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Increased Protein Sorption in Poly(acrylic acid)-Containing Films Through Incorporation of Comb-like Polymers and Film Adsorption at Low pH and High Ionic Strength

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Abstract

In principle, incorporation of comb-like block copolymers in multilayer polyelectrolyte films can both increase film thickness relative to coatings containing linear polymers and provide more swollen films for increased sorption of proteins. In the absence of added salt, alternating adsorption of 5 bilayers of protonated poly(allylamine) (PAH) and comb-like poly(2-hydroxyethyl methacrylate)-*graft*-poly(acrylic acid) (PHEMA-*g*-PAA) leads to ~2-fold thicker coatings than adsorption of PAH and linear PAA, and the difference in the thicknesses of the two coatings increases with the number of bilayers. Moreover, the (PAH/PHEMA-*g*-PAA)_n films sorb 2- to 4-fold more protein than corresponding films prepared with linear PAA, and coatings deposited at pH 3.0 sorb more protein than coatings adsorbed at pH 5.0, 7.0, or 9.0. In fact changes in deposition pH and addition of 0.5 M NaCl to polyelectrolyte adsorption solutions alter protein sorption more dramatically than variations in the constituent polymer architecture. When deposited from 0.5 M NaCl at pH 3, both (PAH/PHEMA-*g*-PAA)₅ and (PAH/PAA)₅ films increase in thickness more than 400% upon adsorption of lysozyme. These films contain a high concentration of free –COOH groups, and subsequent deprotonation of these groups at neutral pH likely contributes to increased protein binding. Lysozyme sorption stabilizes these films, as without lysozyme films deposited at pH 3 from 0.5 M NaCl desorb at neutral pH. Films deposited at pH 9 from 0.5 M NaCl are more stable and also bind large amounts of lysozyme. The high binding capacities of these films make them attractive for potential applications in protein isolation or immobilization of enzymes.

Introduction

This study aims to increase the capacity of polyelectrolyte multilayers (PEMs) for capturing and immobilizing proteins. Over the last 20 years, layer-by-layer (LbL) deposition of complementary polymers has emerged as one of the most versatile tools for forming a wide range of stable, functional thin films.^{1–3} The most common LbL procedure, alternating immersions of a substrate into polycation and polyanion solutions along with rinsing steps,² can occur on a wide range of substrates including flat surfaces,² nanoparticles⁴ and

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[‡]Professor Baker passed away unexpectedly on October 17, 2012. We dedicate this work as a memorial to him.

Supporting Information Available:

Synthesis of PHEMA-*g*-PAA, Table S1 and Figures S1–S24, including ¹H NMR spectra of the polymers, IR spectra of PAH/PHEMA-*g*-PBA and PAH/PHEMA-*g*-PAA, ellipsometric thicknesses of PAH/PHEMA-*g*-PAA multilayers deposited from polyelectrolyte solutions at various pH values, AFM images of films before and after sorption of lysozyme, examination of lysozyme binding to multilayers as a function of time, pH and salt concentration, and FTIR spectra examining film stabilities. This material is available free of charge via the Internet at <http://pubs.acs.org>.

membranes.^{5–7} Furthermore, a variety of charged molecules such as colloids and biomacromolecules can serve as PEM constituents to introduce functionality.²

Although many studies demonstrate LbL adsorption of proteins as constituents of PEMs,^{8–12} this work aims to first deposit PEMs and subsequently sorb large amounts of protein throughout the film.^{13–17} Salloum and Schlenoff showed that protein capture can occur throughout a PEM (absorption), but the equivalent thickness of absorbed protein was only 35% of the thickness of the dry film.¹⁸ Other studies of protein or nanoparticle sorption in preformed PEMs, especially those whose thickness grows exponentially, reported similar loadings,^{19–23} and a recent study of protein binding to a cross-linked polyelectrolyte film with the anion removed revealed absorption of 4–15 monolayers (~30 nm) of human serum albumin.²⁴ This work demonstrates that with appropriate deposition conditions, PEMs containing poly(acrylic acid) (PAA) increase in thickness 4- to 5-fold (as much as 200 nm) upon protein binding.

Such films might prove useful in applications such as protein delivery, protein purification or formation of enzymatic reactors.^{6,14,15,25} At physiological ionic strength, PEMs containing poly(acrylic acid) (PAA) exhibit minimal nonspecific adsorption of proteins, and activation of –COOH groups provides a means for covalent immobilization of antibodies and protein-binding ligands.^{18,26–28} Thus, PAA is very attractive for creating films that selectively sorb specific proteins. Moreover, native PAA-containing films bind highly positively charged proteins such as lysozyme.²⁶ Nevertheless, improving binding capacity is still important in applications of these films for protein purification or immobilization.²⁸

We initially hypothesized that PEMs containing comb-like PAA would be thicker and bind more protein than corresponding multilayer films prepared with linear PAA (Figure 1). Adsorption of comb-like PAA exploits the same interactions as adsorption of linear PAA, but the cylindrical shape of the polymer may lead to thicker films. Expanded surface layers, in particular, could provide increased protein binding. Additionally, cylindrical polyelectrolyte graft copolymer layers might form a more water-swollen multilayer than linear polymers and improve the penetration of proteins into the film.

Strategies for preparation of comb-like graft copolymers include homopolymerization of macromonomers ('grafting through'),²⁹ attachment of side chains to the backbone ('grafting to'),³⁰ and growth of side chains by polymerization from a macroinitiator ('grafting from').³¹ We apply a 'grafting from' strategy with atom transfer radical polymerization (ATRP) to synthesize poly(2-hydroxyethyl methacrylate)-*graft*-poly(acrylic acid) (PHEMA-*g*-PAA).³² ATRP affords control of the length and molecular weight distribution of both the backbone and the side chains, and PHEMA provides a relatively hydrophilic backbone that we can readily modify with densely grafted PAA.

In addition to examining differences between films containing PHEMA-*g*-PAA and PAA, this work investigates the effect of deposition pH on protein-binding properties of the films. Deposition of PAA at a pH 3 leads to free –COOH groups in the coating,^{33,34} and subsequent immersion of these films in pH 7 buffer deprotonates these groups to increase film swelling and create adsorption sites.³³ In fact, deposition pH has a larger effect on protein binding to PAA-containing films than changing the PAA from a linear to a grafted copolymer. Remarkably, films deposited at pH 3 in the absence of salt bind 7-fold more lysozyme than films deposited at pH 5. Addition of 0.5 M NaCl to polyelectrolyte deposition solutions also dramatically increases lysozyme sorption, although films deposited at pH 3 in 0.5 M NaCl are not completely stable at neutral pH.

Experimental

Materials

Unless otherwise noted, all chemicals were obtained from Sigma-Aldrich and used without further purification. 2,2-Bipyridine (bpy, 99%) was recrystallized from hexanes and sublimed prior to use. Triethylamine was distilled from calcium hydride under a nitrogen atmosphere and stored under nitrogen. 2-Hydroxyethyl methacrylate (HEMA) (98%) and *tert*-butyl acrylate (*t*BA) (98%) were passed through a column of activated basic alumina to remove inhibitors (length × diameter: 10 cm × 3 cm). Methyl ethyl ketone (MEK) and isopropanol were stored with 3 Å molecular sieves. The supporting information describes the preparation of PHEMA-*g*-PAA.

Poly(allylamine hydrochloride) (PAH, molecular weight 120,000 ~ 200,000 Da) was purchased from Alfa Aesar, and poly(acrylic acid) was obtained from Polysciences (molecular weight 90 000 Da, 25 wt% solution in water) or Sigma-Aldrich (molecular weight 100 000 Da, 35 wt% solution in water). Aqueous solutions of 0.02 M PAH, 0.01 M PAA and 0.005–0.01 M PHEMA-*g*-PAA were prepared in deionized water (18.2 MΩcm, Milli-Q) or 0.5 M aqueous NaCl, and solution pH values were adjusted by dropwise addition of 0.1 M NaOH or HCl. (Polymer concentrations are given with respect to the repeating unit, or the repeating unit of the PAA side chain for PHEMA-*g*-PAA). In the case of PHEMA-*g*-PAA, the solutions were filtered through cotton to remove any insoluble material prior to adjusting their pH. NMR spectra with an internal standard of d₄-trimethylsilyl propanoic acid (TSP) (see supporting information) revealed the PHEMA-*g*-PAA solution contained 0.005–0.01 M repeat units of PAA. In some cases, the PHEMA-*g*-PAA solutions became slightly cloudy at pH 3. Au-coated silicon wafers (200 nm of sputtered Au on 20 nm of Cr on Si (100) wafers) were cleaned in a UV/O₃ chamber for 15 min prior to use.

Preparation of PEMs

Au-coated Si substrates (24 mm × 11 mm) were immersed in 5 mM 3-mercaptopropionic acid (MPA) in ethanol for 2 h, rinsed with ethanol and dried with N₂ to form a monolayer of MPA and create a negatively charged surface at neutral pH. These substrates were immersed in 0.02 M aqueous PAH (adjusted to the desired pH) for 5 min and subsequently rinsed with 10 mL of deionized water and blown dry with N₂. Substrates were then immersed in a polyanion-containing solution (0.01 M PAA or 0.005–0.01 M PHEMA-*g*-PAA adjusted to the desired pH value) for 5 min followed by the same rinsing and drying procedures. In some cases, the PAH and polyanion solutions also contained 0.5 M NaCl. The process was repeated to form multilayer films.

Characterization of polymers and PEM films

¹H and ¹³C NMR spectra were obtained at room temperature on a Varian UnityPlus-500 spectrometer, with the chemical shifts reported in ppm and referenced to signals from the solvent. Polymer molecular weights were determined by gel permeation chromatography with a multi-angle light scattering detector (GPC-MALLS) at 35 °C using two PLgel 10μ mixed-B columns in series (manufacturer-stated linear molecular weight range of 500 to 10 × 10⁶ g/mol). The eluting solvent was THF at a flow rate of 1 mL/min. An Optilab rEX (Wyatt Technology) refractive index detector and a DAWN EOS 18-angle light scattering detector (Wyatt Technology) with a laser wavelength of 684 nm were used to calculate absolute molecular weights. “Dry” film thicknesses were determined using a spectroscopic ellipsometer (J.A. Woollam M-44) assuming a film refractive index of 1.5. Fitting the refractive index did not significantly alter the calculated thickness. *In situ* ellipsometry in aqueous solutions was performed using a home-built cell described previously.^{35,36} In this case, both refractive index and thickness were fitting parameters. Reflectance FTIR spectra

were obtained with a Thermo Nicolet 6700 FTIR spectrometer using a Pike grazing angle (80°) apparatus. A UV/ozone-cleaned gold wafer served as a background. pKa values for PAA and PHEMA-*g*-PAA in aqueous solutions were calculated from titrations with 0.1 M HCl. The polyelectrolyte was dissolved in aqueous NaOH (pH \sim 12), and the pH was adjusted to 10 before titration. For films, pKa values for PAA and PHEMA-*g*-PAA were estimated based on the ratio of –COOH and –COO[–] absorbances (peak heights) in FTIR spectra.³⁴ AFM images were acquired using a NanoScope IIIa scanning probe microscope (Veeco Instruments Inc., CA) operating in the contact mode.

Lysozyme binding

To immobilize lysozyme (Sigma, from chicken egg white, lyophilized powder, >90%), substrates coated with PEMs were immersed in 1.0 mg/mL lysozyme in 20 mM phosphate buffer (pH 7.4) for 16 h at room temperature. (In kinetics studies, binding times ranged from 1 min to 16 h.) Subsequently, using a Pasteur pipette these substrates were rinsed with 10 mL of washing buffer (20 mM phosphate buffer containing 0.1% Tween-20 surfactant) and 10 mL of water for \sim 1 min each and dried with N₂. (Experiments with different washing times show that a 1-min wash is sufficient to remove unbound lysozyme, see Figure S1. Figure numbers beginning with S refer to the supporting information.) The amount of lysozyme binding was determined by reflectance FTIR spectroscopy and expressed as the equivalent thickness of spin-coated lysozyme that would give the same absorbance.⁵ The equivalent thickness *d* is calculated from the difference of absorbance (ΔA) at 1680 cm^{–1} (amide band I of lysozyme) before and after binding lysozyme, using the equation $d(\text{nm}) = \Delta A / 0.0017$.⁵ Some of these thicknesses were confirmed using ellipsometry. Assuming a protein density of \sim 1 g/cm³, each nm of equivalent thickness corresponds to approximately 1 mg/m² of surface coverage.

Results and discussion

Synthesis of PHEMA-*g*-PAA

Synthesis of the grafted copolymer, PHEMA-*g*-PAA, proceeds in four steps (Scheme 1). Copper-catalyzed ATRP of HEMA gives linear PHEMA, and subsequent PHEMA esterification with 2-bromoisobutyryl bromide yields the macroinitiator, poly(2-(2-bromoisobutyryloxy)ethyl methacrylate) (PBIEM). ¹H NMR spectra indicate essentially 100% esterification (see Figure S2). Based on GPC ($M_n = 119\,000$, $M_w/M_n = 1.03$, see Figure S3), the PBIEM has an average degree of polymerization of 430, which suggest an initiation efficiency less than 25%.

Grafting of poly(*tert*-butyl acrylate) (PtBA) from PBIEM also occurs via ATRP (see Figure S4 for the NMR spectrum). GPC of the grafted copolymer shows a very high average molecular weight with a relatively narrow distribution ($M_n = 7\,990\,000$, $M_w/M_n = 1.44$, see Figure S3), indicating successful grafting of PtBA with reasonable control of the polymerization. The GPC data correspond to an average degree of polymerization of 140 for the PtBA side chains if we assume 100% initiation efficiency from the poly(BIEM), which is consistent with similar reported reactions.³² Thus the average number of tBA units per grafted copolymer is 61,500. Finally, removal of the *tert*-butyl group proceeds during treatment of PHEMA-*g*-PtBA with 8 M HCl in dioxane for 2h. The disappearance of the ¹H NMR signal (1.35 ppm) from the methyl protons of the tBA groups (Figure S4) and the loss of the *t*-butyl vibration in the IR spectrum (Figure S5) confirm deprotection to PHEMA-*g*-PAA.

Formation of (PAH/PHEMA-g-PAA)_n and (PAH/PAA)_n Films

PHEMA-*g*-PAA can serve as an extremely large, 3-dimensional polyanion in PEMs. Chemically, PHEMA-*g*-PAA should behave like linear PAA because of the high degree of polymerization of the PAA side chains. Literature reports show only a slightly higher apparent pK_a value for star-shaped PAA than linear PAA.^{37,38} Unexpectedly, acid titrations of PHEMA-*g*-PAA show a 1-unit decrease in apparent pK_a relative to PAA (Figure S6). The high density of PAA chains in PHEMA-*g*-PAA should lead to increased electrostatic repulsions between $-COO^-$ groups on neighboring chains and thus an increase in the apparent pK_a . However, extension of these crowded chains may decrease the *intrachain* electrostatic repulsion between $-COO^-$ groups to lower the pK_a . On average, the PAA side chains in PHEMA-*g*-PAA are about 1/3 as long as the PHEMA backbone, so the grafted copolymers should be cylindrical. Extended PAA chains in PHEMA-*g*-PAA might even cause more rapid film growth compared to PEMs with linear or even star polymers^{39,40} and dendrimers.^{41,42}

Figure 2 shows the ellipsometric thicknesses of (PAH/PHEMA-*g*-PAA)_n and control (PAH/PAA)_n films as a function of the number of bilayers (n) adsorbed from pH 7 solutions. After adsorption of the first two priming bilayers, the (PAH/PHEMA-*g*-PAA)_n films grow much faster than (PAH/PAA)_n. At pH 7, both linear PAA and PAA side chains in PHEMA-*g*-PAA will extend due to electrostatic repulsion within the deprotonated side chains. For linear PAA, this likely leads to adsorption of thin films with chains extended parallel to the surface. In contrast, PHEMA-*g*-PAA adsorption might occur with either the backbone or the side chains parallel to the surface (or some intermediate orientation), but all orientations will lead to relatively thick films at full surface coverage because of the large size of the copolymer. Nevertheless, the highest increase in thickness on deposition of an additional PAH/PHEMA-*g*-PAA bilayer is <10 nm. Given the average degree of side-chain polymerization of ~140, which corresponds to a fully extended side-chain length of 35 nm (0.25 nm per repeat unit), bilayer thicknesses of 10 nm are well within reason. (Films with more than 10 bilayers were visibly rough, so we could not determine their ellipsometric thicknesses.)

Additionally, Figure 2 suggests that the thickness of (PAH/PHEMA-*g*-PAA)_n films increases exponentially with the number of bilayers, even though (PAH/PAA)_n films show essentially linear growth both in this and other studies.⁴³ (Figures S7 and S8 show that (PAH/PHEMA-*g*-PAA)_n films also grow exponentially under other deposition conditions.) Previous research suggests that diffusion of polymer chains throughout a film leads to exponential growth,^{44,45} and low polyelectrolyte molecular weights favor exponential increases in thickness.⁴⁶ However, the molecular weight of PHEMA-*g*-PAA (4500 kDa, calculated from the molecular weight of PHEMA-*g*-PBA assuming complete hydrolysis) is much higher than that of linear PAA (90 kDa). Perhaps the extended side chains of PHEMA-*g*-PAA allow diffusion of PAH throughout the film. The high molecular weight of the grafted polymer might also increase the thicknesses of PAH/PHEMA-*g*-PAA films,^{46–48} but Shiratori and Rubner reported that the thicknesses of PAH/PAA films deposited at pH 6.5 do not vary with PAA molecular weights from 3000 to 10⁶ Da.³³ Thicknesses vary with molecular weight at other deposition pH values.³³

Effects of pH and Supporting Electrolyte on Film Thickness

Adsorption pH dramatically affects the thicknesses of (PAH/PAA)_n films^{33,43} and should have a similar effect on (PAH/PHEMA-*g*-PAA)_n coatings. Figure 3 shows (PAH/PAA)_n and (PAH/PHEMA-*g*-PAA)_n thicknesses as a function of adsorption pH, and mostly similar trends with greater overall thicknesses and greater differences between (PAH/PAA)₅ and (PAH/PHEMA-*g*-PAA)₅ thicknesses occur for PEMs with 10 bilayers (Figure S9). In the

case of $(\text{PAH}/\text{PAA})_n$ films, decreased ionization of PAA at pH values <7 or of PAH at pH values >7 leads to increases in film thickness as shown previously.³³ Less ionization presumably results in polymer chains with more loops and tails and, hence, greater thickness. Additionally, lower charge densities on polymer chains in solution may require deposition of more polymer to compensate the charge on the surface. The latter explanation might better describe why the thickness of $(\text{PAH}/\text{PHEMA-}g\text{-PAA})_5$ films increases at low deposition pH even though the grafted copolymer should be most extended and occupy the greatest volume at high degrees of deprotonation. At all deposition pH values, $(\text{PAH}/\text{PHEMA-}g\text{-PAA})_5$ films are about twice as thick as $(\text{PAH}/\text{PAA})_5$ films with the same number of bilayers (Figure 3). For ten-bilayer films the $(\text{PAH}/\text{PHEMA-}g\text{-PAA})_5$ can be as much as 4.6-fold thicker than $(\text{PAH}/\text{PAA})_5$ (Figure S9).

A number of studies demonstrate that the presence of salt in adsorption solutions increases the thickness and roughness of PEMs.^{49–51} The excess electrolyte screens the charges along the polyelectrolytes to give more coiled chain conformations and thicker films.⁵² Addition of 0.5 M NaCl to PHEMA-*g*-PAA, PAA, and PAH deposition solutions dramatically changes trends in film thicknesses as a function of pH (compare Figures 3 and 4). With 0.5 M NaCl in deposition solutions, the thicknesses of both $(\text{PAH}/\text{PHEMA-}g\text{-PAA})_n$ and $(\text{PAH}/\text{PAA})_n$ films increase essentially monotonically with pH (Figure 4). Moreover the ratios of the thicknesses of $(\text{PAH}/\text{PHEMA-}g\text{-PAA})_5$ films to the thicknesses of corresponding $(\text{PAH}/\text{PAA})_5$ films range from only 0.9 to 1.6. At a deposition pH of 3, the supporting electrolyte has little effect on film thickness (compare Figures 3 and 4, also see Figures S10 and S11), but at all other adsorption pH values, film thicknesses increase by a factor between 2.4 and 24 when using 0.5 M NaCl in the deposition solution.

The thickness increase due to the presence of 0.5 M NaCl is especially large for $(\text{PAH}/\text{PAA})_n$ films. Compensation of the polymer charge by ions in the electrolyte might result in a much more coiled conformation for linear polyelectrolytes than for the comb-like copolymer. This would explain why supporting electrolyte impacts the thicknesses of $(\text{PAH}/\text{PAA})_n$ films more than $(\text{PAH}/\text{PHEMA-}g\text{-PAA})_n$. Overall, the presence of salt leads to greater film thickness than variation of pH in the absence of salt. However, rinsing of the films with 0.5 M NaCl rather than deionized water leads to visually nonuniform films.

Lysozyme Binding as a Function of Film Deposition pH

Adsorption of active proteins on PEMs is a simple method for creating functional thin films both on flat surfaces and in membranes.^{5–7} This study examines sorption of lysozyme, which due to its protonated amino acids has a net charge of +8 in pH 7.4 phosphate buffer (the isoelectric point of lysozyme is 11.4⁵³). For $(\text{PAH}/\text{PHEMA-}g\text{-PAA})_n$ and $(\text{PAH}/\text{PAA})_n$ multilayers that terminate with the polyanion, we initially thought protein would adsorb via ion exchange in the surface layer, and that the large size of PHEMA-*g*-PAA molecules would enhance protein binding. However, in some cases, sorption of proteins can occur throughout a PEM to yield the equivalent of many monolayers of adsorbed protein.¹⁸ Moreover, a recent study shows that human serum albumin, a protein with a molecular mass of 66 kDa, is highly mobile in water-swollen polyelectrolyte films, at least at low protein concentrations in the film.⁵⁴ With a molecular mass of 14.3 kDa, lysozyme may be even more mobile in swollen films.

We first examined lysozyme sorption from phosphate buffer (pH 7.4) into $(\text{PAH}/\text{PHEMA-}g\text{-PAA})_5$ and $(\text{PAH}/\text{PAA})_5$ films deposited from polyelectrolyte solutions with different pH values but no supporting electrolyte. With the exception of films deposited at pH 3, the $(\text{PAH}/\text{PAA})_5$ films all sorb the equivalent of <5 nm of lysozyme (Figure 5). Thus, these films essentially capture a monolayer or less of lysozyme (the dimensions of this protein are $3 \times 3 \times 4.5$ nm), presumably by adsorption at the film surface.⁵⁵ Strong polyanion-polycation

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ion-pairing in films deposited at pH values 5 likely prevents lysozyme from entering the film.^{33,43,51} (PAH/PHEMA-g-PAA)₅ films deposited at pH values 5 show 2- to 4-fold higher lysozyme sorption than corresponding (PAH/PAA)₅ coatings. This increase could stem from more adsorption in the thicker surface layer or a more swollen film. When normalized to film thickness, lysozyme binding in (PAH/PHEMA-g-PAA)₅ is about twice that in (PAH/PAA)₅ coatings deposited at pH 5, 7, and 9. Although this suggests that (PAH/PHEMA-g-PAA)₅ coatings swell more in water than (PAH/PAA)₅ films, the uncertainties in the film thicknesses prevent a firm conclusion.

(PAH/PHEMA-g-PAA)₅ and (PAH/PAA)₅ films deposited at pH 3 contain free -COOH groups that deprotonate in pH 7.4 buffer. These new carboxylate groups should provide lysozyme binding sites and also increase swelling in water to enhance diffusion of lysozyme into the film. Remarkably, when deposited at pH 3 both (PAH/PHEMA-g-PAA)₅ and (PAH/PAA)₅ capture an amount of lysozyme equivalent to the initial film thickness, indicating binding throughout the film (absorption). These results are consistent with a surface plasmon resonance study that showed a 2-fold increase in lysozyme sorption to PAH/PAA films deposited at pH 2.0 compared to films in which PAH was deposited at pH 7.5 and PAA was deposited at pH 3.5.²⁶ With deposition at pH 3 the (PAH/PHEMA-g-PAA)₅ coating shows 60% more lysozyme binding than (PAH/PAA)₅, but this may stem primarily from the greater thickness of the (PAH/PHEMA-g-PAA)₅.

Lysozyme Binding to Films Prepared in 0.5 M NaCl

The presence of 0.5 M NaCl during adsorption of (PAH/PHEMA-g-PAA)₅ and (PAH/PAA)₅ films greatly enhances lysozyme sorption, regardless of the pH used for film deposition (compare Figures 5 and 6). For coatings deposited in 0.5 M NaCl, the (PAH/PHEMA-g-PAA)₅ and (PAH/PAA)₅ films adsorbed at pH 3 show some of the highest lysozyme binding capacities, even though these films have the lowest thicknesses prior to lysozyme adsorption (Figure 6). Deprotonation of the free -COOH groups in these coatings and lysozyme binding lead to films that contain more than 80% protein. Multilayer films deposited at pH 7 and pH 9 also bind remarkably large amounts of protein, suggesting that when deposited in 0.5 M NaCl these films either contain a larger number of intrinsic ion-exchange sites, or the lysozyme readily disrupts polyanion-polycation ion pairs to create new absorption sites.

The relatively low sorption capacity of coatings prepared at pH 5 in 0.5 M NaCl may indicate that these films have the strongest intrinsic ion pairing between polycations and polyanions. Interestingly, only the film deposited at pH 5 shows significantly more binding to (PAH/PHEMA-g-PAA)₅ than (PAH/PAA)₅. At this deposition pH, the graft copolymer may yield a more swollen structure than linear PAA as well as more binding at the film surface.

As mentioned above, lysozyme sorption likely occurs by ion exchange, and experiments with binding at different pH values and ionic strengths corroborate this mechanism. When lysozyme sorption to (PAH/PAA)₅ films (deposited from 0.5 M NaCl at pH 3) occurs from 20 mM phosphate solutions at pH 3 and 5, the equivalent thicknesses of sorbed lysozyme are only 7.1 ± 1.7 nm and 18.9 ± 0.2 nm, respectively. In contrast, sorption at pH 7.4 and 10 leads to lysozyme equivalent thicknesses around 150 nm (Figure S12). At low pH, protons effectively compete with lysozyme for -COO⁻ ion-exchange sites and decrease binding. Notably, terminating films with PAH rather than PAA does not lead to a significant difference in lysozyme adsorption (Figure S13). Thus, the lysozyme penetrates the film during sorption.

Lysozyme elution with 1 M KSCN is also consistent with binding by ion-exchange. Exposure to the KSCN solution for only 1 min removes 96% of the bound lysozyme. In contrast, elution with 0.1 M KSCN removes only 85% of the lysozyme in 1 h (Table S1). Binding also varies with the ionic strength of the lysozyme solution (Figure S14). In 20 mM phosphate buffer at pH 7.4, addition of 0.1 M NaCl to the adsorption solution decreases the effective thickness of bound lysozyme marginally from 147 ± 29 nm to 133 ± 1 nm. However, further increasing the NaCl concentration to 0.3 or 0.5 M decreases the effective lysozyme thickness to essentially zero (Figure S14). The Na^+ effectively competes with lysozyme for the ion-exchange sites.

The above results employed a 16-h adsorption time for lysozyme sorption to ensure equilibrium, but binding actually occurs in a few minutes. Figure S15 shows how the binding capacity of a $(\text{PAH}/\text{PAA})_5$ film (deposited from 0.5 M NaCl solutions at pH 3) varies with adsorption time. Even after a 4-min exposure to lysozyme, the binding reaches 85 % of the value at 16 h, and the difference in the amount of lysozyme sorption during 1 h and 16 h is negligible. The rapid binding suggests a highly swollen film that affords access to interior binding sites.

Film Swelling and Changes in Morphology Upon Protein Sorption

To examine the extent of film swelling, we performed *in situ* ellipsometry with $(\text{PAH}/\text{PAA})_5$ films immersed in deionized water. After an overnight immersion in water, swelling increases the film thickness 5-fold relative to the thickness in ambient air. (Most of the swelling occurs in the first two hours of immersion). Consistent with ~80% water in the immersed coating, the film refractive index decreases from 1.50 to 1.36 after swelling. (The refractive index of water at the wavelengths of the spectroscopic ellipsometer is about 1.333.) After lysozyme binding, the dry film thickness is essentially the thickness of the water-swollen film. The coating containing bound protein swells by only 30% in water, and the swollen film refractive index is around 1.50, reflective of the large amount of sorbed protein.

Atomic force microscope (AFM) images of $(\text{PAH}/\text{PAA})_5$ multilayers deposited from 0.5 M NaCl at pH 3 suggest that film morphology does not change greatly upon lysozyme sorption (see Figures S16 a and b). The root-mean square roughness values for $(\text{PAH}/\text{PAA})_5$ films increase from 2.0 to 3.4 nm after sorption. Given the large amount of protein binding (150 nm of lysozyme), these images provide another evidence for diffusion of lysozyme into the interior of the film. Adsorption of multilayers of lysozyme at the surface would likely increase roughness.

Interestingly, although $(\text{PAH}/\text{PAA})_5$ films deposited at pH 3 and pH 9 (from solutions containing 0.5 M NaCl) sorb similar amounts of lysozyme, only the latter coating shows a large morphology change upon lysozyme sorption. With $(\text{PAH}/\text{PAA})_5$ deposited at pH 9, the rms roughness increases from 2.7 to 14.7 nm upon lysozyme sorption (see Figures S17 a and b). For these particular films, the large positive charge due to both protonation of amine groups and sorption of lysozyme may cause film reconstruction.⁵⁶

Film Stability and Repetitive Binding Experiments

The high swelling of $(\text{PAH}/\text{PAA})_5$ films deposited from 0.5 M NaCl at pH 3 is attractive for protein binding, but these films are not stable in neutral pH buffers in the absence of lysozyme. (They are stable in deionized water.) Figure 7 shows FTIR spectra of $(\text{PAH}/\text{PAA})_5$ films before and after an overnight immersion in 20 mM phosphate adjusted to pH 3, 5, 7.4, and 10 (no added NaCl). At pH 3, the overnight immersion does not affect the IR spectrum of the film, but after immersion in pH 7.4 or pH 10 phosphate buffer, the

absorbance due to PAA decreases by about 80%. Even after immersion in pH 5 buffer, the absorbance due to PAA may decrease by 20%. However, this 20% decrease is not definitive because although the acid carbonyl absorbance near 1720 cm^{-1} decreases, the carboxylate absorbance increases slightly. (Even though we immersed films in pH 3 prior to obtaining the spectra, the degree of protonation may change slightly.). At neutral pH, deprotonation of -COOH groups leads to electrostatic repulsion as well as increased solubility of PAA, and this apparently leads to dissolution of much of the film. The IR spectra in Figure S18 confirm partial deprotonation of -COOH groups as the ambient pH increases from 3.0 to 6.0.

Lysozyme binding stabilizes the adsorbed PAA in $(\text{PAH/PAA})_5$ films. Although binding occurs at pH 7.4 where the film is unstable, the thickness of films containing sorbed lysozyme is $180 \pm 23\text{ nm}$. The thickness prior to lysozyme sorption is $37 \pm 6\text{ nm}$. However, after elution of lysozyme with 1 M KSCN in 20 mM phosphate buffer (pH 7.4), the spectrum of the film is similar to that after immersion of the film overnight in pH 7.4 buffer, showing that the $(\text{PAH/PAA})_5$ is unstable in 1 M KSCN at neutral pH. The protein binding could involve some replacement of PAH by lysozyme,^{57,58} but given the thickness increase upon binding, there is much more sorbed lysozyme than initially adsorbed PAH.

Interestingly, after immersion in pH 7.4 phosphate buffer, the spectrum of a $(\text{PAH/PAA})_5$ film is similar to that of a $(\text{PAH/PAA})_2$ film (also deposited from a pH 3, 0.5 M NaCl solution). Thus, we investigated repetitive lysozyme binding to $(\text{PAA/PAH})_2$ multilayers deposited at pH 3.0 and pH 9.0. Figure S19 shows that 5 cycles of binding and elution on the same film deposited from a pH 3, 0.5 M NaCl solution lead to only a ~25% decrease in the binding capacity. For the film deposited from a pH 9 solution, the binding capacity decreases by 50% in the same experiment. Our recent experiments with porous nylon membranes modified with (PAA/PAH)PAA films showed a high and constant binding capacity for lysozyme.³² Nevertheless, with films on Au, immersion of a $(\text{PAH/PAA})_2$ film in pH 7.4 buffer overnight results in a 40% decrease in the carbonyl absorbance of the film (Figure S20). In contrast to films deposited from pH 3, 0.5 M NaCl solutions, $(\text{PAH/PAA})_5$ films deposited at pH 9 are relatively stable at neutral pH (compare Figure 7 and Figure S21), as these films contain a small fraction of -COOH groups after deposition.

Variation of Lysozyme Sorption with the Number of Polyelectrolyte Bilayers

The PEMs deposited at pH 3 in 0.5 M NaCl clearly exhibit the highest ratio of bound lysozyme to film thickness (Figure 6), so despite some challenges with film stability, we examined binding to these coatings as a function of the number of layers in the film. For both $(\text{PAH/PHEMA-}g\text{-PAA})_n$ and $(\text{PAH/PAA})_n$, the amount of adsorbed lysozyme generally increases with the number of polyelectrolyte bilayers, but the ratio of lysozyme binding to film thickness decreases with the number of layers (Figure 8). This suggests that access to the film interior or the film swelling decreases with the addition of more layers. In all cases, for the same number of layers the $(\text{PAH/PHEMA-}g\text{-PAA})_n$ films bind more lysozyme than $(\text{PAH/PAA})_n$. However, the ratios of lysozyme binding to film thickness are similar for these two polyelectrolyte systems. This result suggests that the internal structure of $(\text{PAH/PHEMA-}g\text{-PAA})_n$ multilayers is similar to the structure of $(\text{PAH/PAA})_n$ under these deposition conditions (pH 3, 0.5 M NaCl).

The binding capacities of these films are remarkable. The equivalent thickness of the sorbed protein ranges from 4.4 to 10 times the initial film thickness. Previous studies even with typically swollen polyelectrolytes showed an effective bound protein thickness of 60 nm or less.^{18,19,59,60} Clearly, control over polymer structure, especially by variation of deposition conditions can greatly enhance protein sorption by PEMs. For films with more than 1 or 2 bilayers, addition of 0.5 M NaCl to deposition solutions increases lysozyme sorption and the

ratio of lysozyme thickness to film thickness by a factor of 2 or more (see Figure S22 a and b). Our recent work shows that deposition of these films in porous supports yields membranes that selectively bind large amounts of protein.²⁸ Derivatization of the –COOH groups can also introduce specific binding sites that impart more selectivity to the protein binding along with the high binding capacity.²⁸

Conclusions

In the absence of supporting electrolyte, LBL adsorption of PAH and a comb-like copolymer (PHEMA-*g*-PAA) yields thicker films, regardless of deposition pH, than adsorption of PAH and linear PAA. Moreover, with deposition at pH 5, 7, or 9 in the absence of added salt, films containing the graft copolymer may sorb approximately twice as much lysozyme per film thickness as films containing linear PAA. Addition of 0.5 M NaCl to PAA, PHEMA-*g*-PAA, and PAH deposition solutions has little effect on film thickness at a deposition pH of 3, but at pH values of 5, 7, and 9 the added salt increases thicknesses by factors as high 24. Moreover, lysozyme binding capacities of (PAH/PHEMA-*g*-PAA)₅ and (PAH/PAA)₅ films increase as much as an order of magnitude when 0.5 M NaCl is present during film formation at pH 7 or 9. However, for films prepared in the presence of 0.5 M NaCl the ratios of lysozyme binding to film thickness are similar for PAH/PAA and PAH/PHEMA-*g*-PAA. Deposition of either PAH/PAA or PAH/PHEMA-*g*-PAA films at pH 3 in the presence of 0.5 M NaCl leads to remarkably high protein adsorption, as film thickness can increase up to 10-fold upon exposure to lysozyme. We recently showed that this high binding capacity is particularly attractive for modifying porous supports to create membranes that selectively absorb large amounts of desired proteins.²⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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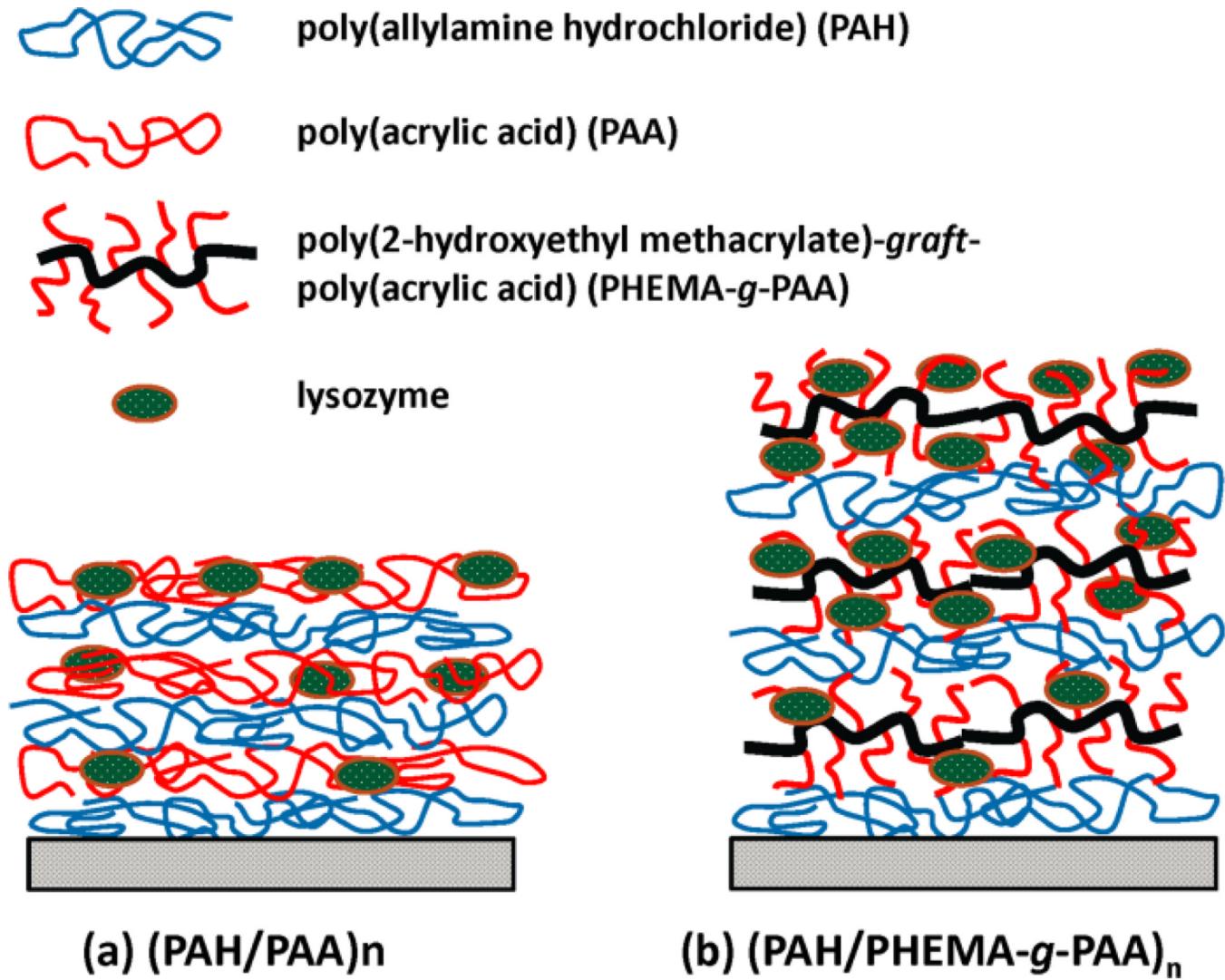
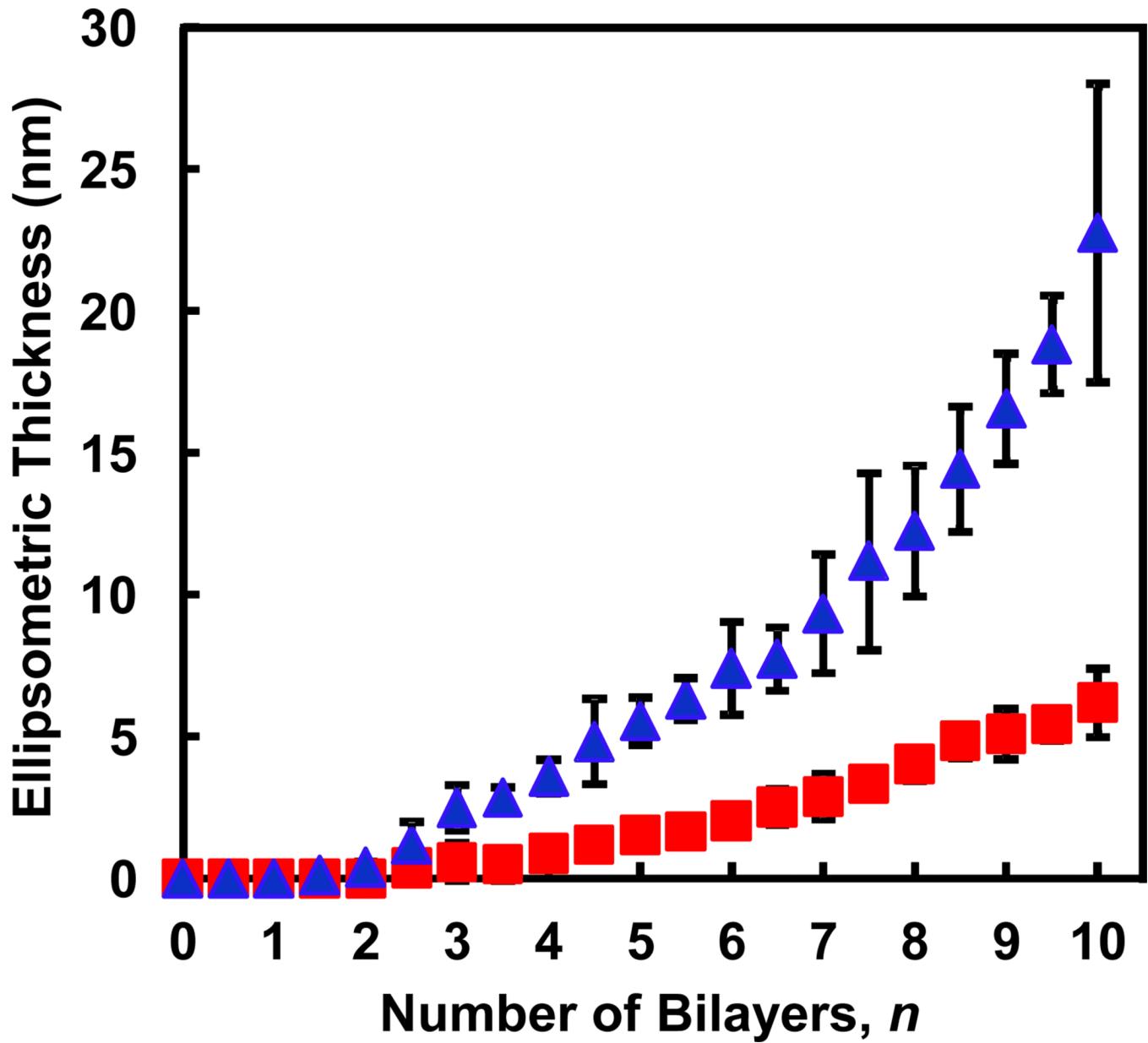
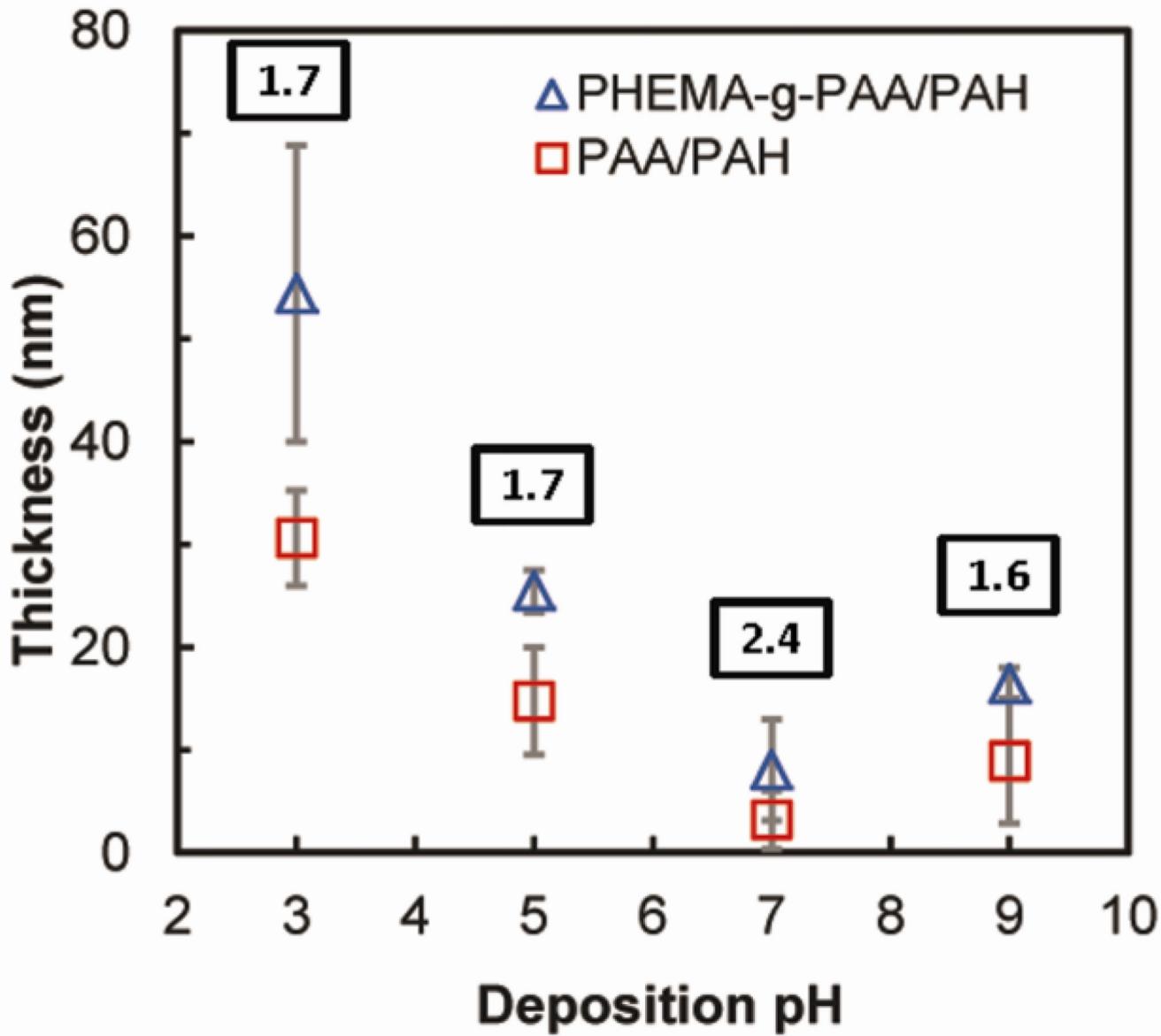


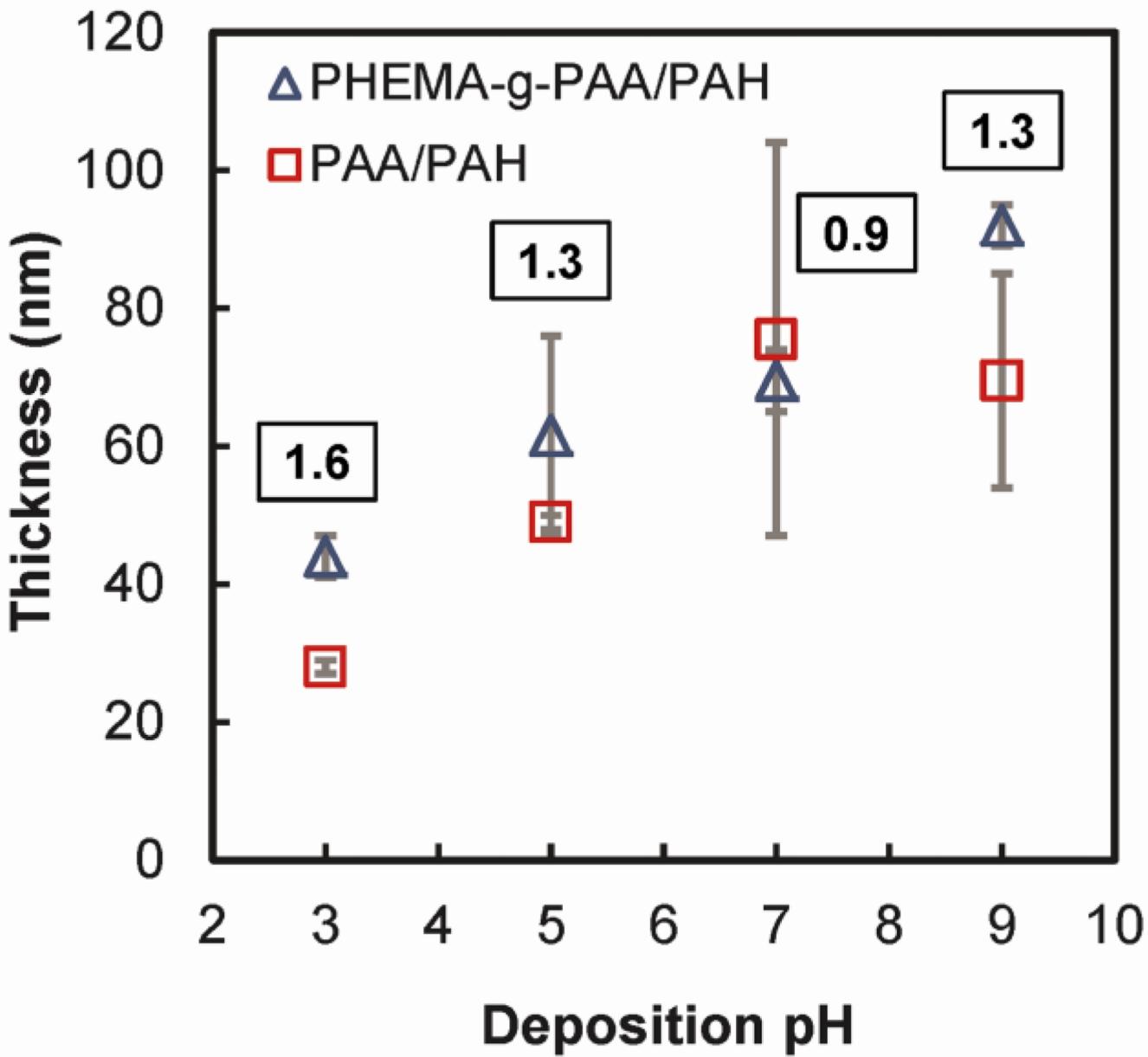
Figure 1.
Schematic cross-sections of lysozyme binding to PEMs prepared with (a) linear poly(acrylic acid) and (b) PHEMA-*g*-PAA.

**Figure 2.**

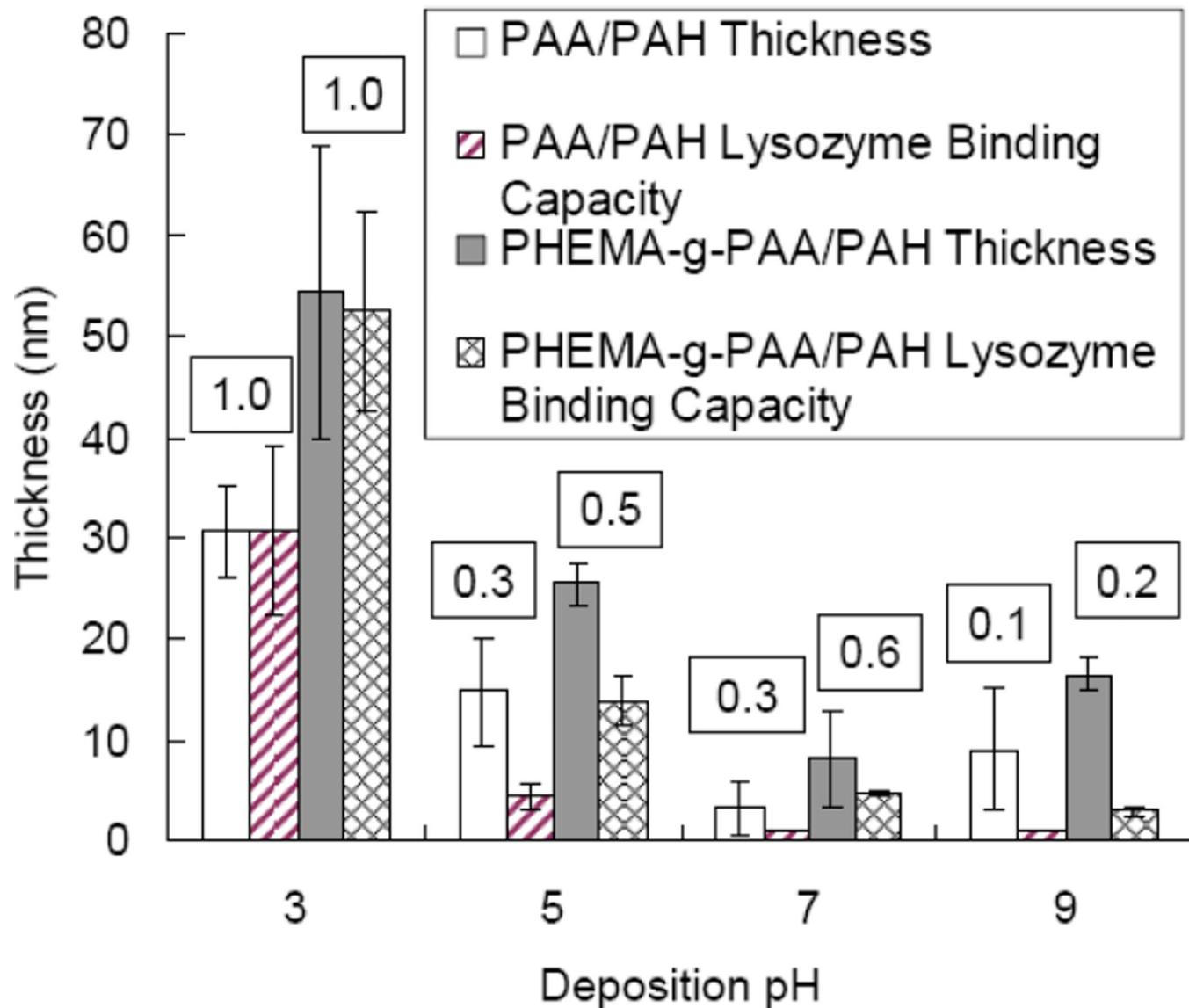
Ellipsometric thicknesses of $(\text{PAH}/\text{PHEMA-}g\text{-PAA})_n$ (triangles) and $(\text{PAH}/\text{PAA})_n$ (squares) films deposited at pH=7 on Au substrates modified with a monolayer of MPA. (The polyelectrolyte deposition solutions contained no NaCl.) Integer numbers of bilayers indicate films terminated with PHEMA-*g*-PAA or PAA, and films with an extra half bilayer end in PAH. Error bars indicate standard deviations of 4 total measurements on two substrates.

**Figure 3.**

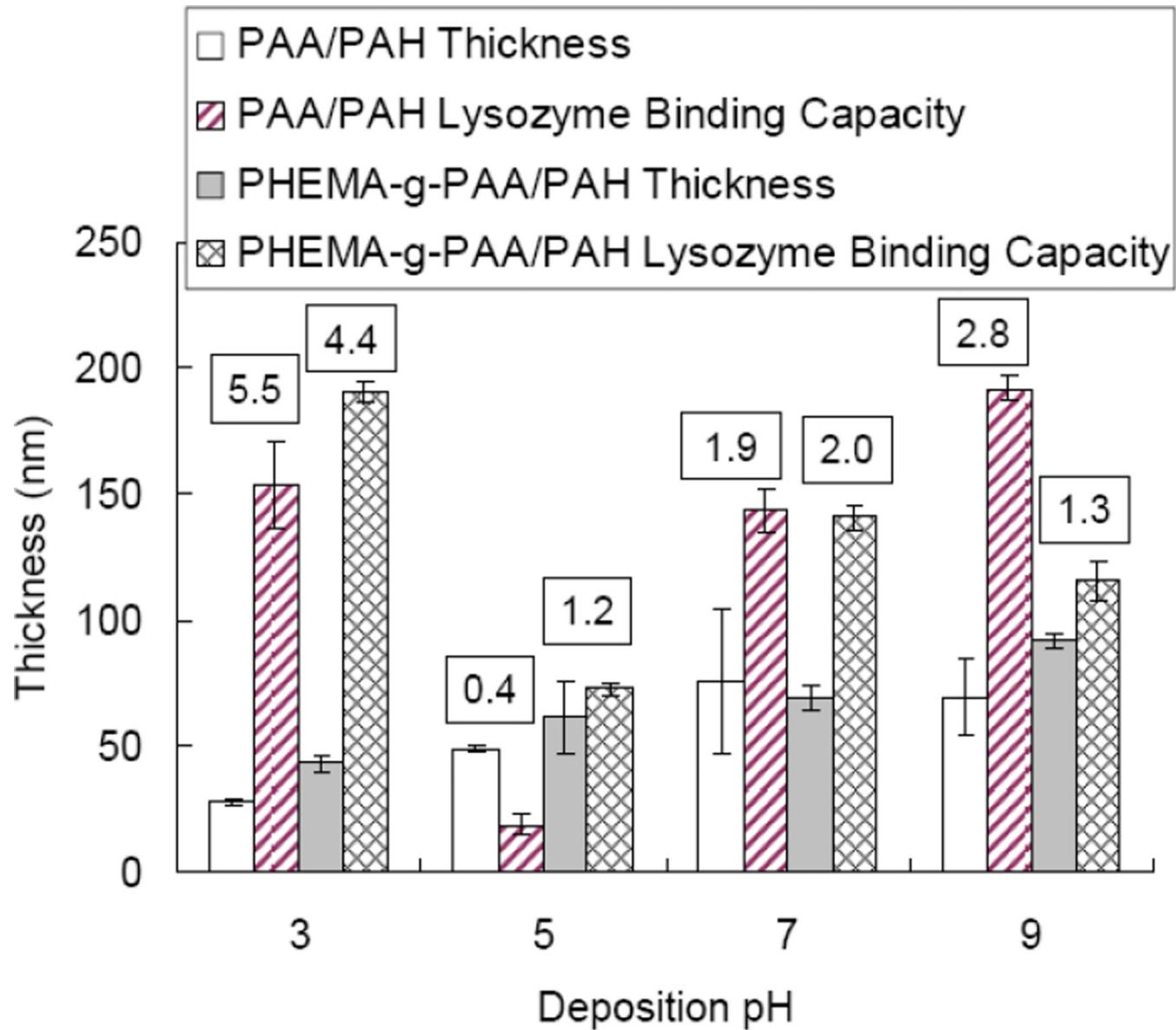
Ellipsometric thicknesses of $(\text{PAH}/\text{PHEMA}-g\text{-PAA})_5$ (triangles) and $(\text{PAH}/\text{PAA})_5$ (squares) films deposited from polyelectrolyte solutions with various pH values and no supporting electrolyte. The numbers above the triangles represent the ratio of the average thicknesses of the two types of films deposited at the same pH. Data points are the average of two trials, and error bars here and below represent the difference between the trials unless specified otherwise.

**Figure 4.**

Ellipsometric thicknesses of $(\text{PAH}/\text{PHEMA-}g\text{-PAA})_5$ and $(\text{PAH}/\text{PAA})_5$ multilayers deposited from polyelectrolyte solutions containing 0.5 M NaCl at various pH values. The numbers above the triangles represent the ratio of the average thicknesses of the two types of films deposited at the same pH.

**Figure 5.**

Film thicknesses and the equivalent thicknesses of lysozyme sorbed in $(\text{PAH}/\text{PHEMA-g-PAA})_5$ and $(\text{PAH}/\text{PAA})_5$ multilayers deposited from polyelectrolyte solutions with various pH values and no supporting electrolyte. The numbers above the bars represent the ratios of the lysozyme equivalent thickness to the film thickness. The equivalent thickness is the thickness of spin-coated lysozyme that would give an FTIR absorbance equivalent to that of the sorbed lysozyme. Error bars show the standard deviations of measurements on at least three different films

**Figure 6.**

Film thicknesses and the equivalent thicknesses of lysozyme sorbed in $(\text{PAH}/\text{PHEMA-g-PAA})_5$ and $(\text{PAH}/\text{PAA})_5$ multilayers deposited from polyelectrolyte solutions containing 0.5 M NaCl at various pH values. The numbers above the bars represent the ratios of the lysozyme equivalent thickness to the film thickness. The equivalent thickness is the thickness of spin-coated lysozyme that would give an FTIR absorbance equivalent to that of the sorbed lysozyme. Error bars represent the standard deviations of measurements on at least three different films.

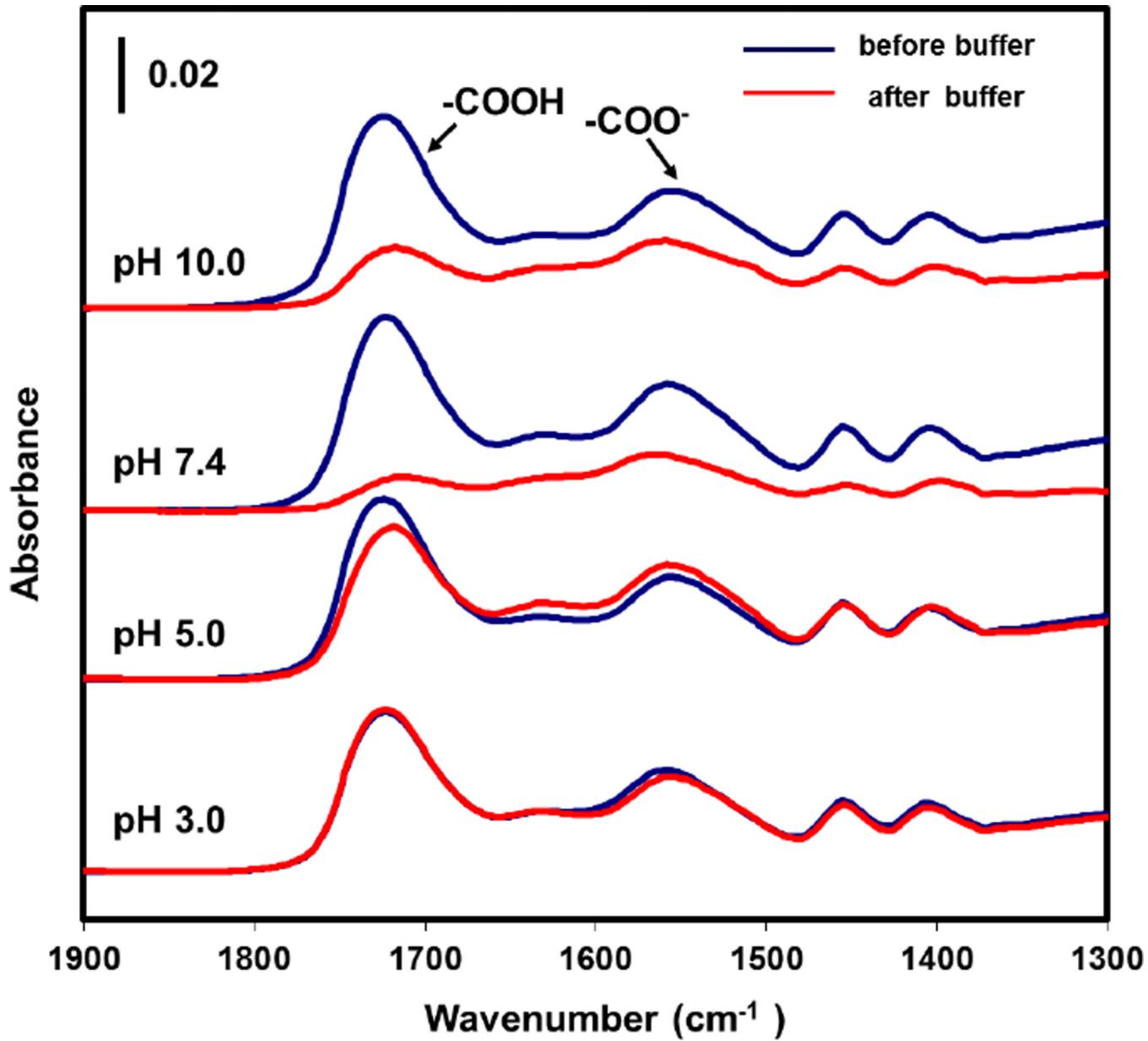
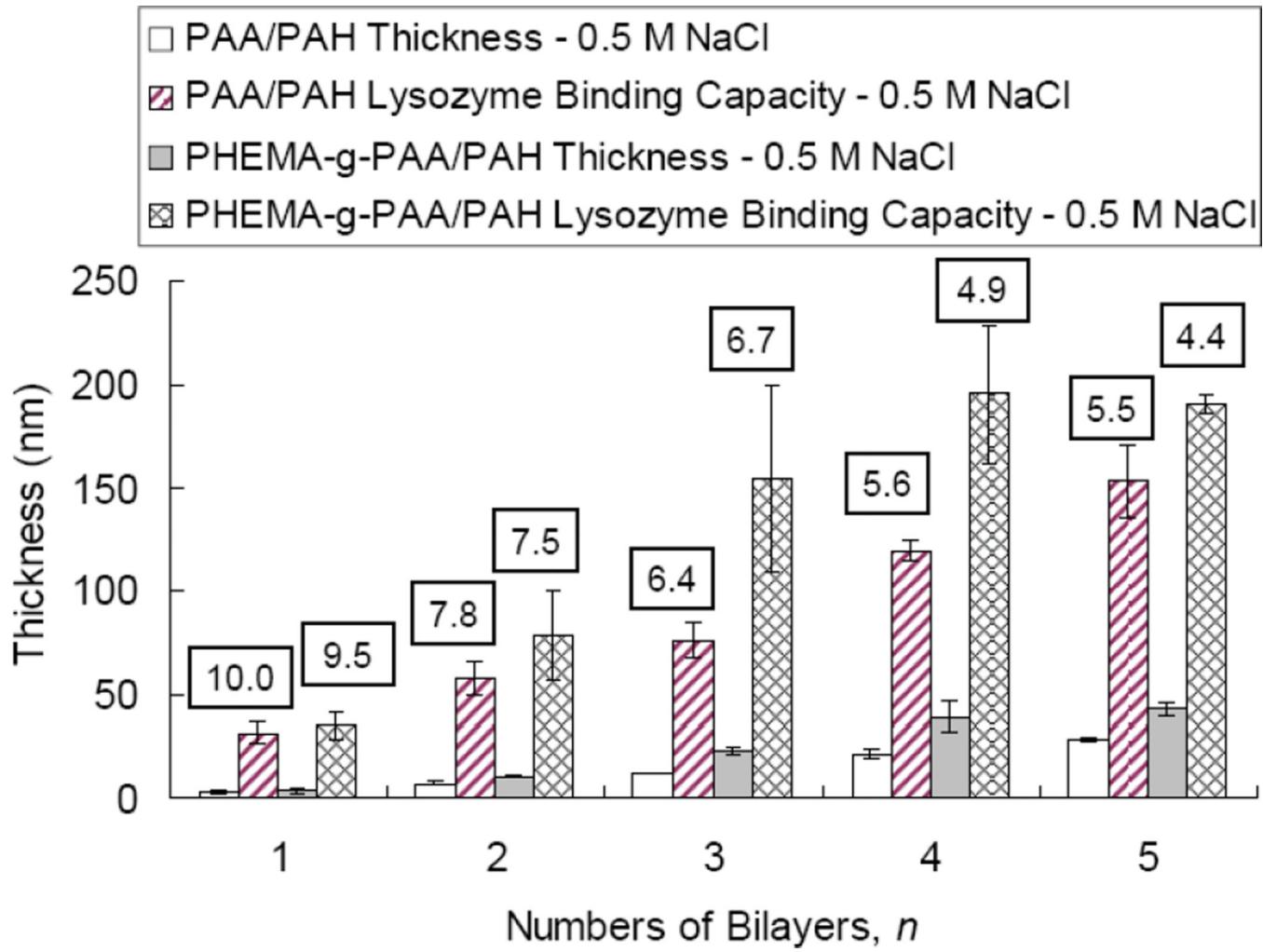
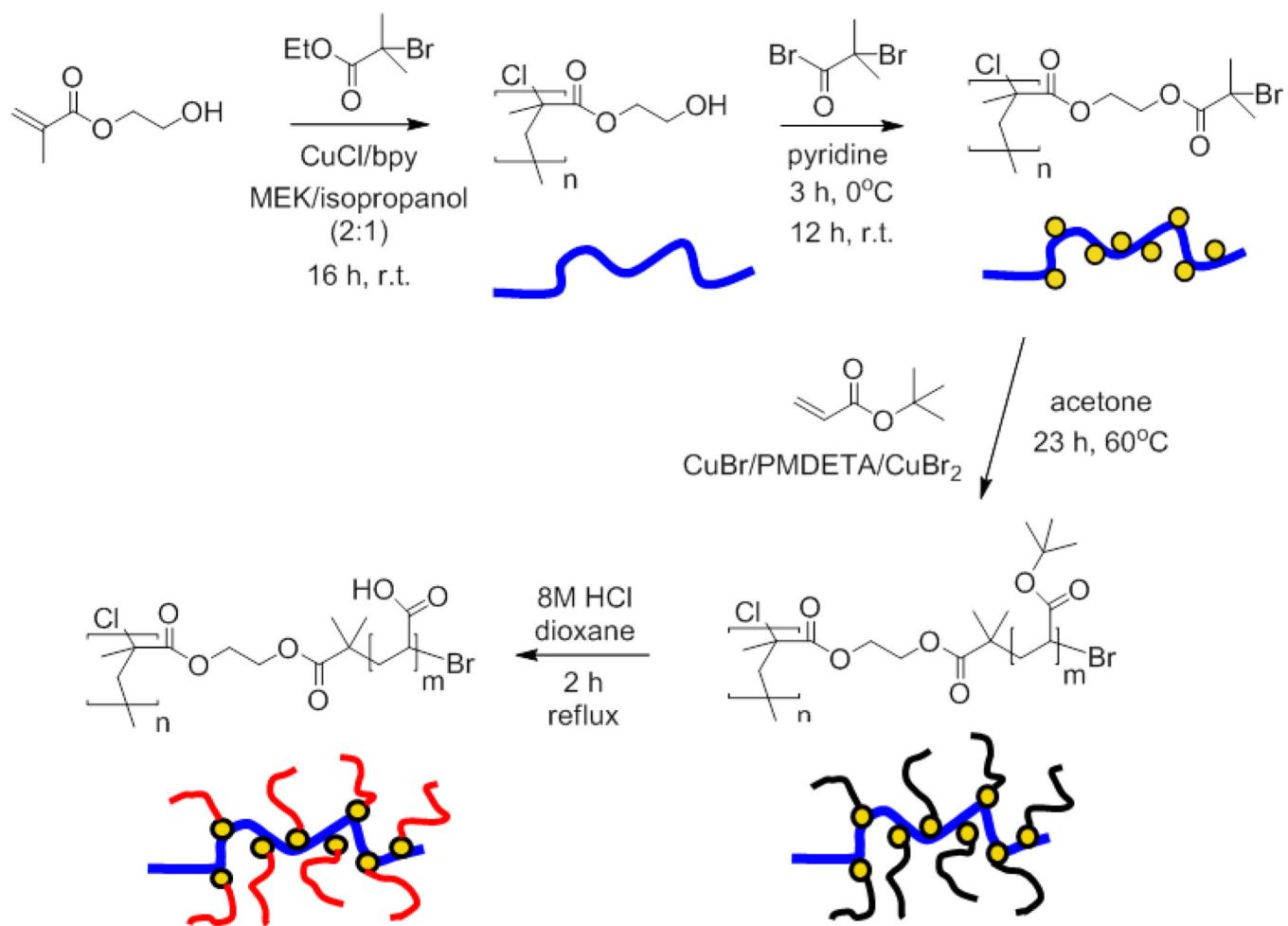


Figure 7.

Reflectance FTIR spectra of $(\text{PAH}/\text{PAA})_5$ films before and after immersion in a 20 mM phosphate solution adjusted to different pH values. $(\text{PAH}/\text{PAA})_5$ films were deposited from 20 mM polyelectrolyte solutions containing 0.5 M NaCl at pH 3.0. These coatings were immersed in the phosphate solutions overnight and washed with pH 3.0 buffer and water before obtaining FTIR spectra.

**Figure 8.**

Lysozyme binding capacities of $(\text{PAH}/\text{PHEMA-}g\text{-PAA})_n$ and $(\text{PAH}/\text{PAA})_n$ multilayers ($n=1\text{--}5$) deposited from polyelectrolyte solutions containing 0.5 M NaCl at pH=3. The numbers above the bars represent the ratios of the lysozyme equivalent thickness to the film thickness. The equivalent thickness is the thickness of spin-coated lysozyme that would give an FTIR absorbance equivalent to that of the sorbed lysozyme.

**Scheme 1.**Synthesis of poly(2-hydroxyethyl methacrylate)-*g*-poly(acrylic acid).