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Control of stability of polypeptide multilayer nanofilms by quantitative control of disulfide bond formation

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Abstract
The crosslinking of polymers in a polymeric material will alter the mechanical properties of the material. Control over the mechanical properties of polyelectrolyte multilayer films (PEMs) could be useful for applications of the technology in medicine and other areas. Disulfide bonds are 'natural' polypeptide crosslinks found widely in wild-type proteins. Here, we have designed and synthesized three pairs of oppositely charged 32mer polypeptide to have 0, 4, or 8 cysteine (Cys) residues per molecule, and we have characterized physical properties of the peptides in a PEM context. The average linear density of free thiol in the designed peptides was 0, 0.125, or 0.25 per amino acid residue. The peptides were used to make 10-bilayer PEMs by electrostatic layer-by-layer self-assembly (LBL). Cys was included in the peptides to study specific effects of disulfide bond formation on PEM properties. Features of film assembly have been found to depend on the amino acid sequence, as in protein folding. Following polypeptide self-assembly into multilayer films, Cys residues were disulfide-crosslinked under mild oxidizing conditions. The stability of the crosslinked films at acidic pH has been found to depend on the number of Cys residues per peptide for a given crosslinking procedure. Crosslinked and non-crosslinked films have been analysed by ultraviolet spectroscopy (UVS), ellipsometry, and atomic force microscopy (AFM) to characterize film assembly, surface morphology, and disassembly. A selective etching model of the disassembly process at acidic pH is proposed on the basis of the experimental data. In this model, regions of film in which the disulfide bond density is low are etched at a higher rate than regions where the density is high.

M Supplementary data files are available from stacks.iop.org/Nano/17/5726

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Multilayer films have been investigated by numerous researchers for use in applications ranging from micro-devices to biomaterials [1]. Examples include polymeric electronic micro-devices [2], ion selective membranes [3], redox-active polyelectrolyte multilayer films [4], and biofunctional microcapsules [5]. LBL is a method of controlled fabrication of multilayer films by the alternate deposition of oppositely charged chemical species on a suitably charged

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substrate [1, 6, 7]. The adsorbing surface both attracts oppositely charged particles in the adsorbing layer and repels like-charged particles. This allows fine-tuning of film properties, notably, layer thickness on the nanometer scale. LBL is a relatively rapid and simple way to produce complex multilayer structures. In PEM assembly, control over variables such as the chemical structure of the polyelectrolyte, pH, ionic strength, temperature, immersion and rinsing times, and post-preparation treatment enable the realization of a wide variety of film architectures [8–13]. The present report discusses PEMs made of designed polypeptides.

Control over PEM stability under different conditions is a significant challenge. A change of solvent, pH, ionic strength, or layer architecture can affect PEM structural integrity [8, 14–19]. Covalent crosslinking of polymers is one means of stabilizing PEMs [17, 20–22]. Crosslinking could potentially result in enhanced physical, chemical, and biological properties of PEMs [23, 24]. For example, crosslinking can influence film permeability and conductivity [25, 26]. There are potential drawbacks, however, to some approaches to crosslinking. Certain chemical crosslinking molecules, for example glutaraldehyde, will not only modify PEM structure in an uncontrolled manner but also affect biocompatibility [16]; glutaraldehyde is toxic [27]. In general, the effects of a particular approach to crosslinking will vary with PEM architecture.

The disulfide bond is a crosslink that forms between two free thiol groups under oxidizing conditions. Such bonds, being covalent, are much stronger than hydrogen bonds, van der Waals interactions, and electrostatic interactions—the types of non-covalent interaction that stabilize folded protein structure. Disulfide bonds play an important role in increasing the stability of many native proteins. In hen egg white lysozyme, for example, disulfide bonds are formed between Cys side chains in the same polypeptide chain [28, 29], whereas in the mature form of the peptide hormone insulin, disulfide bonds are formed between Cys residues in two different chains. Disulfide crosslinking is reversed in a sufficiently reducing environment [30]. Thiol-containing polypeptides have been combined with LBL to form 'naturally' crosslinked polypeptide PEMs [17–19, 31, 32] and polypeptide multilayer microcapsules [33]. Disulfide bond-stabilized PEMs could be useful for microencapsulation, cell culture coatings, or PEMs and microcapsules that are sensitive to the surrounding redox potential [13, 33, 34].

Here, three pairs of oppositely charged polypeptides were designed and synthesized to contain a certain number of Cys residues. The polypeptides were assembled into PEMs by LBL. We have investigated how control over the Cys content of the peptides and disulfide crosslinking enables control over physical properties of the polypeptide PEMs. Research in this direction will also inform the study of non-peptide polymers that can form intermolecular disulfide bonds [34].

2. Experimental section

2.1. Polypeptides

Three pairs of designed peptides were prepared by solid-phase synthesis. The design principles of the peptides for multilayer film assembly are described elsewhere [35]. The synthesis products were analysed by mass spectrometry (Louisiana State University, Baton Rouge, USA) and used without further purification. The sequences were:

P0: $(KVKVKVKV)_3KVKVKVKY$
N0: $(EVEVEVEV)_3EVEVEVEY$
P1: $(KVKGKCKV)_3KVKGKCKY$
N1: $(EVEGECEV)_3EVEGECEY$
P2: $(KCKGKCKV)_3KCKGKCKY$
N2: $(ECEGECEV)_3ECEGECEY$

where K, E, V, G, C, and Y represent the amino acids lysine, glutamic acid, valine, glycine, Cys, and tyrosine, respectively. Designed polypeptides P0 and N0, P1 and N1, and P2 and N2 contain 0, 4, and 8 Cys residues each, respectively. The pK_as of the Lys and Glu side chains are about 10.5 and 4.3 [30]. P0, P1 and P2 will have about the same positive net charge in aqueous solution at pH 7.4; N0, N1 and N2 will have about the same negative net charge at the same pH (figure 1). The charge density of the positive peptides is close to that of the negative peptides under the conditions of the experiments. 4 kDa poly(L-lysine) (PLL) and 5 kDa poly(L-glutamic acid) (PLGA), from Sigma-Aldrich (USA), were used in control experiments.

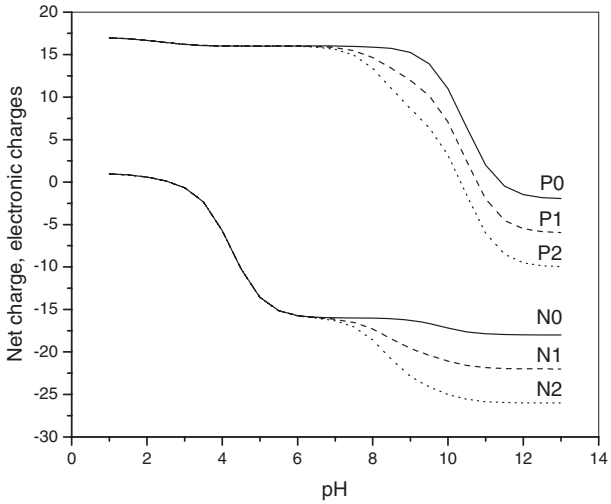

Figure 1. Estimated peptide net charge versus pH [30]. Peptides P0, P1, P2, N0, N1, and N2 all have about the same linear charge density at pH 7.4.

2.2. Multilayer film assembly, oxidization, and disassembly

All chemicals and materials were from Sigma-Aldrich and used without further purification, except as indicated. Lyophilized peptides were dissolved in 10 mM Tris, 50 mM NaCl, 0.1% NaN_3, pH 7.4, bubbled with nitrogen for 0.5 h. The final peptide concentration was 1 mg ml^{-1}. PEMs were assembled on quartz microscope slides (Electron Microscopy Sciences, USA) or on N-type ⟨100⟩ silicon wafers (Montco Silicon Technologies Inc., USA). The substrate cleaning processes are described elsewhere [36]. Substrates were immersed in a solution of P0, P1, P2, or PLL, followed by immersion in a solution of N0, N1, N2, or PLGA, for 15 min

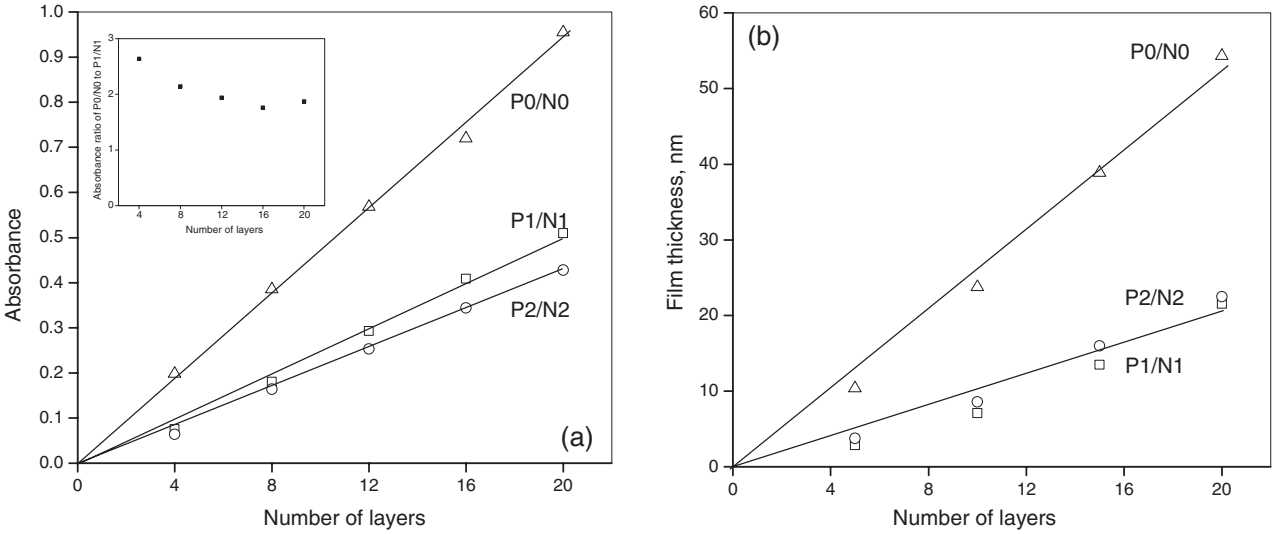

Figure 2. Film thickness. (a) Peptide bond absorbance at 190 nm during the assembly process. Inset: ratio of P0/N0 absorbance to P1/N1 absorbance. The ratio is approximately 2 throughout the 0–10 layer range. The molecular weights of P0, N0, P1 and N1 are 3719, 3733, 3567 and 3581 Da, respectively. The calculated ratio of the sum of the molecular weights of P0 and N0 to the sum of the molecular weights of P1 and N1 is 2.1; two layers of P0 or N0 are deposited per adsorption step, whereas one layer of P1 or N1 is deposited. See [37].
(b) Ellipsometric film thickness during the assembly process. P1/N1 and P2/N2 behave similarly with regard to absorbance and thickness.

per polymer adsorption step. The film was rinsed three times in separate baths of deionized water for 2, 1, and 1 min after each peptide adsorption step and dried with nitrogen gas. The drying step is not necessary for PEM assembly, but it was required for the *ex situ* film characterization described here.

Ten-bilayer PEMs were oxidized in 10 mM Tris, 50 mM NaCl, 0.1% NaN_3, 1 μM $MnCl_2$, pH 7.4 and 40% (v/v) dimethylsulfoxide (DMSO). Fully assembled PEMs were immersed in oxidizing solution for about 48 h, rinsed with deionized water, dried with an air gun, and left in air for about 40 h. This mild oxidization treatment promoted disulfide bond formation between cysteine residues in PEMs P1/N1, P2/N2, P1/PLGA, and PLL/N1 [17, 31]. Then, PEMs were immersed in acidic pH solution to assess film stability. The disassembly buffer was 10 mM KCl, 50 mM NaCl, 0.1% NaN_3, pH 2.0 or 10 mM glycine, 50 mM NaCl, 0.1% NaN_3, pH 3.0, bubbled with air for 0.5 h. At acidic pH, non-crosslinked polypeptide PEMs disassemble spontaneously due to deionization of acidic side chains and electrostatic repulsion between polycations. The efficiency of covalent crosslinking in PEMs was assessed by optical film thickness at defined time points after the initiation of disassembly. PEMs were rinsed with deionized water and dried with an air gun after each disassembly step.

2.3. Analysis of PEMs by physical techniques

Several methods were used to obtain complementary data sets. Circular dichroism spectrometry (CD) enables the determination of structural properties of chiral molecules by detection of the differential absorption of right- and left-circularly polarized light. CD spectra of polypeptide PEMs deposited on quartz microscope slides were recorded in the range 180–260 nm at ambient temperature with a Jasco model J-810 spectropolarimeter (Japan) for the characterization of secondary structure content. Absorbance spectra were recorded in the range 190–300 nm with a Shimadzu UV-1650 PC UV–vis spectrophotometer (Japan) for the determination of optical film thickness. Film assembly and disassembly were assessed by changes in absorbance; film thickness and therefore UV absorbance decrease on the exposure of a polypeptide PEM to an aqueous solution at strongly acidic pH. The film thickness was also measured with a Sentech SE 850 ellipsometer (Germany) at ambient temperature. The spectral range was 350–820 nm, and the angle of incidence was 70°. Surface morphology was determined by atomic force microscopy (AFM) in tapping mode at ambient temperature with a Q-scope TM 250 scanning probe instrument (Quesant Instrument Corp., USA).

3. Results

3.1. PEM assembly

The calculated absolute value of net charge is about the same for P0, P1, and P2 and for N0, N1, and N2 at pH 7.4 (figure 1). Figure 2(a) shows that P1/N1 (4 Cys residues per peptide) and P2/N2 (8 Cys residues per peptide) had about the same UV absorbance increment per peptide deposition step. By contrast, P0/N0 (0 Cys residue per peptide but a comparatively large number of valine residues) had an absorbance increment about twice that of P1/N1 and P2/N2 (inset of figure 2(a)). Nevertheless, the growth of P0/N0 with an adsorption step, like that of P1/N1 and of P2/N2, was approximately linear. Figure 2(b) shows the corresponding ellipsometry data. The results of the two techniques are consistent: P0/N0 is thicker than P1/N1 or P2/N2. The absorbance and therefore the thickness of P0/N0 was close to twice that of P1/N1 or P2/N2 (figure 2). Material deposition per adsorption step of P1 and N1 and of P2 and N2 was more limited than that of P0 and N0. Generally, a high linear density of charge will give a greater extent of intra-layer charge repulsion and therefore thinner layers. Cys titrates in the mildly basic pH range gives a

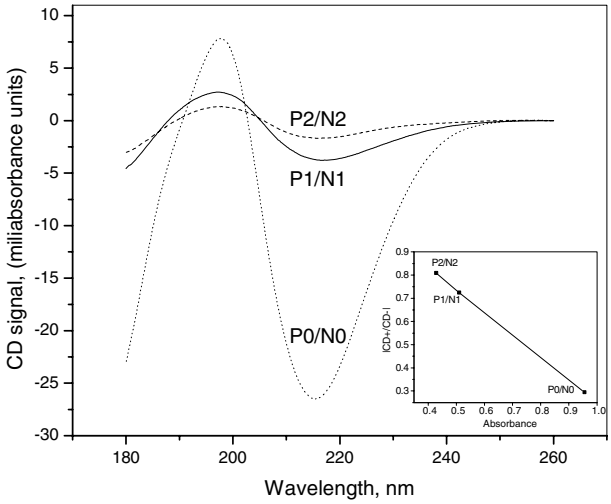

Figure 3. CD spectra of ten-bilayer films. The amplitudes of CD spectra follow the trend P0/N0 > P1/N1 > P2/N2. In P0/N0 the negative Cotton effect amplitude is about 240% larger than the positive one. The asymmetry of the positive and negative Cotton effect amplitudes of P1/N1 and P2/N2 is much smaller, with the negative amplitudes 39% and 25% larger than the positive ones, respectively. The inset illustrates a correlation between CD and UVS data for P0/N0, P1/N1 and P2/N2. |CD+/CD−| is the absolute ratio of the positive Cotton effect amplitude to negative Cotton effect amplitude for ten-bilayer PEMs. Absorbance was measured at 190 nm. CD spectra are sensitive to the secondary structure of the films, which will depend on different amino acid sequences of designed polypeptides.

combination of positive and negative charges, slightly reducing the linear charge density in peptides P1 and P2, and slightly increasing the linear charge density in peptides N1 and N2 (figure 1).

There are other differences in assembly behaviour. Cotton effect amplitudes, for example, vary as P0/N0 > P1/N1 > P2/N2 (figure 3). Deconvolution of the CD spectra suggests that these films had the following respective secondary structure content: β sheet, 54%, 27%, and 43%; β turn, 20%, 26% and 22%; random coil, 26%, 44% and 31%; and α helix, 0%, 3%, 4%. β sheet was predominant in P0/N0, while random coil was more abundant in P1/N1 and P2/N2. All the films showed substantially more β structure than α helix at neutral pH, as in related work on other peptides [33, 36]. Deconvolution suggests that more than half of all residues in P0/N0 are in a β strand conformation. A β sheet can be stabilized by hydrogen bonds formed between different regions of the same molecule or between different molecules.

Further analysis of the CD and UVS spectra for P0/N0, P1/N1 and P2/N2 is shown in the inset of figure 3. |CD+/CD−| represents the absolute value of the ratio of the amplitude of the positive Cotton effect to that of the negative one after baseline subtraction. Differences in this ratio are indicative of differences in the internal structure of polypeptide PEMs. Absorbance was measured at 190 nm. Low UV absorbance correlates with high |CD+/CD−|. The extent of peptide deposition is related to film structural properties for the peptides studied here. The ratio of UV absorbance to |CD+/CD−| ratio varied as P0/N0 > P1/N1 > P2/N2.

Surface characterization of PEMs by AFM showed that the topography of P1/N1 and of P2/N2 was about the same (supplemental material is available at stacks.iop.org/Nano/17/5726, figure S1). It would appear that the number of Cys residues had relatively little effect on peptide assembly under the conditions studied here (see figure 2). The situation could be different at more basic pH, where the probability of ionization of Cys is greater [18].

3.2. PEM disassembly

Experiments were performed to test polypeptide PEM stability and the formation of disulfide crosslinks after film oxidization. Figure 4(a) shows the UVS absorbance of P0/N0, P1/N1, and P2/N2 during film disassembly at pH 2. P0/N0 lost nearly all its material within 0.5 h, whereas half of the optical mass of P1/N1 was still present after 2 h and P2/N2 showed effectively no change in the same time interval. The data suggest that the number of Cys residues per peptide will influence film stability and retention of material at acidic pH under oxidizing conditions.

Figure 4(b) shows the ellipsometric thickness of peptide PEMs built on silicon dioxide. P0/N0 lost all of its material within 15 min at acidic pH; no crosslinks were present. P1/N1 lost 30% of its thickness after 4.5 h, while P2/N2 retained nearly all of its material in the same time interval. The ellipsometry measurements resemble the UVS data in figure 4(a). The results are consistent with the hypothesis that the number of disulfide bonds actually formed in a film during the oxidation step is related to the number of Cys residues present [17], and that film stability increases as the number of disulfide bonds increases.

Control experiments on the role of disulfide crosslinking in PEM disassembly were performed with ten-bilayer films made of different combinations of P1, N1, PLL, and PLGA. Neither PLL nor PLGA can form disulfide bonds because thiol is not present. Figure 5 presents the assembly and disassembly behaviour of the films. As shown in the inset, the assembly process for P1/N1 resulted in the greatest amount of material being deposited in the assembly of 20 layers, and PLL/PLGA the least. The adsorption of highly charged polypeptides is driven primarily by coulombic attraction and the gain in entropy on release of counterions, though it can be influenced by hydrophobic interactions [37], and adsorption is limited primarily by coulombic repulsion but also by thermal energy [38]. Lysine and glutamic acid side chains are charged at pH 7.4: the absolute charge density of PLL or PLGA is substantially higher than that of P1 or N1. P1/N1, P1/PLGA, PLL/N1 and PLL/PLGA were disassembled in glycine–HCl buffer, pH 3.0. P1/N1 showed very little mass loss after 40 min; it was the most stable film at pH 3. P1/PLGA lost 5% of its mass in the same time period, while PLL/N1 lost about 10%. By contrast, PLL/PLGA lost 45% of its optical thickness in the first 5 min of disassembly and about 55% by 40 min. The trend with respect to percentage mass lost at pH 3.0 was PLL/PLGA > PLL/N1 > P1/PLGA > P1/N1. The trend for mass deposited at pH 7.4 was just the opposite.

Disassembly of P1/N1 at pH 2.0 has also been monitored by AFM. Representative surface images and vertical scan profiles representing surface roughness are shown in figure 6.

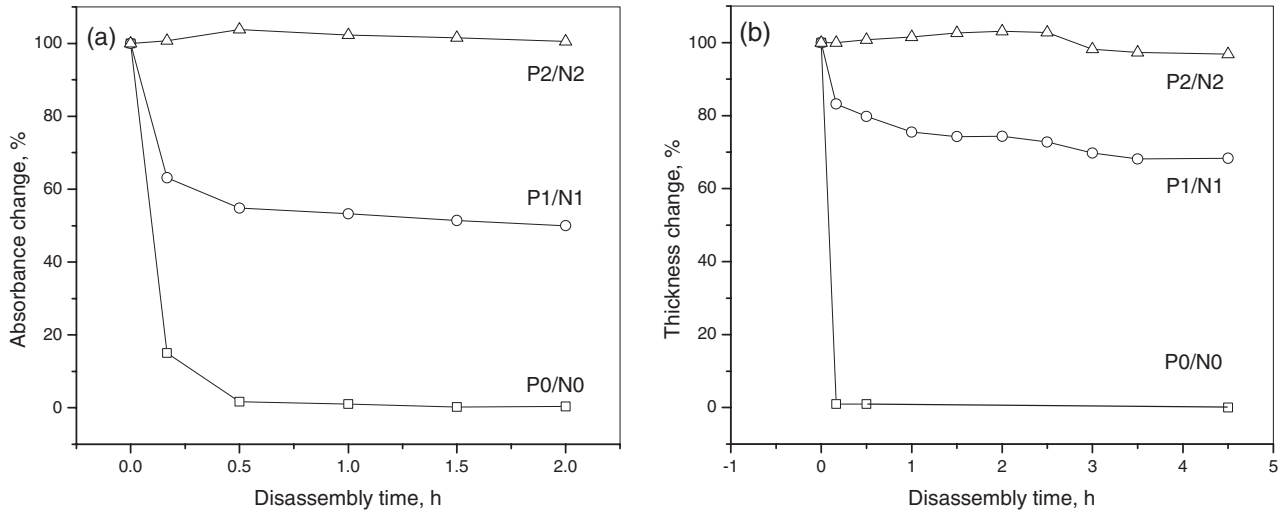

Figure 4. Polypeptide film disassembly in pH 2.0 KCl buffer. (a) Absorbance at 190 nm during film disassembly. P0/N0 loses almost all of its absorbance within 0.5 h; P1/N1 loses about 45% of its absorbance within 0.5 h but only an additional 5% within 2 h; P2/N2 loses practically no material on the same timescale. (b) Ellipsometric thickness during film disassembly. After 4.5 h of disassembly, P2/N2 has retained almost all its thickness and P1/N1 has lost 30% of its thickness. P0/N0 loses all its thickness within 15 min.

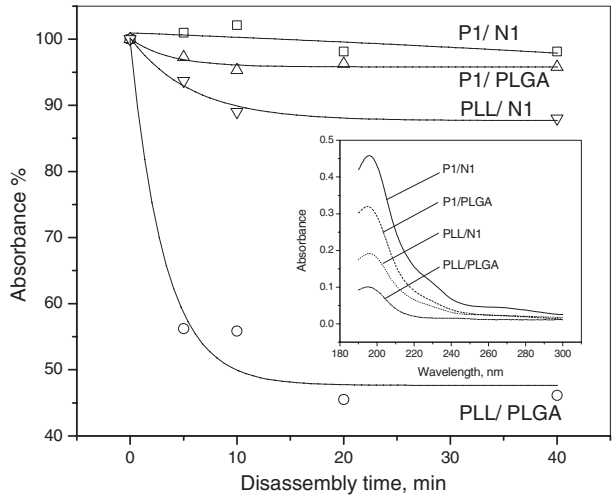

Figure 5. Film disassembly in pH 3.0 glycine–HCl buffer. After 40 min of disassembly, P1/N1 has almost the same mass as at completion of film assembly. P1/PLGA loses 5% of its mass while PLL/N1 loses about 10% in the same time period. PLL/PLGA loses 45% of its mass within the first 5 min of disassembly, and after 40 min it has lost about 55%. The time constants of film disassembly were determined by fitting the data with a first-order exponential decay model. P1/PLGA, PLL/N1 and PLL/PLGA have time constants of 5.8, 3.7, and 3.2 min, respectively. Inset: assembly behaviour of the same four pairs of peptides. The greatest amount of material is deposited in P1/N1; the least in PLL/PLGA.

On oxidation, apparent PEM graininess decreased from about 100 nm to about 50 nm in 'granule' diameter. Practically no film material was lost during oxidation (data not shown). PEM roughness was on the order of 6 nm. After 30 min of film disassembly (figure 6(c)), however, the PEM surface was smoother than before disassembly and graininess was less obvious. After 1 h of disassembly (figure 6(d)), the PEM had a roughness on the order of 10 nm, apparently greater than the roughness after 30 min of disassembly (7 nm, figure 6(c)).

4. Discussion

Films based on biopolymers or polypeptides are hydrogels; they are 'soft' and sensitive materials [16, 23, 39–41]. Common polymers in multilayer film assembly are organic homopolyelectrolytes of high water solubility but low biocompatibility. Polypeptides are a type of weak polyelectrolyte with inherent biocompatibility [13]. In organisms, polypeptides are metabolized into amino acids, which are essential precursors of protein synthesis. In previous work we have used designed polypeptides to prepare multilayer films [17–19, 31–33, 35, 42]. Amino acid composition and sequence have been found to influence LBL film assembly and disassembly, providing both an explanation for observed film assembly behaviour and a foundation for the development of applications of polypeptide PEM technology [13, 37, 38].

Crosslinking of PEMs presents advantages for maintaining film properties under different conditions. The effects of crosslinking ploy(allylamine hydrochloride)/poly(acrylic acid) films by heat-induced amidation have been investigated in considerable depth by Bruening and co-workers [43]. Crosslinking can also be achieved with photo-reactive diazo-resin and sulfonate groups under UV irradiation [44]. These methods, though useful, cannot be achieved under mild reaction conditions. Moreover, they are irreversible.

In previous work we have studied some aspects of polypeptide PEM stabilization by disulfide bond formation [17–19, 31, 33]. Disulfide bonds are important for the stability of some native protein structures [30]. The stability of disulfide crosslinked polypeptide multilayer films has been tested in various harsh physical and chemical conditions [32]. The reversibility of disulfide bond formation has been demonstrated for polypeptide multilayer films [17, 18, 31] and microcapsules at neutral pH [33]. Such reversibility is potentially useful for the preparation of biodegradable PEMs and microcapsules that are responsive to a change of environmental redox potential [13, 33]. The purpose of the present study

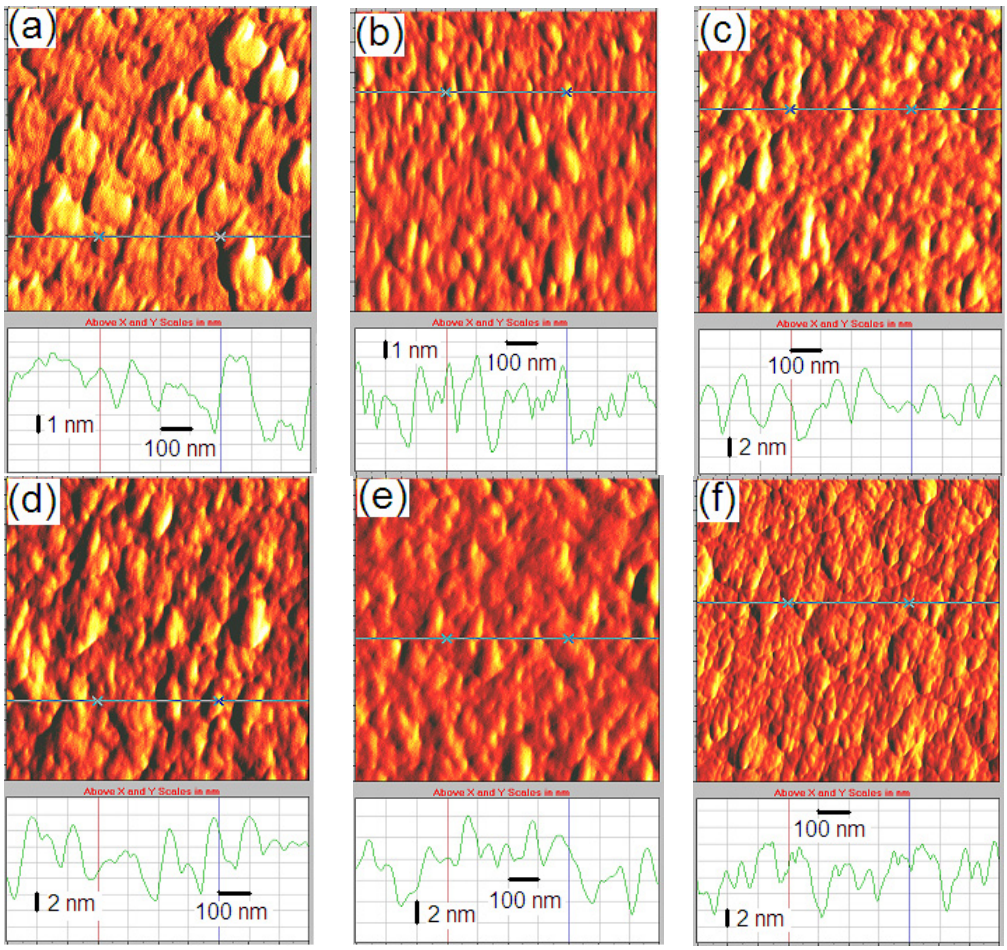

Figure 6. AFM images of P1/N1 during disassembly at pH 2.0: (a) after assembly, (b) after oxidation, (c) after 30 min of disassembly, (d) after 1 h of disassembly, (e) after 2 h of disassembly, (f) after 3 h of disassembly. Image dimensions are $1 \times 1\ \mu m^2$, and the z-axis scale is 24 nm for (a)–(f). Surface roughness (from section profiles) is about 6, 6, 7, 10, 6, and 8 nm, respectively.

was to assess control over polypeptide PEM stability by control over both peptide structure and disulfide bond formation. The data show that amino acid composition and sequence influence polypeptide PEM assembly, and that the number of Cys residues per peptide influences film stability, as assessed by the rate and extent of film disassembly at acidic pH.

4.1. Film assembly

The accessible surface area (ASA) of a peptide molecule can be defined as the sum of the mainchain ASA and the cumulative ASA of side chains. Contributions to the ASA are nonpolar or polar. The hydrophobicity/hydrophilicity of the amino acids used in this work is given in table 1. The average ASA per polypeptide (table 1(b)) was calculated from the ASA of the individual amino acids (table 1(a)) and the polymer sequence. From table 1(b), the calculated hydrophobicities of the designed peptides vary as P0/N0 > P1/N1 > P2/N2. Solubility of the peptides in aqueous solvent will influence the adsorption behaviour in a multilayer film context. The valine side chain in particular is hydrophobic, giving it very different electronic properties from Lys or Glu. P0 and N0, P1 and N1, and P2 and N2 have 15, 7, and 3 valine residues per molecule, respectively. Figure 2 shows that the percentage of valine in P0/N0 is quite large, approximately 50%; both electrostatic interactions and hydrophobic interactions will influence PEM formation at neutral pH and disassembly at acidic pH. In aqueous solution, P0 or N0 molecules will form aggregates due to hydrophobic interactions (valine side chain), with the charged side chains (from lysine or glutamic acid) pointing toward the polar solvent [45]. As a result, two layers of peptide will form in each deposition step, as determined experimentally [37] and illustrated in figure 7. This is consistent with the UVS, CD, and ellipsometry experiments discussed here, which show twice as much P0/N0 deposition as P1/N1 or P2/N2.

The polypeptide backbone, though constrained by the rigidity of the peptide bond, is exceptionably flexible in the vicinity of glycine residues [30]. There is no glycine in P0 or N0, whereas P1, N1, P2 and N2 have four glycine residues each. During the peptide assembly process, then, the adsorption of P1, N1, P2 and N2 will be less favoured than that of P0 and N0 from the point of view of chain entropy; that is, P1, N1, P2 and N2 must give up more backbone degrees of freedom to become deposited on a surface than P0 or N0. This could have a significant effect on peptide adsorption, consistent with the results and conclusions of Flory and colleagues [46].

Table 1. Hydrophobicity/hydrophilicity of polypeptides.

(a) Accessible surface area and hydropathy of amino acid residues

Amino acid residue	Total (Å^2)	Main-chain atoms (Å^2)	Side-chain atoms (Å^2)			Hydropathy index
			Total	Nonpolar	Polar	
Cys (C)	140	36	104	35	69	2.5
Glu (E)	183	45	138	61	77	−3.5
Gly (G)	85	85	—	—	—	−0.4
Lys (K)	211	44	167	119	48	−3.9
Tyr (Y)	229	42	187	144	43	−1.3
Val (V)	160	43	117	117	—	4.2

(b) Physical properties of designed peptides

Polypeptide	Average surface area of side chains (Å^2)	Fraction nonpolar	Average hydropathy	Average thiol density per residue
P0	144	0.83	−0.02	0
N0	130	0.69	0.18	0
P1	128	0.73	−0.8	1/8
N1	113	0.57	−0.6	1/8
P2	126	0.66	−1.0	1/4
N2	112	0.49	−0.8	1/4

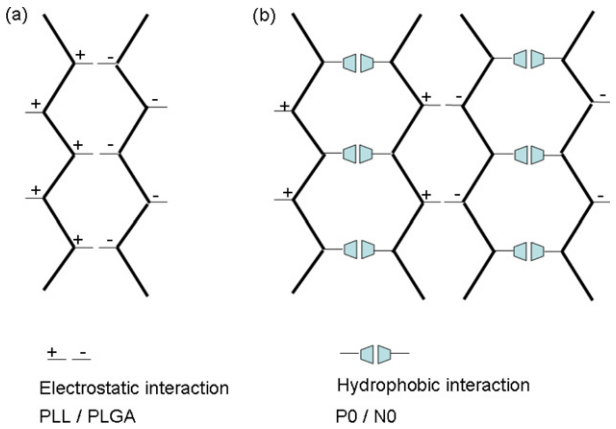

Figure 7. LBL assembly mechanism. (a) The main driving force in LBL is coulombic attraction and the gain in entropy on the release of counterions to solution. Effectively one peptide layer only is formed during each adsorption step. (b) Assembly of peptide molecules with alternating charged and hydrophobic side chains. In aqueous solvent, two peptide layers will form in each deposition step [42].

4.2. Film oxidation

DMSO promotes oxidation of thiols to disulfides, the rate depending on DMSO concentration and temperature. Increasing the DMSO concentration in the oxidizing solution of a Cys-containing polypeptide PEM at pH 7.5 will increase PEM stability [31]. A simpler oxidation process requires atmospheric oxygen only. In the present work, PEMs were oxidized for 40 h at ambient temperature in 40% DMSO, pH 7.4 and 48 h in air.

Designed polypeptides P1 and N1 have four Cys residues per molecule, P2 and N2 have eight Cys residues, and P0 and N0 have none. It is plausible that more intermolecular disulfide bonds will form in P2/N2 than P1/N1 for the same extent of film oxidation, judging by the approximately equal number of molecules deposited per adsorption step (figure 2), the greater number of Cys residues in P2 and N2 than in P1 and N1, and the absence of disulfide bonds in P0/N0. Although the formation of disulfide bonds has not been detected directly in the experiments described here, it has been tested indirectly in the PEM disassembly experiments, since crosslinks increase film stability and retard film disintegration under various conditions [17, 32, 47, 48]. The key structural difference between P2 (or N2) and P1 (or N1) is the number of Cys residues.

4.3. Disassembly behaviour

At pH 2.0, P0/N0 lost almost all of its mass and thickness within 15–30 min. The physical cause of disassembly in P0/N0 is protonation of carboxylate groups in the negatively charged peptide and electrostatic repulsion between the positively charged peptides, leading to disintegration of the deposited multilayer film in the absence of crosslinking [17, 31]. By contrast, oxidized P1/N1 (four cysteine residues per molecule) retained about 50% of its mass and 70% of its thickness, and P2/N2 (containing eight cysteine residues) retained nearly 100% of both mass and thickness under the same conditions (figure 4). Disulfide bonds stabilized these polypeptide PEMs despite charge repulsion, consistent with our earlier work [17]. Oxidation of a thiol-containing film will promote the formation of disulfide bonds, presumably crosslinking peptides of like charge within layers and peptides of opposite charge between layers [31]. When crosslinked P1/N1 was treated with a reducing reagent, the film disintegrated within 2 h from the rupture of disulfide bonds [17]. By controlling the number of Cys residues per unit length in a polypeptide chain, it is possible to control the extent of disulfide bond formation in a peptide PEM and film stability at acidic pH. Controlled

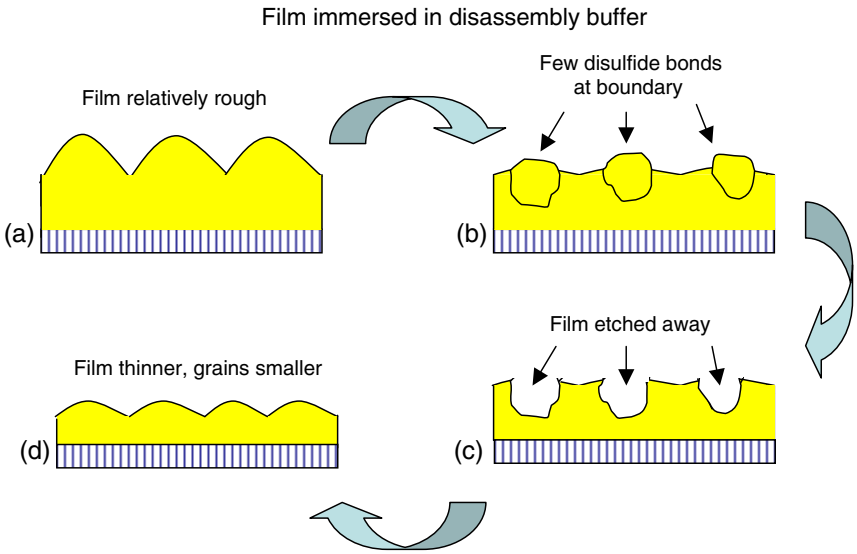

Figure 8. Model of disassembly of Cys-containing peptide PEMs at acidic pH. (a) LBL multilayer peptide film assembled on substrate. After oxidation, disulfide bonds form within and between layers. (b) Film immersed in acidic solution. The solvent etches the film, protuberant parts first. The film becomes smoother. (c) Regions with few or no disulfide bonds are etched away. The film surface becomes rougher, but the grains are smaller than in (a). (d) Acidic disassembly solution etches protuberant parts of the PEM. The surface becomes smooth.

film disintegration in response to a change in environmental pH or reducing potential could be useful for the sustained release of encapsulated materials, drug delivery, or other applications [13, 33, 34].

Analysis of the disassembly behaviour of P1/N1, P1/PLGA, PLL/N1 and PLL/PLGA at acidic pH further illustrates the ability to control peptide PEM stability depending on peptide structure and the extent of crosslinking. During oxidization under mild conditions, P1/N1 will form disulfide bonds in three dimensions: within layers and between layers. P1/PLGA and PLL/N1, by contrast, will form disulfide bonds within layers only, at least to the extent that polymers from neighbouring layers do not interdigitate; PLL/PLGA cannot form 'natural' covalent intermolecular crosslinks. The experimentally determined stability of the peptide PEMs at acidic pH was P1/N1 $>$ P1/PLGA $\approx$ PLL/N1 $>$ PLL/PLGA. The results provide further indirect evidence for the existence of disulfide bonds in the PEMs and indicate the influence of the type of disulfide crosslinking on film stability.

Film surface morphology data, obtained by AFM, suggest how disulfide bonds could influence film stability. The surface of P1/N1 appears to have changed from 'smooth' to 'rough' to 'smooth' to 'rough' during disassembly at acidic pH. A schematic depiction of the data is given in figure 8. When a polypeptide PEM is immersed in acidic disassembly solution at pH 2.0, protuberances on the film surface, which have a large radius of curvature, are etched first. As a result, film roughness decreases. At the same time, regions of film not connected to the surrounding regions by a sufficient number of S–S bonds are etched, leaving the surface rougher. Grain size, however, decreases in this process. P2/N2 will disassemble in roughly the same way. There is less overall change, however, in P2/N2 than in P1/N1, because the extent of crosslinking is greater.

5. Conclusion

32mer peptides containing different numbers of Cys residues are useful for the fabrication of polypeptide PEMs by LBL. Disulfide bonds form between thiol groups under mild oxidizing conditions, increasing film stability at acidic pH. Combining control over the linear density of Cys residues in a peptide chain with film architecture and '2D' or '3D' disulfide bond formation provides quantitative control over the mechanical properties of films, notably film stability. The incorporation of Cys makes polypeptide PEM stability sensitive to the redox potential of the surrounding environment. This is potentially advantageous for the control of drug release from polypeptide multilayer films or capsules.

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