

Formation and Degradation of Layer-by-Layer Assembled Polyelectrolyte Polyrotaxane Capsules

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ABSTRACT: We report the preparation of degradable capsules via layer-by-layer assembly using polyelectrolyte (PE) polyrotaxanes (PRXs). The PRX capsules were prepared by the sequential deposition of PRXs onto silica particles followed by dissolution of the silica cores. The colloidal stability of the PRX capsules formed depends on the salt/buffer solution used in the assembly process. Various salt/buffer combinations were examined to avoid aggregation of the core-shell particles during PRX assembly and core dissolution. Using appropriate assembly conditions, colloidally stable and robust capsules were prepared. PRX capsules consisting of eight layers of PE PRXs had a wall thickness of ~15 nm. Degradation of the PRX capsules was demonstrated through disassembly of the PE PRXs using glutathione, which cleaves the disulfide bonds linking the end capping groups of the PE PRXs. Given the supramolecular non-covalent structure of PRXs and their adjustable properties, it is expected that PRXs will be used as building blocks for assembling advanced capsules with unique and tailored properties.

Introduction

For a number of biological applications, including drug delivery, gene therapy, and microreactors, polymeric capsules are of high importance. The layer-by-layer (LbL) assembly technique makes it possible to engineer capsules with high precision,¹⁻⁶ allowing the tuning of structural characteristics such as porosity, hydrophobicity, and cargo release properties. These functionalities are key characteristics for the successful delivery of therapeutics and need to be specifically engineered. In this regard, the molecular building blocks that constitute the capsules play an important role, as changing the components can lead to capsules with vastly different properties. A variety of molecular building blocks, including DNA,^{1,2,5,6} peptides,^{1-4,6} and various polymers have been used for the preparation of capsules with a range of properties. The use of α -cyclodextrin (α CD)-poly(ethylene glycol) (PEG) polyrotaxanes⁷⁻⁹ (herein referred to as PRXs) as macromolecular building blocks for the preparation of LbL capsules has not been reported yet. The formation of capsules via the LbL assembly of PRXs is of interest since they exhibit a number of unique and tunable properties¹⁰⁻¹² that originate mostly from their non-covalent nature (vide supra).

PRXs are formed via a self-assembly (threading) process in water followed by capping of the PEG end groups with bulky moieties. This process allows for relatively easy control over their supramolecular structure, and structural properties such as their size and rigidity. Their size can be tuned by adjusting the PEG length and their rigidity by varying the threading degree.¹³⁻¹⁵ The non-covalent nature of the PRX structure endows them with disassembly properties.¹⁶⁻²¹ Disassembly can be initiated by uncapping the PRXs so that the α CDs will dethread. A number of methods have been used to cleave end-capping groups and to effect disassembly, including reducing conditions,¹⁶⁻¹⁸ pH changes,¹⁹ enzymatic conditions,²⁰ and UV light.²¹ The threaded α CDs in PRXs can be readily postmodified to introduce additional functionalities such as

charged groups,^{16,22,23} targeting ligands,^{24,25} and for loading drugs.^{26,27} Inspired by such opportunities, we focused our research on the development of responsive nanostructures based on PRX building blocks. In our recent study, we reported the LbL formation of multilayers of polyelectrolyte PRXs of opposite charge on planar supports and showed that degradation of the films was initiated by disassembly of the PRXs via disulfide bond cleavage.¹⁶

In this article we report the formation of LbL-assembled degradable capsules consisting solely of PRXs and examine a range of different salt/buffer combinations to obtain colloiddally stable PRX multilayer-coated particles and hence PRX capsules. It should be emphasized that our aim, at this time, is not the in-depth study of salt and/or buffer effects on the layering properties of the formed capsules other than to achieve the aim of this research. Positively charged PRX-dimethylethylenediamine (PRX-DMEDA), containing on average 2.4 tertiary amine groups per α CD, and negatively charged PRX-glycine (PRX-Gly), containing 1.7 carboxylate groups per threaded α CD (Supporting Information, Figure S1),¹⁶ were used as the molecular building blocks. These PRXs are rigid; however, the charged α CDs retain some mobility, which results from their non-covalent nature. This endows them with the ability to slide across the PEG chain and to rotate about their axis. This constitutes a major difference in comparison to conventional PEs and could be a contributing factor to spatial matching of multivalent interactions with oppositely charged PEs, thus leading to differences in adsorption behavior and physicochemical properties (e.g., permeability) of the capsules. The PRX LbL capsules can be degraded by disassembly of the PRXs, which can be initiated by the reductive cleavage of the PRX capping groups with glutathione (GSH). The main degradation products of PRX structures are linear PEG²⁸ chains and the relatively small α CDs,^{29,30} both of which show excellent biocompatibility. Furthermore, drugs that are conjugated to threaded α CDs can be released following degradation of PRX-based materials, as demonstrated by our recent study on PRX

capsules formed by grafting PRXs, containing alkyne groups at their ends, to azide-functionalized silica particles using copper catalyzed ‘click’ chemistry.³¹ The current study utilizes oppositely charged polyelectrolytes, allowing the assembly of PRX layers through LbL assembly, which provides control over the thickness, composition, and ultimately the capsule properties. These characteristics can potentially lead to capsules with substantial differences in terms of their responsiveness, functionality, and applicability and constitute important advantages for the construction of PRX-containing materials for drug delivery.

Experimental Section

Materials. SiO₂ particles were purchased from MicroParticles GmbH as a 50 mg mL⁻¹ dispersion in water and were used as received. *N,N'*-Disuccinimidyl carbonate (diNHS) and GSH were obtained from Sigma-Aldrich. Alexa Fluor 488 (AF488) and Alexa Fluor 633 (AF633) dyes were purchased from Invitrogen. High-purity water with a resistivity greater than 18 MΩ cm was obtained from an in-line Millipore RiOs/Origin water purification system.

Methods. The capsules were imaged on an Olympus IX 71 inverted fluorescence microscope using a fluorescein isothiocyanate (FITC) filter. The sizes of the capsules were estimated using ImagePro software. The ζ-potentials were measured on a Zetasizer 2000 (Malvern) instrument. The quoted values were calculated by taking the average of five successive measurements. The pH of solutions was measured with a Mettler-Toledo MP220 pH meter. Atomic Force Microscopy (AFM) images were obtained with an MFP-3D Asylum Research instrument. Typical AFM scans were conducted in AC mode with ultrasharp SiN gold-coated cantilevers (NT-MDT). Samples were prepared by dropping 1 μL of a concentrated capsule solution on a freshly cleaned silicon wafer slide followed by drying at 24 °C before the measurements commenced. Transmission electron microscopy (TEM) images were obtained with a FEI Tecnai F30 microscope operated at 200 kV. TEM samples were prepared by dropping 1 μL of a con-

centrated capsule solution on carbon-coated 300-mesh copper grids (ProSciTech, Australia) followed by drying at 24 °C before measurement.

Preparation of Charged PRXs. PRX-DMEDA and PRX-Gly were synthesized according to the method described in literature.¹⁶ Briefly, PRXs were formed by threading multiple α CDs on bis(*o*-pyridyldisulfide)-PEG (M_w 3400) followed by reaction with a thiol containing capping group in DMF. The PRXs were functionalized with charged groups by activating the hydroxy groups on the threaded α CDs with diNHS followed by reaction with either *N,N*-dimethylaminoethylene for PRX-DMEDA or glycine for PRX-Gly.

Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D). QCM-D measurements (Q-sense E4, Sweden) were used to determine the adsorption and desorption characteristics of PRX-DMEDA and PRX-Gly. The silica-coated crystals (QSX 300, Q-sense) were soaked in 2% sodium dodecylsulfate overnight, washed extensively with water, dried, and placed in a UV cleaner (Bioforce Nanosciences, USA) for 30 min. Immediately after cleaning, the crystals were mounted into the liquid-exchange chambers of the instrument and a baseline in the buffer solution was established. For all experiments a flow rate of 0.2 mL min⁻¹ was used. The temperature was kept at 24 ± 0.02 °C throughout all of the experiments. A PRX-DMEDA solution (0.2 mL, 1 mg mL⁻¹) in buffer was used to assemble the initial layer. After the surface was saturated, the chambers were washed with buffer solution. A PRX-Gly solution (0.2 mL, 1 mg mL⁻¹) in buffer was then injected into the chamber for adsorption. When the surface was saturated, the chambers were washed with buffer solution. After each adsorption and rinsing step with buffer the system was allowed to come to equilibration, that is, close to a plateau in f and D signals. PRX layering was continued by the alternate adsorption of PRX-DMEDA and PRX-Gly until the required number of layers was obtained. Following this, GSH (0.005 M) in phosphate buff-

ered saline (PBS) was injected into the chamber at a flow rate of 0.02 mL min^{-1} and degradation of the layers was monitored. Normalized frequencies using the third overtone are presented.

Assembly of PRX-DMEDA and PRX-Gly Capsules. Capsules were formed by the sequential adsorption of charged PRXs on SiO_2 microparticle templates ($d = 2.5\text{-}3.5 \text{ }\mu\text{m}$). The layering procedure was commenced with a dispersion of $400 \text{ }\mu\text{L}$ (50 mg mL^{-1}) of SiO_2 -particles in water, which was combined with an equal volume of 1 mg mL^{-1} PRX-DMEDA in buffer followed by constant shaking for 15 min to allow adsorption to take place. Three washing cycles were applied following the adsorption of each layer to remove excess PRX. Washing cycles consisted of centrifugation of the layered particles at $2000 g$ for 30 s, at which time the supernatant was removed and the particles dispersed in an equal volume of buffer by vortex shaking for 30 s.

Following PRX layering, poly(vinyl pyrrolidone) (PVP) (M_w 10 kD, 10 mg mL^{-1} in 0.05 M acetate buffer at pH 4) and poly(methacrylic acid) (PMA, M_w 15 kD, 1 mg mL^{-1} in 0.05 M acetate buffer at pH 4) were adsorbed. The above-described adsorption and washing procedures were used.

After completion of the layering process the template silica cores were dissolved by treatment with a $2.5 \text{ M HF}/6.7 \text{ M NH}_4\text{F}$ (1:1) solution at $24 \text{ }^\circ\text{C}$ for 5 min, followed by multiple centrifugation ($60 g$, 15 min)/buffer washing cycles. The washing cycles were repeated until the pH of the capsule suspension was identical to the pH of the fresh washing buffer.

Capsule Degradation. The stability and degradation of a dispersion of $\sim 10^8$ capsules labeled with AF488 dye in PBS was monitored in the presence or absence of 0.005 M GSH at $37 \text{ }^\circ\text{C}$. At specified time intervals, the number of capsules was quantified using flow cytometry. A Cyflow Space (Partec GmbH, Germany) flow cytometer (excitation wavelengths of 488 nm) was used

to measure the number of capsules. These experiments were performed in triplicate. Data were analyzed using Partec CyflowMax software.

Results and Discussion

The PE PRXs were prepared by conjugating charged groups to the threaded α CDs of the PRXs (Figure S2).¹⁶ PRX capsules were prepared by the sequential adsorption of positively charged PRX-DMEDA and negatively charged PRX-Gly on silica template particles of 2.5-3.5 μm in diameter in PBS (Figure 1), followed by dissolution of the template cores. The free OH functionalities present on the threaded α CDs (each α CD contains in total 18 OH groups) were used to conjugate fluorescent dyes to visualize the capsules formed. Conjugation involves the activation of carboxylic acid groups of the negatively charged layer (PRX-Gly) with diNHS, followed by the addition of an amine-functionalized dye. Conjugation was performed after the adsorption of a positively charged layer, as labeling with a negatively charged outer layer resulted in desorption of this layer. This is the result of the positively charged intermediate that is formed after activation of the OH groups with diNHS.

A minimum of five PE PRX layers was necessary for the successful formation of capsules. These capsules shrink ($d = 1.88 \pm 0.18 \mu\text{m}$) about 38% relative to the size of the particles (Figure S1). However, capsules formed by dissolution of the cores after eight layers only shrink by 14% ($d = 2.60 \pm 0.19 \mu\text{m}$). The layered particles were found to be colloidally stable in PBS; however, the formed capsules were not. A majority of the capsules aggregated directly after dissolving the cores with buffered HF. It was found that capsules formed from template-multilayer systems having an outer layer consisting of negatively charged PRX-Gly were generally less prone to aggregation (ζ -potential = -48 mV, Table S1). However, we found that capsules prepared in PBS were not robust enough to withstand the centrifugation steps in the

washing procedures, resulting in additional aggregation and collapse or deformation of a significant number of the capsules. A contributing factor to this instability is the pH of the HF buffer solution (pH=5). Protonation of the outer PRX-Gly layer of the capsules at pH 5 removes most of the negative charges at the surface of the capsules, hence, reducing the electrostatic repulsion between particles and increasing their potential to form hydrogen bonds, which promotes aggregation. The concentration of salt present in the buffer solution is known to influence the multilayer properties in terms of architecture and rigidity by changing the amount of PE and coordinated water that is adsorbed onto a surface.³²

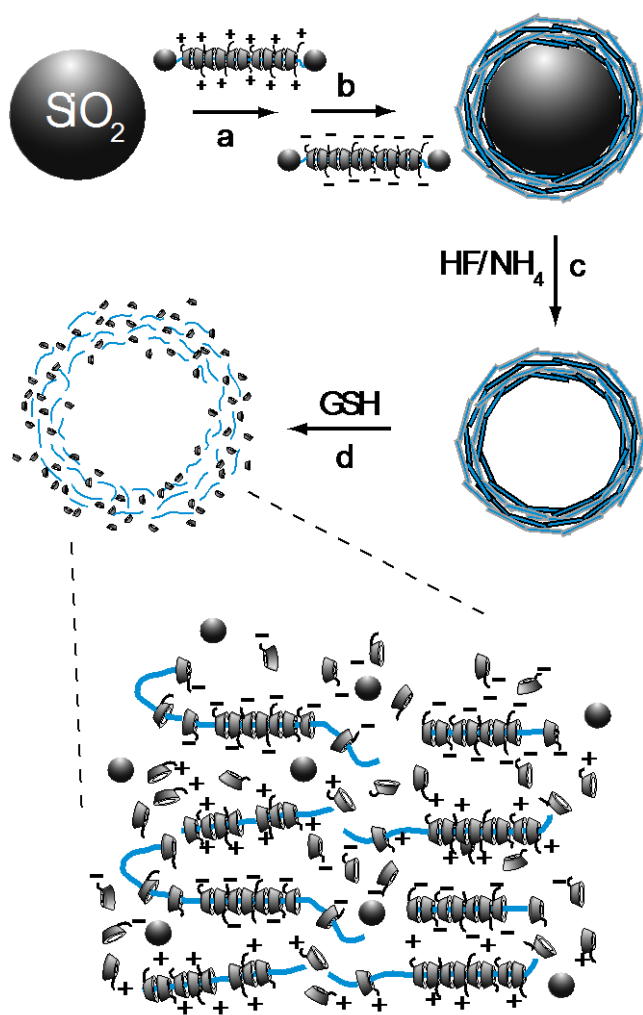


Figure 1. Formation of PRX capsules via the sequential deposition of positively charged PRXs (step **a**) and negatively charged PRXs (step **b**), followed by dissolution of the core with buffered HF (step **c**). The capsules can be

degraded by reductive cleavage of the end-capping groups using GSH (step **d**). Degradation results from dethreading of the α CDs (bottom).

In an attempt to increase the robustness of the multilayer structure and avoid collapse or deformation of the capsules during the centrifugation process we examined the formation of multilayers on planar silica substrates and capsules in 0.01 M phosphate buffer (pH 7.4) containing different concentrations of NaCl. QCM-D confirmed the successful formation of multilayers in 0.01 M phosphate buffer in the presence of 0.14 M (i.e., PBS), 0.3 M, and 0.5 M NaCl on planar substrates (Figure S3). In the presence of 1 M NaCl only one PRX-DMEDA layer could be adsorbed (-18 Hz) and with 2 M NaCl no significant adsorption was observed. For all phosphate buffer solutions the value $(-\Delta f/n)$, in which Δf is the frequency difference and n is the overtone number, is frequency dependent and, therefore, the Sauerbrey relation is not valid and these films can be considered to be non-rigid.³³

The multilayer formation in 0.01 M phosphate buffer containing 0.5 M NaCl on planar surfaces resulted in a significantly increased amount of adsorbed material compared with the multilayers formed in the presence of 0.14 M NaCl. To examine whether the increase in adsorbed material results in improved capsule characteristics we examined the formation of capsules in 0.01 M phosphate buffer containing 0.5 M NaCl. In this buffer mixture the adsorption of four layers is sufficient to give a multilayer structure that is robust enough to allow the formation of capsules. The diameter of these capsules is $2.08 \pm 0.16 \mu\text{m}$, which is 37% smaller than the SiO_2 template particles ($d = 3.25 \pm 0.19 \mu\text{m}$). Capsules formed after the deposition of eight layers do not shrink ($d = 3.01 \pm 0.19 \mu\text{m}$, ζ -potential = -43 mV (Table S1)). However, the colloidal stability and robustness of the capsules formed at this salt concentration did not significantly increase.

As a means to improve these characteristics we examined multilayer formation in 0.01 M phosphate buffer in the presence of NaI or Na₂SO₄. The Hofmeister series arranges kosmotropic anions (i.e., SO₄²⁻), those that have a salting-out effect on aqueous solutions of polymers, to chaotropic salts (i.e., I⁻), which have a salting-in effect.^{34,35} It has been generally noted that anions have a more pronounced effect on the solution properties of polyelectrolytes compared with cations; for anions the Cl⁻ ion can be considered as a median. These anions have a strong influence on the adsorption characteristics of PEs, with the effects linked to conformational changes in the PEs induced by the anions³⁶ and differences in matching water affinity of PEs and counterions.³⁷ Characteristics such as thickness,^{38,39} growth profiles,^{39,40} and the stiffness of multilayers⁴¹ depend on the type of counter anion used during the layering process.

PRX multilayers could be successfully formed in 0.14 M NaI, 0.14 M Na₂SO₄ and in 0.5 M Na₂SO₄, all buffered with 0.01 M phosphate (pH 7.4) (Figure S4). In 0.5 M NaI the adsorption of a negatively charged second layer was not possible. It can be observed that the total shift in Δf after the deposition of eight layers is significantly higher for the chaotropic I⁻ (-780 Hz, $\Delta D = 40$) ions than when using Cl⁻ (-310 Hz, $\Delta D = 7.5$). This is a similar trend to that observed by Liu et al. for the assembly of poly(diallyldimethylammonium)/poly(4-styrene sulfonate) (PDDA/PSS) multilayers.³⁹ The Δf (848 Hz) and ΔD (63) for the assembly of eight layers of PE PRXs in the presence of SO₄²⁻ both increase, as compared to the Cl⁻ system at the same concentration or ionic strength of 0.3 M NaCl (-612 Hz) (Figure S4). It is, however, unclear whether the contribution of water is significant in the total mass adsorbed since we were unable to obtain a reliable dissipation signal for the multilayer formation in 0.3 M NaCl despite multiple measurements.

The formation of capsules in the presence of chaotropic or kosmotropic anions was also successful. That is capsules consisting of eight layers of PE PRXs assembled from 0.01 M phos-

phate buffer and 0.14 M NaI ($d = 3.65 \pm 0.19 \mu\text{m}$), or 0.14 M Na₂SO₄ ($d = 3.44 \pm 0.19 \mu\text{m}$), or 0.5 M Na₂SO₄ ($d = 3.35 \pm 0.17 \mu\text{m}$) (template particle $d = 3.25 \pm 0.19 \mu\text{m}$). The usage of either the chaotropic I⁻ or kosmotropic SO₄²⁻ anion at these concentrations resulted in the formation of structurally stable capsules, i.e., centrifugation did not destroy these capsules. Also their colloidal stability is increased as compared to the capsules prepared in phosphate buffer and NaCl. Furthermore, we found that changing the buffer from phosphate to 2-(*N*-morpholino)ethanesulfonic acid (MES), in the presence of 0.14 or 0.2 M Na₂SO₄, results in improved colloidal stability of the PRX capsules. A fraction (~20%) of the formed capsules aggregated; this number includes dimeric capsules as well and is less than the extent of aggregation that was present in the previously prepared batches of capsules. However, we sought a method to improve the colloidal stability of the PRX capsules in buffered HF, as this is the buffer/salt combination where aggregation occurs. The colloidal stability was improved by overcoating the capsules that were prepared in MES buffer containing Na₂SO₄ with a stabilizing outer PVP/PMA bilayer. Our previous work showed that capsules consisting of PVP and PMA are colloiddally stable under the conditions of template core removal.⁴² The PVP-PMA bond formation is largely based on H-bonding and can be disrupted by deprotonating PMA at pH >7. Therefore, it can be expected that PVP will also bind to the carboxylic acid moieties of the outer PRX-Gly layer and is removed upon increasing the pH. The adsorption of PVP and PMA was performed in 0.05 M acetate buffer at pH 4. After dissolving the core template particles with HF, the pH of the capsule dispersion was gradually increased to neutral (through several washing steps), upon which the PVP and PMA layers were removed, yielding capsules consisting solely of PRXs. The removal of the PVP and PMA layers was verified by using a terminally AF488 dye labeled PVP and monitoring the fluorescence of the capsules formed, as has been described previously.⁴² Capsules formed via this approach are colloiddally stable and are struc-

turally robust with a uniform spherical morphology ($d = 2.84 \pm 0.14 \mu\text{m}$, template $d = 2.76 \pm 0.17 \mu\text{m}$) (Figure 2).

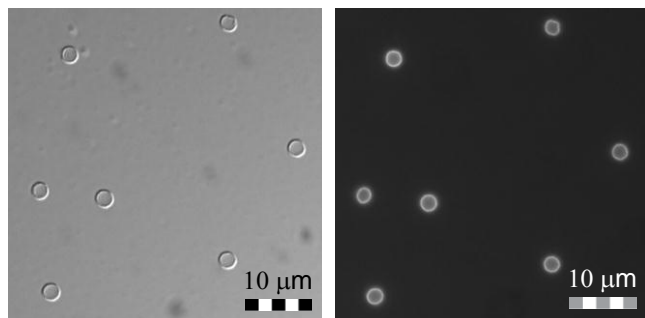


Figure 2. Fluorescence microscopy images of capsules ($d = 2.84 \pm 0.14 \mu\text{m}$) consisting of (PRX-DMEDA/PRX-Gly)₄ assembled in 0.01 M MES buffer and 0.2 M Na₂SO₄ at pH 6.8 using SiO₂ particle templates with $d = 2.76 \pm 0.17 \mu\text{m}$. The capsules were labeled with Alexa Fluor 633 dye. a) Bright field image of capsules. b) Fluorescence microscopy image of capsules.

These capsules were imaged using AFM and TEM by adsorbing them on a planar substrate followed by air-drying. This results in collapse of the capsules (Figure 3). The size of the air-dried capsules, as determined by AFM and TEM, are considerably smaller ($d \sim 2 \mu\text{m}$) than the size of capsules in solution ($d = 2.84 \pm 0.14 \mu\text{m}$). By taking the minimum height thickness of the imaged capsules and dividing this by two, an approximate thickness of 15 nm was obtained for the capsule wall. This corresponds to a thickness of about 1.9 nm per layer. It is likely that neighboring layers interpenetrate, as the thickness of a single PE PRX is $\approx 2.8 \text{ nm}$ when fully extended charged residues are included.

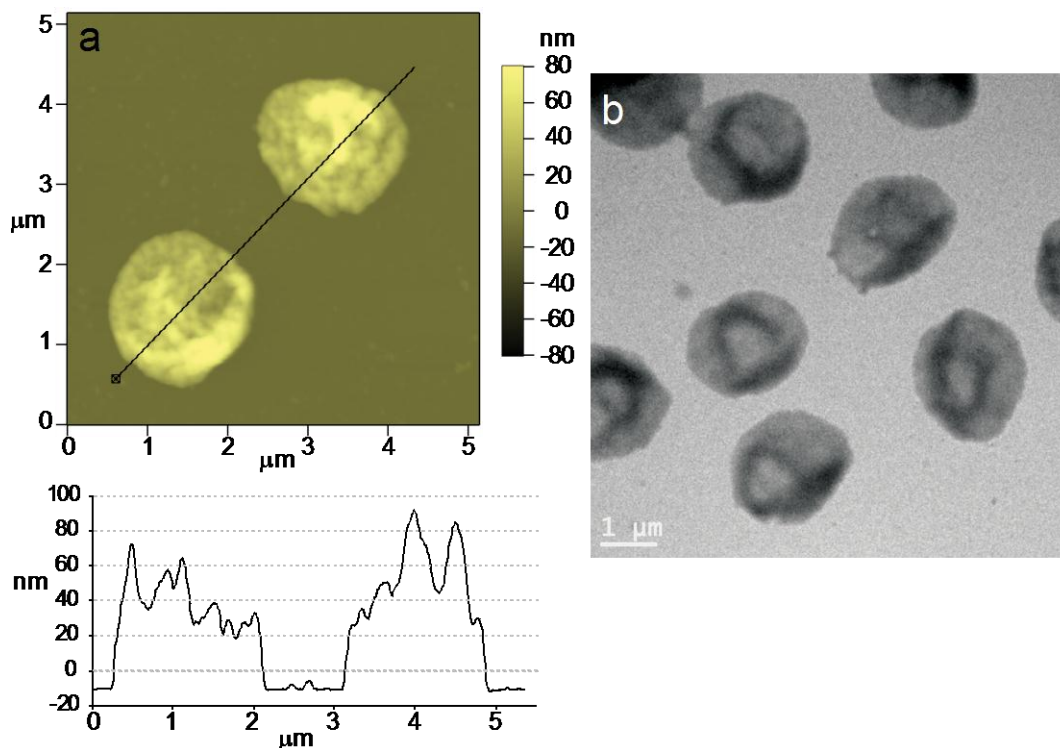


Figure 3. Capsules consisting of (PRX-DMEDA/PRX-Gly)₄, assembled in 0.01 M MES and 0.2 M Na₂SO₄ at pH 6.8 using SiO₂ particle templates with $d = 2.76 \pm 0.17 \mu\text{m}$. a) AFM images (top) of air-dried capsules adsorbed on silica and the corresponding height profile (bottom). b) TEM images of air-dried capsules.

Degradation of PRX Multilayers and Capsules

Capsule degradation under conditions that simulate the intracellular environment is an important consideration for drug delivery applications. Blood is slightly oxidative; however, the cytoplasm is reductive, which provides a means for the specific degradation of the PRX capsules within cells. Capsules that degrade as result of disulfide bond cleavage have been reported previously.⁴³ Our group has described the preparation of degradable capsules consisting of thiol-modified PMA that are stabilized by intermolecular disulfide cross-linkages.⁴² Also, we have prepared capsules that are stabilized by post cross-linking the layered polymers with disulfide containing cross-linkers.⁴⁴ Both of these types of capsules were found to be degradable via reduction of the disulfide bonds in a physiological concentration of GSH (5 mM). Other

groups have used the cleavage of intermolecular disulfide linkages between cysteine containing peptides as a means for degrading LbL capsules.⁴⁵

Reduction of the disulfide linkers that connect the capping groups leads to PE PRX disassembly and degradation of the capsules. Previously, we have shown that PRX multilayers assembled in PBS could be degraded by reductive cleavage of the disulfide linkers using 0.005 M GSH in PBS.¹⁶ The degradation profiles of multilayers consisting of eight layers formed in 0.01 M phosphate buffer and salt were followed using QCM (Figure S5). The multilayer degradation profiles are non-linear with rapid initial degradation. Degradation of the multilayers constructed in 0.14 M Na₂SO₄ proceeded the fastest, within approximately 20 h.

As a result of the absence of a stabilizing template surface the rate of capsule degradation is expected to be faster than for planar multilayers. PE PRX capsules formed in 0.01 M MES and 0.2 M Na₂SO₄ consisting of eight layers labeled with AF488 were placed in a 0.005 M GSH solution in PBS (pH 7.4) at 37 °C. The fluorescence of the capsules was monitored as a function of time using flow cytometry. The control consisted of a sample without GSH. The fluorescence of these capsules showed a negligible change over time. From Figure 4 it can be observed that within 2 h more than 80% of the capsules degrade.

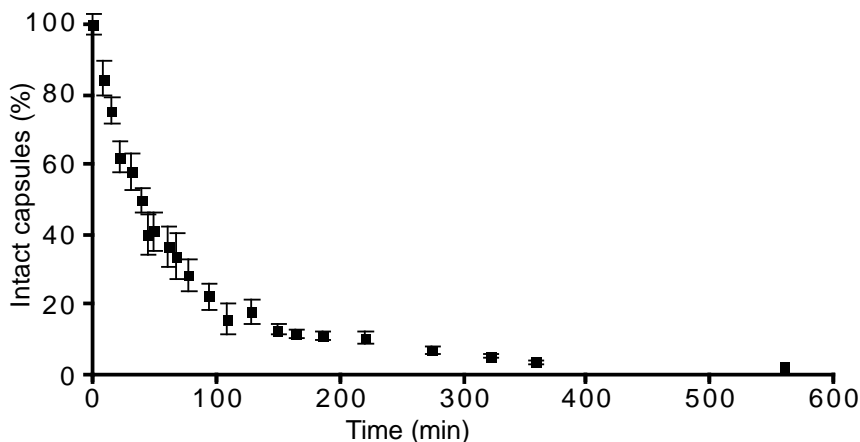


Figure 4. Degradation of capsules consisting of eight layers prepared in 0.01 M MES buffer and 0.2 M Na₂SO₄, monitored by flow cytometry (37 °C). The degradation was initiated by the addition of 0.005 M GSH to a dispersion of the capsules in PBS.

Conclusions

We have demonstrated the assembly of oppositely charged α CD-PEG PRXs for the formation of PRX capsules. The multilayer formation is determined by the type and concentration of anion used in the assembly process. We have shown that it is possible to prepare structurally robust and colloiddally stable capsules that consist solely of PRXs. The layered PRXs in these capsules were functionalized with dyes by conjugating them to the free OH groups of the threaded α CDs. The labeled capsules were also degraded under reducing conditions (GSH), with the main degradation products being α CDs and PEG chains – materials that have low toxicity. This, as well as the ability to conjugate various drugs to threaded α CDs, makes them of interest in biomedical applications.

ASSOCIATED CONTENT

Supporting Information. Fluorescence microscopy images of capsules, structures of the PRXs, QCM data for multilayer formation and degradation, and ζ -potentials of the PRX-coated silica particles. This information is available free of charge via the Internet at <http://pubs.acs.org>.

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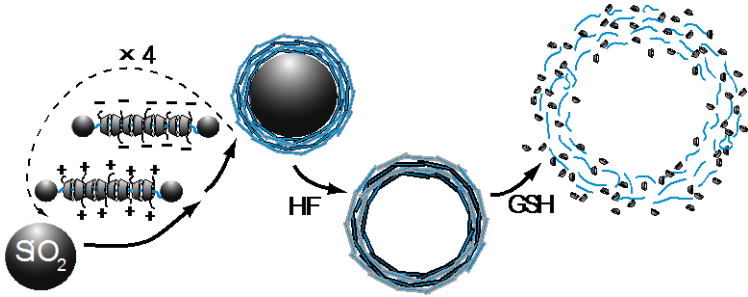
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