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Ultrathin Layered Myoglobin−Polyion Films Functional and Stable at Acidic pH Values

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Abstract: Cross-linking of myoglobin (Mb) promoted by 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide within films of polystyrene sulfonate after layer-by-layer self-assembly provided remarkable stabilization. Cross-linking greatly improved adhesion of the films to fused silica slides and allowed extensive optical studies over a wide pH range. Circular dichroism and visible absorbance spectra showed that Mb retained its native conformation when films were placed in solutions of pH as low as 2 and up to pH 11. Linear dichroism revealed an average orientation of the Mb iron heme cofactors of 58° to the film normal. High concentrations of urea did denature the protein in the films, however. At pH 1, Mb in solution is fully unfolded but retained considerable α-helical content in the cross-linked films. Both the polyion film environment and cross-linking seem to play roles in stabilizing protein secondary structure and function at low pH. Cross-linked myoglobin−polyion films on pyrolytic graphite electrodes were used in strongly acidic solutions for the electrochemical catalytic reduction of trichloracetic acid, hydrogen peroxide, and oxygen. The pH-dependent catalytic reduction of trichloracetic acid was faster in 0.1 M HCl than in the medium pH range.

Introduction

Nature employs biomolecular superstructures incorporating enzymes and redox proteins to shuttle electrons to sites where they are needed to support life processes. Mimicking these superstructures offers a viable approach to fundamental studies of protein redox chemistry and to designing biosensors and bioreactors.[1−3] Thin films containing redox proteins and enzymes on electrodes can facilitate driving biological electron-transfer events by controlling the applied voltage of an electronic source or sink of electrons. Electron exchange between electrode and enzyme eliminates the need for natural electron donors and reductase enzymes and can simplify device construction.

A number of film technologies have been developed in recent years to facilitate direct electron exchange between electrodes and proteins.[3] However, little attention has been paid to films that allow the utilization of redox proteins in extreme environments, such as high or low pH, or in unusual media, such as microemulsions or ionic liquids. For proteins, the definition of "extreme environment" is much more restrictive than for typical synthetic polyelectrolytes. Many proteins decompose or denature at pH values less than 5 or greater than 10. However, utilization of proteins with native structures and activities at low or high pH or in other unusual environments may foster unique applications of biosensors and bioreactors. In this paper, we apply simple techniques to construct films of the protein myoglobin and polyions, and show that these films are stable and useful at high- and low-pH values.

Electrostatic layer-by-layer alternate adsorption of proteins and polyions is a versatile general method of film construction that has emerged over the past decade.[4−6] It provides excellent control over film thickness and architecture and has been used to make ultrathin films of a wide variety of proteins with oppositely charged polyions.[3−8] To illustrate, suppose a positively charged solid is immersed into a 0.5−3 mg mL^{-1} solution of negatively charged polyions. The polyanions adsorb at saturation coverage in about 15−20 min, effectively reversing the charge on the solid surface. The solid is rinsed in water and then immersed in a 0.5−3 mg mL^{-1} solution of positively charged proteins in a buffer of pH smaller than the protein's isoelectric point. The surface develops a positive charge as a layer of protein is adsorbed. Adsorption cycles can be repeated many times to obtain desired thicknesses on the nanometer scale in a multilayer film.

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We used this layer-by-layer adsorption method to make stable films of myoglobin (Mb) and cytochrome P450cam with alternate layers of DNA, polyanions,[9,10] and metal oxide nanoparticles[11] on gold and pyrolytic graphite electrodes. These films featured direct, chemically reversible electron transfer between the electrodes and the iron heme groups of the proteins. The films were used for electroenzyme catalyzed epoxidation of styrene and *cis*-methylstyrene.[9,10a] Also, we utilized the orientation of electrostatic binding of several cyt P450s to charged outer layers of polyion films to monitor their binding to natural redox partners using a quartz crystal microbalance and atomic force microscopy.[12]

The genesis of the present paper was our desire to further characterize protein structure and orientation in layered polyion films by optical techniques and to explain the unusual pH dependence of cyt P450cam and Mb formal redox potentials ($E^{\circ\prime}$). In solution and in surfactant films, Mb and cyt P450cam show very different slopes of $E^{\circ\prime}$ vs pH below and above pH 5,[13] and this is correlated with the partial denaturation of the proteins at pH < 5 observed by optical methods. However, in layered polyion and nanoparticle films we found a single linear $E^{\circ\prime}$ vs pH relationship between pH 3 and 11 consistent with a one-proton, one-electron transfer.[14] We felt that this behavior might be interpreted in terms of stable protein secondary structure in the films in acidic solutions. In this paper, we employ UV−vis circular and linear dichroism, UV−vis absorption spectra, and cyclic voltammetry to show that films of myoglobin and polystyrene sulfonate that are cross-linked after construction are remarkably stable in acidic solutions, even to the point of maintaining protein secondary structure. Good stability in acidic media can be obtained on pyrolytic graphite even when the films are not cross-linked. We demonstrate the use of these films for several catalytic reductions at pH 1 and 11.

Experimental Section

Chemicals and Materials. Horse heart myoglobin (Mb, Sigma, MW 17 400) in pH 5.5 buffer was passed through a YM30 filter (Amicon, 30,000 MW cutoff).[15] Sodium poly(styrene sulfonate) (PSS, MW 70 000) and poly(dimethyldiallylammonium chloride) (PDDA) were from Aldrich and used at 3 and 2 mg mL^{-1}, respectively. 1-[3-(Dimethylamino)propyl]-3-ethylcarbodimide hydrochloride (EDC) was from Aldrich, and solutions were prepared fresh before use. Fused silica slides (1 mm thick) were from BES Optics, RI. Water purified with a Barnstead Nanopure system had specific resistance >15 MΩ cm.

Film Assembly. For optical studies, multilayer films were assembled on fused silica slides that had been soaked in pirhana solution (H_2O_2:H_2SO_4, 30:70) for 30 min, followed by thorough rinsing and ultrasonication in water *(Safety note: Pirhana solution is highly corrosive and reacts violently with organics; it must be handled with care).* Cleaned slides were sonicated in KOH:ethanol:water (1:59:40) and then rinsed with water. Films were prepared by repeated alternate adsorption from aqueous solutions of PDDA, PSS, and myoglobin as described previously.[9] To form a smooth bed for myoglobin adsorption, three PDDA/PSS layers were adsorbed first.[5,6] Polyion adsorption was done from 0.5 M NaCl solutions, which coils the polyion, gives thicker layers, and allows more protein to be adsorbed.[5,6] Adsorption times of 15 min were used to achieve saturation,[9] and surfaces were rinsed thoroughly with water between adsorption steps to remove weakly adsorbed molecules.

After assembly, protein/polyion films were cross-linked by immersing in fresh 25 mM aqueous EDC, reacting for 20 min, washing, and then storing in buffer. This procedure is similar to one that we used previously to link poly(L-lysine) to carboxylate groups on carbon surfaces and to metal catalyst complexes.[16] Here it promotes formation of amide linkages[17] between the protein molecules. Films wet by immersion in buffer for 20 s were used for absorption spectroscopy and CD. Films of PDDA/PSS with equivalent numbers of layers were used for optical background correction.

Instrumental Procedures. Unless otherwise noted, all measurements were at ambient temperature, 21.5 ± 0.1 °C. Absorption, and linear dichroism spectroscopy were done using a Hewlett-Packard 8453 UV−vis spectrophotometer with a diode array detector. The setup for linear dichroism was described previously.[18] A JASCO 710 spectropolarimeter was used to obtain CD spectra. Solutions used to treat films before spectral measurements were aerobic. Cyclic voltammetry (CV) procedures were described previously.[9−11] Voltammetry was done in anaerobic solutions, unless otherwise noted.

For voltammetry, basal plane pyrolytic graphite disk electrodes (PG, $A = 0.17$ cm^2, Advanced Ceramics) were abraded on 400-grit SiC paper while being flushed simultaneously with water, followed by ultrasonication in water for 1 min. Preparation of films on these rough PG surfaces was done as described above.

Results

Film Assembly. In previous studies, we weighed individual layers of PSS and myoglobin during construction of Mb/PSS films made under the same conditions used here employing a quartz crystal microbalance (QCM).[9] Layer weights were converted to nominal thicknesses by reference to high-resolution SEM cross-sections.[7] These studies showed reproducible layer formation from layer to layer. Individual layers of Mb on PSS deposited from 0.5 M NaCl weighed 1.7 μg cm^{-2} and were nominally 9 nm thick.[19] PSS layers were 3 nm thick, and PDDA layers were 1.3 nm thick. On the basis of these results, the nominal thickness of a typical film with four Mb layers used for the our studies, i.e., $(PDDA/PSS)_3(Mb/PSS)_4$, is estimated at about 50 nm. Reproducibility of layer formation was confirmed in the current work by measuring the Mb Soret bands after deposition of each Mb layer on the fused silica slides, which showed a reproducible increase in absorbance at 410 nm with deposition of each Mb layer (see Supporting Information).

Mb and cyt P450cam films prepared on gold surfaces coated with a mercaptopropanesulfonate monolayer or on pyrolytic graphite electrodes were stable at 4 °C in buffer for more than a month.[9,10] While Mb/PSS films on fused silica were stable enough to obtain spectra immediately after preparation, immersion in and out of various buffers and repeated washing required

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(19) A monolayer of close packed Mb is 0.2 μg cm^{-2}. The larger value found by QCM reflects the adsorption onto the layers of coiled PSS which have a much larger capacity for binding Mb than linear PSS (see refs 5, 6, and 9). As discussed later, the final film structure is intermixed.

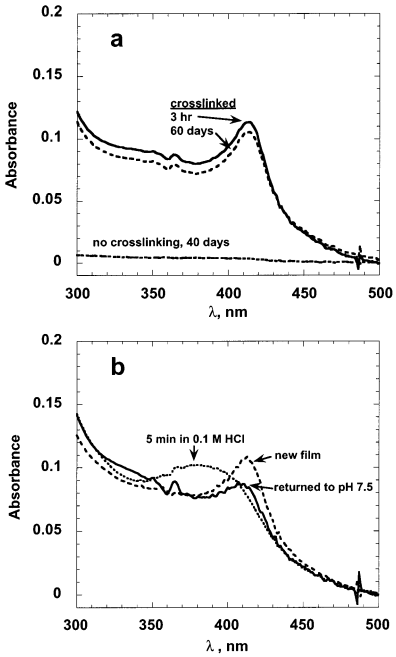

Figure 1. UV−vis spectra of $(PDDA/PSS)_3(Mb/PSS)_4$ films on fused silica: (a) influence of cross-linking on stability of films stored at 5 °C in pH 7 buffer; (b) a film initially at pH 7.5, immersed for 5 min in 0.1 M HCl, and then returned to pH 7.5 buffer.

to study the influence of pH on protein structure caused the films to be removed from the fused silica surface at unacceptable rates. Films stored in buffer were also slowly lost from the fused silica surface. Thus, we assembled Mb/PSS films on fused silica slides and subsequently cross-linked them using EDC, a water-soluble carbodiimide and standard promoter of amide linkages.[17]

Figure 1 shows typical spectra of an $(Mb/PSS)_4$ film[20] after storage in buffer for 1 and 60 days. Dry films gave high backgrounds, probably from scattering. After 30−60 min in buffer, the background in the 300−400 nm region decreased and a well-defined Soret band of the Fe^{III} heme group of Mb at 411 nm emerged. The cross-linked films on fused silica slides were stable for up to 60 days with little loss in the absorbance (Figure 1a) when compared to the film without cross-linking. In 0.1 M HCl, broadening and a blue shift in the Soret band in the films were observed (Figure 1b). Return of the acid treated films to pH 7.5 buffer regenerated the band at 411 nm. In the film shown, a small loss in absorbance was observed, but for some films the absorbance was nearly constant. All of the optical studies reported below were done on cross-linked films.

Influence of pH on UV−Vis and Circular Dichroism Spectra. For pH studies, films were immersed in various buffer solutions, and spectra were recorded immediately after the slide was removed from the buffer. While recording spectra after immersion in each of the buffers, care was taken to expose the films to the medium-pH range first before exposure to high or low pH. Cross-linked (Mb/PSS) films showed a slightly increasing Soret band absorbance with minimal shifts in the maximum wavelength over the range of pH 2−11 (Figure 2). After exposure to high- or low-pH solutions, a return to a medium-pH buffer resulted in recovery of the original spectrum

(20) Films with three or four (Mb/PSS) bilayers were used to obtain adequate signal/noise in spectral and voltammetric measurements, but qualitatively similar results for all experiments were found with up to seven (Mb/PSS) bilayers.

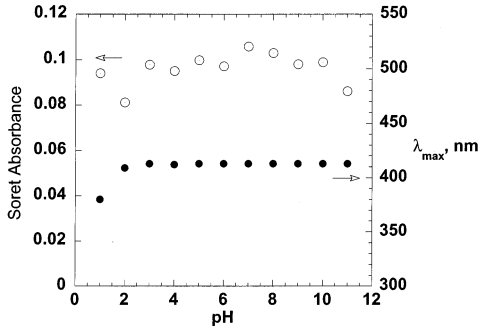

Figure 2. Influence of buffer pH on spectral characteristics of the Soret band of cross-linked $(PDDA/PSS)_3(Mb/PSS)_4$ films on fused silica.

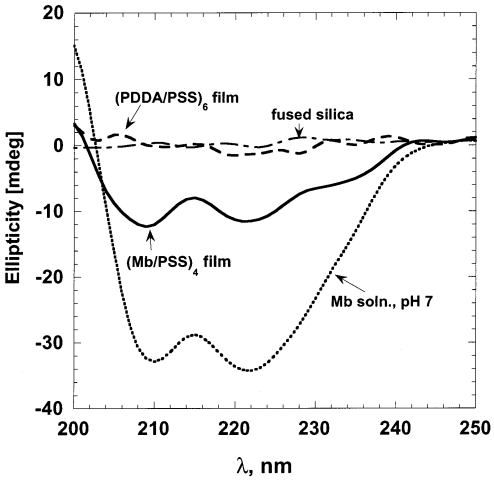

Figure 3. Circular dichroism spectra of cross-linked $(PDDA/PSS)_3(Mb/PSS)_4$ films on fused silica and Mb solution at pH 7.

at that pH. In contrast, myoglobin in solution denatures by partial unfolding beginning at pH < 5, and the Soret band is significantly broadened and blue shifted.[21] For example, at pH 3 the Soret maximum for dissolved myoglobin is at 375 nm.

Circular dichroism (CD) was used to monitor the secondary structure of myoglobin in the films. Figure 3 shows the UV CD spectra of Mb in films compared to the spectrum of Mb in solution. The double minima at 210 and 222 nm represent the α-helical portions of Mb,[22] comprising 76% of the polypeptide backbone in the native conformation in solution.[23] Similar minima in the film CD spectra are found at 208 and 222 nm. Spectra for fused silica and a $(PSS/PDDA)_6$ film did not reveal any ellipticity features in this region.

Upon exposure of the films to buffers of pH 2−11, the double minima are influenced only to a minor extent (Figure 4). The spectra are so similar between pH 2 and 11 that the small differences are difficult to see.[24] Careful inspection shows that the spectra at pH 7 and 9 are nearly identical, while those at pH 2, 5, and 11 show close similarities. Mb in solution is fully

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(24) Quantitative estimates of the secondary structural features of Mb in the films were impossible because of large errors in Mb concentration and path length. For a discussion of problems in quantitative analysis of CD spectra of films, see: McPhie, P. *Anal. Biochem.* **2001,** *293*, 109−119.

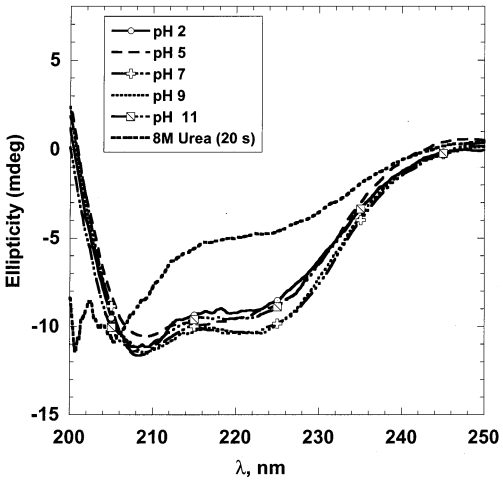

Figure 4. Influence of solution pH and urea treatment on circular dichroism spectra of cross-linked $(PDDA/PSS)_3(Mb/PSS)_4$ films on fused silica.

denatured when exposed to 8 M urea,[25,26] and CD spectra clearly show that Mb in the films was denatured after 20 s immersion in 8 M urea (Figure 4). This also shows that the 20 s immersion is sufficient for the protein in the film to respond to solution composition. Remarkably, little change in the CD spectrum was found after Mb films were immersed in pH 2 buffer or 0.1 M HCl solution for up to 5 min (Figure 5).

While the usual pH of the Mb solution used for film assembly was 5.5, films were also assembled using Mb solutions of pH 2 and 3. The UV−vis absorbance spectra of these solutions showed blue-shifted Soret bands indicating changes from the Mb native structure. Visible spectra of the films also showed a blue shift to about 380 nm. The solution CD spectra (Figure 6) with a minimum at 200 nm, clearly showed the loss of nearly all α-helical structure. However, the (PSS/Mb) *films*, assembled from the very same solution, show a partial restoration of the double minima characteristic of partial recovery of the α-helical content (Figure 6). However, the Soret bands of the films grown starting with pH 2 and 3 Mb solutions were broad with maxima at about 380 nm, confirming that only partial recovery of secondary structure was achieved.

Linear Dichroism Spectroscopy. The difference in absorbance with parallel and perpendicular plane polarized light for the Soret band of Mb was measured as linear dichroism (LD) to obtain information about orientation of Mb in the films.

$$\mathrm{LD} = A_{\parallel} - A_{\perp} \tag{1}$$

LD was measured at a series of angles ω between the direction of light propagation and the film plane. Equation 2, with the assumption of uniaxial distribution of the heme,[27] was used to obtain order parameter S:

$$\frac{\mathrm{LD}}{(A + \mathrm{LD}^{\omega=0}/3)} = 3S\frac{\cos^2\omega}{n^2\sqrt{1 - \dfrac{\cos^2\omega}{n^2}}} \tag{2}$$

where $\mathrm{LD} = (A_{\parallel} + A_{\perp})/2$ and n is the refractive index of the fused silica. $\mathrm{LD}^{\omega=0}$ is obtained by extrapolation of LD vs $\cos^2\omega$ to $\omega = 0$. The order parameter S is

$$S = (1 - 3\cos^2\varphi)/2 \tag{3}$$

where φ is the angle between the transition moment vector in the heme plane and the normal to the film plane.

Random errors in LD were rather large for the films because the absorbance is small and LD is the difference between two absorbance spectra. Nevertheless, the quantity $\mathrm{LD}/(A + \mathrm{LD}^{\omega=0}/3)$ plotted vs the angular factor $= \cos^2\omega/[n^2(1 - (\cos^2\omega/n^2))^{1/2}]$ gave reasonable linearity for the (PSS/Mb) films (Figure 7) as predicted by eq 2, with a correlation coefficient of 0.78. The slope of this plot is proportional to the order parameter S, which was used to obtain values of φ for the films using eq 3. The LD at $\omega = 90°$ was close to zero, as expected from eq 2. Analysis of these LD results with eqs 2 and 3 gave $S = 0.081 \pm 0.01$ and $\varphi = 58 \pm 1°$.

Electrochemical Catalytic Reductions. As found previously for non-cross-linked polyion films of heme proteins,[3] cross-linked films of Mb/PSS gave chemically reversible cyclic voltammetry characteristic of the reduction of the heme Fe^{III} form of the protein on the forward scan and oxidation of the Fe^{II} form on the reverse scan (Figure 8a). The midpoint potentials of these CVs shifted negative with increasing pH

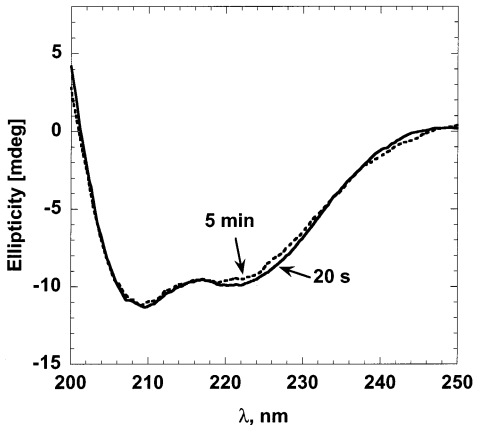

Figure 5. Circular dichroism spectra of cross-linked $(PDDA/PSS)_3(Mb/PSS)_4$ film on fused silica after immersion in 0.1 M HCl solution.

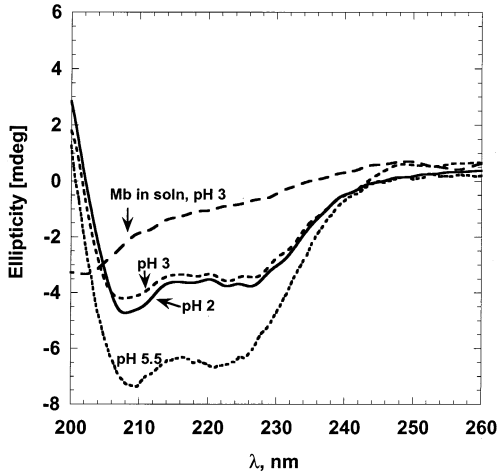

Figure 6. Influence of the pH of the protein adsorbate solution during film deposition on the circular dichroism spectra of the resulting cross-linked $(PDDA/PSS)_3(Mb/PSS)_3$ films.

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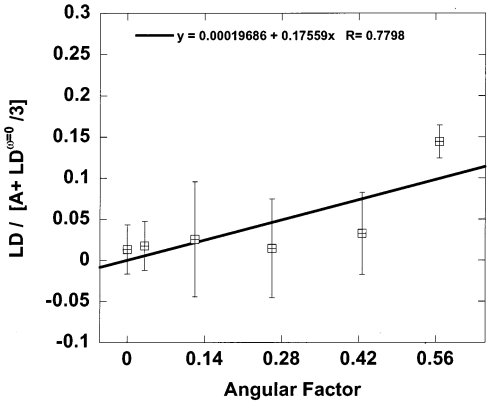

Figure 7. Influence of angular factor AF = $\cos^2 \omega/[n^2(1 - (\cos^2 \omega/n^2))^{1/2}]$ on linear dichroism of the Soret band as LD/(A + LD$^{\omega=0}$/3) for cross-linked (PDDA/ PSS)$_3$(Mb/PSS)$_4$ films on fused silica at pH 5.5 (average of 4 films).

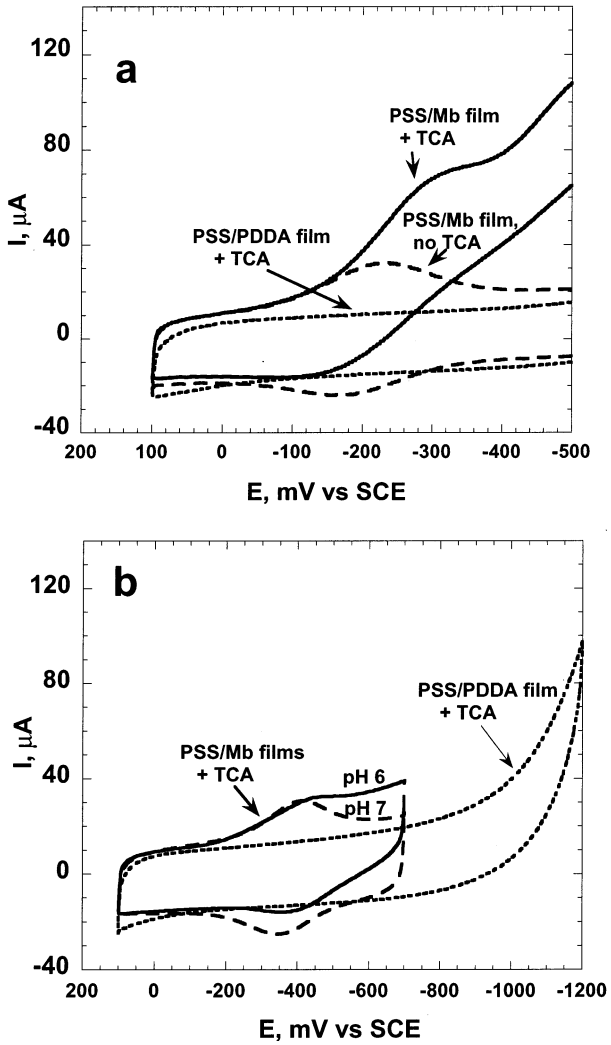

Figure 8. Cyclic voltammograms of cross-linked (PDDA/PSS)$_3$(Mb/PSS)$_4$ and (PDDA/PSS)$_3$ films on PG electrodes at 300 mV s^{-1} illustrating catalytic reduction of 10 mM trichloroacetic acid (TCA): (a) in 0.1 M HCl; (b) (PDDA/PSS)$_3$(Mb/ PSS)$_4$ films in pH 6 and pH 7 buffers, and (PDDA/ PSS)$_3$ at pH 7.

similar to those of the non-cross-linked films (ca. 50 mV pH^{-1}),[14] and peak currents decreased by about 20% after cross-linking, consistent with increased film rigidity. No significant

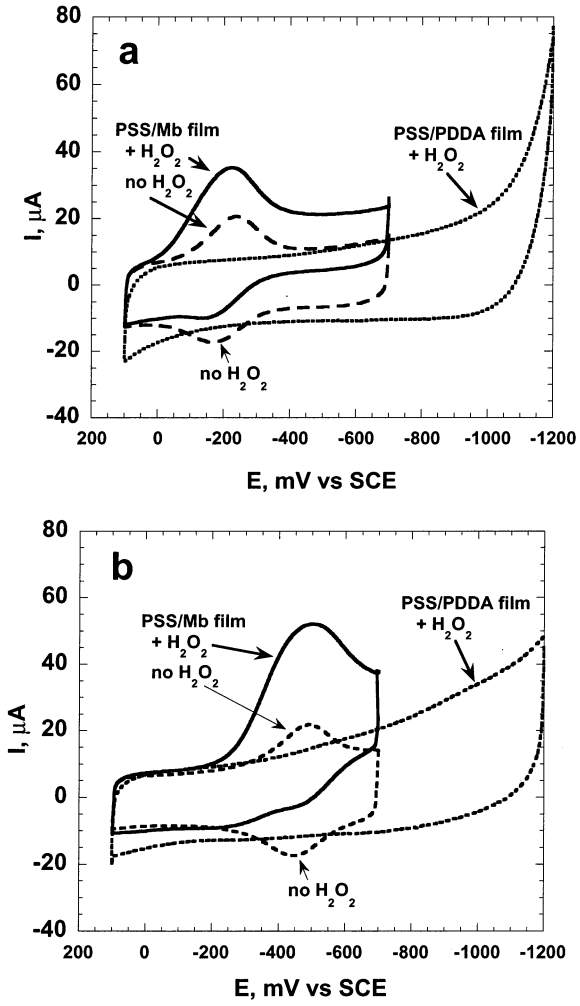

Figure 9. Cyclic voltammograms of cross-linked (PDDA/PSS)$_3$(Mb/PSS)$_4$ and (PDDA/PSS)$_3$ films on PG electrodes at 300 mV s^{-1} illustrating catalytic reduction of 200 μM hydrogen peroxide: (a) in 0.1 M HCl; (b) in pH 11 buffer.

changes in the CVs were found for temperatures between 4 and 60 °C (see Supporting Information).

Myoglobin in films has been used to reduce trichloracetic acid (TCA). Dechlorination occurs in a stepwise fashion to yield acetic acid as the final product.[28] Figure 8 shows cyclic voltammograms characteristic of catalytic reduction of TCA by cross-linked (PDDA/PSS)$_3$(Mb/PSS)$_4$ films on PG electrodes. In 0.1 M HCl with no TCA present, the film shows the reversible oxidation−reduction peak pair by CV for the Mb Fe^{III}/Fe^{II} redox couple at about −200 mV vs SCE. With 10 mM TCA present, the reduction current increases and shows a plateau. The Fe^{II} oxidation peak disappeared because this form of the protein reacted with TCA, giving the Fe^{III} form and the catalytic increase in current (Figure 8a). Direct of reduction of TCA on (PDDA/ PSS)$_3$ electrodes in 0.1 M HCl is obscured by the final current rise caused by reduction of protons that begins at about −900 mV.

The catalytic TCA reduction is pH dependent, and at pH 6 only a small catalytic reduction current increase was found for TCA with all other conditions the same. At pH 7, the catalytic reaction is not observed under our experimental conditions, and

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only the reversible CV of the Mb Fe^{III}/Fe^{II} redox couple was observed. Direct, noncatalytic reduction of TCA using a PG electrode coated with $(PDDA/PSS)_3$ is the cause of the increase in current at about −1000 mV in Figure 8b. These films are relatively stable at pH 1, and multiple scans can be made with observation of only the usual small decrease of catalytic current on subsequent scans due to depletion of TCA reactant in the film and in the solution close to the electrode. Surprisingly, films that were not cross-linked also showed this relatively stable repetitive CV behavior (see Supporting Information). Following multiple scans, when films were allowed to reequilibrate in the TCA solution for several minutes without scanning, the initial catalytic CV was obtained for both cross-linked and non-cross-linked films.

Myoglobin has peroxidase activity and can be used in films to reduce hydrogen peroxide by electrochemical catalysis.[29] Figure 9 shows characteristic catalytic voltammograms for cross-linked films of $(PDDA/PSS)_3(Mb/PSS)_4$ on PG electrodes. In 0.1 M HCl, addition of 0.2 mM hydrogen peroxide resulted in an increase in the catalytic reduction peak and a large decrease in the oxidation peak (Figure 9a). Control CVs using $(PDDA/PSS)_3$ films on PG showed no obvious peaks for peroxide reduction. The catalytic reduction of hydrogen peroxide by Mb/PSS films was observed throughout the pH range up to pH 11 (Figure 9b), where a catalytic peak larger than at pH 1 was found. Behavior at pH 7 was similar to pH 11. The catalytic peak current increased with concentration of hydrogen peroxide (see Supporting Information).

Myoglobin in films also reduces oxygen to hydrogen peroxide in an electrochemical catalytic pathway.[9,10] CV signatures for catalytic reduction of oxygen with cross-linked films of $(PDDA/PSS)_3(Mb/PSS)_4$ on PG electrodes were found at pH 1, 7, and 11 with characteristics (see Supporting Information) similar to those for reduction of hydrogen peroxide.

Discussion

Film Stability and Structure. While protein−polyion films grown layer-by-layer are quite stable on gold or pyrolytic graphite electrodes, cross-linking was investigated initially because of poor long-term stability on fused silica. Similar cross-linking was successful in stabilizing enzyme crystals for synthetic applications[30] and for stabilizing biocompatible albumin/heparin coatings.[31]

Figures 1, 4, and 5 illustrate the remarkable stability imparted to Mb/PSS films by cross-linking after being grown layer by layer. Even after 60 days of storage in buffer, the cross-linked films on fused silica retain the characteristic Soret band with only a small decrease in absorbance from that observed on the day of film formation. Comparison of Figure 5 with Figure 4 shows that myoglobin in the cross-linked films on fused silica retains most of its α-helical structure even in strongly acidic solution, where myoglobin in solution would be completely denatured.[32] In contrast, after 40 days storage of films on fused silica at pH 7, no protein was observed by absorbance spectroscopy. Cross-linking can only occur in these films between carboxylate and amine side chains in the proteins and can be imagined to weave a network holding the layers together and preventing removal from the fused silica surface.

The first few layers adsorbed during electrostatic layer-by-layer film assembly are thought to exhibit nonlinear growth[5,6] resulting in formation of polymer islands, which we visualized by atomic force microscopy (AFM).[12] Successive layers of adsorbed polyions eventually fill in the gaps between these islands after several polyion layers are adsorbed, resulting in a more uniform landscape on which to assemble the protein layers. This is why we first adsorbed $(PDDA/PSS)_3$ precursor films onto surfaces before adding the protein layers. Cross-linking after adding the protein layers increased the adhesion of the entire film to the fused silica surface and rendered the films reusable with a long storage life (Figure 1). The reason for the success of the cross-linking is likely to be related to the film structure. Neutron scattering studies of Mb/PSS films on flat silicon suggested 50% mixing between neighboring layers.[6] Thus, we expect that Mb molecules interpenetrate the precursor film and all of the PSS layers. When the Mb is cross-linked, it then stabilizes much of the entire film structure. This is also consistent with the reversible electrochemistry of the $(PDDA/PSS)_3(Mb/PSS)_4$ films. If there were absolutely no Mb interpenetration, the $(PDDA/PSS)_3$ layers would represent a 13 nm insulating layer, based on QCM results quoted above, between the electrode and the first Mb layer. If this layer contained no Mb, electron exchange with the electrode would be quite slow across such a distance. Furthermore, it is likely that the rough PG surface used for electrochemistry provides a template for increased layer disorder that could facilitate interlayer mixing.

Figures 2, 4, and 5 indicate very little change of visible or CD spectra with pH, showing that Mb in the cross-linked films retains the essential features of its native secondary structure at values as high as pH 11 and as low as pH 2. The position of the Soret band at 411 nm and the double CD minima, very similar to Mb in solution, indicate that Mb in the film has not denatured and retains its native state over a wide pH range. Layer-by-layer protein−polyion films are permeable to water and contain about 40−50% water when immersed in aqueous solutions.[6] Mb in the cross-linked films is susceptible to the properties of the contacting solutions, and this is reflected in the complete denaturation in concentrated urea solutions (Figure 4) and by the shifts in the formal potential with pH of the reversible Fe^{III}/Fe^{II} couple (Figures 8 and 9). Despite these facts, a different internal film pH from that in solution cannot be completely ruled out.

In any case, low pH does not fully denature the protein in the cross-linked films as it does for the protein dissolved in solution. Furthermore, it seems that the polyion film environment has some ability to stabilize folded, partly helical structures of Mb, since a partly helical structure is evident in films grown by starting with denatured protein solutions (Figure 6). This can be understood as a refolding to a "local minimum" structure instead of the global minimum of the native state.[35a]

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Linear dichroism results showed (Figure 7) that in the medium-pH range, the Mb heme plane is oriented at an average of 58 ± 1° to the film normal. While few data on orientation of proteins on such films have been reported, this LD result is consistent with binding studies of cyt P450s and its redox partners on polyion films.[12] These studies used QCM and AFM to show that cyt P450s with positive and negative charges on different sides of the protein could be oriented for proper electrostatic binding to a redox partner by adsorption onto an underlying polyion layer of a charge such that the binding site for the redox partner was not blocked.

Enzyme-like Electrochemical Catalysis. Voltammetric studies showed that cross-linked $(PDDA/PSS)_3(Mb/PSS)_4$ films on PG could be used to catalyze enzyme-like reactions in strongly acidic solutions. The reduction of trichloroacetic acid was pH dependent and gave the largest catalytic current with the films at pH 1 (Figure 8), with a total disappearance of the catalytic current at pH 7 under the conditions used. Considering the removal of the first chloride ion in this reaction, the following pathway is likely

$$Cl_3C{-}COO^- + H^+ = Cl_3C{-}COOH \quad (4)$$

$$MbFe^{III} + e^- = MbFe^{II} \quad (5)$$

$$Cl_3C{-}COOH + 2MbFe^{II} + H^+ \rightarrow 2MbFe^{III} + Cl_2CH{-}COOH + Cl^- \quad (6)$$

The pK_a of TCA[33] is 0.7, and at pH 1 it exists 67% in the dissociated form. However, the acid form of an organic acid is more easily reduced than the anionic form.[34] It follows that pH dependence can arise from protonation of the TCA anion predominating at pH 1 (eq 4). Also, there may be a requirement for a proton as in eq 6. It is also of interest that this reaction was catalyzed at pH 1 for non-cross-linked $(PDDA/PSS)_3(Mb/PSS)_4$ films as well (see Supporting Information), suggesting that the non-cross-linked films on PG, unlike on fused silica, are stable even in the acidic pH range. These results also suggest that the polyion matrix may play a major role in holding the protein in fixed near-native conformations under extreme conditions.

Reduction of hydrogen peroxide and oxygen were also catalyzed by the $(PDDA/PSS)_3(Mb/PSS)_4$ films at values from pH 1 to 11. The occurrence of these reactions under very acidic and basic conditions suggests that Mb retains its catalytic activity at these pH values and supports the spectroscopic studies, suggesting nearly intact native secondary structure between pH 2 and 11. The pathways for these reactions can be quite complex.[29] Addition of H_2O_2 to Mb causes the formation of an oxyferrylheme radical of the protein that may be reduced electrochemically to the ferric form. However, the oxyferrylheme radical also reacts with H_2O_2 to give the ferric protein and oxygen. Since oxygen is reduced electrocatalytically by Mb in layered polyion films,[3,9,10] its reduction may also contribute to the catalytic current for peroxide. Furthermore, catalytic reduction of oxygen yields hydrogen peroxide, which can then initiate the peroxide reduction cycle.

Conclusions

Cross-linking greatly stabilized self-assembled films on fused silica slides. Mb in these films remained in a near native conformation over a wide range of solution pH. In the acidic pH range, there is spectroscopic evidence for a partly modified Mb, but this form has not lost a significant fraction of its α-helical character and is thus different from the "molten globule" form observed in Mb solutions[35] and in surfactant films.[36] In Mb solutions, histidines in hydrophobic pockets of the native structure become protonated below pH 5, leading to partial unfolding to the molten globule structure with a smaller helical content than the native conformation. Apparently, these histidine protonations are inhibited in the cross-linked films.

Both the polyion film environment and cross-linking seem to play roles in stabilizing the protein in its highly helical secondary structure and retaining its catalytic functions. The remarkable stability allowed myoglobin−polyion films to be used in strongly acidic solutions for catalytic reduction of trichloracetic acid, hydrogen peroxide, and oxygen, suggesting future applications in sensing in acidic media, and for bioreactors usable under unusual solution conditions not feasible for proteins dissolved in solutions.

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Supporting Information Available: Four additional figures documenting film growth, stability of catalytic voltammograms, and catalytic reduction of oxygen. This material is available free of charge via the Internet at http://pubs.acs.org.

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