Biomimetic Nanostructured Materials: Inherent Reversible Stabilization of Polypeptide Microcapsules

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Received August 30, 2004. In Final Form: October 30, 2004

Introduction

Layer-by-layer self-assembly (LBL) is a powerful tool for fabricating nanometer-thick multilayer thin films from a wide variety of materials.[1−3] Polyelectrolyte films are particularly promising.[4,5] The scope of suitable polyelectrolytes encompasses organic polymers and some types of biomacromolecules;[6,7] the materials involved can be environment-friendly and indeed edible. Potential applications of LBL films and coatings include sensors, drug delivery systems, and catalytic microreactors.[8,9] Here, we combine LBL, spherical colloidal "templates", and custom-designed oppositely charged polypeptide chains to create nanostructured polypeptide microcapsules with properties desirable for a range of purposes.

The assembly process developed for macroscopic planar surfaces has been adapted to colloidal particles 0.1−10 μm in diameter for the preparation of capsules.[10,11] To form capsules, a polycation solution is added to a suspension of colloidal particles; following saturation of polymer adsorption, the particles are separated from free polycations in solution; then, a polyanion layer is deposited in the same way. One thus can deposit any number of polyion layers. Dissolution of the template particles[12] will yield empty capsules with a wall thickness of 5−50 nm, depending on the assembly procedure, materials, and cycles of polyion adsorption. In principle, a large variety of polyelectrolytes could be used as capsule components to yield a correspondingly broad range of microcapsule functionalities.

In previous studies, we used poly(L-lysine) and poly(L-glutamic acid) to investigate the behavior of polypeptides in LBL.[13−15] The conclusions of this work resemble those of other investigators[16−18] but differ in a few key respects,[13,19] for instance, the type and quantity of secondary structure present under the given conditions. The thickness of such films is ∼2 nm per layer,[19] giving a total capsule thickness of well below 100 nm. We have also shown that the inclusion of cysteine in designed polypeptides enables the preparation of multilayer films of enhanced stability,[20,21] providing a novel means of cross-linking polyelectrolyte multilayers. The indicated references provide proof of disulfide bond formation under the conditions studied here.

The present report is the first one on the formation of microcapsules by LBL and polypeptides. The results and methods of our initial cysteine peptide studies[20,21] were adapted for this purpose. Polypeptide layers were cross-linked by oxidation, forming disulfide-bond-"locked" capsules. This process, which is reversible,[20,21] stabilized the capsules in "harsh" environments. The approach mimics the known stabilization by disulfide bonds of secreted hormones and proteins, for example, insulin and lysozyme. The reversibility of disulfide bond formation is important in the metabolism of insulin—the two chains undergo rapid proteolysis following reduction by glutathione—insulin transdehydrogenase—and other bioactive peptides and proteins.

Experimental Section

Polyelectrolyte films of 12 bilayers were fabricated on 3-μm-diameter melamine formaldehyde (latex) particles (Microparticles GmbH, Germany) at pH 7.4, using cysteine-containing positive and negative peptides,[20,21] sequential adsorption, and centrifugation. The peptide sequences were KVKG KCKV KVKG KCKV KVKG KCKV KVKG KCKY and EVEG ECEV EVEG ECEV EVEG ECEV EVEG ECEY, where K = lysine, V = valine, G = glycine, C = cysteine, E = glutamic acid, and Y = tyrosine. Tyrosine was included for spectroscopic detection in the near-UV. The outermost layer was poly-(L-lysine) (PLL, MW 80 kDa, Sigma, U.S.A.) labeled with fluorescein isothiocyanate (FITC), a fluorescent dye, enabling characterization by epifluorescence microscopy or confocal laser scanning microscopy. Latex templates were dissolved at pH 1.6 to obtain polypeptide microcapsules. Confocal micrographs, shown in Figure 1, indicate that FITC-labeled poly-(L-lysine) was present before and after the latex cores had dissolved. Prior to the deposition of poly-(L-lysine), each batch of peptide-coated latex particles was divided, one sample for reducing conditions and the other for oxidizing conditions. Disulfide bond formation was promoted by incubating polypeptide-coated microparticles for 1 week in 1% dimethyl sulfoxide (DMSO), a common oxidant at low concentrations, and 1 μM $MnCl_2$ prior to the dissolution of the latex particles. Both sets of capsules were subjected to "harsh" environments, and fluorescence microscopy was used to assess capsule stabilization

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10.1021/la047833d CCC: $30.25
Published on Web 12/30/2004

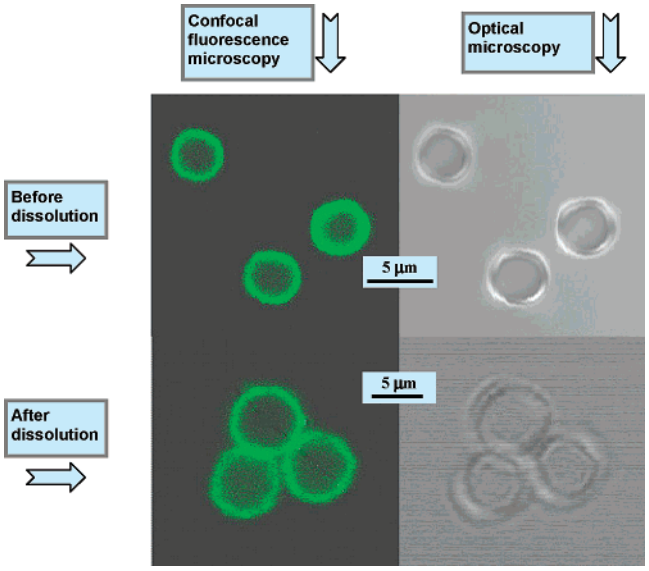

Figure 1. Confocal scanning micrographs of capsules. The images show spherical films of polypeptides 1 and 2 before and after dissolution of MF particle cores. Visualization by fluorescence microscopy was made possible by using FITC-labeled PLL as the outer coating. A study of the control of polypeptide capsule aggregation will be reported elsewhere.

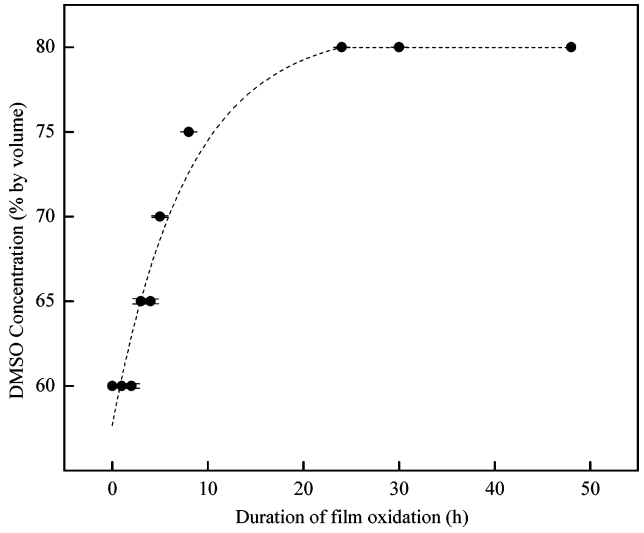

Figure 2. Stability of locked capsules in DMSO. The amount of DMSO required to dissolve locked capsules as a function of duration of film oxidation and, therefore, extent of locking. Disulfide cross-links enhanced capsule stability in this harsh environment. Data were taken at 0, 1, 2, 3, 5, 8, 24, 30, and 48 h.

due to disulfide bond formation. There were 100−200 capsules in the field of view at the beginning of each experiment.

Results and Discussion

Three parts by volume locked or "unlocked" capsules were added to two parts DMSO, a chemical denaturant of protein structure at high concentrations, and mixed manually at room temperature for 10 min. Stable FITC-labeled capsules were visualized by fluorescence microscopy and counted. The concentration of DMSO was incremented and counting repeated until all capsules had disintegrated (Figure 2). Under reducing conditions, capsules readily dissolved in 60% (v/v) DMSO. Disintegration of oxidized capsules required a higher concentration of DMSO: a plateau of ~80% was reached after 24 h of oxidation. "Locking" the capsules by oxidation stabilized them in the harsh environment of concentrated organic solvent.

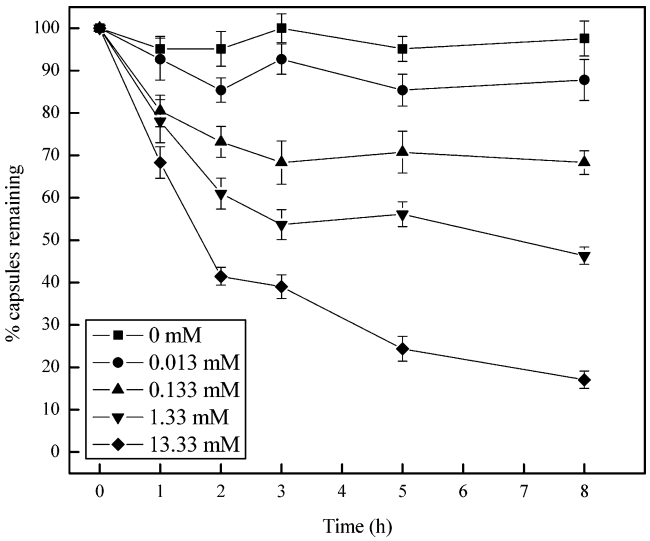

Figure 3. Effect of DTT on the stability of locked capsules at acidic pH. The rate of the disappearance of capsules depended on DTT concentration. The number of capsules decreased as the concentration of DTT increased. Some capsules remained intact, however, indicating an enhanced stability of locked capsules in a highly acidic environment.

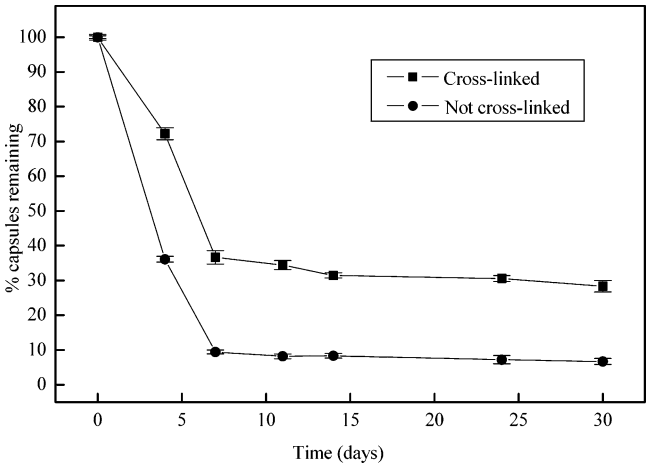

Figure 4. Effect of time on the stability of locked capsules at acidic pH. Capsules dissolved rapidly during the first week of observation. A much larger percentage of capsules remained intact in the oxidized sample than the reduced one. This resulted from disulfide bond formation during film oxidation. The number of capsules was approximately constant after the first week.

Dithiothreitol (DTT), a common reductant, was used to assess both the stability of locked microcapsules maintained at 4 °C for an extended period of time, in this case, 30 days, and the reversibility of S−S capsule stabilization. Aliquots of DTT were added to aliquots of locked capsules, giving a final concentration of 0.013, 0.13, 1.3, or 13.3 mM DTT. Reference samples contained no DTT. All samples were mixed manually. Capsules were visualized by fluorescence microscopy and counted for at least three randomly chosen fields of view for each set of conditions. The results are shown in Figure 3. DTT reduced the disulfide cross-links between polymers, decreasing capsule stability at acidic pH. As the concentration of DTT increased, the number of capsules detected at a given time point decreased. Disulfide cross-

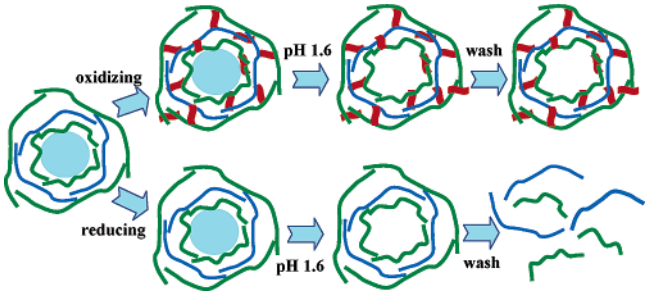

Figure 5. Proposed disulfide locking of polypeptide microcapsules. Polycations are represented by solid lines and polyanions by dashed lines. The polypeptides contain cysteine which can form a disulfide bond represented as a cross-link between layers under oxidizing conditions. The scheme is idealized, as oxidation did not result in complete oxidation under the conditions discussed here. In principle, it should be possible to optimize the locking process for a particular harsh environment, maximizing the yield of capsules of desired longevity.

links stabilized cysteine-containing polypeptide microcapsules in the harsh environment of high reducing potential.

Disulfide-locked and -unlocked capsules were maintained at pH 1.6 after the dissolution of latex particle cores as a further test of capsule stability. Capsules were visualized by fluorescence microscopy as described above and counted at intervals during a 30-day period. The results are shown in Figure 4. Capsules dissolved rapidly during the first week. The extent of dissolution was substantially lower, however, for locked capsules than unlocked ones. The number of capsules was relatively constant during the remainder of the observation period, but the percentage of unlocked capsules was lower than that of locked ones. Disulfide bond formation stabilized polypeptide microcapsules in a low-pH environment. The results closely resemble those of thin films fabricated from the same peptides on quartz following treatment at acidic pH.[17,18]

The mechanism proposed to explain the results is depicted in Figure 5. Treatment of a capsule in an oxidizing environment promotes disulfide bond formation between polypeptides, stabilizing the structure at acidic pH. By contrast, a polypeptide microcapsule without disulfide cross-links disintegrates at acidic pH, as decreasing the charge per unit length on negative peptides leads to increasing charge repulsion between the positive peptides. Disulfide-locked microcapsules sustain exposure to harsh environments much better than capsules without cross-linking.

Disulfide stabilization of capsules is expected to increase their lifetime *in vivo* for a variety of reasons, including increased resistance to proteolysis because of decreased fluctuations of structure; the extracellular environment is oxidizing. The reversibility of disulfide bond formation may be advantageous for microparticle disassembly and drug delivery, as the intracellular environment is reducing.

To summarize, we have shown how cysteine-containing polypeptides can be used to prepare microcapsules. The capsules are stabilized under harsh conditions by the formation of disulfide bonds between polymers. Disulfide bond formation in cysteine-containing polypeptide microcapsules is reversible, suggesting that the controlled formation and cleavage of disulfides could be an element of the polypeptide microcapsule design and engineering process for various projected applications. The biocompatibility and immunogenicity of polypeptide microcapsules will be more favorable for biomedical applications of LBL structures than those made from more usual organic polyelectrolytes, for example, poly(allylamine hydrochloride) and poly(styrene sulfonate), particularly if the sequences are based on genomic information.[22]

Acknowledgment. We thank Tatsiana Shutava for assistance with CLSM and Zheng-liang Zhi, Satish Bharadwaj, and Yuri Lvov for helpful discussions. This research was supported by a seed grant from the Center for Entrepreneurship and Information Technology, a Nanoscale Exploratory Research award, "Structural Basis of Stability of Nanoengineered Polypeptide Thin Films and Microcapsules" (DMI-0403882), an enhancement grant from the Louisiana Space Consortium (Louisiana NASA EPSCoR, project R127172), and the 2002 Capital Outlay Act 23 of the State of Louisiana (Governor's Biotechnology Initiative).

Supporting Information Available: Details of the material preparation, microcapsule preparation, fluorescent labeling, and microcapsule characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

LA047833D

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