Multilayer Biomimetics: Reversible Covalent Stabilization of a Nanostructured Biofilm

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Designed polypeptides and electrostatic layer-by-layer self-assembly form the basis of promising research in bionanotechnology and medicine on development of polyelectrolyte multilayer films (PEMs). We show that PEMs can be formed from oppositely charged 32mers containing several cysteine residues. The polypeptides in PEMs become cross-linked under mild oxidizing conditions. This mimicking of disulfide (S−S) bond stabilization of folded protein structure confers on the PEMs a marked increase in resistance to film disassembly at acidic pH. The reversibility of S−S bond stabilization of PEMs presents further advantages for controlling physical properties of films, coatings, and other applications involving PEMs.

Introduction

PEMs are of broad and growing interest to scientific research in areas ranging from electronics to biomaterials.[1,2] Layers of nanometer-scale thickness can be readily fabricated by electrostatic layer-by-layer self-assembly (LbL),[3] the variation on the general theme when the linear charge density of the self-assembling polyelectrolytes is high (∼0.5−1 electronic charges per monomer). The simplicity and universality of LbL holds much promise for commercial exploitation of PEMs. Common polymers in LbL are organic homopolyelectrolytes of high water solubility but low biocompatibility. Our work in bionanotechnology has aimed to broaden the frontier of PEMs research by initiating and developing the use of designed polypeptides for LbL.

Analysis of PEM properties has been largely concerned with chemical, mechanical, or thermal stress.[4a,4b] PEMs show reduced structural integrity or tend to dissolve[4c,4d] at a pH near the pK_a of the ionizable groups of the polycations or polyanions, owing to electrostatic repulsion. In the present work, we have assessed the structural integrity of polypeptide PEMs in a model harsh environment, viz. strongly acidic pH, in the presence and absence of S−S cross-links. Harsh conditions are common in industrial chemistry; they are also pertinent to some situations in biology.

There are two main ways of stabilizing a PEM: choosing polyelectrolytes of high intrinsic structural integrity and cross-linking the polyelectrolytes following assembly. As to the latter various approaches are known: thermal/photo-induced cross-linking, for instance by diazonium groups; glutaraldehyde cross-linking; other; but the modification process will be irreversible or involve a toxic chemical or structure-damaging ultra-violet radiation. The present work is concerned with cross-linking and stabilization of polypeptide PEMs by formation of S−S bonds. The approach is attractive for a host of reasons: polypeptides and proteins are important to biology and medicine, S−S bonds can be formed under relatively mild conditions, the number and distribution of S−S bonds can be controlled in a variety of ways, and S−S bond formation is reversible.

Development of films and coatings in food science, pharmaceutics, waste disposal, and other areas has involved proteins to a substantial extent.[5] This pertains to the present work because a protein comprises one or more polypeptide chains. At the same time, however, the present work is far more general in scope, because all the different protein sequences on Earth are but a very tiny fraction of all of the polypeptide sequences that are both possible and realizable by molecular biology or synthetic chemistry.

A key amino acid in the present work is cysteine (Cys), which can form an S−S bond in an oxidizing environment. S−S bonds occur naturally in biological matter, where they are important for stabilizing folded structure of proteins, e.g., lysozyme.[6] To the best of our knowledge, this report is the first one in the scientific literature where PEMs of any sort are strengthened by mimicking S−S bond stabilization of proteins and, moreover, where PEMs have been fabricated from such short polyelectrolytes. The results suggest a multitude of new possibilities for developing polypeptide PEMs. Areas of research and development most likely to be affected by this work are biotechnology and medicine. Applications of this technology include implanted device coatings, drug delivery systems, and artificial cells.

Materials and Methods

Polypeptides. The sequences were

(1) KVKG/KCKV/KVKG/KCKV/KVKG/KCKV/KVKG/KCKY

(2) EVEG/ECEV/EVEG/ECEV/EVEG/ECEV/EVEG/ECEY

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where K, E, V, G, C, and Y represent, respectively, the amino acids lysine, glutamic acid, valine, glycine, cysteine, and tyrosine. Peptide synthesis was by F-moc chemistry. The products were confirmed by mass spectrometry and analyzed by high-performance liquid chromatography and used without further purification.

LbL Assembly and Disassembly. Peptide multilayers were assembled at room temperature on quartz crystal microbalance (QCM) resonators (Sanwa Tsusho Co., Ltd, Japan) or quartz microscope slides as described previously.[7] The peptide concentration was 2 mg/mL in each case, and the buffer was 10 mM tris-HCl, pH 7.4. The rinsing solution was 10 mM tris-HCl, pH 7.4. Following polyelectrolyte adsorption, each sample was dried using a stream of dry gas, nitrogen for non-oxidized samples and air for oxidized ones. The film fabrication process was (1) prepare solutions of peptides as described above; (2) sequentially immerse the QCM resonator in each of these solutions for 20 min; (3) rinse the resonator for several seconds after each adsorption step; (4) dry the resonator in a stream of gas; (5) monitor the deposition of material by QCM. An illustration of film assembly is provided in the Supporting Information. QCM resonator frequency shift, Δf, was converted to mass of adsorbed material, Δm as Δm (ng) $\approx -0.87 \times \Delta f$ (Hz) (see, for example, ref 1d and citations therein).

The efficiency of covalent cross-linking in multilayers was measured by determining the resonant frequency at defined time points following exposure of the film to acidic pH. Samples were exposed for a defined length of time to an oxidizing environment or a reducing environment, and then immersed in a 10 mM KCl solution buffered at pH 2.0. This pH is well below the pK_a of the side chains of the negatively charged peptide in aqueous solution; glutamic acid titrates at pH 4.0−4.5.[8] After treatment in acidic pH for 240 min, oxidized samples were or were not exposed for several hours to 50 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) buffered at pH 2.0; TCEP is an effective sulfhydryl reducing agent in the range 1.5−9.0.[9] QCM was used to determine mass of the PEM at defined time points. All samples were dried in a stream of gaseous nitrogen at each time point prior to QCM measurement to minimize the dependence on frequency of the viscoelastic properties of a PEM. An illustration of film disassembly is provided in the Supporting Information.

Determination of S−S and S−H Groups. Ellman's reagent, (5,5′-dithiobis[2-nitrobenzoic acid], DTNB), is used in a standard method of determining reactive sulfhydryls in proteins (pH 8). It can also react with S−S linkages when one free sulfhydryl group is available for reaction (pH 10.5). The product, TNB^{2-}, has an absorbance maximum at 412 nm. Titration of cysteinyl residues with DTNB was used to characterize the relative abundance of S−H and S−S bonds. Detailed information on procedure can be found in ref 10. Briefly, in the present context two sets of sample films, oxidized and non-oxidized, were assembled on quartz slides. For each sample or reference, 1 mL of DTNB solution (50 mM sodium acetate, 2 mM DTNB in H_2O, pH 8.0), prepared fresh, was added to 2 mL of 1 M tris, pH 8.0 and 17 mL of H_2O, mixed thoroughly, and dispensed into a UV cuvette. Oxidized and non-oxidized PEMs on quartz slides were incubated in the resulting solution for 30 min. The slides were removed, and the absorption spectra of the solutions were measured in the range 190−1100 nm. The pH of the solutions was adjusted to 10.5, and the samples were immersed for another 30 min. The slides were removed and the absorbance spectra were measured and compared.

Results and Discussion

We have designed peptides (1) and (2) on the basis of principles described elsewhere.[11] Briefly, at the pH of PEM assembly all ionized side chains of a peptide were required to have the same sign and polypeptide charge per unit length was required to be above 0.5 in solution, assuming complete ionization for the sake of simplicity; quantitative determination of a probable shift in side chain pK_a on PEM formation was beyond the scope of the present study. The sequence designs were intended to test whether S−S bonds could stabilize polypeptide PEMs in a manner resembling S−S bond stabilization of ordered structure of a protein at acidic pH.[6] Sequences resembling peptides (1) and (2) but encoded by a genome could be especially useful for maximizing biocompatibility or minimizing immunogenicity of a film, coating, or microcapsule in the respective organism.[11]

We have shown that peptides (1) and (2) can be used to fabricate chemically cross-linked polypeptide PEMs. The peptides were dissolved in 10 mM Tris-HCl, pH 7.4 to a final concentration of 2 mg/mL. Figure 1a displays film assembly on QCM resonators as monitored by QCM. The change in polypeptide mass deposited per adsorption step was linear. This implies that the surface density of charge of the substrate reversed sign on peptide adsorption, consistent with the assumed net charge at neutral pH based on amino acid sequence composition. It is remarkable that such a large quantity of designed 32mers was deposited, over 285 ng per layer; the adsorption of poly-L-lysine and poly-L-glutamic acid, which is qualitatively different, is discussed in ref 7. The behavior of the designed peptides may reflect the relative importance of hydrophobic interactions in polypeptide PEM assembly.

We have developed a novel way of using QCM to measure the effectiveness of cross-linking in polypeptide PEMs. Following assembly, PEMs of peptides (1) and (2) were exposed for several hours to an oxidizing aqueous environment (air-drying for 30 min, then immersion in 20 v/v % DMSO, 10 mM Tris-HCl saturated with air, pH 7.5) or a reducing environment. Figure 1b shows that acidic pH treatment of non-oxidized polypeptide PEMs led to film disassembly with relatively rapid kinetics. The absolute net charge of the negative polymers declined on protonation, and the positive polymers repelled each other, weakening attraction between layers. By contrast, oxidized polypeptide PEMs containing Cys retained most of their mass after 240 min at acidic pH. (Optimization of the extent of mass retention will be discussed elsewhere.) All retained material was lost from the oxidized PEMs, however, on immersion of the sample in a reducing aqueous environment: TCEP reduced S−S bonds in the oxidized PEMs, leading to complete film

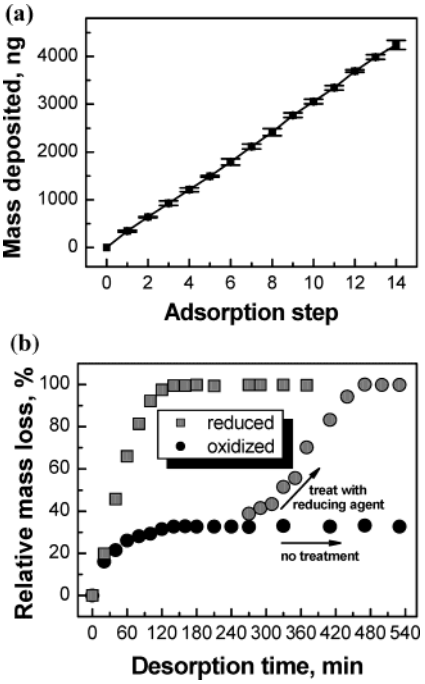


Figure 1. Polypeptide PEM assembly and disassembly monitored by QCM. Symbols indicate the average of three independent trials. (a) LbL of designed 32mers 1 and 2. Deposited polymer increases linearly with adsorption step. The mass sensitivity constant for conversion of frequency shift into mass deposited was assumed to be 1.83×10^8 Hz cm^2 g^{-1}.[1d] (b) Representative time course of loss of polypeptide from QCM resonator at acidic pH. Substantially less material was lost from S−S bonded PEMs (oxidized) than ones stabilized by van der Waals interactions alone (reduced). After treatment with a reducing agent at acidic pH, however, complete disassembly of the film was observed; S−S bond formation in polypeptide PEMs was reversible.

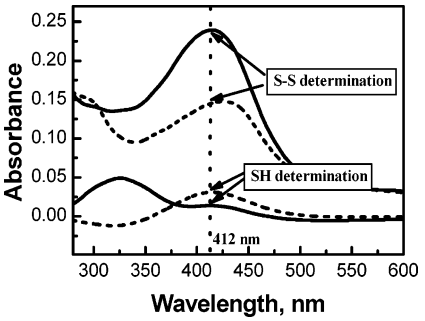


Figure 2. Confirmation of S−S bond formation in polypeptide PEMs. S−H and S−S determination using Ellman's reagent. Absorption spectra of non-oxidized samples are shown with dashed lines, oxidized samples with solid lines. The proportion of S−S is much higher in the oxidized sample than the non-oxidized one, and vice versa for S−H.

disassembly at acidic pH, as in the reduced sample. This resembles the known reversibility of S−S bond formation in proteins.[8]

DTNB was used to demonstrate the formation of S−S bonds in oxidized polypeptide PEMs. At pH 8, free S−H reacts with DTNB, giving a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB^{2-}). Figure 2 shows that at pH 8 (S−H determination) TNB^{2-} absorbance was higher in the non-oxidized film than the oxidized one, indicating more S−H groups in the former than the latter. At pH 10.5 (S−S determination), DTNB also reacts with S−S in the presence of S−H. In this case, TNB^{2-} absorbance was higher in the oxidized sample than the non-oxidized one; there were more S−S bonds in the former than the latter. The data thus indicate that more S−S bonds were present in samples after than before oxidation. Quantitative determination of S−S bonds per unit mass of PEM, though possible, was beyond the scope of the present work, as the primary concern was demonstration of proof of principle.

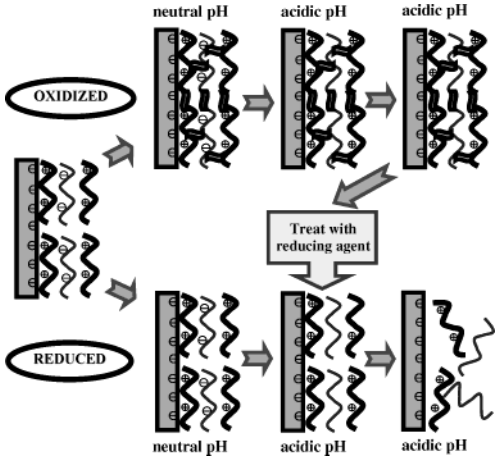
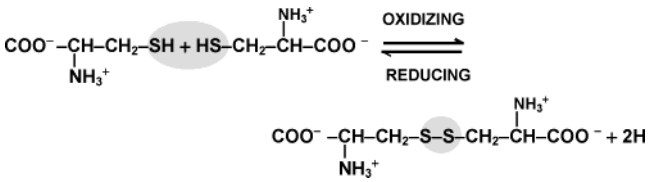


Figure 3. Effect of S−S bond formation on Cys-containing polypeptide PEM stability under extreme conditions, in this case strong acid. The cross-linked polymers would also be stable in different harsh conditions, e.g. an organic solvent. S−S cross-links stabilize the oxidized film. The formation of S−S bonds is reversible. On addition of a reducing agent, the disulfide-bonded PEM becomes unstable at acidic pH. Counterions are omitted for clarity.

The mechanism proposed for the results of this work is presented in Figure 3. Exposure of a Cys-containing PEM to an oxidizing solution promotes S−S bond formation; cross-links form between like-charged polypeptides within a single layer and between oppositely charged polypeptides in adjacent layers. The product is a cross-linked PEM of three-dimensional structure which resists disintegration in a harsh environment, in the present case acidic pH. By contrast, non-crosslinked ("reduced") samples readily disintegrate at acidic pH, due in large measure to charge repulsion between positively charged polypeptides. S−S cross-link formation increases PEM stability against chemical degradation, substantially reducing the kinetics and extent of disintegration of oxidized Cys-containing polypeptide PEMs relative to non-oxidized ones (cf Figure 1b).

Taken together, the experimental results indicate that oxidation of a PEM assembled from a Cys-containing polypeptides increases film stability by the following reaction:



Other researchers have cross-linked PEMs by thermal or photoinduced processes for various purposes, e.g., micropattern polymer films, stabilize enzyme crystals for synthetic applications, enhance ion-transport selectivity of polyelectrolyte membranes, and stabilize biocompatible albumin/

heparin coatings.[12] In all such cases, however, cross-linking was irreversible. S−S cross-linking of polypeptide PEMs is both distinctive and advantageous for fabrication, it can be achieved under mild reaction conditions, for example on air oxidation in the absence of added chemicals, and advantageous for controlling properties of PEMs, it is chemically reversible. The PEM fabrication methodology outlined in this work thus is promising for development of biocompatible coatings for implanted devices, edible food coatings,[13a] artificial cells intended for use in vivo,[13b] and other applications.

Conclusion

Short custom-designed polypeptides (32mers) can be used to create PEMs by electrostatic LbL. Polypeptide PEMs have exceptional potential for functional design and a vast range of possible uses in areas involving protein-based films, coatings, and microcapsules, for example pharmaceutical science, biotechnology, food science, and waste disposal. Inclusion of Cys in a polypeptide suitable for electrostatic LbL permits controlled and reversible cross-linking between polypeptide chains under mild conditions. Stability of polypeptide PEMs to disintegration at acidic pH is substantially increased on film oxidation. Further investigation is needed to determine whether S−S cross-linking will stabilize polypeptide PEMs in other types of harsh environment and the extent to which the character and extent of cross-linking can be optimized for a specific application.

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Supporting Information Available. Illustrations of film assembly and disassembly. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) (a) Eckle, M.; Decher, G. *Nano Lett.* **2001**, *1*, 45. (b) Zheng, H. P.; Lee, I.; Rubner, M. F.; Hammond, P. T. *Adv. Mater.* **2002**, *14*, 569. (c) Rmaile, H. H.; Schlenoff, J. B. *J. Am. Chem. Soc.* **2003**, *125*, 6602. (d) Lvov, Y. *Protein Architecture: Interfacing Molecular Assemblies and Immobilization Technology*; Marcel Dekker: New York, 2000. (e) Decher, G.; Schlenoff, J. B. *Multilayer Thin Films*; Wiley-VCH: Weinheim, Germany, 2003.

(2) (a) Sinani, V. A.; Koktysh, D. S.; Yun, B. G.; Matts, R. L.; Pappas, T. C.; Motamedi, M.; Thomas, S. N.; Kotov, N. A. *Nano Lett.* **2003**, *3*, 1177. (b) Decher, G.; Lehr, B.; Lowack, K.; Lvov, Y.; Schmitt, J. *Biosensors Bioelectron.* **1994**, *9*, 677. (c) Möhwald, H.; Lichtenfeld, H.; Moya, S.; Voigt, A.; Bäumler, H.; Sukhorukov, G.; Caruso, F.; Donath, E. *Macromol. Symp.* **1999**, *145*, 75.

(3) (a) Decher, G. *Science* **1997**, *277*, 1232. (b) Tripathy, S. K.; Kumar, J.; Nalwa, H. S. *Handbook of Polyelectrolyte-based Thin Films for Electronic and Photonic Applications*; American Scientific Publishers: Stevenson Ranch, CA, 2002; Vol. 1, p 1. (c) Caruso, F.; Möhwald, H. *J. Am. Chem. Soc.* **1999**, *121*, 6039. (d) Lvov, Y.; Caruso, F. *Anal. Chem.* **2001**, *73*, 4212.

(4) (a) Mao, G.; Tsao, Y. H.; Tirrell, M.; Davis, H. T. *Langmuir* **1995**, *11*, 942. (b) Gao, D. Y.; Leporatti, S.; Moya, S.; Donath E.; Möhwald, H. *Langmuir* **2001**, *17*, 3491. (c) Kharlampieva, E.; Sukhishvili, S. A. *Langmuir* **2003**, 19, 1235. (d) Blodgett, K. B. *Phys. Rev.* **1935**, *55*, 391.

(5) Gennadios, A. *Protein-based Films and Coatings*; CRC Press: Boca Raton, FL, 2002; p 1.

(6) (a) Cooper, A.; Eyles, S. J.; Radford, S.; Dobson, C. M. *J. Mol. Biol.* **1992**, *225*, 939. (b) Morozova, L. A.; Haynie, D. T.; Arico-Muendel, C.; Van Dael, H.; Dobson, C. M. *Nat. Struct. Biol.* **1995**, *2*, 871. (c) Morozova-Roche, L. A.; Arico-Muendel, C.; Haynie, D. T.; Emelyanenko, V.; Van Dael, H.; Dobson, C. M. *J. Mol. Biol.* **1997**, *268*, 903.

(7) Haynie, D. T.; Balkundi, S.; Palath, N.; Chakravarthula, K.; Dave, K. *Langmuir* **2004**, *20*, 4540.

(8) (a) Creighton, T. E. *Proteins: Structures and Molecular Properties*, 2nd ed.; Freeman: New York, 1993; p 6. (b) Mendelsohn, J. D.; Barrett, C. J.; Chan, A. J.; Mayes, A. M.; Rubner, M. F. *Langmuir* **2000**, *16*, 5017. (c) Boulmedais, F.; Bozonnet, M.; Schwinte, P.; Voegel, J. C.; Schaaf, P. *Langmuir* **2003**, *19*, 9873. (d) Zhi, Z. L.; Haynie, D. T. submitted.

(9) Han, J. C.; Han, G. Y. *Anal. Biochem.* **1994**, *220*, 5.

(10) Robyt, J. F.; White, B. J. *Biochemical Techniques: Theory and Practice*; Waveland Press: Prospect Heights, IL, 1990; p 237.

(11) (a) Zheng, B.; Haynie, D. T.; Zhong, H.; Sabnis, K.; Surpuriya, V. *J. Biomater. Sci. Polym. Ed.*, in press.

(12) (a) Stair, J. L.; Harris, J. J.; Bruening, M. L. *Chem. Mater.* **2001**, *13*, 2641. (b) Park, M. K.; Xia, C. J.; Advincula, R. C.; Schutz P.; Caruso, F. *Langmuir* **2001**, *17*, 7670. (c) Yang, S. Y.; Rubner, M. F. *J. Am. Chem. Soc.* **2002**, *124*, 2100. (d) Singh, A.; Santos, J. P.; Stanish, I.; Lee, Y. W. *Abstr. Papers Am. Chem. Soc.* **2002**, *223*, U429. (e) Brynda, E.; Houska, M. *J. Colloid Interface Sci.* **1996**, *183*, 18.

(13) (a) Rudra, J. S.; Haynie, D. T., submitted. (b) Haynie, D. T.; Liu, Y.; Li, B.; Palath, N. Manuscript in preparation.

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