Disulfide Cross-Linked Polymer Capsules: En Route to Biodeconstructible Systems

Alexander N. Zelikin, John F. Quinn, and Frank Caruso*

Centre for Nanoscience and Nanotechnology, Department of Chemical and Biomolecular Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

Received November 1, 2005; Revised Manuscript Received November 24, 2005

Hydrogen-bonded multilayer thin films were constructed using poly(vinylpyrrolidone) and poly(methacrylic acid) functionalized with cysteamine. The resulting films included thiol moieties that were cross-linked to render the films stable at physiological pH. Film buildup was followed using quartz crystal microgravimetry, which was also used to demonstrate the improved stability imparted by reacting the thiol moieties to form disulfide bonds. Films without disulfide bonds were readily deconstructed at physiological pH, while those with disulfide bonds were swollen upon exposure to this pH (7) but remained intact. Addition of a common thiol−disulfide exchange reagent, dithiothreitol (DTT) at pH 7 led to disassembly of the multilayer films. The films were also prepared on colloidal substrates (as demonstrated using confocal microscopy) and were used to retain a model drug (fluorescently labeled transferrin) and release this molecule when triggered by the addition of DTT. This approach has potential for the in vivo applications of hollow capsules, as thiol−disulfide exchange leading to deconstruction of the capsules can occur with the assistance of intracellular proteins.

Introduction

Hollow polymer capsules prepared by layer-by-layer (LbL) templating of colloidal particles hold promise for diverse applications, including drug delivery, diagnostics, and microreactors.[1−3] These capsules can be engineered with respect to their size, shape, composition, and permeability. A key challenge for the application of LbL capsules is to stabilize them at physiological conditions while also being able to trigger capsule disassembly with an external stimulus or in an intracellular environment, releasing encapsulated substances.

The propensity of hydrogen-bonded multilayers to undergo rapid deconstruction at physiological pH[5] makes them ideal components for drug delivery vehicles. Several groups have reported that cross-linking hydrogen-bonded films containing polycarboxylic acids through amide chemistry endows the multilayers with improved stability at physiological pH.[6,7] We hypothesized that cross-linking of hydrogen-bonded multilayers could also be achieved via reversible thiol−disulfide chemistry, which has been shown to stabilize polypeptide capsules under highly acidic (nonphysiological) conditions.[8]

Herein, we report the preparation of capsules from hydrogen-bonded polymer multilayers that are cross-linked through disulfide (S−S) bonds. These capsules are *both* stable at physiological pH and amenable to deconstruction under reducing conditions or via thiol−disulfide exchange, such as those occurring within cells.[4] We employed a pair of polymers that form stable multilayers when alternately deposited at moderately acidic conditions, pH 4: poly(methacrylic acid) containing thiol moieties, PMA_{SH}, and poly(vinylpyrrolidone), PVPON (Figure 1A).[5,9] We also demonstrate that hydrogen-bonded, disulfide cross-linked capsules can be loaded with a protein (transferrin) that is retained at physiological conditions. This encapsulated transferrin is subsequently released by cleavage of the S−S bonds.

* fcaruso@unimelb.edu.au.

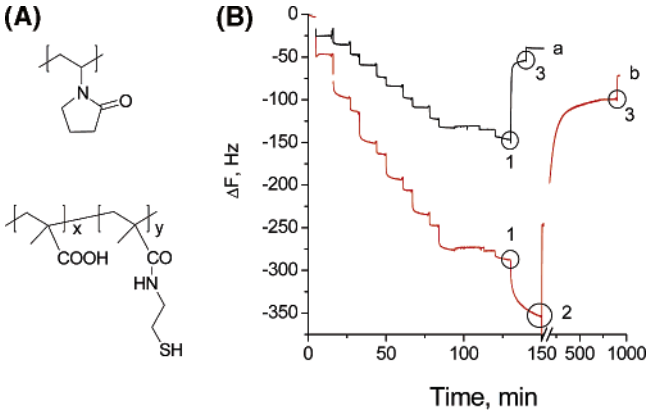

Figure 1. (A) Chemical structures of PVPON, PMA ($x = 100$ mol % and $y = 0$ mol %), and PMA_{SH} ($x = 82$ mol %, $y = 18$ mol %). (B) QCM ΔF vs time for the assembly of PMA/PVPON (a) and PMA_{SH}/PVPON (b) multilayers on PEI-modified gold QCMs. Frequency changes for treatment of the films with buffers at pH 7 (curves a and b, point 1), pH 4 (curves a and b, point 3), and 0.1 M DTT solution at pH 7 (curve b, point 2) are also shown.

Experimental Section

Materials. Poly(methacrylic acid, sodium salt) (PMA), $M_w = 15$ kDa, was purchased from Polysciences, and poly(vinylpyrrolidone) (PVPON), $M_w = 55$ kDa, was from Sigma-Aldrich. Cysteamine hydrochloride, dithiothreitol (DTT), *N*-hydroxysuccinimide (NHS), and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) were purchased from Sigma-Aldrich and used as received.

High-purity water with a resistivity greater than 18 MΩ cm was obtained from an in-line Millipore RiOs/Origin system. QCM electrodes (Q-Sense AB, Västra, Frölunda, Sweden) were cleaned four times with Piranha solution (70/30 v/v % sulfuric acid−hydrogen peroxide). [*Caution! Piranha solution is highly corrosive. Extreme care should be taken when handling Piranha solution, and only small quantities should be prepared.*] The pH of the buffer solutions was measured with a Mettler-Toledo MP220 pH meter.

10.1021/bm050832v CCC: $33.50
Published on Web 12/10/2005

Polymer Synthesis (PMA_{SH}). In a typical experiment, a PMA solution (250 mg of 30 wt % solution) was diluted into 5 mL of potassium phosphate buffer (0.1 M, pH 7.2). The resulting solution was charged with EDC (70 mg) and NHS (45 mg) and the mixture stirred for 15 min. After this time, cysteamine hydrochloride (7.5 mg, target modification = 20 mol %) preoxidized in air for several days was added to the mixture, and the reaction was allowed to proceed overnight. The resulting mixture was dialyzed extensively against distilled water, and the polymer was isolated via freeze-drying. The resulting solid was purified further by reprecipitation from water into dioxane. The degree of functionalization was estimated from elemental analysis of the polymer (C% 45.38, S% 3.35), which corresponds to 18.3 mol % modification.

Fluorescein Isothiocyanate (FITC)-Labeled Polymers. FITC (5 mg, 12.84 μmol) and butanediamine (11 μL of 1 M solution, 11 μmol) were combined in 200 μL dimethylsulfoxide (DMSO) and allowed to react for 2 h. In a separate vial, PMA (285 mg of 30 wt % solution), EDC (10 mg), and NHS (10 mg) were dissolved in 2.5 mL of phosphate buffer (0.1 M, pH 7.2) and allowed to react for 15 min. The two mixtures were then combined and allowed to react overnight. The resulting mixture was filtered through a 0.2 μm filter and extensively dialyzed against distilled water, monitoring the completion of dialysis via the UV−visible absorption of the outer solution. The final polymer was isolated by freeze-drying.

PMA_{SH} labeled with FITC was synthesized by adopting the two outlined protocols with the simultaneous addition of oxidized cysteamine and product of interaction between FITC and butanediamine. PMA and PMA_{SH} were labeled with FITC at the same fluorescent dye-to-polymer ratio.

Multilayer Preparation. A stock solution of PVPON (50 mg mL^{-1}) was prepared in 10 mM sodium acetate buffer (pH 4). The PMA and FITC−PMA stock solutions (10 mg mL^{-1}) were prepared in pH 3 water. The stock solution of PMA_{SH} (10 mg mL^{-1}) was prepared in 10 mM phosphate buffer (pH 8), charged with DTT (100 mg mL^{-1}), and left for at least 12 h to ensure separation of the polymer chains. All adsorption steps were conducted using 0.5 mg mL^{-1} polymer solutions prepared from the stock solutions, diluted with 10 mM sodium acetate buffer (pH 4).

Quartz Crystal Microgravimetry (QCM). QCM measurements were conducted using a Q-sense D300 device with a flow cell (Q-Sense AB, Västra Frölunda, Sweden). The temperature was kept constant at 23.4 °C throughout the experiments. A gold-coated 5 MHz AT-cut crystal was excited at its fifth overtone at ~25 MHz, and the change in the resonance frequency was recorded. The resulting frequencies were divided by 5 to be comparable to the results at the base frequency (5 MHz). After deposition of the initial layer of polyethyleneimine (1 mg mL^{-1} with 0.5 M NaCl added), the chamber was washed with 10 mM sodium acetate buffer (pH 4), after which the solution of PMA or PMA_{SH} was introduced into the chamber and allowed to adsorb for 10 min. After this, the chamber was washed again with 10 mM sodium acetate buffer (pH 4) and then filled with PVPON solution, allowing an adsorption time of 5 min. The multilayer film was then assembled by alternating between PMA or PMA_{SH} and PVPON adsorption.

Assembly on Colloidal Particles. In a typical experiment, 20 μL of a 5 wt % suspension of SiO_2 particles (diameter of 3 μm) (MicroParticles, GmbH) were dispersed in 80 μL of 10 mM sodium acetate buffer (pH 4.0). To this suspension was added 500 μL of the first adsorbing solution (PVPON), and adsorption was allowed to proceed for 15 min with constant shaking of the mixture. After this time, the particles were isolated by centrifugation (350 *g* for 1 min). 580 μL of supernatant was removed and replaced with fresh buffer, and the particles were redispersed and centrifuged again. The particles were then redispersed in 80 μL of the buffer, and adsorption of PMA_{SH} (or PMA) was then performed, followed by the same washing protocol. Multilayers were formed via the alternate adsorption of PVPON and PMA_{SH} (or PMA). No particle aggregation was observed at any point throughout the multilayer assembly, and only gentle vortexing was required to redisperse the particles. After completing the multilayer buildup, the particles were exposed to 10 mM hydrogen peroxide in 10 mM sodium acetate buffer at pH 4. To form hollow capsules, the silica core was dissolved by adding 5 M HF solution at 20 °C for 5 min, followed by three centrifugation (2000 g for 10 min)/buffer washing cycles. The multilayers were assembled on the FITC−transferrin loaded particles as described above, substituting bimodal mesoporous SiO_2 particles with preadsorbed labeled protein[10] in place of conventional SiO_2 particles.

Fluorescence Imaging and Quantification. The particles were imaged on an Olympus IX 71 inverted fluorescence microscope using a FITC filter cube. To quantify the fluorescence of the particles, a Leica TCS SP2 AOBS confocal microscope equipped with a picosecond pulsed diode laser (excitation at 405 nm, fluorescence emission detected in the range 500−550 nm) was used.

Results and Discussion

To introduce thiol functionality, PMA was conjugated with cysteamine ($NH_2-(CH_2)_2-SH$) in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS),[11] yielding PMA with 18 mol % of thiol-containing units, (PMA_{SH}). PMA_{SH} was alternately deposited with PVPON in 10 mM sodium acetate buffer at pH 4 to give PMA_{SH}/PVPON thin films. Films were first assembled on macroscopic planar supports, and quartz crystal microgravimetry (QCM) was used to monitor the film growth. Regular frequency changes (ΔF) were observed (Figure 1B), indicating that both PMA_{SH} and PMA form hydrogen-bonded multilayers with PVPON on a gold substrate precoated with polyethyleneimine.[12] In comparison with PMA, PMA_{SH} exhibited a larger ΔF at each adsorption step (29 ± 4 Hz vs 15 ± 1 Hz), which is attributed to a greater mass per hydrogen-bonded group for PMA_{SH}. The ΔF for the PVPON deposition steps was the same in both the PMA and PMA_{SH} films (16 ± 2 Hz).

Deconstruction of the multilayers at various pHs of the medium was assessed after exposing the films to 25 mM hydrogen peroxide at pH 4 to effect disulfide cross-linking. Both cross-linked and non-cross-linked films were stable when exposed to pH 5 and pH 6 buffers (Figure 1B, time range 110−130 min). However, introduction of 10 mM potassium phosphate (pH 7) led to completely different behavior between the two films (Figure 1B, point 1). In agreement with the reported threshold pH value for the disintegration of PMA/PVPON films (pH 6.9),[5] the PMA/PVPON film completely deconstructed within 60 s (curve a). Returning the sample to pH 4 (Figure 1B, curve a, point 3) provides evidence that the residual film consists of a layer of PEI with a single adsorbed layer of PMA. In contrast, the disulfide cross-linked film was stable at these conditions: introduction of pH 7 buffer resulted in a significant decrease in ΔF, i.e., increase in film mass (Figure 1B, curve b, point 1), reflecting the uptake of water and swelling of the film. A corresponding increase in the dissipation factor (the sum of all energy losses in the system per oscillation cycle) confirms that the film becomes increasingly water-swollen upon elevation of pH (see Supporting Information). The decrease in ΔF continued throughout the observation time (20 min), and no deconstruction of the PMA_{SH}/PVPON multilayers was observed.

The triggered deconstruction of the PMA_{SH}/PVPON film was examined by exposing the film to a solution of a thiol−disulfide exchange reagent, dithiothreitol (DTT)[13] at pH 7 (Figure 1B, curve b, point 2). A marked increase in ΔF was observed, indicating that cleavage of the interpolymer disulfide bonds leads to deconstruction of the film. Further support for this was

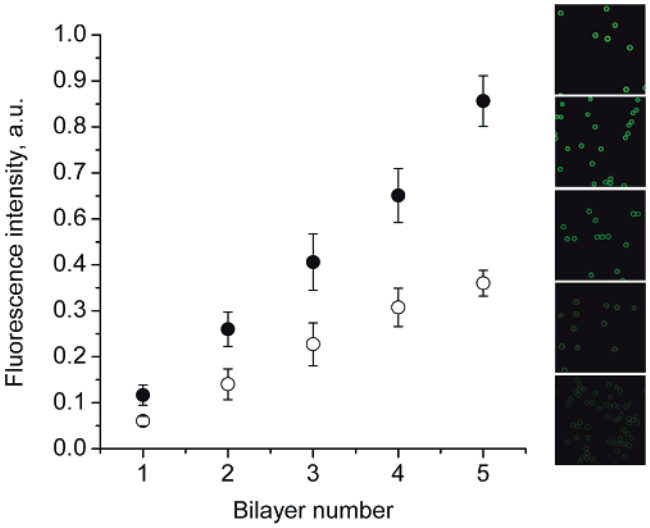

Figure 2. Fluorescence intensity of 3-μm-diameter SiO_2 particles as a function of the number of deposited PVPON/PMA (open circles) and PVPON/PMA_{SH} (filled circles) bilayers. CLSM images show the systematic increase in fluorescence observed for the PVPON/PMA multilayers on the SiO_2 particles (bottom to top: 1 to 5 bilayers). The images are 60 μm $\times$ 60 μm.

provided by returning the film to the starting pH 4 conditions (Figure 1B, curve b, point 3). The ΔF suggests that the remaining film corresponds to a PEI layer with an adsorbed PMA_{SH} layer. The QCM data demonstrate that modification of PMA with thiol-containing groups provides a simple route for preparing cross-linked PMA/PVPON hydrogen-bonded multilayers that are stable at pH 7 and undergo triggered deconstruction by using an external chemical stimulus.

We next examined the formation of PVPON/PMA multilayers on monodisperse SiO_2 particles (3 μm in diameter) at pH 4.[14] To quantify multilayer buildup, PMA and PMA_{SH} were conjugated with fluorescein isothiocyanate (FITC) using a diamine linker, and the fluorescence intensity of the particles was measured using confocal laser scanning microscopy (CLSM) after deposition of the labeled polymer. In both cases, the buildup of the multilayers was regular and linear, and the amount of FITC−PMA_{SH} adsorbed was twice that compared to FITC−PMA (Figure 2). This is in excellent agreement with the QCM data for the buildup on planar substrates.

The core−shell particles were exposed to 10 mM hydrogen peroxide at pH 4 to cross-link the shell, followed by incubation in 10 mM potassium phosphate buffer (pH 8) for 30 min. For the $(PVPON/FITC-PMA)_5$-coated particles, this resulted in deconstruction of the shell, which showed fluorescence only in bulk solution (data not shown). In contrast, the multilayers of the $(PVPON/FITC-PMA_{SH})_5$-coated particles remained intact after exposure to pH 8 (Figure 3A). However, incubation of these particles at pH 8 in the presence of 0.1 M DTT resulted in deconstruction of the multilayers and release of FITC−PMA_{SH} into the bulk solution (Figure 3B).

To demonstrate the potential of the proposed approach for the creation of colloidal carriers, PVPON/PMA and PVPON/PMA_{SH} multilayers were assembled on mesoporous SiO_2 particles[10] loaded with the fluorescently labeled protein, FITC−transferrin. In these experiments, the polymers were not fluorescently labeled—the fluorescence signal originates only from the FITC−transferrin. Removal of the core particles after multilayer assembly yielded capsules loaded with FITC−transferrin (Figure 3C). These capsules were stable and retained the protein at pH up to 10 (highest pH tested). Upon treatment

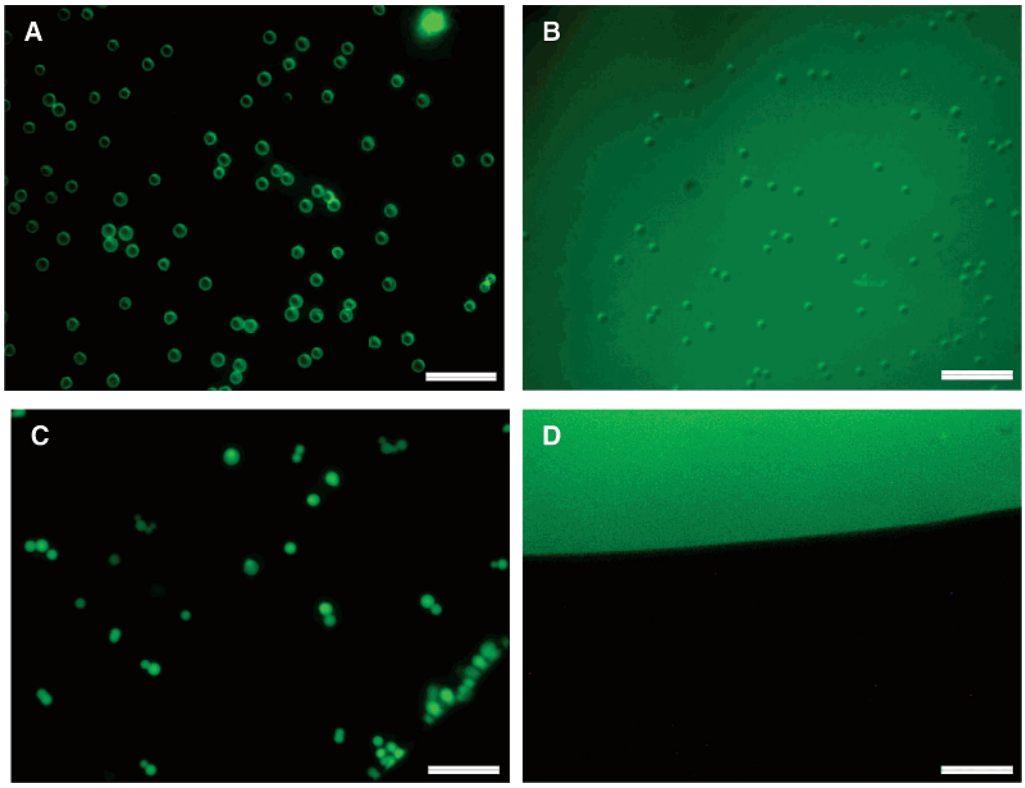

Figure 3. Fluorescence images of 3-μm-diameter SiO_2 particles with five bilayers of PVPON/PMA_{SH} at pH 8 before (A) and after (B) treatment with DTT. PMA_{SH} was fluorescently labeled with FITC. Fluorescent images of $(PVPON/PMA_{SH})_4$ capsules loaded with FITC−transferrin at pH 10 before (C) and after (D) treatment with 0.1 M DTT. The scale bars are 25 μm.

with DTT, the protein was released into the bulk solution (Figure 3D), indicating disassembly of the multilayers.

Conclusions

We have shown that the coupling of PMA_{SH}/PVPON hydrogen-bonded multilayers and thiol−disulfide chemistry for film stabilization provides a viable route to responsive polymer delivery vehicles that are stable at physiological pH and which can undergo deconstruction and release encapsulated materials based on the cleavage of S−S bonds. This offers new opportunities for the application of LbL polymer capsules in delivery applications, for example, in cells where the reducing intracellular environment, and protein-assisted thiol−disulfide exchange may trigger the release of cargo drugs. We are currently examining the deconstruction behavior of these capsules under different biochemical and chemical reducing conditions for both delivery and microreactor applications.

Acknowledgment. We thank A. P. R. Johnston for assistance with confocal microscopy. This work was supported by the Australian Research Council (Discovery Project and Federation Fellowship schemes) and by the Victorian STI Initiative.

Supporting Information Available. Dissipation curves for the multilayered films. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Caruso, F., Caruso, R. A.; Möhwald, H. *Science* **1998**, *282*, 1111.
(2) Donath, E.; Sukhorukov, G. B.; Caruso, F.; Davis, S. A.; Möhwald, H. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 2202.
(3) Peyratout, C. S.; Dähne, L *Angew. Chem., Int. Ed.* **2004**, *43*, 3762.
(4) Saito, G.; Swanson, J. A.; Lee, K.-D. *Adv. Drug Deliv. Rev.* **2003**, *55*, 199.
(5) Sukhishvilli, S. A.; Granick, S. *Macromolecules* **2002**, *35*, 301.
(6) Yang, S. Y.; Lee, D.; Cohen, R. E.; Rubner, M. F. *Langmuir* **2004**, *20*, 5978.
(7) Kozlovskaya, V.; Ok, S.; Sousa, A.; Libera, M.; Sukhishvilli, S. A. *Macromoloecules* **2003**, *36*, 8590.
(8) (a) Haynie, D. T.; Palath, N.; Liu, Y.; Li, B.; Pargaonkar, N. *Langmuir* **2005**, *21*, 1136. (b) Li, B.; Haynie, D. T. *Biomacromolecules* **2004**, *5*, 1667.
(9) Yang, S.; Zhang, Y.; Yuan, G.; Zhang, X.; Xu, J. *Macromolecules* **2004**, *37*, 10059.
(10) Wang, Y.; Caruso, F. *Chem. Mater.* **2005**, *17*, 953.
(11) Zhang, H.; Ito, Y. *Langmuir* **2001**, *17*, 8336.
(12) In the case of PMA_{SH}, regular film buildup was observed without a PEI prelayer. PEI priming was used here only for consistency.
(13) Cleland, W. W. *Biochemistry* **1964**, *3*, 480.
(14) Multilayer formation was accomplished without PEI priming, which is important for biomedical applications, as PEI is known to be toxic.

BM050832V