Notes

Internal Structure of Wet and Dry Polypeptide Multilayer Nanofilms

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Introduction

Layer-by-layer self assembly (LBL) is a method of multilayer film fabrication by alternate dipping of a charged solid support into solutions of oppositely charged species.[1] Diverse linear synthetic polyelectrolytes and natural biomacromolecules have been used to form multilayer films by LBL. Conventional polyelectrolytes such as poly(allylamine) hydrochloride (PAH) and poly(styrenesulfonate) (PSS) have flexible backbones and are not known to form regular structure in multilayer films. Layers of such polymers in the films are "fuzzy".

In a polypeptide molecule, by contrast, the partial double-bond character of the peptide unit places severe limits on polymer backbone flexibility.[2] In addition, each residue contributes to the polymer backbone an amide hydrogen atom and a carbonyl oxygen atom. These hydrogen-bonding groups play a key role in so-called secondary structures, α helices and β sheets.[3,4] Hydrogen bond donors and acceptors can influence the self-assembly of nonpeptide polymers in a multilayer context and the stability of the resulting film.[5,6]

The α-carbon atoms of the polypeptide backbone are chiral centers. Polypeptides therefore show differential absorption of left- and right-circularly polarized light, enabling analysis of structure by circular dichroism spectrometry (CD). The far-UV CD signal is particularly sensitive to the conformation of the peptide backbone, whether the molecules are in solution[7−9] or in a thin film.[10−13]

Here, we have used CD to monitor changes in internal film structure on film drying and rewetting. Peptides of various properties have been studied—high or low degree of polymerization, and large or moderate $|\lambda|$ near neutral pH. The reversibility of structural changes on film drying and rewetting has been investigated.

Experimental Section

Polymers. Lyophilized poly(L-lysine) (PLL, 93.8 kDa, "P0") and poly(L-glutamic acid) (PLGA, 97.8 kDa, "N0"; 5.0 kDa, "N2") were from Sigma (U.S.A.) and used without further purification. Designed 24mer polypeptides, $(KV)_{11}KY$ ("P1") and $(EV)_{11}EY_8$ ("N1"), were prepared by solid-phase synthesis, analyzed by high-performance liquid chromatography and mass spectrometry (Louisiana State University, Baton Rouge), and used without further purification.

Multilayer Film Assembly. Substrates were quartz microscope slides from Electron Microscopy Sciences (U.S.A.). Slides were cleaned by agitation in hot 1% sodium dodecyl sulfate (SDS) for 30 min, followed by immersion in 1% NaOH in 99.8% CH_3OH/H_2O (50/50, v/v) for 2−3 h, followed by agitation in a hot 1:3 (v/v) mixture of 98% H_2SO_4 and 27% H_2O_2 for 5 min ("piranha"). Slides were stored in piranha solution and rinsed thoroughly with ultrapure water and dried with nitrogen gas immediately before film assembly. Films were assembled from peptide solutions at a concentration of 1 mg/mL for 15 min per deposition step, rinsed thrice in ultrapure water, and dried with a gentle stream of nitrogen gas after each adsorption step unless stated otherwise. Phosphate buffer (8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 0, 10, or 50 mM NaCl, pH 7.4) was used for assembly and rewetting of $(P0/N0)_8$; 8 bilayers of peptides P0 and N0 were deposited in this film in 8 adsorption cycles (16 adsorption steps). Polypeptide aggregation and strong UV absorption was avoided in the assembly of $(P1/N1)_8$ by use of trishydroxymethylaminomethane (Tris) buffer (10 mM Tris, 10 mM NaCl, pH 7.4); 10 mM NaCl was used for rewetting. A mixture of 10 mM Tris, 50 mM NaCl, pH 7.4 was used for assembly of $(P1/N2)_6$; water was used for rewetting.

Multilayer Film Characterization. Spectra were collected with a Shimadzu UV-1650 PC UV−vis spectrophotometer (UVS, Japan) or a Jasco J-810 spectropolarimeter (Japan). For dry films, film-coated quartz microscope slides were placed in a sample holder and fixed in place. For wet films, film-coated slides were immersed in aqueous solution in a quartz cuvette with a 1 cm path length. CD data were collected every 1.0 nm. In general, signal intensity was excellent and the signal-to-noise ratio was high. None of the optical spectra presented here is smoothed.

Far-UV CD spectra were deconvoluted into contributions from secondary structures with the CONTIN/LL algorithm.[38−40] Different reference sets were used to analyze the dry and wet film spectra. As much as possible of each raw CD spectrum was included in the analysis, and the largest reference set was used: IBasis 1 for dry films (178−260 nm) and IBasis 4 for wet films (190−240 nm). Failure to ensure that the wavelength range of a spectrum matches the range of the reference set can lead to significant artifacts in the deconvoluted secondary structure content.

Further details of instrument settings for UVS and CD, and principles and methods of deconvolution of polypeptide multilayer film CD spectra, are reported in refs 13−16.

Results and Discussion

UV spectra of dry $(P0/N0)_8$ and dry $(P1/N1)_8$ are presented in Figure 1. As the concentration of NaCl in phosphate buffer increased from 0 to 50 mM, the optical mass of $(P0/N0)_8$ decreased by about 20% (Figure 1a). About 25% more $(P0/N0)_8$ than $(P1/N1)_8$ was deposited under the same conditions, for example, 10 mM NaCl (Figure 1a). Figure 1b shows that the wavelength of maximum absorption, due to the peptide bond, was ∼193 nm for the $(P0/N0)_8$ films and ∼196 nm for $(P1/N1)_8$. Signal intensity in the 210−250 nm range, which

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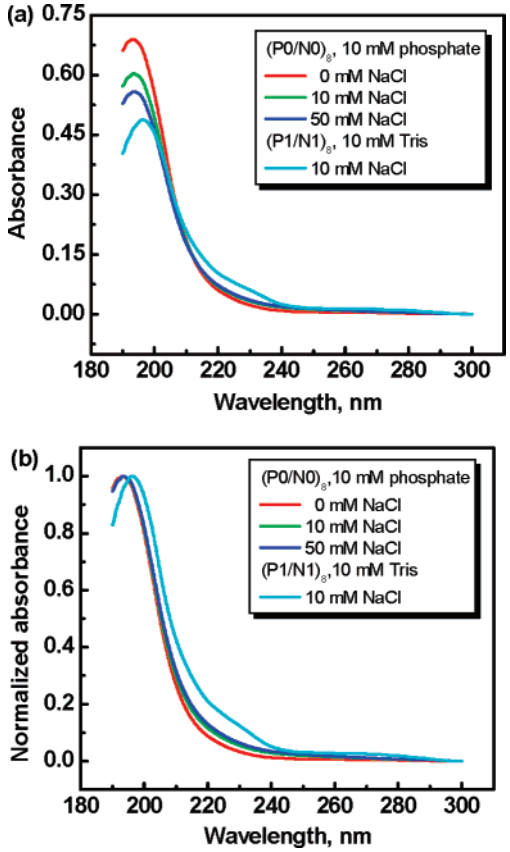

Figure 1. (a) As-obtained and (b) amplitude-adjusted UV spectra of dry films of $(P0/N0)_8$ and $(P1/N1)_8$. The qualitative difference in the P1/N1 spectrum from the P0/N0 spectra suggests a difference in internal film structure.

contains relatively little contribution from tyrosine, was stronger for $(P1/N1)_8$ than $(P0/N0)_8$. Salt ions increased the absorption of $(P0/N0)_8$ to a slight extent in the same range, possibly by influencing the solubility of polymer.

The average degree of polymerization of P0 (~448) and N0 (~647) was over 10-fold larger than for P1 and N1 (~24). This led to more extensive mass deposition of polymer in P0/N0 than P1/N1:[14,17,18] a single P0 or N0 can be partly adsorbed and partly extended into solution, whereas a P1 or N1 chain is effectively either completely adsorbed or in solution. Effects of ions in solution on the formation and dissolution of weak polyelectrolyte multilayers have been investigated previously.[19,20] It was found that the self-assembly of layers from weak polyelectrolytes can involve polymer adsorption and resolubilization, the balance depending on the salt and ionic strength. Phosphate ions in particular can induce the erosion of a multilayer film at relatively low ionic strength. The decrease in UV absorbance in $(P0/N0)_8$ on increase of NaCl concentration (Figure 1a) could therefore result from an increase in the solubility of polyion complexes or a change in the organization of molecules in the film. The extinction coefficient of a polypeptide chain in solution and therefore of a polypeptide multilayer film will depend somewhat on backbone conformation.[21,22] A qualitative difference in the shape of UV spectra of films which contain peptides from an identical number of adsorption steps suggests a qualitative difference in internal film structure.

Are changes in polyelectrolyte structure that occur during multilayer film drying and rewetting reversible? CD analysis of polypeptide multilayer films provides a means of addressing the matter. Figure 2 displays CD spectra of $(P1/N2)_6$ fabricated in 10 mM Tris, 50 mM NaCl, pH 7.4. The film was not dried

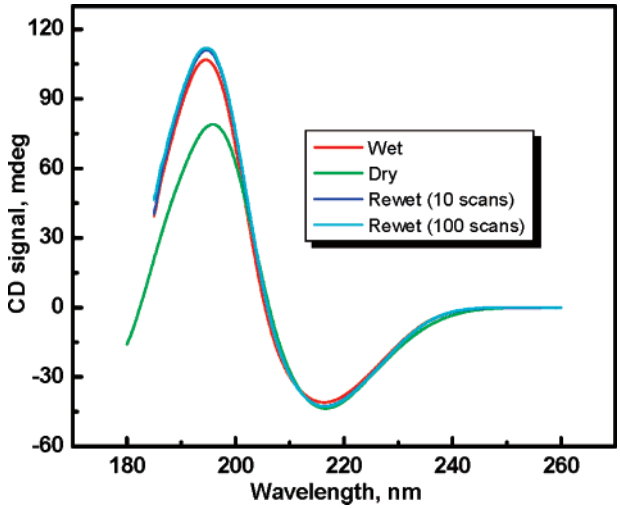

Figure 2. CD spectra of $(P1/N2)_6$ when wet, dry, and after rewetting. The buffer was 10 mM Tris, 50 mM NaCl.

between successive polymer adsorption steps. The wet and dry spectra show minor differences in shape, but the wet film spectrum and the spectrum after 16 min of rewetting are practically the same. The duration of each CD scan was about 1 min. The data provide clear evidence of a limited structural change on film drying and subsequent rewetting and of a substantial degree of reversibility of structural changes in the film. The data are consistent with a previous analysis of the reversibility of structural changes in PLL/PLGA films[13] and of the reversibility of swelling on dehydration.[23,24] Analysis by CD of multilayer films made of conventional polyelectrolytes, e.g., poly(styrene sulfonate) and poly(allylamine), is not possible because the polymers are achiral.

CD spectra and deconvolution data for $(P0/N0)_8$ when wet and when dry are shown in Figure 3. The positive band was incomplete for wet films due to the well-known interference of chloride ions below 200 nm. Hydration of $(P0/N0)_8$ resulted in no shift of the negative band at ~216 nm, but it did give a substantially reduced signal strength. The amplitude of the positive band, nearly zero in dry films, became comparable to that of the negative band in wet films. Nevertheless, deconvolution suggests that dry films and wet films contain a large amount of β sheet, β turn, and random coil but only a small amount of α helix, consonant with previous studies of PLL/PLGA films by CD and Fourier transform infrared spectroscopy (FTIR).[13−15,25,26] Apparently, random coil dominates in dry films and the percentage of β turn is relatively large, whereas in wet films β sheet is preponderant and the fraction of β turn is comparatively small. Deconvolution thus suggests that some molecules pass spontaneously from an irregular conformation to a more ordered β sheet structure on film rehydration.

CD spectra and deconvolution results of a multilayer film of $(P1/N1)_8$ under dry and under wet conditions are shown in Figure 4. On film rehydration, the negative band shifted to the UV by about 1 nm but the amplitude remained about the same. Such a small shift in wavelength is credible because the signal intensity and signal-to-noise ratio were extremely good, the data were not smoothed, and a shift in band position is clearly evident. The positive band, by contrast, showed no wavelength shift, but its amplitude increased by about 80%. Deconvolution suggests that both dry and wet films contain a large amount of β sheet and that β sheet and α helix content increase on film rehydration at the expense of random coil.

Closer analysis of CD spectra reveals possible differences in secondary structure content between dry $(P0/N0)_8$ and $(P1/N1)_8$

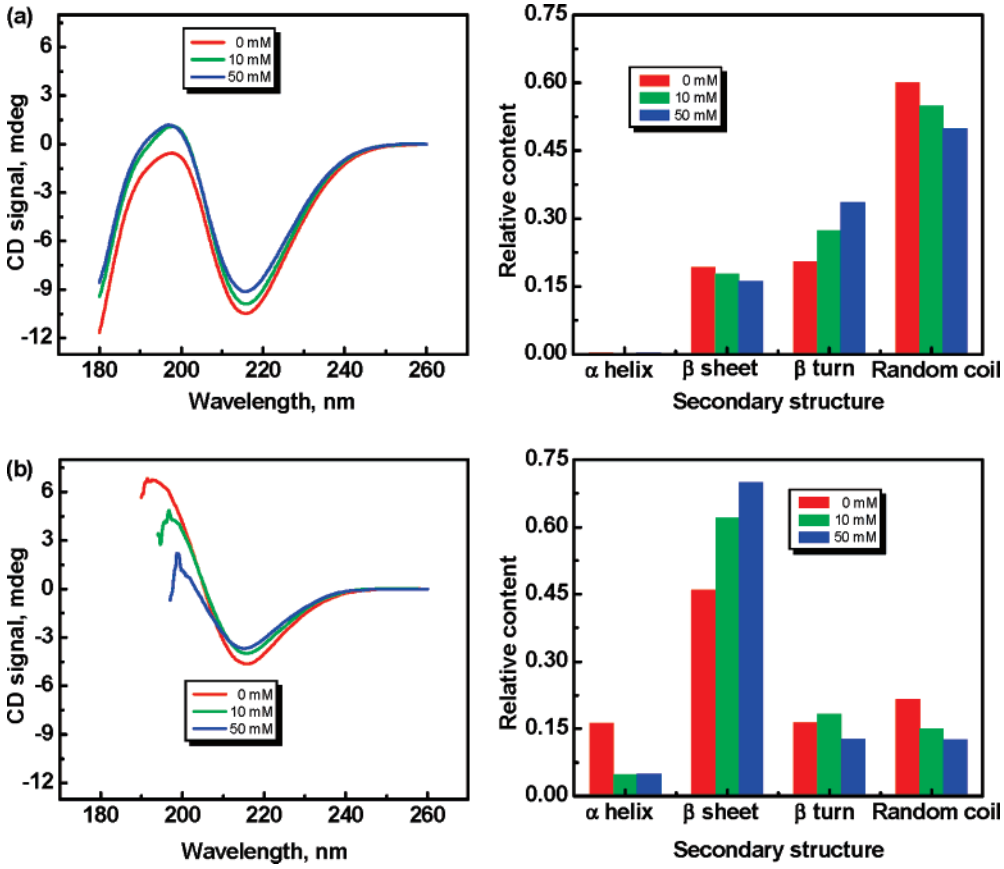

Figure 3. CD spectra and secondary structure deconvolution for (a) dry and (b) wet multilayer films of $(P0/N0)_8$. The buffer was 10 mM phosphate. The added salt at 0, 10, or 50 mM was NaCl.

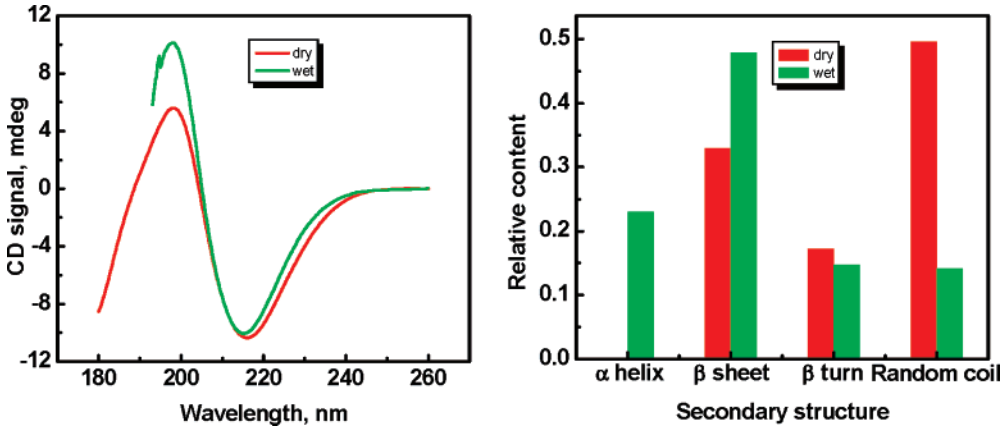

Figure 4. CD spectra and secondary structure deconvolution for dry and wet multilayer films of $(P1/N1)_8$. The buffer was 10 mM Tris, 10 mM NaCl. The large percentage of α helix in the wet spectrum may be an artifact of deconvolution.

films (Figures 3a and 4). The CD spectrum of a β sheet is characterized by a negative band at ~216 nm and a positive band in the range of 195−200 nm, while that of random coil has a negative band at ~200 nm.[27−29] Although the amplitude of the negative band was similar in both films, the amplitude of the positive band was close to zero in $(P0/N0)_8$ but about 55% of that of negative band in $(P1/N1)_8$. Deconvolution suggests that the former is dominated by random coil, the latter by β sheet. At neutral pH the absolute linear charge density $|\lambda| \approx 1$ for P0 and N0, and $|\lambda| \approx 0.5$ for P1 and N1. P0 and N0 are relatively long polymers and therefore prone to form loop and tail structures on the substrate surface. This may explain the relatively large amount of random coil found for $(P0/N0)_8$ by deconvolution. Polypeptides P1 and N1, by contrast, for which $|\lambda| \approx 0.5$ and the degree of polymerization is comparatively small, tend to form β sheet-containing fibrils in aqueous solution,[30,31] consistent with the large amount of β sheet in $(P1/N1)_8$ by deconvolution.[32]

Effects of drying and rewetting polyelectrolyte multilayer films have been investigated in earlier studies. Dry PSS/PAH films swell in humid air, increasing both thickness and mass as water molecules permeate the polymer matrix.[33] Mendelsohn et al. have proposed that ionic cross-linking between nonpeptide weak polyelectrolytes can greatly influence multilayer film swellability under physiological conditions.[34] Halthur et al. have found that intermediate drying steps in air during the assembly of PLL/PLGA films do not affect continuation of film buildup.[23] Instead, such films prepared at neutral pH display a reversible collapse upon drying, the original film thickness being regained on rewetting without loss of mass. The water content of dry

PLL/PLGA films can be significant.[23,24] Richert et al. have reported that PLL/PLGA films show greater swellability in water when made at acidic pH than basic pH,[35] which may reflect differences in the amount of material deposited under the different conditions (compare ref 14).

Complexation of highly charged polypeptides in aqueous solution and polypeptide deposition in a multilayer film are predominantly entropy-driven processes.[36,37] That is, the decrease in entropy on fixing some degrees of freedom of the polypeptide chain is overcompensated by the gain in entropy on release of water molecules and counterions. The enthalpy gain on formation of electrostatic interactions, hydrophobic interactions, and hydrogen bonds on peptide deposition, complexation, and secondary structure formation will also play some role in film assembly,[32,38] but the relative importance is generally smaller than for entropy.

Differences in spectra in the present work, and therefore in secondary structure content between dry and wet films of $(P0/N0)_8$ or of $(P1/N1)_8$, were small. Deconvolution suggests that a significant percentage of irregular structure is present in the films, along with a large amount of β sheet and β turn but little or no α helix. More configurations of β sheet than α helix are possible for a polypeptide of a given degree of polymerization, promoting a tendency to form β sheets instead of α helices in multilayer films.[37] Whether the film is wet or dry, polypeptides in the film will organize noncovalent interactions so as to minimize free energy.

Regarding CD deconvolution in general, the largest reliable reference set that is available for the wavelength range of the spectra should be used to represent the greatest range of different structures and spectra, particularly for β sheets and turns.[39−41] The CONTIN/LL reference sets IBasis 1 and IBasis 4 include 29 and 43 soluble and membrane proteins, respectively. IBasis 4 was used for wet films in the present because of the lack of data in the 180−190 nm range. The secondary structure percentages obtained with the two reference sets were comparable for the spectra reported here.

Further aspects of CD deconvolution should be mentioned and addressed. The IBasis 1 and IBasis 4 reference sets do not contain spectra of peptides in a thin film of any type. The CD spectra of different secondary structures in a multilayer film will not necessarily be the same as for soluble proteins or membrane proteins. Artifacts could therefore arise when using either of the indicated reference sets to analyze polypeptide multilayer film spectra. Extensive comparison can be made between CD spectra of soluble proteins and the corresponding X-ray or NMR structures, enabling assessment of the significance and utility of a deconvolution method of CD spectra. No such comparison can presently be made in the case of polypeptides in multilayer films.

The points so far adduced, however, do not invalidate the deconvolution method used in the present work. Indeed, the CD spectrum of a typical peptide multilayer film closely resembles that of model β sheet structures in solution (for example, see ref 42); the similarity can hardly be fortuitous. Moreover, under conditions where soluble PLGA forms helical structure, namely, acidic pH, the CD spectrum of a dry PLL/PLGA film becomes increasingly like that of model α helix structures in solution.[14,42] This strongly suggests—though it clearly does not prove—that it should be possible to use existing deconvolution approaches and soluble protein reference sets to obtain at least a rough indication of the secondary structure content of a polypeptide multilayer film. It is also possible to compare CD spectra of polypeptide multilayer films with structural information obtained by some other experimental method, e.g., FTIR.[43] Polypeptide multilayer film FTIR spectra are present in the open scientific literature,[25,26,44] and a comparison has been made with corresponding CD data.[14] The cited FTIR data were interpreted by the authors of those reports as indicating that wet PLL/PLGA films at neutral pH contain a substantial proportion of residues in a β sheet conformation, consistent with the present work. CD has been used to analyze the structure of peptides and protein films since the late 1960s.[11,12,45,46]

Regardless of the secondary structure content of a dry polypedtide multilayers film, the dielectric constant inside will be relatively low, making electrostatic attraction and repulsion relatively strong. Polypeptide chains will therefore be able to orient themselves less freely than when wet, and some weak hydrogen bonds in secondary structures will be broken to optimize electrostatic interactions during the drying process. Dry films could therefore show a larger percentage of random coil structure than wet films. The data suggest that β sheets are shorter in dry films than wet films, based on the relative percentage of turns.

Film rewetting results in the return of water molecules and counterions from the surrounding solution. This weakens internal electrostatic attractions due to the rise in dielectric constant of the local environment, and it increases the ability of polypeptide chains to self-organize and form more ordered secondary structures. The resulting films have comparatively less random coil structure and longer β sheets and, apparently, a small amount of α helix. In the work reported here rehydration resulted in an apparent increase in the order of internal structure in the films according to deconvolution, perhaps owing to the ability of peptide chains to associate more freely in the presence of charge screening. By contrast, film rewetting at extremes of pH will generally lead to film disintegration,[13] though disulfide bonds formed between peptides will reduce the rate and extent of film delamination.[16,38,47]

Conclusion

Polypeptide multilayer films made of high molecular weight homopolypeptides display differences in internal structure from films made of low molecular weight heteropolypeptides, according to UV and CD measurements. Dry and wet films show similarities and differences in secondary structure. Both show a large percentage of β sheet some random coil structure but relatively little α helix. Hydrated films may be more ordered than dry films, according to deconvolution of CD spectra.

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