important role for successful delivery of encapsulated cargo, while another significant functionality is subsequent release [15]. Various mechanisms of release from microcapsules are possible [5,16,17] including a combination of dual [18] mechanisms such as external triggers and biodegradability [2,14,19,20], which together with decomposition at physiological pH [21] and aptamer-binding [22] are some of the mechanisms for controllable release [14,23–25]. Here, enzymes provide an essential functionality [26–29] for controlling the kinetics of release, essentially complementing directionality [30] of release triggered by external stimuli.

Controlled permeability, an essential property of multilayer films, was achieved in films of synthetic polymers consisting of polyvinyl pyrrolidone/polyglutamic acid [31,32] or in polysaccharide films [8,9], by stimuli such as electrochemical ones [33], temperature [34] or light [35,36]. Microcapsules composed of synthetic polyelectrolytes were shown transiently permeable by laser light exposure of gold nanoparticles incorporated inside their shells [37,38]. The controlled release of payload from microgel capsules has been achieved under physiological conditions by enzymatic reaction using various types of enzymes which were specifically bound to polymer chains [39], and by methods based on click-chemistry [31].

In addition to the necessity of controlling release of cargo from biodegradable microcapsules consisting of only a single compartment, controlled biodegradation and release [40] can also be applied to multicompartments [41], anisotropic capsules [42,43], and particles [44], and their combination, and thus give rise to additional potential applications [45]. Development of such carriers was spurred by efforts of increasing the degree of shell complexity and extending the range of surface functionalities. Primarily, these systems have been developed for simultaneous delivery of several different types of molecules [46], while they can be also used for

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http://dx.doi.org/10.1016/j.jconrel.2012.08.006

carrying out biochemical reactions, as well as sensory functions [47]. Furthermore, strategies for performing enzyme-catalyzed reactions in multicompartment particles have recently been shown [48,49]. Microgel [50,51] and hydrogel delivery vehicles [52] represent another promising system; for hydrogel microcapsules, selective degradation of subcompartments was realized with thiolated poly(methacrylic acid) and poly(N-vinyl pyrrolidone) polymers [53].

It has been previously shown that the non-specific protease Pronase loaded inside $CaCO_3$ microcapsules disrupts shells of capsules made of poly(L-arginine) and poly(L-aspartic acid) [54], and thus was used for release of DNA [54,55], while the dextran sulfate and poly-arginine pair of polymers was shown to degrade inside living cells [56]. Although $CaCO_3$-templated capsules [57,58] represent some of the most biocompatible (which is an essential property [59]), accessible and inexpensive, and widely used carriers, investigation of their properties remains elusive. This is partially attributed to difficulties in characterizing such capsules due to non-uniformities of their shape, sizes, pores, and morphology. In sharp contrast to mono-disperse SiO_2-templated capsules, which are widely employed by other groups and are used by our group for precise characterization of their properties, some applications have been developed [60], but no systematic attempts have been made to-date to study properties of $CaCO_3$-templated capsules. Yet, there are a growing number of applications and impending needs where the use of such biocompatible carriers is of paramount importance. Herein, we fill this niche and present results of our studies on degradation of microcapsules composed of the biodegradable polymers dextran sulfate and poly-(L-arginine). The degradation rate is controlled by different thicknesses of the polymeric shells and conditions of degradation. Unlike the case of microcapsules with synthetic polymeric layers wherein their size, and as a consequence the shell thickness, can be controlled by temperature, for microcapsules comprised of biodegradable polymers we study degradability as a function of the number of layers which determine the shell thickness. This number is also expected to affect mechanical properties of capsules [2], and therefore is a significant variable since only mechanically strong capsules can be used for successful delivery into living cells [61]. In previous work, intracellular delivery has been achieved by using thermally shrunk capsules composed of synthetic polymers [61,62]. Thus, in the case of biodegradable microcapsules, increasing the number of layers on one hand can influence their mechanical properties, which is an essential parameter [63–65], while on the other hand enabling controlled release of cargo by biodegradability. We compare here the conditions of capsule degradation for purely synthetic, purely biodegradable, and mixed-type (comprising a combination of synthetic and biodegradable polymers) capsules. Another novelty of this work is that of the concept of biodegradability to disassemble multicompartment capsules. We apply here the biodegradability principle for detachment of the outer compartments of multicompartment capsules. The latter approach can be used for theranostics, that is delivery of materials which simultaneously act as sensors and curing agents [66].

2. Experimental section

2.1. Materials

Silica particles with sizes of 4.80 ± 0.19 μm and 0.58 ± 0.02 μm were purchased from Microparticles GmbH, Berlin, Germany. Poly(allylamine hydrochloride) (PAH, Mw 70 kDa), poly(sodium 4-styrenesulfonate) (PSS, Mw 70 kDa), poly(L-arginine) (pArg, Mw 70 kDa), poly(L-aspartic acid) (pAsp, Mw 15 kDa), poly(L-glutamic acid) (pGlu, Mw 50–100 kDa), tetramethylrhodamine isothiocyanate-dextran (TRITC-dextran, 70 kDa), fluorescein isothiocyanate-dextran (FITC-dextran, 70 kDa) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich, Germany. Pronase was purchased from Roche, Switzerland.

2.2. Preparation of hollow capsules

$CaCO_3$ particles were fabricated using the previously described method [67]. The homo poly(L-amino acids) pArg and pGlu were adsorbed on the surface of particles using the layer-by-layer (LbL) deposition technique from solutions with concentration of 2 mg/mL each in 0.15 M NaCl. For visualization, dye-labeled molecules were incorporated in the capsules. One type of capsules contained FITC-dextran inside the shell. Before adsorption of polyelectrolytes, $CaCO_3$ cores were incubated with FITC-dextran (1 mg/mL) for 30 min at constant shaking. The other type of capsules contained one layer of TRITC-pArg in the shell. To obtain hollow capsules, the $CaCO_3$ core was decomposed by EDTA. For monitoring in a glass chamber, a drop of particle suspension was placed onto a glass slide. After the particles had sedimented, water was removed from the top of the drop. Subsequently, EDTA solution (0.2 M, pH 7) was added to the particles for dissolution of $CaCO_3$. Excess of EDTA was removed, and few drops of water were added for washing. For monitoring in an Eppendorf tube, particles were mixed with EDTA two times for 20 min, and then were washed with deionized water. For controlling the kinetics of release, hybrid capsules were made containing biodegradable (pArg/pGlu) and synthetic (PAH/PSS) polyelectrolytes.

2.3. Preparation of multicompartment particles

Silica microparticles with sizes of 4.80 μm and 0.58 μm, coated with a polyelectrolyte shell, were used as inner and outer subcompartments for fabrication of multicompartment particles, respectively. Both synthetic (PAH and PSS) and biodegradable (pArg and pAsp) polyelectrolytes were adsorbed on the particles by the LbL deposition technique from 2 mg/mL solutions in 0.5 M and 0.15 M NaCl, respectively. For visualization, one layer of TRITC-dextran was incorporated in the shell of the outer subcompartments, and one layer of FITC-PAH was incorporated in the shell of the inner ones. The larger particles had the shell composition $(PAH/PSS)_2$/FITC-PAH/PSS/PArg/PAsp/PArg, and the smaller ones $(PAH/PSS)_2$/PAH/TRITC-dextran/PArg/PAsp. To adsorb outer subcompartments onto the surface of the inner ones, equal volumes of micro- and nanoparticle suspensions (0.1 mL) with concentrations of 7.5 mg/mL and 1.5 mg/mL, respectively, were mixed and shaken for 10 min.

2.4. Enzymatic degradation of capsules

1 mL of capsule suspension was collected by centrifugation and supernatant removal. Then, 0.5 mL of 1 or 5 mg/mL Pronase solution was added into the tube and incubated at 37 °C for 2 h. After 30, 60, 90, and 120 min of incubation, the capsules were centrifuged and washed with deionized water.

2.5. Disassembly of multicompartment particles

Before starting the enzymatic reaction, 0.1 mL of multicompartment suspension was collected by centrifugation, and the supernatant was removed. Then, 0.1 mL of 20 mg/mL Pronase solution was added into the tube, and particles were incubated at 37 °C for 1 h. During incubation for defined time intervals (5, 15, 30, and 60 min), aliquots of multicompartment particles were analyzed under a Leica TCS SP confocal laser scanning microscope (CLSM) equipped with a 100×/1.4–0.7-oil immersion objective.

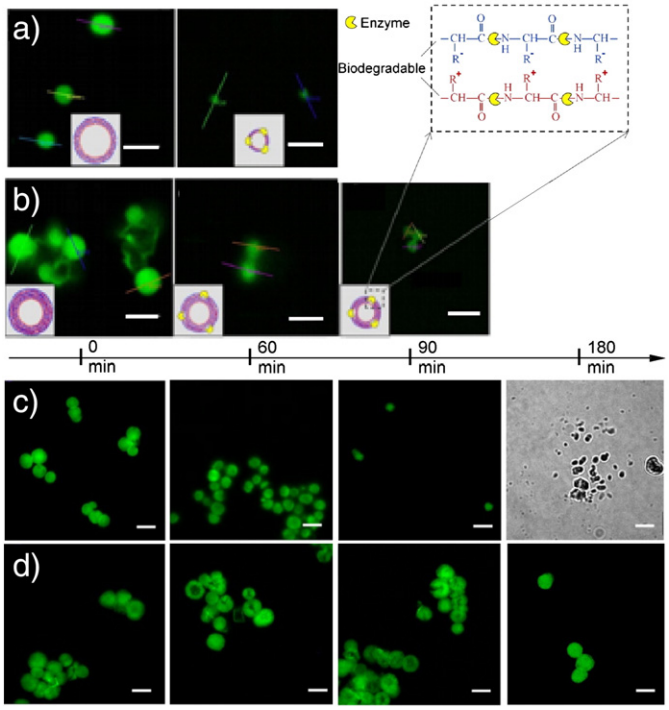

Fig. 1. Confocal laser scanning microscope (CLSM) images of enzyme-catalyzed degradation of: (a) four layer-pair $(pArg/pGlu)_4$ polyelectrolyte capsules with encapsulated FITC-dextran before and 60 min after incubation in a solution containing Pronase; (b) $(pArg/pGlu)_8$ capsules before (at 0 min), after 60 and 90 min of enzyme-catalyzed degradation; (c) $(PAH/PSS)(pArg/pGlu)_3$ capsules before (at 0 min), after 60 and 90, and 180 min of enzyme-catalyzed degradation; (d) $(pArg/pGlu)_3(PAH/PSS)$ capsules before (at 0 min), after 60, 90, and 180 min of enzyme-catalyzed degradation. The scale bars correspond to 5 μm. The full analysis including profiles taken through several capsules is shown in the Supporting information (Fig. S2). Schematic of enzyme cleavage is shown in the left-upper corner.

3. Results and discussion

Simons and Blout [68] reported that poly-L-lysine, poly-L-aspartic acid, and even poly-L-proline were hydrolyzed by Pronase. In comparison to other available proteases, Pronase causes the most complete hydrolysis of native or denatured proteins, breaking them down into individual amino acids. This is thought to be due to the concerted action of various proteolytic enzymes coexisting in Pronase, which is isolated from the extracellular fluid of *Streptomyces griseus*. For investigation of enzyme-dependent decomposition of biodegradable polyelectrolyte shells, capsules consisting of the following polypeptides were formed: poly(L-arginine) (pArg) as a polycation, poly(L-aspartic acid) (pAsp), poly(L-glutamic acid)(pGlu) as polyanions. These polyelectrolytes possess peptide bonds which can be cleaved by an enzyme-catalyzed reaction. Specific enzymes can be used for different types of proteins, polypeptides, or polysaccharides. We have chosen a non-specific proteolytic enzyme, Pronase, for our model system of microcapsules composed of polypeptide shells [46,69] (Fig. 1). We conducted a series of experiments to study the dependence of the integrity of capsule shells and their sizes on the number of polyelectrolyte layers and enzyme concentration. Capsules consisting of four and eight bilayers of pArg/pGlu were constructed (Fig. S1); degradation of the shell was studied using two approaches: a) taking probes from samples placed under continuous shaking in a reaction tube, and b) in a glass chamber placed under a confocal laser scanning microscope (CLSM). In the latter approach, the same capsules can be continuously monitored.

Pronase has its maximum proteolytic activity at 30–50 °C in the pH range of 6–9. Therefore, experiments were performed at 37 °C. Upon degradation, the size of microcapsules decreases. Fig. 1 shows that encapsulated FITC-dextran escapes from microcapsules already after 60 min of incubation for both shell thicknesses. After 1 h of

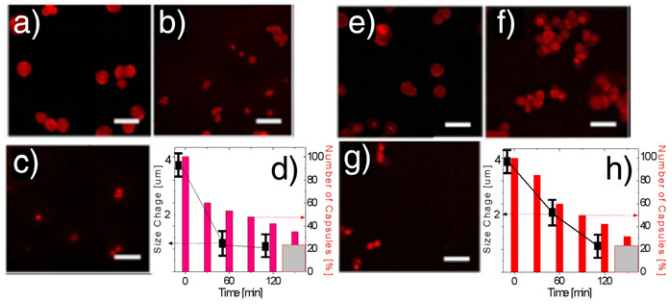

Fig. 2. CLSM images of four layer-pair capsules composed of pArg and pGlu and containing TRITC-labeled pArg in the shell. Images are shown as follows: (a) before the incubation, (b) after 90 and (c) 150 min of incubation; (d) time dependence of the average size (black squares) and the number (red bars) of capsules incubated in a 1 mg/mL Pronase solution. CLSM images of eight layer-pair capsules composed of pArg and pGlu and containing TRITC-labeled pArg in the shell. Images are shown as follows: (e) before the incubation, (f) after 90 and (g) after 150 min of incubation; (h) time dependence of the average size (black squares) and the number (red bars) of capsules incubated in a 1 mg/mL Pronase solution. Scale bars correspond to 8 μm. Gray rectangles demonstrate proportion of capsules with incomplete degradation of cores as measured upon drying before conducting experiments. Statistics was collected on more than hundred capsules.

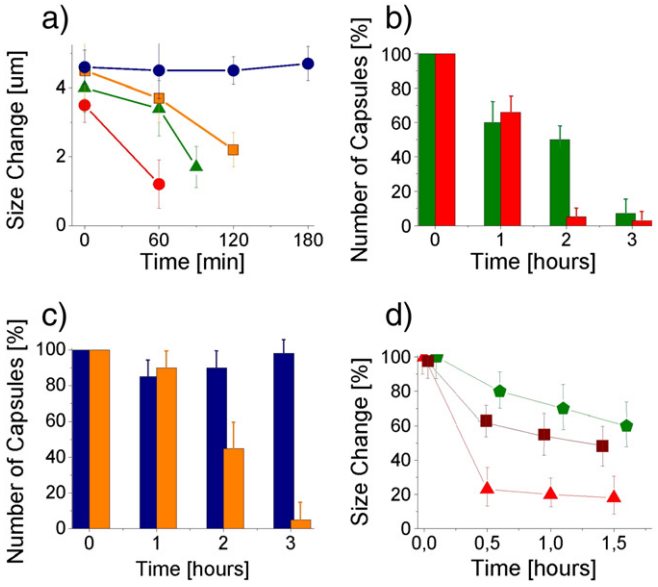

Fig. 3. a) The average size decrease of capsules depicted in Fig. 1 upon biodegradation. The following notations are used: degradation of $(pArg/pGlu)_4$ is shown in the curve with red circles, $(pArg/pGlu)_8$ with green triangles, $(PAH/PSS)(pArg/pGlu)_3$ with orange squares, while degradation of $(pArg/pGlu)_3(PAH/PSS)$ capsules is represented by the curve with blue circles. Size decrease incurred from sampling kinetics of degradation for: b) $(pArg/pGlu)_4$ (red) $(pArg/pGlu)_8$ (green), c) $(PAH/PSS)(pArg/pGlu)_3$ (orange) and $(pArg/pGlu)_3(PAH/PSS)$ (dark blue). Data in (d) are collected in an in-situ measurement when the same capsules are continuously monitored under CLSM (Fig. S4, S5) for $(pArg/pGlu)_4$ (red triangles) and $(pArg/pGlu)_8$ (green pentagons) both at 1 mg/mL of Pronase as well as for $(pArg/pGlu)_4$ (dark red squares) at 5 mg/mL Pronase. Statistics was collected for over thirty capsules and three experiments.

enzyme reaction, the average diameter of the capsules decreases by more than 3 μm for four layer-pair capsules, and by less than 2 μm for eight layer-pair capsules (Fig. 1 and Fig. S2). This indicates re-arrangements of the polymeric shell after some polymers have been peeled off. These experiments were conducted by sampling an aliquot of capsules from the reaction tube.

Additional insights into the process of degradation were obtained by labeling the shells by polyelectrolytes labeled with fluorescent dyes. The polyelectrolyte shell was fluorescent-labeled by adsorbing TRITC-pArg in the multilayers of the capsule walls. In the following experiments, capsules consisting of four and eight layer pairs of biodegradable polyelectrolytes (with one layer of TRITC-pArg substituted for pArg in the shell) were incubated in the Pronase solution of 1 mg/mL for 2 h. Confocal images recorded at various incubation times are shown in Fig. 2. Indeed, degradation of capsules with an eight layer-pair shell occurred more slowly than those with four layer pairs. For example, after 1 h of enzyme reaction, the capsule average size decreased from initial 4 μm to ~2–2.5 μm for eight layer-pairs (Fig. 2h), and to 1–1.5 μm for four layer-pairs (Fig. 2d). We noticed in our studies that upon incorporation of TRITC-labeled pArg in the shell of capsules, the $CaCO_3$ templates dissolved differently than those without TRITC-labeling. Fig. S3 shows scanning electron microscope (SEM) images of microcapsules: some undissolved cores can be clearly seen here (the number of undissolved cores depends on the concentration of the solvent, EDTA and time of the dissolution). Therefore, for monitoring degradation kinetics of this type of capsules, we have also determined the proportion of capsules which do not completely dissolve (~20%), and marked these numbers by gray rectangles in Fig. 2 d) and h).

Under the same dissolution conditions, capsules which did not contain labeled polymer in the shell did not exhibit leftover of cores. Reduction of permeability by incorporation of additional material in capsule shells agrees with a similar trend observed upon addition of nanoparticles in the shell [70].

Fig. 3 shows that the degradation process is retarded by about 1 h by increasing the number of polyelectrolyte layers from four layer-pairs to eight layer-pairs. Further decrease of release kinetics can be induced by incorporating synthetic polyelectrolytes into biodegradable polyelectrolyte multilayer shells (Fig. 1, bottom panels). When replacing the last two layers of capsules (the composition $(PAH/PSS)(pArg/pGlu)_3$) (Fig. 3) the kinetics of release is slowed down by one more hour (i.e. to 180 min now). We found in our studies that the position of synthetic polyelectrolyte layers also affects degradation. Indeed, $(pArg/pGlu)_3(PAH/PSS)$ capsules demonstrated substantially reduced degradability (Fig. 3c). This can be assigned to the fact that the protease first encounters synthetic polyelectrolytes which impede the degradation reaction. Peculiarly, if the pair of synthetic polyelectrolytes is placed first on the core, and subsequently covered by three layer-pairs of biodegradable polyelectrolytes, the kinetics of degradation is slower as compared to purely synthetic capsules, but it is still faster than that when synthetic polyelectrolytes are in the outer layers (Fig. 3c).

In subsequent experiments, we investigated the influence of the concentration of Pronase by conducting experiments at concentrations of 1 mg/mL and 5 mg/mL. In the course of these experiments, Pronase was added to the solution containing the capsules (i.e. the degradation proceeded from the outside of capsules) to monitor capsule degradation. Fig. S4 (top) shows the time-dependent degradation process of four layer-pair capsules in 1 mg/mL of the Pronase solution. Under these conditions, the degradation of capsules led to a decrease of their average size by ~1 μm after 30 min. Also, during the enzyme's action on the capsules, their shells showed deformation due to decrease of the amount of polyelectrolytes inside the shell as a result of proteolytic digestion. Increasing the concentration of Pronase up to 5 mg/mL caused faster degradation of capsules composed of four bilayers (Fig. S4, bottom).

Four bilayer-capsules degraded in the first few minutes (around 10 min), and the degradation was accompanied by a decrease of their size. Under the same conditions, the average size decreased by more than 3 μm after 30 min of the reaction. A similar trend was observed for microcapsules containing a different number of polyelectrolyte layer pairs (Fig. S5). In a separate experiment, we monitored degradation of these samples by incubation with Pronase solution in an Eppendorf tube, under the same condition as in glass chambers. After 2 h of incubation in the tube, only few capsules were found

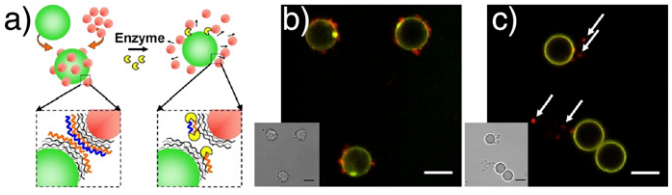

Fig. 4. Schematic illustration of detachment of small compartments from the surface of an inner particle by proteolytic activity (a) (blue and orange lines – biodegradable polyelectrolytes, gray lines – synthetic polyelectrolytes). Overall CLSM images of multicompartments (b) before the enzyme reaction, and (c) after 1 h of incubation with Pronase (20 mg/mL). Arrows show detached particles. Transmission CLSM images are shown in the insets. The scale bars correspond to 4 μm. An image of the same multicompartment particles with a larger field of view is shown in Fig. S7(c).

(Fig. S6); the capsules showed a deformed shell, and their size decreased more than that of those tested in the glass chamber. This difference can be assigned to enhanced intermixing and diffusion upon incubation in Eppendorf tubes. In a control experiment, capsules were shaken at 37 °C for 1 h in the absence of Pronase, and in this case, capsule size did not change. In summary, at higher Pronase concentration the capsule size reduces faster than at a lower one. These results are expected, and they agree with earlier data obtained by Borodina et al. [54] who performed the experiment in a different way by encapsulating Pronase. Yet, they observed that capsules built with a larger number of layer pairs are more stable. The influence of the concentration of Pronase is also reflected in Fig. 3(d).

Before enzymatic degradation, the $CaCO_3$ core was dissolved by EDTA. Some EDTA might remain in the reaction solution, and after adding Pronase, those remnants could influence the enzyme-catalyzed process. Indeed, it was shown in previous work [26] that EDTA can inhibit Pronase activity. To remove these doubts, we performed a control experiment with the same capsules as reported in Fig. 1(a,b). However, this time, we added 10 mM of calcium chloride solution to circumvent the potential problem of EDTA-induced inhibition of Pronase. The result was similar to the experiment performed without adding calcium chloride. Since degradation is initiated after the core removal and is followed by washing for at least two times in deionized water, we conclude that the remnants of EDTA are insignificant. Also, more intense diffusion in such a case might lead to more effective degradation of the capsule shell. We note that Pronase is a mixture of several proteolytic *S. griseus* enzymes including endopeptidases and exopeptidases. These show differences in their proteolytic activities toward various substrates when acting like an aminopeptidase (substrates L-Leucyl-glycine, LG, L-Leucyl-β-naphthylamide, LNA), or like a carboxypeptidase (carbobenzoxyglycyl-L-leucine, CGL). The catalytic properties of the peptidase which hydrolyzes LG and LNA are in many respects different from those of proteases and peptidases hydrolyzing CGL. In general, endopeptidases hydrolyze peptide bonds within the polypeptide chain, whereas exopeptidases break them down from either of their ends (amino and carboxy peptidases).

Another novelty of this work is the application of the concept of enzyme-dependent biodegradation to controllable detachment of multicompartment capsules (Fig. 4a). This is envisioned to be an important concept for intracellular sensing and for simultaneous intracellular delivery of several types of molecules [41]. In these experiments, the polyelectrolyte shell was deposited onto the surface of particles including both synthetic and biodegradable polyelectrolytes (Fig. 4). For fabrication of multicompartment particles, silica microparticles with sizes of 4.80 μm and 0.58 μm coated with a polyelectrolyte shell were used as inner and outer subcompartments, respectively. Two types of polyelectrolyte shells were made: (i) capsules for a control experiment used only synthetic polymers ($(PAH/PSS)_2$/FITC-PAH and PAH/PSS/PAH/TRITC-dextran), and (ii) those for the detachment experiment consisted of both synthetic polymers and biopolymers. In the latter case, the large silica particles were composed of PAH/PSS/PAH/TRITC-dextran/pArg/pAsp polyelectrolytes, and the small containers carried the multilayer sequence $(PAH/PSS)_2$/FITC-PAH/PSS/pArg/pAsp/pArg on their surface. In all cases, the outer layers of the larger and smaller particles had opposite charges. Thus, the small silica particles are connected to the surface of the bigger ones due to electrostatic interaction of their outer layers (Fig. 4b). Here, small particles (red color) are bound to the inner spheres (green color) and are randomly distributed on their surface.

For detachment of nanoparticles from the surface of microspheres, an aqueous Pronase solution (20 mg/mL, final concentration) was used. The CLSM image of multicompartment particles after Pronase incubation is presented in Fig. 4(c). This figure shows that nanoparticles (red color) detached from the inner microparticles. Although not all small particles were separated from the inner particles, using this approach more than 60% of the outer containers were detached (Fig. S7c). In a control experiment, the suspension of such multicompartment containers was incubated at 37 °C in the absence of Pronase. In addition, multicompartment capsules composed of only synthetic polyelectrolytes, the first type of capsules, were incubated in the Pronase solution. In both cases, after 1 h of incubation, small particles were still seen on the surface of inner spheres and were found intact (a control experiment with synthetic microcapsules after 1 h of incubation is shown in Fig. S8). We observed that detached small containers were spread out at the surface, but in future experiments, they may be collected by functionalization with magnetic nanoparticles [71]. Although it is important to study biocompatibility of all types of capsules [72,73], synthetic and biodegradable, we note that microcapsules comprised of synthetic polyelectrolytes have been found to be non-toxic for cells [62,74].

4. Conclusions

In the present study, we investigated the influence of the number of layers, the incorporation of synthetic polymers into capsules comprised of biodegradable polymers, as well as conditions of enzyme-catalyzed degradation of microcapsules made of biodegradable polyelectrolytes. Using confocal laser scanning microscopy, and electron microscopy, we found that capsules with a fewer number of layers degrade significantly faster than capsules with a larger number of layers. Introduction of labeled polyelectrolytes in the shell of capsules was found to affect template dissolution. Incorporation of synthetic polyelectrolyte layers in the shell of capsules composed of otherwise biodegradable polymers significantly slows down degradation and release of encapsulated material. On the other hand, increasing the concentration of Pronase, which causes polypeptide degradation, enhances the degradation rate; a similar trend was observed by mixing the capsules upon shaking. The concept of degradation has also been applied to detachment of sub-compartments of multicompartment capsules. With regard to potential biomedical applications, we demonstrated disassembly of multicompartment particles by controlled enzyme-catalyzed degradation. This approach has potential for application in biosensors [47,75,76]. It is also of relevance for drug delivery, as degradation is inevitably connected with cargo release, and the corresponding times (minutes to hours) are significant for many applications [77].

Acknowledgments

We thank Anneliese Heilig for the AFM measurements, and Rona Pitschke for SEM images. I. Marchenko thanks the FEBS scholarship for support. A. Yashchenok acknowledges the Alexander von Humboldt fellowship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2012.08.006.

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