Designing Hyaluronic Acid-Based Layer-by-Layer Capsules as a Carrier for Intracellular Drug Delivery

Anna Szarpak,† Di Cui,† Frédéric Dubreuil,† Bruno G. De Geest,‡ Liesbeth J. De Cock,‡ Catherine Picart,§ and Rachel Auzély-Velty*†

Centre de Recherches sur les Macromolécules Végétales (CERMAV-CNRS), BP53, 38041 Grenoble Cedex 9, France, Laboratory of Pharmaceutical Technology, Department of Pharmaceutics, Ghent University, Havenhuisstraat 72, 9000 Ghent, Belgium, Minatec, Grenoble Institute of Technology and LMGP, 3 parvis Louis Nèel, F-38016 Grenoble Cedex, France

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Polyelectrolyte microcapsules were prepared by the layer-by-layer assembly of hyaluronic acid (HA) and a polycationic polymer, poly(allylamine) (PAH) or poly(lysine) (PLL). The influence of the polycationic partner on the morphology, stability, permeability properties, and enzymatic degradation of microcapsules was thoroughly analyzed. It was found that these properties could be tuned by shell cross-linking. Confocal microscopy studies of cellular uptake of the capsules showed that the polyelectrolyte shells remain stable outside the cells but readily break open once internalized by cells, suggesting their potential as carrier for intracellular drug delivery.

Introduction

The use of polyelectrolyte microcapsules is currently being studied as a method to deliver drugs to cells in a controlled and selective way. Such systems, having a shell thickness in the nanometer range, are made by the layer-by-layer (LbL) assembly of oppositely charged polyelectrolytes on a sacrificial template, followed by its decomposition. Several advantages of polyelectrolyte capsules have been noted. First, they can be prepared under mild conditions which allow encapsulation of large amounts of fragile biologically active molecules.3–4 Second, by varying the layer number and the multilayer composition, a high degree of functionality within their shell can be obtained.5–9 Third, the capsule surface can be designed with a wide variety of functionalities, such as lipids,10 polyelectrolytes (ethylene glycol),11 antibodies,12 or sugars.13 Such surface modifications allowed to obtain biofunctional capsules having the potential not only to resist to nonspecific adsorption of proteins11 but also to target cells specifically.12 Moreover, cellular uptake of capsules was demonstrated12,14–20 In this context, several efforts have been made toward the development of smart capsules that are able to release their content inside the cell in response to external or internal stimuli. It was thus shown that after internalization by cells, capsules made of nonbiodegradable synthetic polymers (poly(diallyl dimethylammonium chloride), PDADMAC, and poly(styrene sulfonate), PSS) remained intact but, upon light illumination, could be opened and thereby release the cargo from their cavity.17,19 This light-activated release was due to local disruption of the shell resulting from the heat produced by irradiated gold nanoparticles that were incorporated during multilayer assembly construction. Another strategy consisted in the synthesis of degradable polyelectrolyte capsules containing polyelectrolytes that can be degraded either enzymatically (polypeptides or polysaccharides) or through hydrolysis.14,21–23 After cellular uptake, such capsules were degraded over approximately 24 h, contrary to capsules made of the synthetic polyelectrolytes which remained quasi-intact.14 Regarding enzymatic degradation, it was also shown that the wall properties of capsules can be readily changed upon varying enzyme concentrations.24 In view of drug delivery applications, polyelectrolyte capsules templated on porous inorganic cores such as calcium carbonate (CaCO3) and mesoporous silica have recently emerged as promising carriers for macromolecular drugs such as protein antigens and DNA, providing high encapsulation efficiency.24–30

In a previous paper, we reported on the synthesis of capsules from hyaluronic acid (HA), a highly hydrated natural polysaccharide which plays an important structural and biological role in the living organisms.31 In spite of the property of HA to form soft hydrogel-type multilayered films and its weak polyelectrolyte character, we developed conditions for the construction of stable hollow capsules in combination with poly(allylamine hydrochloride) (PAH), a synthetic polyelectrolyte.31 Such capsules did not show cytotoxicity after contact at a high concentration with in vitro cultured myoblast cells. However, they contain PAH as a nonbiodegradable component which impairs their future use in a clinical setting. In this work, we focused on the design of fully biodegradable capsules based on HA with the purpose to create carriers for intracellular drug delivery. Poly(t-lysine) (PLL), a biocompatible and biodegradable polypeptide, was used as polyelectrolyte in combination with HA as polyanion to form multilayer microcapsules. We first investigated the morphology and stability properties of these microcapsules under physiological conditions using several complementary techniques. Because disruption of the multilayer was observed for HA/PLL capsules, we investigated a possible route to improve the shell stability by chemical cross-linking. These chemical modifications and their effect on the permeability properties of the capsule membrane in the absence and in the presence of endogenous enzymes are described in the second section of this paper. Finally, we also report on the intracellular uptake and intracellular fate of the capsules by macrophages. Our data pave
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Figure 1. Scanning electron microscopy images showing the surface of spherical CaCO₃ microparticles (A) before and (B,C) after deposition of 4.5 HA/PLL and 4.5 HA/PAH bilayers. The first and outermost deposited layer is HA.

Figure 2. Film growth monitored in situ by QCM-D for (PLL/HA; □) and (PAH/HA; ■) films. (a) Difference in the frequency shifts ∆f measured at 15 MHz after each polycation and polyanion deposit. (b) Film thickness as a function of the number of deposited layers deduced from the QCM-D data for the two films.

Figure 3. Scanning electron microscopy images showing (HA/PLL)₄,₅ (A) and (HA/PAH)₄,₅ (B) dried hollow capsules. The first and outermost deposited layer is HA. Holes can be distinguished on the HA/PLL shell as indicated by an arrow.

diaminocarboxylic acid (EDTA), calcium chloride (CaCl₂), sodium carbonate (Na₂CO₃), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich-Fluka. LysoTracker Red was purchased from Invitrogen. All chemicals were used without any further purification. The water used in all experiments was purified by a Millipore Milli-Q Plus purification system, with a resistivity of 18.2 MΩ cm.

Capsule Preparation. Microparticles were prepared using calcium carbonate microparticles as a sacrificial template. CaCO₃ microparticles were synthesized from solutions of CaCl₂ and Na₂CO₃ as reported in the literature. The CaCO₃ microparticles were coated using the layer-by-layer technique, by incubating them at a concentration 2% (w/v) in an aqueous solution (0.15 M NaCl, pH 6.5) of HA (Cᵥ = 5 g/L) and PAH (Cᵥ = 2 g/L) or PLL (Cᵥ = 2 g/L). After shaking for 10 min, the microparticles were collected by centrifugation and the residual nonadsorbed polyelectrolyte was removed by washing twice with an aqueous solution of 0.01 M NaCl (pH 6.5).

After deposition of 4.5 layers, the CaCO₃ core was removed by treatment with a 0.1 M EDTA solution (pH 7.5). To avoid mechanical damages of “soft” polyelectrolyte shells, the dissolved ions resulting from the decomposition of CaCO₃ were removed by dialysis against pure water, using spectra Por dialysis bags with a molar mass cut off of 6–8 kDa.

Cross-Linking of the HA/PAH Multilayers. Chemical cross-linking of the multilayer shells was performed by activation of the carboxylic acid groups of HA using the water-soluble carbodiimide, EDC, and sulfo-NHS in 0.15 M NaCl. Various EDC concentrations (50, 100, 200 mM) were used, whereas the concentration of sulfo-NHS was kept constant at 50 mM. The core-templated HA/PAH multilayers were incubated in the EDC/sulfo-NHS solution (pH 6.5) overnight. The cores were dissolved by shaking the particle dispersion in 0.1 M EDTA (pH 7.5).

Isothermal Titration Calorimetry (ITC). ITC experiments were carried out on a Microcal VP-ITC titration microcalorimeter (Northampton, U.S.A.). All titrations were made in 0.01 M Tris-HCl buffer pH 7.4 with 0.15 M NaCl at 25 °C. The reaction cell (V = 1.45 mL) contained the PAH solution (Cᵥ = 2 g/L). corresponding to [PAH] = 21.3 mM, calculated from the average molecular weight of the repeating unit) or PLL solution (5 g/L corresponding to [PLL] = 25 mM). A series of 10 injections of 10 µL from the computer-controlled 300 µL microsyringe at an interval of 10 min of the solution of HA (Cᵥ = 3 g/L) corresponding to [HA] = 7.4 mM) were performed into the polyelectrolyte solution while stirring at 300 rpm at 25 °C. Under such conditions, endothermic heat was produced after each injection of HA and the magnitude of the released heat was nearly constant for both polyanion/polycation systems, as the chains of HA established a maximum number of interactions with the PLL and PAH chains. Indeed, the polycations are in large excess compared to HA, even after the ten injections. The amount of heat produced per injection was calculated by integration of the area under individual peaks by the instrument software, after taking into account heat of dilution. The ΔH value for PAH/HA and PLL/PAH complexation given in this paper is an average value of the ΔH values derived from the ten peaks.

Experimental Section

Materials. Hyaluronic acid under the sodium salt form, having a molar mass Mᵥ of 820 × 10³ g/mol, was a gift from ARD (Pomacle, France). We selected this HA sample for the preparation of capsules according to our previous work showing that diffusion of the initial polyelectrolyte layers in the porous carbonate core became limited when the HA molar mass was equal to or higher than 820 × 10³ g/mol. Poly(allylamine hydrochloride) (Mᵥ ≈ 70 × 10³ g/mol), poly(1-lysine hydrobromide) (Mᵥ ≈ (15–30) × 10³ g/mol), dextran samples labeled with fluorescein isothiocyanate with molar mass of 4 × 10⁴, 500 × 10⁴, and 2000 × 10⁴ g/mol (dextranFITC-4, –500 and –2000), 2-(N-morpholino)ethanesulfonic acid sodium salt (MES sodium salt), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), ethylene-

the way for the development of tailor-made capsules based on hyaluronic acid.
Films Characterization by Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D). The (PAH/HA), and (PAH/PLL), film buildup (where \( i \) denotes the number of layer pairs) was followed by in situ quartz crystal microbalance (QCM with dissipation monitoring, D3000, Qsense, Sweden). The quartz crystal was excited at its fundamental frequency (about 5 MHz, \( v = 1 \)) as well as at the third, fifth and seventh overtones (\( v = 3, 5, \) and 7 corresponding to 15, 25, and 35 MHz, respectively). Changes in the resonance frequencies \( \Delta f \) and in the relaxation of the vibration once the excitation is stopped were measured at the four frequencies. As the silica coated quartz crystal is negatively charged, depositions always started with the positively charged polyelectrolyte. The QCM-D data have been analyzed using a Voigt based model implemented in the Qtools software (Q-Sense) using predetermined values for the film density (1009 kg/m\(^3\)), buffer density (1000 kg/m\(^3\)), and viscosity (1 mPa.s). Three parameters (film thickness, viscosity, and shear modulus) are then deduced.

Confoocal Laser Scanning Microscopy (CLSM). Capsules suspensions were observed with a Leica TCS SP2 AOBs (Acoustico Optical Beam Splitter) confocal laser scanning system and, an inverted fluorescence microscope equipped with an oil immersion objective lens. FITC-labeled dextran samples were visualized by excitation of the fluorochrome with a 488 nm argon/krypton laser and the emitted fluorescence was collected between 497 and 576 nm, precisely defined by the AOBS.

Scanning Electron Microscopy (SEM). Drops of capsules suspensions were deposited onto copper stubs and allowed to air drying. The samples were sputtered with Au/Pd and observed in secondary electron imaging mode with a Jeol JSM6100 microscope using an accelerating voltage of 8 kV. For high resolution SEM analysis, the specimens were coated by 2 nm of electron beam evaporation carbon and observed in secondary electron imaging mode with a Zeiss ultra 55 FEG-SEM (CMTIC-INPG, Grenoble) at an accelerating voltage of 3 kV, using an in-lens detector.

Incubation with FITC-Labeled Dextran. Suspensions of HA/PAH and HA/PLL capsules in 0.02 M MES buffer (pH 6.5) were left overnight at room temperature. The FITC-labeled dextran samples were dissolved in the same buffer at a concentration of 2 mg/mL. For permeability tests in the presence of salt, NaCl was added into MES buffer to obtain a concentration of 0.15 M. Typically, 20 \( \mu \)L of a dextran\(^{FITC} \) solution was mixed with 20 \( \mu \)L of capsule suspension on a glass slide. After 20 min, the capsules were observed by CLSM.

The images were analyzed using Leica Confocal Software by the measurement of the light emitted by the capsule interior \( (I_{int}) \) and surrounding solution \( (I_{ext}) \). The permeability coefficient \( (I_{int}/I_{ext} \times 100\%) \) was estimated as an average value from 7–10 capsules.

Cell Culture Experiments. RAW 264.7 mouse macrophages were purchased from ATCC and cultured in DMEM (Invitrogen) supplemented with 10% FBS, 1% penicillin-streptomycin (Invitrogen) and 1% glutamine. The cells were seeded onto round steril cell culture chambers, equipped with a microscopy coverslip as bottom, and allowed to attach overnight. Subsequently, the cells were incubated with capsules for 2 h followed by lysosomal staining with LysoTracker Red according to the manufacturer’s instructions.

Results and Discussion

1. Synthesis of HA/PAH and HA/PLL Hollow Capsules. HA/PAH and HA/PLL multilayer capsules were fabricated by using CaCO\(_3\) microparticles as a template and the optimal conditions reported previously. Thus, high \( M_w \) HA (\( M_w = 820 \times 10^3 \) g/mol) and the polycation (PAH or PLL) were alternatively deposited at a concentration of 5 and 2 g/L in water containing 0.15 M NaCl at pH 6.5, respectively. Deposition of the polyanions and polycations on calcium carbonate cores could be demonstrated by SEM analysis. As can be seen in Figure 1, the surface of CaCO\(_3\) particles coated with 4.5 bilayers of HA/PLL or HA/PAH becomes smoother than the initial porous particles. It exhibits in both cases bumpy islets which, in the case of the surface of HA/PAH coated particles, appear to be larger and coalesce to form an almost uniform film. Such differences in film pattern suggest different rate of multilayer growth depending on the polycation. To get more information about the multilayer build-up, the step-by-step deposition of HA/PAH and HA/PLL under identical polymer concentrations as for capsule synthesis was followed on a solid planar substrate by QCM-D (Figure 2). As previously reported, HA/PAH film growth is exponential in our working
conditions (0.15 M NaCl at pH 6.5). Actually, HA/PAH films have already been described as exponentially growing films in a previous study performed at the same ionic strength for a HA sample of intermediate molecular weight (4 × 10^6 g/mol).36 but a linear growth was reported at low ionic strength conditions (0.01 M NaCl).37 Exponential growth could be related here to the diffusion of PAH within the film architecture, as previously observed for films containing HA in combination with polysaccharides such as chitosan (CHI)38 or polypeptides, such as PLL.39 In comparison with HA/PAH, HA/PLL film growth was slower, presumably due to the fact that we are still in the early stages of the buildup. Indeed, for these films, several investigators have shown a transition in growth rates between a precursor regime and “the regular” growth regime.40-43 The number of bilayers for which the crossover occurs (∼8 layer pairs for HA/PLL)44 was found to coincide with the stage at which the film surface is fully covered with polyelectrolyte complex.41 In contrast, it can be assumed from Figure 2 that in the case of HA/PAH, full surface coverage is achieved after adsorption of 3 or 4 bilayers.

We also measured by ITC the complexation enthalpy for both polyelectrolyte couples to investigate if differences in film growth could be attributed to different affinities between the polyelectrolytes. The complexation enthalpy (∆H) values derived from ITC experiments (cf. Supporting Information, Figure S1) performed with our polyelectrolyte pairs were respectively of 560 ± 55 J/mol for PAH/HA and 1479 ± 145 J/mol for PLL/HA complexes in 0.01 M Tris-HCl buffer with 0.15 M NaCl (pH 7.4). Of note, the entropy gain is not measurable by ITC but it is accepted that polyelectrolyte complexation is associated with an entropy gain (positive ΔS) due to the release of associated counterions and water.42 For the polyelectrolyte complexation to take place, the free energy of interaction ∆G (∆G = ∆H − TΔS) has to be negative, which indicates that, in our case, the entropic contribution has to drive the formation of the complexes.

Interestingly, Laugel et al.45 recently reported a correlation between the nature of the growth process and the heat of complexation between the polyanions and the polycations constituting the multilayer film. These authors found that an endothermic process is rather characteristic of an exponential film growth, whereas a strongly exothermic process is rather characteristic of an exponential growth regime and “the regular” growth regime.40-43 The number of bilayers for which the crossover occurs (∼8 layer pairs for HA/PLL)44 was found to coincide with the stage at which the film surface is fully covered with polyelectrolyte complex.41 In contrast, it can be assumed from Figure 2 that in the case of HA/PAH, full surface coverage is achieved after adsorption of 3 or 4 bilayers.

As carbonate particles showed a high tendency to aggregate when coated with more than five HA/PAH or HA/PLL bilayers, the production of capsules by core dissolution was performed from carbonate cores coated with 4.5 bilayers. In a first step, we imaged by SEM the morphologies of the different capsules obtained after core dissolution by treatment with a 0.1 M buffered EDTA solution (Figure 3). As previously reported in our first work on HA/PAH capsules,31 the capsules are collapsed after drying with a diameter of ∼5 μm, providing evidence of core removal. On the other hand, the HA/PLL capsules appear much smaller (∼2.5 μm). These observations suggest that the HA/PLL exhibit an important shrinkage, which is maybe related to HA/PLL complexation. Capsules shrinkage was previously reported by Mauser et al.,46 when Ca^{2+} was added to capsules made of poly(methacrylic acid) (PMA) and PAH. Such a phenomenon was attributed to the competitive binding of Ca^{2+} to PMA -COO− groups, thereby reducing the electrostatic interactions between PMA and PAH, which stabilize the multilayer wall. This may also partly explain the reduced stability of the HA/PLL capsules after treatment with EDTA. Indeed, due to the fact that HA/PLL electrostatic interactions are weaker than HA/PAH, we can assume that Ca^{2+} ions may act as a competitor of PLL by forming a HA/Ca^{2+} complex, causing precipitation of HA and capsule shrinkage.

Based on these results and the hypothesis, we decided in the next step to cross-link the HA/PLL shell to increase the stability of capsules after core dissolution.

2. Manipulating the Shell Properties by Chemical Cross-Linking. The capsule shells were cross-linked following the procedure already developed for HA-based polyelectrolyte multilayers on planar surfaces and HA/PAH capsules, consisting in an amine-carboxyl coupling reaction. The carboxylate groups of HA are thus converted by reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in combination with N-hydroxysulfosuccinimide into activated esters, which can then be reacted with the amine groups of the polycation to form amide bonds. The cross-linking reactions were performed on HA/polycation multilayers deposited on CaCO_{3} particles under mild conditions (pH 6.5), allowing to maintain the polyelectrolyte complex integrity. The multilayer coated particles were incubated in freshly prepared EDC/sulfosuccinimide solutions overnight at room temperature followed by core dissolution and washing with water. Considering that the concentration of the coupling agent can influence the degree of cross-linking and, hence, the film’s mechanical properties,47 48 different concentrations of EDC (50, 200, and 400 mM) were tested for HA/PAH and HA/PLL capsules. Structural and morphological changes after cross-linking were analyzed by means of FTIR-ATR spectroscopy. Several modifications of the FTIR-ATR spectrum of HA/PLL capsules after cross-linking could be observed (cf. Supporting Information, Figures S2 and S3). The carbonyl peaks of HA (at 1400 and 1610 cm⁻¹) decreased, while the amide I and amide II bands (at ∼1670 and ∼1540 cm⁻¹, respectively) increased.

The cross-linked capsules were further subjected to SEM and AFM characterizations to reveal their morphological changes. Figure 4A,B shows that the cross-linked HA/PAH and HA/PLL capsules have smooth surface and a compact thin wall. They exhibit the typical folds and creases observed for synthetic polyelectrolyte multilayer capsules as well as biopolyelectrolytes capsules (Figure 4C,D). The swelling of capsule wall thus seems to be restricted. Of note, all the cross-linked capsules exhibited well-formed spherical shapes with a size of ∼5 μm in aqueous solution (see Figure 5B,D). Thus, the shrinkage of HA/PLL capsules could be prevented by cross-linking the multilayer assembly. One can also notice that cross-linked capsules have lower tendency to agglomerate (Figure 5B,D) than the un-cross-linked (Figure 5A,C).

By using AFM, the thickness of the dried polyelectrolyte shells could be estimated according to the method reported by Leporatti et al. They are reported in Table 1. It can be noticed that the thickness values are in the same order of magnitude of those measured by ellipsometry on planar films in the dry state.57,58 Of note, higher thickness values were obtained in the wet state by QCM-D on planar films, which is consistent with the fact the films are hydrated due to the presence of HA.

The permeability of the un-cross-linked and cross-linked capsules toward hydrophilic dextrans was next investigated
dextranFITC-4 solution (2 g/L in 0.02 M MES buffer, pH 6.5). The two small molecules investigated, DextranFITC-4, were only permeable for the lowest concentration of EDC used (50 mM) while they were impermeable for the highest ones (200 and 400 mM). Of note, at a low concentration of coupling agent (50 mM), the diffusion of dextranFITC-4 molecules was totally impermeable.

Furthermore, we studied the effect of the cross-linker concentration on the shell permeability for dextran molecules of different molar masses (4 × 10^3, 500 × 10^3, 2000 × 10^3 g/mol; Figure 6). Interestingly, the permeability of HA/PAH and HA/PLL cross-linked capsules toward dextranFITC-500 and dextranFITC-2000 remains very low (equal or near to zero) for all cross-linked capsules incubated in MES buffer without NaCl. In fact, only the smallest molecule investigated, DextranFITC-4, partially diffused into the capsules interior, with permeability values depending on the amount of EDC used. The capsules were only permeable for the lowest concentration of EDC used (50 mM) while they were impermeable for the highest ones (200 and 400 mM). Of note, at a low concentration of coupling agent (50 mM), the diffusion of dextranFITC-4 molecules was higher in the case of HA/PLL (32%) than HA/PAH capsules (∼25%).

Also and interestingly, the cross-linked capsules containing closed pores in 0.02 M MES buffer were found to open pores by increasing salt concentration (0.15 M NaCl) in the surrounding medium. This dramatically increased diffusion of molecules into capsules, especially that of dextranFITC-4. As previously reported,31 treatment of a HA/PAH film on a planar surface by EDC at a concentration of 200 mM leads to a cross-linking degree of 0.35. This indicates that many charged groups may be still involved in electrostatic interactions within the multilayer wall. This salt effect can be thus related to a weakening of the electrostatic binding between the oppositely charged polyelectrolytes.53,54

Thus, based on these data, it seems possible to tune the permeability of the capsules by shell cross-linking. Cross-linking endows the capsules with a stronger ability to reduce the extent of capsule wall swelling. Our results are in line with others from the literature. A reduction of permeability in the shells after cross-linking was observed for the (PAH/PSS)5 capsules which in the native form were permeable for dextran at Mw 460 × 10^3 g/mol and became impermeable after treatment with glutaraldehyde as a cross-linking agent.59 Cross-linking of alginate in the (alginate/chitosan)3 capsule shells with calcium ions decreased the insulin release rate.55 It was also shown that the drug release could be tailored by the cross-linking density of dextran-based multilayer capsules prepared by click chemistry.56

### Table 1. Comparison of the Thickness in the Dry State of Cross-Linked Capsules and Planar Films Prepared from HA/PAH and HA/PLL

<table>
<thead>
<tr>
<th>Multilayer</th>
<th>Capsules* in the dry state</th>
<th>Planar films in the dry state</th>
<th>Planar films in the wet state</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA/PAH</td>
<td>(50 ± 13) nm (4.5 bilayers)</td>
<td>~40 nm* (5 bilayers)</td>
<td>~140 nm</td>
</tr>
<tr>
<td>HA/PLL</td>
<td>(26 ± 5) nm (5 bilayers)</td>
<td>~20 nm*</td>
<td>~100 nm</td>
</tr>
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*Thicknesses were measured by AFM. b Thicknesses were measured by ellipsometry. c Thicknesses were measured by QCM-D.

(Figure 5). These experiments revealed that shell cross-linking has a drastic effect on the capsule’s permeability. In fact, the diffusion of a low molar mass dextranFITC was very high inside the un-cross-linked capsules, while the cross-linked ones (prepared using an EDC concentration of 200 mM) were almost totally impermeable.

Figure 5. Effect of shell cross-linking on the morphology and permeability toward dextranFITC-4 of (HA/PAH)4.5 and (HA/PLL)4.5 capsules. Top: CLSM images of un-cross-linked (A,C) and cross-linked (B,D) (HA/PAH)4.5 and (HA/PLL)4.5 capsules incubated in dextranFITC-4 solution (2 g/L in 0.02 M MES buffer, pH 6.5). The two left images corresponding to the HA/PAH capsules and the two right images, to the HA/PLL capsules. Bottom: comparison of the permeability properties of (HA/PAH)4.5 and (HA/PLL)4.5 capsules in 0.02 M MES buffer (pH 6.5). Permeability is expressed as the ratio of fluorescence intensities of the capsules interior (Iint) and surrounding solution (Iext) 20 min after mixing capsules and solutions of dextranFITC-4. Cross-linking was performed using EDC at a concentration of 200 mM. The average value was taken from 10 capsules.

Figure 6. Ratio of the fluorescence intensities of the capsules interior (Iint) and surrounding solution (Iext) 20 min after mixing capsules and solutions containing dextranFITC with different molar masses (4 × 10^3 g/mol (○), 500 × 10^3 g/mol (□), and 2000 × 10^3 g/mol (▲)) for HA/PLL (A) and HA/PAH (B) capsules at different cross-linker concentration. Experiments were performed either without NaCl (open symbols) or with 0.15 M NaCl (filled symbols) in a buffer (0.02 M MES buffer, pH 6.5).

### 3. Enzyme-Responsive Shell Permeability.

Owing to the potential applications of HA capsules as drug delivery systems,
we investigated their degradation by a tissue enzyme. Cross-linked and un-cross-linked capsules were thus incubated at 37 °C in the presence of testicular hyaluronidase (Hase) at concentrations in the range of 10 to 500 U/mL in MES buffer (Figure 7). To exclude a possible effect of the temperature jump from 25 to 37 °C, we verified that it did not affect that wall permeability (Figure 7D). The contact with Hase at concentrations of 10 and 50 U/mL did not lead to noticeable changes of HA capsules (data not shown). In the presence of 500 U/mL Hase, only the un-cross-linked HA/PLL capsules with initial shrunken morphology lose their structural stability; the shell were disintegrated (Figure 7B). Increase in permeability of HA/PLL capsules can be related to more “porous” structure of these capsules allowing the enzyme to diffuse deeper into polymer complex/shell, resulting in a higher degradation of the multilayer. This higher sensitivity of the HA/PLL capsules may be due to weaker interactions between the polyelectrolytes compared to the HA/PAH systems. Moreover, it can be noted that the concentration used for HA/PLL degradation is in the same range of magnitude of concentrations required for the degradation of planar multilayer films and gels. Degradation of cross-linked capsules evidenced in Figure 7D shows that permeability of HA/PAH shells does not change after contact with enzyme contrary to the HA/PLL capsules of which permeability increased ∼10 folds. However, the cross-linked capsules did not change their spherical shape after contact with enzyme (Figure 7C). These results may suggest that the shells are only partially degraded due to the presence of covalent bonds between polyelectrolyte partners which hinder accessibility to glycosidic bonds. In a recent work, Lee et al. found that BSA is released faster from cross-linked HA/PLL as soon as Hase concentration is equal to or greater than 10 U/mL. However, these capsules, having a diameter of ∼16 µm, were prepared under different conditions from a HA sample of $M_w 64 \times 10^3$ g/mol and were cross-linked with EDC only, a reaction which is much less efficient than in the presence of sulfo-NHS. It can be noted that the Hase concentration in the human serum is of the order of 2.6 U/mL. However, the physiological

Figure 7. Effect of hyaluronidase on the permeability toward dextranFITC-4 of (HA/PAH)$_{4.5}$ and (HA/PLL)$_{4.5}$ un-cross-linked and cross-linked capsules (200 mM EDC). Top: transmission images of un-cross-linked (A, B) and cross-linked (C) (HA/PLL)$_{4.5}$ microcapsules in the absence (A) and in the presence of hyaluronidase (B, C) at a concentration of 500 U/mL. In both cases, capsules were left overnight at 37 °C in 0.02 M MES buffer. Bottom: ratio of the fluorescence intensities of the capsules interior ($I_0$) and surrounding solution ($I_{eq}$) 20 min after mixing cross-linked capsules and MES buffer containing dextranFITC-4.

Figure 8. Confocal microscopy images of (A) cross-linked HA/PAH and (B) cross-linked HA/PLL capsules after 2 h coincubation with RAW mouse macrophages. Capsules are stained green fluorescent using HA FITC, 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. Colocalization between the green and red channel is observed as a yellow/orange color.
concentrations of Hase depend on the location in the body and other factors such as thermal degradation and attack by free radicals are also responsible for the degradation of hyaluronic acid. The greater resistance of our HA-based capsules to the enzymatic hydrolysis could be due to the formation of a compact network with a lower permeability to enzymes as suggested for chemically cross-linked HA hydrogels. The accessibility of HA in the multilayer assembly may thus depend on the molar mass of HA chains and on the cross-linking extent. This assumption regarding the influence of the molar mass is supported by degradation studies performed on un-cross-linked HA/CHI planar films, which showed that assemblies made of CHI with \( M_w = 100 \times 10^3 \) g/mol are more resistant to degradation by Hase (500 U/mL) than those containing CHI with \( M_w = 5 \times 10^3 \) g/mol.

This work thus demonstrated that the stability and permeability properties of the HA capsules can be tuned by controlled chemical modifications of the shell.

4. In Vitro Capsule–Cell Interactions. To assess whether the cross-linked HA-based capsules can be taken up by phagocytizing cells, RAW mouse macrophages were cultured for 2 h in the presence of capsules and observed by confocal microscopy imaging. For visualization purpose and to assess the microcapsules intracellular fate, the capsules were stained using HA\( ^{F I T C} \) (green fluorescence) while the cellular lysosomes were stained with LysoTracker Red (a fluorescent dye emitting red fluorescence, which selectively accumulates in acidic cellular vesicles). As depicted in Figure 8, both HA/PAH and HA/PLL capsules are intact while being outside cells and become largely deformed and break-up when internalized within the cells.

Moreover, for all phagocyted capsules, complete colocalization between green capsule fluorescence and red lysosomal fluorescence is observed, indicating that the capsules end-up in lysosomal vesicles. This is in accordance with previous reports by the Parak and De Geest groups. However, the deformation kinetics of the internalized capsules (few hours) is remarkably faster compared to earlier reported capsules. Bedard et al. studied the deformation upon cellular uptake of synthetic nondegradable PSS/PDADMAC capsules. It was shown that, after 15 h, capsules deformation occurred, however the capsules did not break. De Geest and De Koker investigated intracellular degradation in vitro and in vivo of capsules based on dextran sulfate and poly(L-arginine), but in contrast to the HA-based capsules in this paper it took more than 24 h before capsule deformation and degradation occurred. The fast capsule break-up observed in our present study clearly offers perspective to use the capsules as an intracellular delivery carrier that stably encapsulates its payload in the extracellular space but which readily opens upon cellular uptake.

Conclusions

In this work, we designed fully biodegradable capsules based on hyaluronic acid and poly(L-lysine). By investigating the morphology and permeability properties of capsules, we assessed which parameters are important to obtain capsules that are stable under physiological conditions.

Contrary to capsules containing the synthetic polycation PAH, capsules containing PLL exhibited strong shrinkage after core dissolution. By chemically cross-linking the shell before template dissolution, we could prevent this shrinkage and stable capsules with limited permeability to relatively small hydrophilic molecules (dextran\( ^{F I T C} \)) were obtained. Regardless of the chemical cross-linking, the HA/PLL capsules were still responsive to a tissue enzyme, hyaluronidase, showing higher permeability toward dextran\( ^{F I T C} \). Such a behavior suggests potential to control drug release from these capsules through a biodegradation process. Finally we demonstrated that both HA/PLL as well as HA/PAH capsules become rapidly (i.e., within 2 h) internalized in endo/lysosomal vesicles upon incubation with in vitro cultured macrophages. Subsequently, we observed a remarkably fast intracellular rupturing of the capsules, while extracellular capsules remained intact. Such fast intracellular opening of capsules without external triggering has, to the best of our knowledge, not yet been reported and could offer a distinct advantage for the delivery of, for example, protein antigen, where the encapsulated payload becomes readily available for processing in an early stage of endo/lysosomal acidification.

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Supporting Information Available. Thermograms obtained from the calorimetric titration of PLL and PAH with HA, FTIR-ATR spectra of (HA/PLL)\(_{4.5}\) and (HA/PAH)\(_{4.5}\) hollow dried capsules without cross-linking and cross-linked with 50, 200, and 400 mM EDC. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes
