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Nano-Scale Biomimetics: Fabrication and Optimization of Stability of Peptide-Based Thin Films

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The method of thin film preparation known as layer-by-layer assembly is of growing interest for current and envisioned developments in bionanotechnology. Here, cysteine-containing 32mer peptides have been designed, synthesized, purified, and used to prepare polypeptide films. A range of methods—quartz crystal microbalance, Fourier transform infrared spectroscopy, circular dichroism spectroscopy, and high-performance liquid chromatography—have been used to probe the effect of ionic strength and polymer secondary structure in solution on peptide self-assembly, and on secondary structure formation and disulfide bond cross-linking in the multilayer film. The amount of designed peptide deposited per adsorption step of film fabrication increased with increasing ionic strength, as with conventional polyelectrolytes. Secondary structure content changed from random coil to β sheet on incorporation of peptides into a film. "Peptide-inherent" cross-linking by disulfide bond formation increased film stability at acidic pH. Conditions for disulfide stabilization have been optimized. The results contribute to exploration of the physical basis of peptide self-assembly broaden the scope of applications of layer-by-layer assembly, particularly where biocompatibility and stability are key design concerns, and provide a basis for mass production of custom polypeptide thin films of high stability, even in harsh environments.

Keywords: Crosslinking, Cysteine, Disulfide Bond, Layer-by-Layer Assembly, Multilayer Thin Film, Nanomaterials, Polypeptide, Secondary Structure, Self-Assembly.

RESEARCH ARTICLE

1. INTRODUCTION

Three methods of fabricating a thin film or coating on a solid support are the Langmuir-Blodgett technique, monolayer adsorption, and layer-by-layer assembly (LbL). The last of these has proved to be the most appropriate one for preparation of multilayer films of both controlled thickness and defined architecture.[1] Nanometer-scale order can be achieved by LbL, independent of the surface area and shape of the support. Moreover, the layering process is repetitive, so robots could perform it. Furthermore, the versatility and convenience of LbL make it attractive for the development of a variety of applications—in optics, electronics, medicine, biotechnology, and other areas.[2–6] This suggests that LbL has favorable prospects for commercialization and explains why there has been rapid growth in this area since the early 1990s, when Decher and colleagues began preparing multilayer films from polyelectrolytes.[7]

A concern about polyelectrolyte films, especially ones prepared from biopolymers, is film stability in different environments, particularly harsh ones. Immersion in a highly polar solvent, for example water at a pH near the intrinsic pK_a of a side chain, can destabilize film structure.[8] Increase in film stability can be achieved by chemical modification, for example thermal- or photo-induced crosslinking. Most such treatments, however, are irreversible, limiting the potential for responsiveness of film properties to environmental conditions. Nevertheless, such crosslinking has successfully been applied to various non-biological film applications.[9–16] Examples include micropatterning and enhancement of ion-transport selectivity of polyelectrolyte membranes.

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The present work concerns stabilization of polypeptide multilayer films by disulfide (S–S) bonds. Attractive features of S–S crosslinking are wide and natural occurrence in biological macromolecules, formation under mild reaction conditions, and reversibility. The folded structure of the anti-microbial enzyme lysozyme, found in tears and other secretions in humans, is stabilized by S–S bonds.[17] S–S bonds can be formed in the presence of dimethylsulfoxide (DMSO) or, even simpler, by air oxidation. S–S bond formation is widely used in protein chemistry and biotechnology, for example in the conjugation of a peptide hapten to a carrier molecule in immunological studies.[18] We have demonstrated the basic suitability for LbL of designed peptides containing cysteine.[19] The present work extends our previous study and provides details of how we have optimized the S–S film crosslinking process.

Proteins and polypeptides are important in products in many different areas of science and technology, not only medicine.[20] This class of biological macromolecule seems particularly suitable for LbL films and coatings applications where biocompatibility, edibility, or environmental benignity is a concern, for example food science. Moreover, the structural and functional properties of polypeptides and proteins can be exploited in the development of films for specific applications, in implant medicine, biosensor design, and other areas.[21] Proteins studied in this context include myoglobin, hemoglobin, lysozyme, and glucose oxidase.[22, 23] The sequence of a peptide designed for LbL could be based on human genome information for the sake of optimizing biocompatibility of the thin film[24] or chosen to provide a specific biochemical functionality. The effectively unlimited number of realizable polypeptide sequences greatly broadens the scope of the potential of LbL film design for different applications.

Our initial work in polypeptide LbL was concerned more with fundamental aspects of the subject than applications.[25] The present study discusses a means of forming thin films using cysteine-containing custom-designed peptides in place of common polypeptides, for example poly(L-lysine) (PLL) and poly(L-glutamic acid) (PLGA), enabling "polymer-inherent" crosslinking. The data show that S–S bond crosslinking stabilizes polypeptide LbL films. Other features of this study include the use of short peptides to form thin films and a comparison of the secondary structure of peptides in aqueous solution and in thin films.

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2. EXPERIMENTAL DETAILS

2.1. Materials

NaCl, KCl, tris(hydroxymethyl)aminomethane (Tris), DMSO, PLL (mass MW 13.6 kDa), and PLGA (mass MW 14.6 kDa) were from Sigma-Aldrich (USA). All solvents (1,3-diisopropyl-carbodiimide, *N*, *N*-dimethylformamide, HOBt hydrate, and piperidine) and amino acids (Fmoc-Lys(Boc)-OH, Fmoc-Glu(Obut)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gly-OH, Fmoc-Val-OH, and Fmoc-Tyr(But)-Wang resin) for solid-phase peptide synthesis were from Advanced ChemTech (USA). HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were from Sigma-Aldrich. All chemicals from commercial sources were used as received. All aqueous solutions were prepared with deionized (DI) water. Quartz microscope slides (Electron Microscopy Sciences, USA) for circular dichroism spectroscopy (CD) analysis of peptide films were cut into $10 \times 25 \times 1$ mm^3 pieces, cleaned overnight with 1% NaOH in ethanol-H_2O (60%/40%, v/v), rinsed extensively with DI water, and dried with nitrogen gas before use.

2.2. Peptide Design and Solid-Phase Synthesis

Cysteine-containing peptides were designed to be positively-charged or negatively-charged at neutral pH on the basis principles adduced earlier.[22] Cysteine was included to introduce free thiol (SH) groups into polypeptide films. The peptide sequences were as follows:

(1) KVKG/KCKV/KVKG/KCKV/KVKG/KCKV/KVKG/KCKY
(2) EVEG/ECEV/EVEG/ECEV/EVEG/ECEV/EVEG/ECEY

where K, E, V, G, C, and Y represent the amino acids lysine, glutamic acid, valine, glycine, cysteine, and tyrosine, respectively. This design provides a uniform distribution of charge at neutral pH, important for controlled LbL, and sulfhydryl groups, important for crosslinking. Tyrosine was included for quantitative determination of peptide concentration in aqueous solution by absorption at 274 nm. Peptides were synthesized by standard Fmoc solid phase synthesis on the Advanced ChemTech Apex 390 peptide synthesizer at Louisiana Tech. The synthesis products were lyophilized, analyzed by HPLC (Beckman, USA) and mass spectrometry (Louisiana State University, Baton Rouge, USA), and stored at −20 °C until use.

2.3. Kinetic Study of Peptide Cross-Linking by HPLC

HPLC separates molecules in solution on the basis of differences in physical properties of the analytes. An estimate of the oxidization kinetics of thiol was obtained by dissolving Peptide 1 in H_2O/DMSO (80%/20%, v/v) and analyzing a fixed volume of peptide solution at given time points. A Beckman Coulter stainless steel C18 guard column preceded a C18 reversed-phase column (5 μm, 300 Å, 250 mm × 4.6 mm, Adsorbosphere XL, Alltech, USA). Absorbance was measured at 214 nm and ambient temperature. Samples were eluted using a linear acetonitrile–water gradient. The water contained 0.1% (v/v) TFA, and the gradient began at 2% (v/v) acetonitrile, increasing at a rate of 1%/min. The total solvent flow rate was 1 mL/min.

2.4. Multilayer Assembly, Oxidization, and Disassembly

Peptide films were assembled layer by layer at room temperature on quartz crystal microbalance (QCM) resonators (Sanwa Tsusho Co., Ltd., Japan) or quartz microscope slides. A series of NaCl solutions was prepared to study influence of ionic strength on peptide conformation and assembly characteristics. In each case the peptide concentration was 2 mg/mL. The buffer was 10 mM Tris-HCl, pH 7.4, as was the rinsing solution. Following polymer adsorption, each sample was dried using a stream of dry gas, nitrogen or air. Film fabrication proceeded as follows: (1) prepare solutions of peptides as described above; (2) immerse the substrate in these solutions sequentially for 20 min; (3) rinse the substrate for several seconds after each adsorption step; (4) dry the substrate in a stream of dry gas; (5) monitor deposition of material. See Figure 1a. QCM resonator frequency shift, Δf, was converted to mass increment of adsorbed material as Δm (ng)$\approx -0.87 \times \Delta f$ (Hz).[23]

The oxidization conditions for crosslinking were optimized by varying the DMSO concentration or ionic strength, and films were dried with air instead of nitrogen gas.

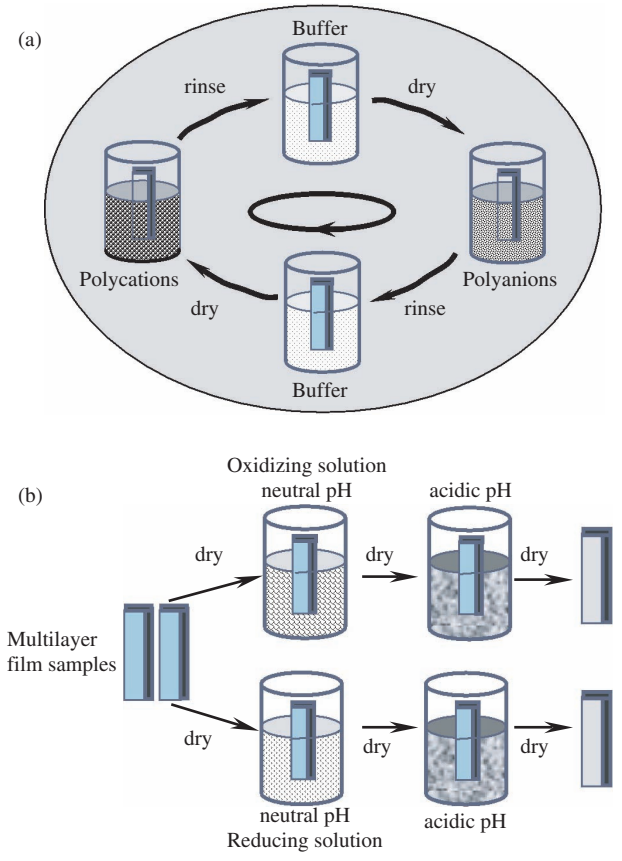

Fig. 1. Schematic diagrams of (a) the LbL process, and (b) subsequent film disassembly. The latter illustrates the process used to determine the efficiency of cysteine crosslinking.

A simple but effective approach to QCM was used to measure efficiency of covalent crosslinking in films.[19] Following assembly, a film was exposed to an oxidizing or reducing aqueous environment for several hours and immersed in a 10 mM KCl solution buffered at pH 2.0, i.e., 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. See Figure 1b. At defined time points the resonator was rinsed and dried, and QCM was used to determine resonant frequency. This provided a measure of film stability in a "harsh" environment. A negative control experiment was done under the same conditions using the non-cysteine containing polypeptides PLL and PLGA.

2.5. Film Characterization by CD and Fourier Transform Infrared Spectroscopy

CD provides structural information on chiral compounds by measuring the differential absorption of right- and left-circularly polarized light. The far-UV signal in particular is sensitive to the backbone conformation of a polypeptide. A Jasco J-810 spectropolarimeter (Japan) was used to characterize the secondary structure of peptides in solution (1 mm path length cell) or in polypeptide thin films (1 mm-thick quartz microscope slide). Measurements were taken every 0.5 nm with an average sampling time of 1 s. A minimum of 20 scans were accumulated and averaged for each spectrum. Baseline spectra were collected and subtracted from the respective sample spectra. In some cases raw CD data were converted to mean molar residue ellipticity. Spectra were analyzed qualitatively: the negative 222/208 nm doublet (n—π^*, π—π^* transitions, respectively) was assumed to indicate α helix, and the negative 216 nm and positive 197 nm absorption bands (π—π^*, n—π^*, transitions, respectively) β sheet.[26]

Fourier transform infrared spectroscopy (FTIR) is widely used to identify chemical groups by specific absorption bands. Here, spectra were recorded using a Thermo Nicolet Nexus 470 (USA). Polypeptide films were prepared on a 25 mm × 12 mm × 2 mm slide of polished CaF_2. The baseline was subtracted from each film spectrum.

3. RESULTS AND DISCUSSION

3.1. Peptide Design and Calculated PH-Dependence

The ability to control the structure of peptides and mass produce and assemble them into structures of desired architecture could be of substantial value for development and commercialization of thin films and coatings.[27] The amino acid sequence of a peptide employed in LbL could in principle be designed on any desired basis, e.g., human genome information.[22] Of particular interest in the present work is the amino acid cysteine, as its highly reactive thiol group in the side chain can form a covalent crosslink. Moreover, such crosslink formation is reversible,

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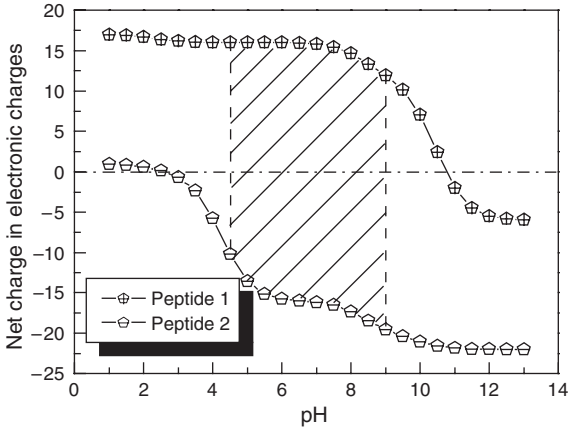

Fig. 2. Influence of pH on calculated net charge of designed Peptides 1 and 2. The pH range 5–8 seems well-suited to LbL.

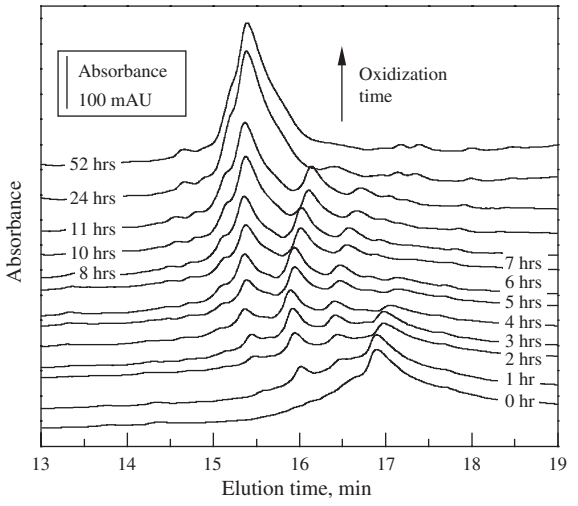

Fig. 3. Kinetics of S–S bond formation in designed peptide solution (Peptide 1) studied by analytical C18 reversed-phase HPLC and monitored at 214 nm.

conferring sensitivity to the reducing potential of the surrounding environment. A polypeptide will ordinarily be charged in aqueous solution due to the presence of amino or carboxyl groups. Deprotonation of the former or protonation of the latter will change the net charge on the molecule—another form environmental sensitivity. The pH-dependence of the net charge of a designed peptides can be estimated using amino acid composition and intrinsic pK_a values.[28] Figure 2 indicates that Peptide 1 and Peptide 2 had a relatively high charge per unit length at neutral pH. The calculation further suggests that the designed polypeptides will be useful for LbL assembly in the pH range 5–8. Experimental data presented here have confirmed this.

3.2. Estimate of Kinetics of S–S Bond Formation

The rate, yield, and specificity of oxidation of two cysteine residues to cystine can be influenced by the reagents used, solvent, presence of chemical denaturant, and temperature. DMSO, a mild oxidizing agent for simple organic thiols, produces harmless byproducts.[29] Other advantages of DMSO oxidation are rate of reaction and applicability over a wide range of pH (3–8). Figure 3 shows HPLC analysis of oxidization of designed cysteine-containing peptides in aqueous solution. The elution profile shifted on increasing oxidation of polypeptide, reaching a plateau after about 24 h. This provides a rough indication of S–S bond formation between designed peptides in a thin film, and it constitutes a starting point for determining the conditions for optimizing the extent of crosslinking for a particular application. The data also suggest that simple overnight oxidization will yield increased film stability at acidic pH.

3.3. Assembly Behavior of Designed 32mer Short Peptides

LbL involves kinetic trapping of polymers by sequential adsorption on an oppositely-charged surface. In previous work we showed that chain length and ionic strength influence the assembly behavior of the common polypeptides PLL and PLGA under different conditions.[25] No peptide film adsorption was apparent at neutral pH during 32 adsorption steps of polydisperse PLGA (MW 3,000) and PLL (MW 3,800) when the ionic strength was below 0.02. It may be that short, highly-charged peptides form a soluble complex with peptides from solution in successive adsorption steps. Figure 4a shows the contrasting behavior of Peptides 1 and 2, which have a net charge per unit length of about 0.5 at neutral pH. Not only do these peptides adsorb, but the amount of material deposited per adsorption step increases significantly with adsorption step. The increase in film mass is linear. No "precursor" layers of strong polyelectrolyte, for example poly(styrenesulfonate), were needed to initiate film growth or control film assembly. It is clear that the designed 32mers, monodisperse and of MW about 3.6 kDa, are suitable for LbL thin film production. The result suggests a promising future for polypeptide LbL biofilms in basic research and technology development.

Figure 4a also shows that increasing the NaCl concentration increases the amount of peptide adsorbed. This is consistent with previous results for PLL and PLGA.[25] Slopes of the best-fit straight lines for assembly at a given ionic strength are plotted versus ionic strength in Figure 4b. Apparently, a transition in assembly behavior occurred near 20 mM NaCl. The increase in adsorption with ionic strength may be due to charge-screening, as this decreases repulsion between like-charged peptides and increases the relative importance of hydrophobic interactions and hydrogen bonds. To the extent that polypeptides behave as common polyelectrolytes,[30, 31] for instance poly(styrenesulfonate), adjustment of ionic strength represents a simple means of manipulating thickness and morphology of polypeptide films.

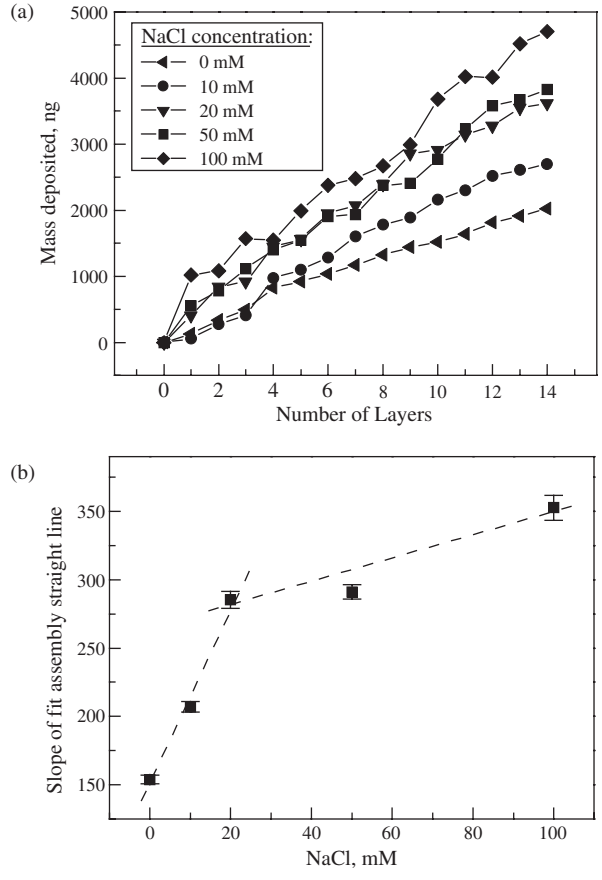

Fig. 4. Adsorption of Peptides 1 and 2 as a function of NaCl concentration as monitored by QCM. (a) Mass deposition, deduced from resonant frequency, is plotted against adsorption step. (b) Slope of best-fit straight line for each salt concentration in (a).

Figure 5 shows FTIR spectra from 3700 to 1250 cm^{-1} of a thin film of Peptides 1 and 2 assembled on a CaF_2 slide. The spectra indicate that a polypeptide film formed on this substrate, as absorbance increases with layer number.

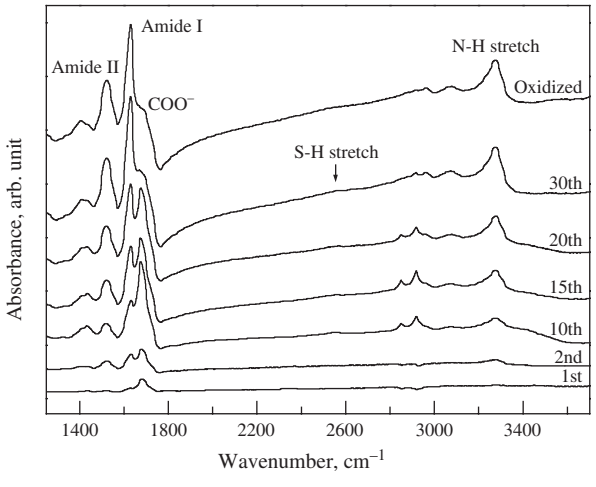

Fig. 5. FTIR study of polypeptide film assembly. The spectra are baseline-subtracted. Signal intensity increases with adsorption step. Various bands pertinent to peptide structure, including S–H stretch, are indicated.

Amide I (1620 cm^{-1}), amide II (1550 cm^{-1}), COO^- (1650 cm^{-1}), and N–H stretch (3280 cm^{-1}) bands appear in all spectra; the S–H stretch absorbance peak (2550 cm^{-1}) is very weak. The large COO^- peak suggests that the films will be hydrophilic.

3.4. Secondary Structure of the Designed Peptides in Solution and in Multilayer Films

Non-covalent polypeptide structure is known to depend on van der Waals interactions, electrostatic interactions, and hydrogen bonding. The chemical nature of a polypeptide allows it to form an α helix, β sheet, or some other type of "higher-order" structure depending on conditions. An α helix is stabilized by intramolecular hydrogen bonds; a β sheet can be stabilized by intermolecular hydrogen bonds, hydrogen bonds formed between different regions of the same molecule, or a combination of these. There are many more ways of forming a β sheet than an α helix for a given length or amount of peptide. Peptides are chiral, and CD is widely used to assess structure and provide a relatively accurate determination of content of α helix, β sheet, and random coil.

Both of the designed 32mer peptides were mainly in a random coil conformation in solution, according to far-UV CD (Fig. 6a and 6b). Ionic strength had some effect on conformation, but little (insets in Fig. 6). It would appear, though, that a small structural transition occurred in solution around 20 mM NaCl, similar to finding for PLL and PLGA.[25] Small and therefore relatively mobile ions may influence the structure of the peptides in solution. At low ionic strength like charges in a short polyelectrolyte will repel each other, making the chain relatively stiff. The energetic barrier to formation of persistent regular structure in solution is high under such conditions. At high ionic strength, small counterions will screen some of the polymer charges, giving van der Waals interactions and hydrogen bonds relatively more influence over peptide structure and reducing the energetic barrier to formation of persistent secondary structure. The local concentration of counterions in the vicinity of a peptide will be higher than in bulk solution. Mobile ions will influence peptide structure in a variety of ways, for example electrostatic interaction of a counterion with a charged group in the peptide and alteration of the structure of the solvent on increasing the ionic strength. It is not yet clear in the present case which physical cause results in the apparent transition, nor how any structural change of the peptides in solution will translate into a change in assembly behavior.

A negative Cotton effect near 205 nm indicates irregular backbone structure. This band was significantly more negative for Peptide 1 than Peptide 2 at the same concentration (Fig. 6a and 6b), suggesting a more random-like conformation for the former under some conditions. By contrast, the peptide film shows a negative $\pi—\pi^*$ transition at c. 216 nm and a positive $n—\pi^*$ transition at c. 197 nm,

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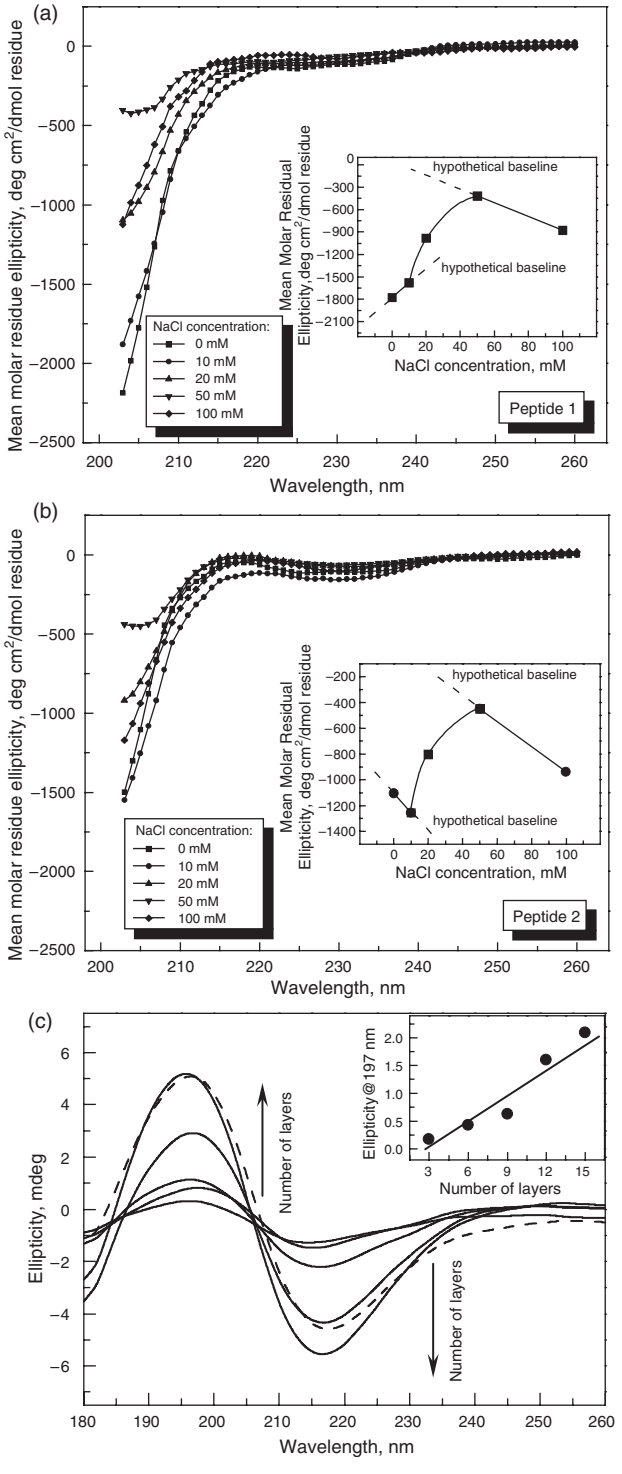

Fig. 6. Far-UV CD study of polypeptide film assembly. (a) Peptide 1 in solution, and (b) Peptide 2 in solution. Insets indicated ellipticity at 205 nm, which suggests a phase transition near 20 mM NaCl for both peptides. (c) Peptide 1/Peptide 2 film assembly on a quartz slide. Spectra are shown for 3, 6, 9, 12, and 15 layers. The dashed line shows the spectrum after oxidization. The inset is the corresponding ellipticity at 197 nm, which shows quantitative mass adsorption with increasing number of layers.

indicative of β sheet structure (Fig. 6c). The CD spectra give little doubt that a considerable degree of induction and therefore rigidification of structure occurs in these peptides during LbL film preparation at neutral pH.

Either the adsorption process itself or interactions between peptides following adsorption induce and stabilize a β sheet conformation in the peptides. The increase in local concentration of peptide in the film[32] may promote β sheet formation, despite the substantial energetic barrier to complexation of peptides of like charge in solution. A β sheet consists of aligned β strands, which interact by hydrogen bonds between a carbonyl group of one strand and an amide group of the other. A β sheet is a type of nano-organized structure. A β sheet can be configured in a film in one of two ways: with positive peptides and negative peptides forming separate β sheets which attract each other by electrostatics, or with individual positive peptides forming a β sheet with negative peptides. A likely possibility is that both types are present in films to some extent. If the blending of different peptide layers occurs, as seems likely in order to maximize entropy, it may be that oppositely-charged peptides will form a highly-integrated and uniform β sheet during relaxation to equilibrium. It is not clear from the data presented here whether the β-sheet content of the films was predominantly parallel or anti-parallel in character, or whether most if not all such structure occurred exclusively at layer interfaces.

Optical activity increased with number of adsorption steps. The increasing signal at 197 nm (inset of Fig. 6c) could be used as a semi-quantitative measure of multilayer growth, as with QCM (Fig. 4a). There was no obvious change in secondary structure content on film oxidation, according to CD and QCM. In a different context the views provided by CD and QCM might not be redundant (Zhang and D. T. H., unpublished results).

3.5. The Role of S–S Crosslinking on Multilayer Film Stability at Acidic pH and Optimization of S–S Crosslinking

Polypeptide LbL films could exhibit reduced stability or the tendency to dissolve in a strongly acidic or basic environment, or in the presence of an organic solvent, limiting commercial potential. The situation could be improved by stabilizing film structure, for example by crosslinking. This can be achieved using glutaraldehyde or some other type of covalent crosslinking agent, or by hydrogen bonding.[11–16] Glutaraldehyde crosslinks polypeptides by forming covalent bonds between free amino groups. The approach has been used to crosslink human serum albumin to heparin in LbL multilayers.[9] The result was a film of high durability and efficient passive protection of the underlying surface from direct contact with blood.

We have developed a means of stabilizing polypeptide multilayer films and coatings by S–S bonding.[19] Conditions

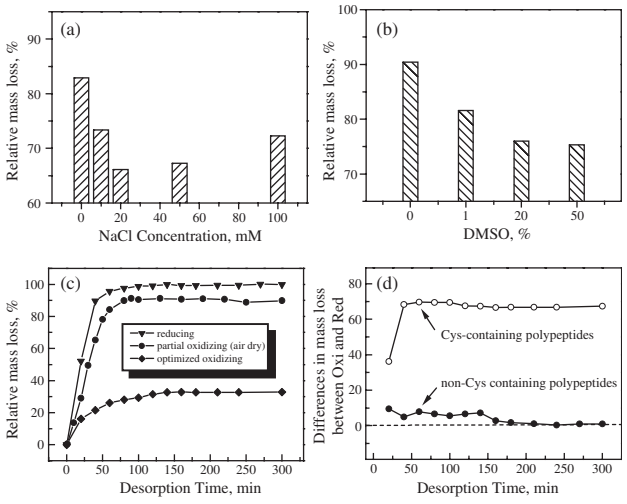

Fig. 7. Optimization of oxidizing conditions for S–S locking. (a) Effect of salt. (b) Effect of DMSO. (c) Effect of air drying versus DMSO oxidation during film assembly. (d) Negative control involving non-cysteine-containing polypeptides (PLL and PLGA).

for crosslinking have been optimized. Figures 7a, b and c show the influence of different variables on polypeptide thin film stability. To some degree stability depended on NaCl concentration (Fig. 7a). Best apparent stability was obtained at 20 mM NaCl. As shown in Figure 4, NaCl concentration influenced the amount of peptide deposited per adsorption step, and a transition in assembly behavior (Fig. 4b) and conformation (Fig. 5a and 5b) was observed around 20 mM NaCl. Taken together, the data would suggest that the stability of layer-by-layer assembled films of Peptides 1 and 2 is particularly favored at this salt concentration. Apparently, charge shielding influences the deposition, density, and conformation of peptides in the films and, consequently, formation of S–S bonds between molecules.

DMSO promotes oxidation of thiols to disulfides, the rate depending on DMSO concentration at a given temperature. Figure 7b shows that increasing DMSO in the oxidizing solution at pH 7.5 led to increasing film stability. Maximum stability was reached at about 20% DMSO during overnight treatment. A simpler but slower oxidation process for S–S bond formation would be to rely on atmospheric oxygen. Air-drying a sample partially stabilizes the film in comparison with nitrogen-drying: about 10% less loss of material was found after acidic-pH treatment. In other words, S–S bond formation does occur in the film during air drying but is relatively limited for such a short drying period (less than 1 min). Even in the presence of a mild oxidizing agent, in this case DMSO, the kinetics of S–S bond formation are relatively slow (Fig. 3). Our results suggest that conditions for optimizing S–S crosslinking of Peptides 1 and 2 at ambient temperature are: 20 mM NaCl for film assembly at neutral pH, air drying, and 20% DMSO oxidation overnight at pH 7.5. Films prepared under such conditions lose considerably less material at acidic pH after 300 min than films stabilized by electrostatic and van der Waals interactions alone (Fig. 7c). Presumably this reflects the relative magnitude of bond energies.

Our results would suggest that the physical cause of film disassembly in reducing samples was protonation of carboxylate groups in the negatively-charged peptide and electrostatic repulsion between the positively-charged peptides, leading to disintegration of the deposited film in the absence of crosslinking. Oxidation of cysteine-containing films led to retention of about 70% more mass than non-oxidation, despite charge repulsion. S–S bond formation after DMSO oxidation was confirmed using Ellman's method (see Ref. [33] for an example). Treatment of the film in an oxidizing solution promotes the formation of S–S bonds from free sulfhydryls, presumably by crosslinking intralayer like-charged peptides as well as interlayer oppositely-charged peptides. The result is a 3-D structured film which remains on the substrate even in acidic solution. By contrast, no difference in mass loss was observed with the non-Cys-containing polypeptides PLL and PLGA after oxidizing treatment compared to reducing treatment (Fig. 7d). Taken together, the results provide strong evidence in support of the claim that the designed polypeptide multilayer films were stabilized by S–S crosslinking, and that film stability can be optimized by adjusting conditions.

4. CONCLUSIONS

We have shown that S–S bond formation in a polypeptide multilayer is a powerful tool for increasing film stability. S–S bonding is much stronger than hydrogen bonding ($\sim$350 kJ $>$ $\sim$10 kJ). In view of this, it is perhaps not surprising that S-S bonds are found in many secreted proteins, for example lysozyme and antibodies, stabilizing folded structure in an oxidizing environment and increasing resistance to proteolytic degradation. This form of polypeptide film stabilization, which is biomimetic in nature, can be achieved simply by means of exposure to oxygen; it requires no "additional" treatment involving a toxin, as in glutaraldehyde crosslinking.

The mass of peptide deposited per adsorption step increases with increasing ionic strength. FTIR suggests that a multilayer film made of the designed peptides will be hydrophilic. The short designed peptides studied here display mainly random coil structure in solution but form predominantly β-sheet structures in thin films deposited at neutral pH, according to CD. There was no obvious change in secondary structure content on S–S crosslinking. Optimal oxidation of free thiols to S–S in polypeptide films was achieved by assembling the polymers at 20 mM NaCl, drying the films in air, and treating the films with 20% DMSO overnight at pH 7.5.

The present work could advance experimental and theoretical studies on adsorption of polyelectrolytes on a charged surface and provide a broader basis for technological exploitation of peptide LbL. Peptide thin films are

expected to be useful in biomedicine, bioengineering, food science, and other areas of technology development.

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