Soft Matter

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/softmatter

Soft Matter

RSCPublishing

ARTICLE

Stiff Chains Inhibit and Flexible Chains Promote Protein Adsorption to Polyelectrolyte Multilayers†

Cite this: DOI: 10.1039/xoxxoooooox

Bo Wu,[ab] Guangming Liu,*[a] Guangzhao Zhang[c] and Vincent S. J. Craig*[b]

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

We tested the hypothesis that the level of protein adsorption onto polyelectrolyte multilayers (PEMs) is influenced by the chain stiffness of the polymers forming the multilayer. The implication being that by altering the chain stiffness, PEMs can be formed that promote or inhibit protein adsorption. Protein adsorption to PEMs consisting of flexible and semi-flexible polyelectrolytes was investigated. The flexible polyelectrolytes were poly(sodium 4-styrene sulfonate) (PSS) and poly(diallyldimethylammonium chloride) (PDDA) and the semi-flexible polyelectrolytes were sulfated chitosan (SC) and cationic guar gum (CGG). Polyelectrolytes were used in pairs to produce four types of polyelectrolyte multilayer films. Moreover, each of these films could be terminated with either of the polyelectrolytes resulting in protein adsorption being studied on 8 systems. Protein adsorption was investigated by optical reflectometry and quartz crystal microbalance with dissipation using bovine serum albumin as the test protein. We found that when a pair of semi-flexible polyelectrolytes were used very little protein adsorption took place, irrespective of which polyelectrolyte was used to terminate the film. When the film was formed by flexible polyelectrolytes, significant protein adsorption took place and the degree of adsorption depended strongly on which polyelectrolyte was used to terminate the film. We explain these observations by considering the conformation of the polyelectrolyte in the outermost region of the film and relate this to the flexibility of the polyelectrolyte chains employed to produce the polyelectrolyte multilayer.

I Introduction

Protein adsorption at interfaces plays an important role in numerous applications including biosensors, immunoassays, medical implants, drug delivery and food processing.[1-5] The promotion of protein adsorption is desired in some cases while the inhibition of protein adsorption is the goal in other cases.[6] For example, selective adsorption of specific proteins is required for biological sensors.[7] On the other hand, preventing nonspecific protein adsorption reduces thrombus formation in blood-contacting devices and limits the initial stages of bacteria adhesion and hence biofilm formation.[8,9] Furthermore, protein-resistant properties will extend the service life and biocompatibility of these devices. Thus, understanding and controlling protein adsorption remains an important technical and practical challenge offering significant economic and societal rewards.

Electrostatic interactions have a strong influence on protein adsorption. Surfaces of unlike charge attract proteins and surfaces of like charge repel them, but other factors also influence protein adsorption. For example, protein adsorption can be promoted by hydrogen bonding and by hydrophobic interactions between protein molecules and surfaces. In contrast, protein adsorption can be reduced by specific interactions between a surface and solvent such as employed in the antifouling material poly(ethylene glycol) (PEG) or polyzwitterion-based films.[10-15]

Polyelectrolyte multilayer films, formed by exposing a surface alternately to anionic and cationic polymers, can also be used to control protein adsorption.[16-22] Protein adsorption to a multilayer surface of the opposite charge can be significantly enhanced by the incorporation of protein molecules into the inner region of PEM.[18,19] That is when *adsorption* is accompanied by *absorption*, the total amount of protein associated with a surface can be significantly increased. In contrast, protein molecules only adsorb to the exterior of the multilayer surface when it carries an identical charge to the protein.[17] Protein resistant properties are also exhibited by PEMs when a hydrophilic PEG block is incorporated into the multilayer due to the strong PEG-water interactions.[23] When the polymers themselves do not possess such antifouling properties it is argued that protein resistance can accrue if the PEM film is organized to form a zwitterionic net neutral surface.[14] Our hypothesis is that the relative chain stiffness of

the polyelectrolytes is important in the organization of the films and this is significant in determining the level of protein adsorption. Polyelectrolyte multilayers consisting of relatively stiff chains will be less susceptible to fouling by protein adsorption, as PEMs consisting of stiff chains present a flat, near electrically neutral surface with few favorable configurations for protein adsorption, whereas flexible chains do not form electrically neutral domains and favour protein adsorption.

Protein adsorption is largely determined by interactions between the protein and the terminating surface of the PEM. The properties of the outermost region of the film will be influenced by the flexibility of the polyelectrolytes that make up the PEM.[24-28] When a flexible polyelectrolyte is used in the terminating layer of a PEM the outermost region of the film will be characterized by more loops than when a stiff polyelectrolyte is employed, which will tend to lie flat on the surface. Flexible chains with many loops, will promote charge reversal of the film through charge overcompensation.[27,28] By contrast, chains lying flat on the surface will result in surfaces of low charge in comparison to flexible chains with many loops. Moreover, flexible chains in loops have more degrees of freedom and are therefore more able to adopt conformations that promote favorable interactions with an adsorbing protein.

The growth behavior and surface topography of four types of PEMs which are constructed from flexible and semi-flexible polyelectrolytes in pairs with different intrinsic chain persistence lengths has been extensively characterized by QCM-D and AFM imaging of the films previously.[27] In this previous work, two flexible polyelectrolytes, i.e., anionic poly(sodium 4-styrenesulfonate) (PSS) and cationic poly(diallyldimethylammonium chloride) (PDDA) and two semi-flexible polyelectrolytes, i.e., anionic sulfated chitosan (SC) and cationic guar gum (CGG) were employed to construct the PEMs and the results showed that PEMs will exhibit different surface roughness, distinct interfacial chain conformations and different extents of surface charge overcompensation at the terminating surface due to the different chain stiffness.[27] Thus, it is expected that these features will also influence the protein adsorption on the PEMs interface. Here, we extend this investigation to the adsorption of bovine serum albumin (BSA) using optical reflectometry (OR) and quartz crystal microbalance with dissipation (QCM-D) on the surfaces of those preformed four types of PEMs and in order to understand how these changes influence protein adsorption.

II Materials and methods

Materials

Two anionic and two cationic polyelectrolytes were chosen based on their flexibility as characterized by the chain persistence length. PSS ($M_w \sim 1.0 \times 10^6$ g·mol^{-1}) and PDDA ($M_w \sim 4.5 \times 10^5$ g·mol^{-1}), were sourced from Sigma-Aldrich and used as received. Details about the purification of CGG ($M_w \sim 1.5 \times 10^6$ g·mol^{-1}) and the preparation of SC ($M_w \sim 6.2 \times 10^4$ g·mol^{-1}) have been described previously.[27] Poly(ethyleneimine) (PEI, $M_w \sim 2.5 \times 10^4$ g·mol^{-1}) and BSA ($\geqslant$98%, $M_w \sim 6.8 \times 10^4$ Da, pI ~ 4.8) were purchased from Sigma-Aldrich and used as received. Sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), and potassium phosphate (KH_2PO_4) were all AR grade and were used as received. The water used was purified by filtration through a Millipore Gradient system after distillation, giving a resistivity of 18.2 MΩ·cm. Phosphate buffered saline (PBS, 0.14 M, pH 7.4) solution was prepared using NaCl, KCl, Na_2HPO_4, and KH_2PO_4.

Preparation of Polyelectrolyte Multilayers

Our previous study showed that the structure of polyelectrolyte multilayer is significantly influenced by ionic strength in a complex manner and by the specific nature of the ions.[27] In the present study, BSA dissolves in PBS buffer which has the ionic strength of 0.14 M similar to that of the physiological environment. We chose the ionic strength of 0.1 M to construct the PEMs because this ionic strength is close to that of the PBS buffer. Thus, the addition of BSA solution to the multilayer during protein adsorption will not generate a significant disturbance to the structure of the PEMs. If the PEMs are constructed under much higher or lower ionic strengths than 0.14 M, the addition of BSA solution will change the structure of PEMs dramatically, which will be disadvantageous for the investigation of protein adsorption. Therefore, all the PEMs in this study were constructed at an ionic strength of 0.1 M. The detailed procedure for preparing PEMs is as follows.

Pre-cleaned substrates were immersed in PEI solution (1.0 mg·mL^{-1}) for ~ 20 min and then rinsed with water to produce a uniform positively charged coating which is known to minimize the influence of the substrate on the growth of the multilayers.[29] Multilayers were grown by alternately depositing first a polyanion and then a polycation onto the surface by immersing the substrate in a 0.1 M NaCl solution containing 0.1 mg·mL^{-1} of the polyanion or polycation for 20 min. Three rinses using 0.1 M NaCl solution were used between depositions to avoid the formation of loose polyelectrolyte complexes on the surface. The process was repeated to produce a polyelectrolyte multilayer (PEM) with the desired number of layers. We use the following abbreviations to describe the PEMs produced. When the PEM is terminated by a polycation and *n* bilayers are formed, we use $(\text{polyanion/polycation})_n$ to represent the PEM. Here the total number of layers is 2*n*. When the PEM is terminated by a polyanion the multilayer is denoted as $(\text{polyanion/polycation})_n$polyanion and the total number of layers is 2*n*+1.

Optical Reflectometry

The dry mass of BSA adsorbed on the PEMs was measured using a custom built, fixed-angle optical reflectometer.[30] The term 'dry mass' is used to distinguish it from measurements with QCM-D, which includes the mass of water associated with

the protein. OR relies upon the change in reflective properties of a substrate that occur upon adsorption of the species of interest.[31,32] To convert changes in optical properties to surface excess, an optical model is required to calculate the sensitivity parameter which is used to convert changes in the polarization of reflected light to a measure of surface excess (Γ).[33] In a typical reflectometry experiment, the cell initially contains only PBS buffer while a stable baseline is recorded for the PEM coated surface. 1.0 mg·mL^{-1} BSA in PBS buffer solution was then injected into the cell via a two-way valve and the change in the ratio of the two perpendicular polarizations of the laser beam was recorded which is proportional to the surface excess, Γ

$$\Gamma = \frac{\Delta S}{S_0 A_s} \quad (1)$$

where S_0 is the baseline ratio of the reflectivities of the *p* and *s* components, i.e. $S_0 = R_p/R_s$ (usually ~ 1), $\Delta S = S\text{-}S_0$ is the change in the polarization, and A_s is the sensitivity parameter. The sensitivity parameter (A_s) was determined from a five-layer Fresnel optical model by taking the refractive indices of PBS buffer, silica, and silicon as 1.34, 1.46, and 3.80, respectively, and by using a *dn/dc* value of 0.19 cm^3·g^{-1} for the BSA/PBS buffer solution.[34] The bulk solution and silicon substrate were treated as semi-infinite. Here, silicon wafers (50 mm × 10 mm) with a well-defined 320 nm oxide layer were employed in the OR experiments (Silicon Valley Microelectronics, CA). All the OR measurements were conducted at 25 ± 0.2 °C.

QCM-D Measurements

QCM-D (Q-sense E1) and the AT-cut quartz crystals were from Q-sense AB.[35] The quartz crystal resonator with a fundamental resonance frequency of 5 MHz was mounted in a fluid cell with one side exposed to the solution. The resonator has a mass sensitivity constant (C) of 17.7 ng·cm^{-2}·Hz^{-1}.[36] When a quartz crystal is excited to oscillate in the thickness shear mode at its fundamental resonance frequency (f_0) by applying a RF voltage across the electrodes near the resonance frequency, a small layer added to the electrodes induces a decrease in resonance frequency (Δf) which is proportional to the mass change (Δm) of the layer. In vacuum or air, if the added layer is rigid, evenly distributed and much thinner than the crystal, Δf is related to Δm and the overtone number (n = 1, 3, 5….) by the Sauerbrey equation[37]

$$\Delta m = -\frac{\rho_q l_q}{f_0}\frac{\Delta f}{n} = -C\frac{\Delta f}{n} \quad (2)$$

where f_0 is the fundamental frequency, ρ_q and l_q are the specific density and thickness of the quartz crystal, respectively. The dissipation factor is defined by[35]

$$D = \frac{E_d}{2\pi E_s} \quad (3)$$

where E_d is the energy dissipated during one oscillation and E_s is the energy stored in the oscillating system. The measurement of ΔD is based on the fact that the voltage over the crystal decays exponentially as a damped sinusoidal wave when the driving power of a piezoelectric oscillator is switched off.[35] By switching the driving voltage on and off periodically, a series of changes of the resonance frequency and the dissipation factor are obtained. In the protein adsorption measurements, the PBS buffer was used to establish the baseline and the concentration of BSA was fixed at 1.0 mg·mL^{-1}. The Sauerbrey mass of the adsorbed protein was calculated from the change in Δf at the third overtone (n = 3). As the Sauerbrey equation might not be valid for a viscoelastic protein layer, the hydrodynamic mass (M_h) was obtained by fitting the changes of Δf and ΔD at n = 3, 5, and 7 on the basis of the Voigt model using Q-tools software from Q-sense AB.[38] All the QCM-D experiments were performed at 25 ± 0.02 ºC. It is important to recognize that QCM-D gives an effective wet mass of the film, which includes a considerable contribution from coupled water.[39] Therefore OR and QCM-D are complementary techniques. The OR provides quantitative measures of adsorption and the QCM-D data provides a measure of the conformation of the adsorbed layer.[39]

III Results

Polyelectrolyte multilayers were produced using polyelectrolytes of different chain stiffness as indicated by the chain persistence length. The important properties of these polyelectrolytes are summarized in Table 1. The PSS and PDDA are termed flexible because of their short intrinsic chain persistence lengths. SC and CGG are stiffer and are called semi-flexible because of their longer intrinsic chain persistence lengths.

Protein adsorption to PEMs consisting of semi-flexible polyelectrolytes

Optical reflectometry gives a direct measure of protein adsorption in real time, allowing the protein adsorption process to be followed. Protein adsorption onto PEMs consisting of the relatively stiff (semi-flexible) polyelectrolytes SC and CGG are shown in Fig. 1 for anionic terminated and cationic terminated PEMs. A baseline is obtained in the first 20 minutes before the BSA is introduced. After a further 30 minutes, buffer without BSA is introduced and the loosely bound BSA is removed from the surface. The surface excess obtained corresponds to the amount of protein that wasn't readily rinsed from the surface. For both the anionic and cationic terminated PEM the surface excess was found to be ~ 0.08 mg·m^{-2}.

QCM-D was also employed to follow protein adsorption. The QCM-D data is complementary to the OR data as the OR data reflects the amount of protein adsorbed whilst the QCM-D data combines the mass of the adsorbing protein along with any change in the amount of coupled water. The change in frequency and dissipation that accompanies the adsorption of BSA on the $(SC/CGG)_4SC$ and $(SC/CGG)_5$ surface is shown in Fig. 2.

Protein adsorption on PEMs was studied as a function of layer number by both OR and QCM-D. The results of these experiments are summarized in Fig. 3 for anionic and cationic terminated PEMs. Both Γ_{eq} from OR and M_h from QCM-D decrease as the layer number increases from 1 to 5 and then remain constant with increasing layer number.

Protein adsorption to PEMs consisting of flexible polyelectrolytes

Protein adsorption onto PEMs consisting of the flexible polyelectrolytes PSS and PDDA are shown in Fig. 4 for anionic terminated and cationic terminated PEMs, measured using OR. A baseline is obtained in the buffer solution before introduction of BSA (in buffer) at approximately 20 minutes. The protein adsorbs over a substantial period of time before buffer alone is introduced into the cell and loosely bound BSA is removed from the surface. The surface excess of BSA on the anionic terminated PEM saturates at 5.8 mg·m^{-2} before rinsing decreases it to 4.8 mg·m^{-2} after rinsing. The surface excess of BSA on the cationic terminated PEM saturates at 1.0 mg·m^{-2} before rinsing decreases it to 0.8 mg·m^{-2} after rinsing.

Table 1. Polymers used to form polyelectrolyte multilayers in this study

Polyelectrolyte	Abbrev.	Molecular Weight (g·mol^{-1})	Charge	Intrinsic Chain Persistence Length (nm)
Poly(sodium 4-styrenesulfonate)	PSS	~ 1.0×10^6	Anionic	~ 0.9[40]
Poly(diallyldimethylammonium chloride)	PDDA	~ 4.5×10^5	Cationic	~ 2.7[41]
Sulfated Chitosan	SC	~ 6.2×10^4	Anionic	~ 10.0[42]
Cationic Guar Gum	CGG	~ 1.5×10^6	Cationic	~ 10.0[43]

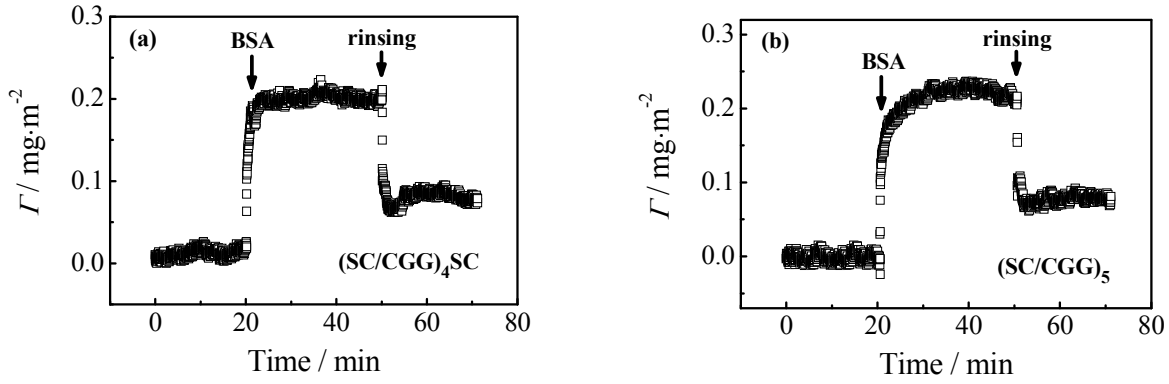


Fig. 1 Surface excess (Γ) of BSA as a function of time for the adsorption of BSA on the SC/CGG polyelectrolyte multilayer surface measured using OR. For $(SC/CGG)_4SC$, panel (a) and $(SC/CGG)_5$, panel (b); PEMs.

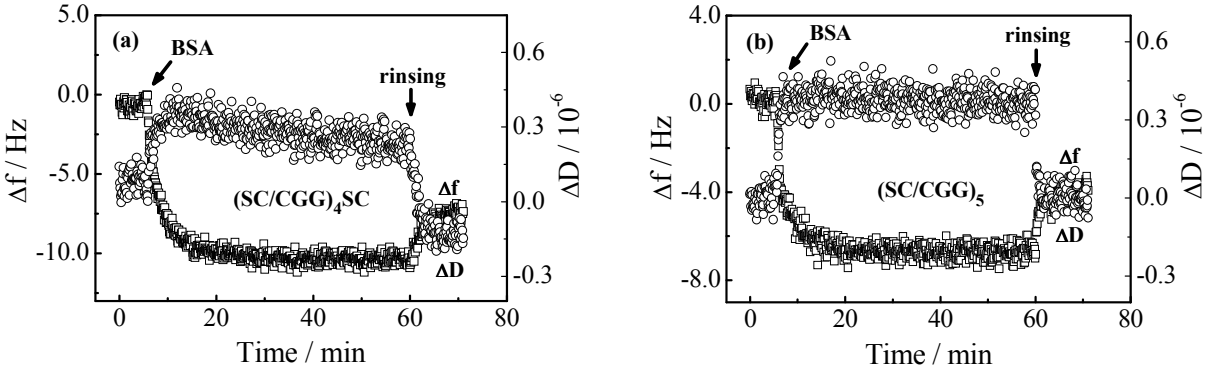


Fig. 2 Changes in frequency (Δf) and dissipation (ΔD) as a function of time for the adsorption of BSA on the SC/CGG polyelectrolyte multilayer surface measured using QCM-D, where the overtone number (n) is 3, for $(SC/CGG)_4SC$, panel (a); and $(SC/CGG)_5$, panel (b); PEMs.

Protein adsorption onto PEMs consisting of the flexible polyelectrolytes PSS and PDDA were also studied using QCM-D and the results are shown in Fig. 5. As with OR, both anionic terminated and cationic terminated PEMs were investigated. BSA adsorption on the anionic terminated surface decreased the resonance frequency by 176 Hz before rinsing and 138 Hz after

rinsing, which corresponds to a Sauerbrey mass of 8.1 mg·m^{-2} (Fig. 5a). Similarly, BSA adsorption increased the dissipation by 4.0 × 10^{-6}. BSA adsorption on the cationic terminated surface decreased the resonance frequency by 16 Hz before rinsing and 14 Hz after rinsing, which corresponds to a Sauerbrey mass of 0.8 mg·m^{-2}. This was accompanied by a small change in dissipation.

Protein adsorption on PEMs consisting of the flexible polyelectrolytes was studied as a function of layer number by both OR and QCM-D. The results of these experiments for the flexible polyelectrolyte system are summarized in Fig. 6 for anionic and cationic terminated PEMs. For the anionic terminated PEM, both Γ_{eq} from OR and M_h from QCM-D are unaffected by the number of layers in the PEM. In contrast, for the cationic terminated PEM, both Γ_{eq} from OR and M_h from QCM-D decrease as the layer number increases up to 6 layers, and then remain constant with increasing layer number.

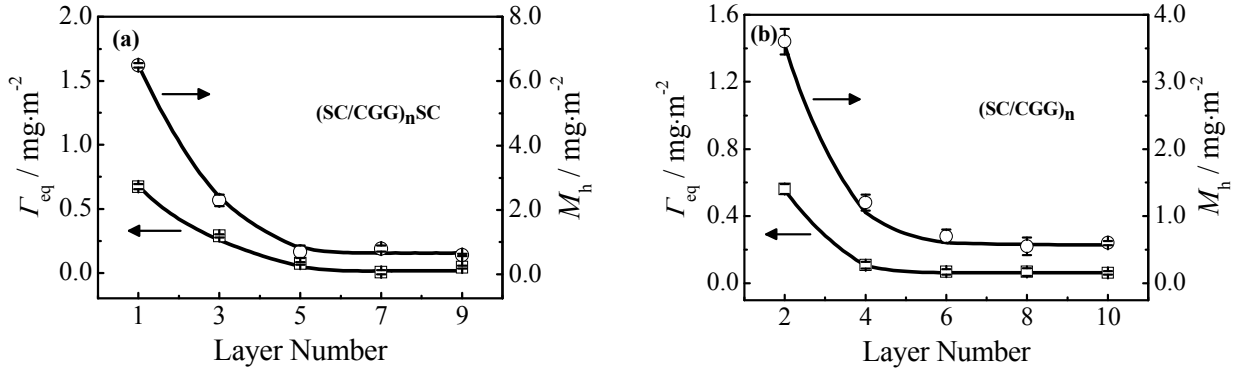


Fig. 3 Surface excess (Γ_{eq}) from OR and hydrodynamic mass (M_h) from QCM-D as a function of layer number for the adsorption of BSA on the SC/CGG polyelectrolyte multilayer surface. For $(SC/CGG)_nSC$, panel (a); and $(SC/CGG)_n$, panel (b); PEMs. Lines are provided to guide the eye.

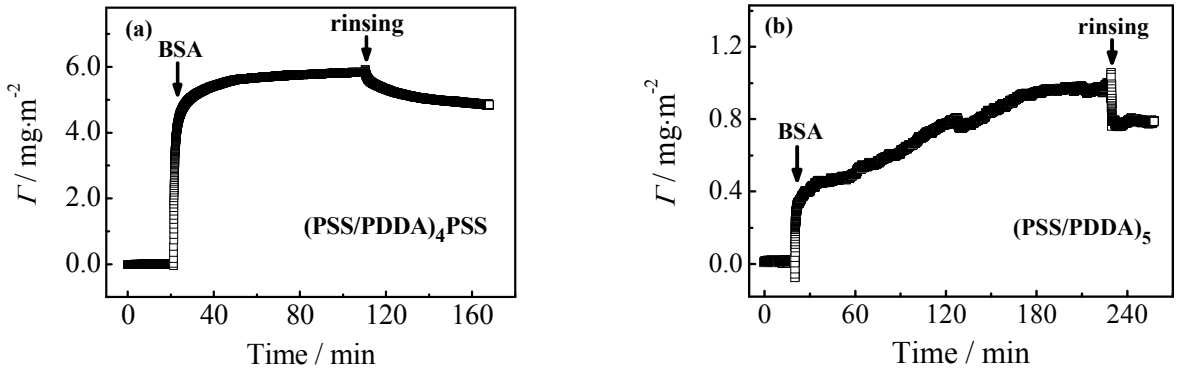


Fig. 4 Change in surface excess (Γ) as a function of time for the adsorption of BSA on the PSS/PDDA polyelectrolyte multilayer surface. For $(PSS/PDDA)_4PSS$, panel (a) and $(PSS/PDDA)_5$, panel (b); PEMs.

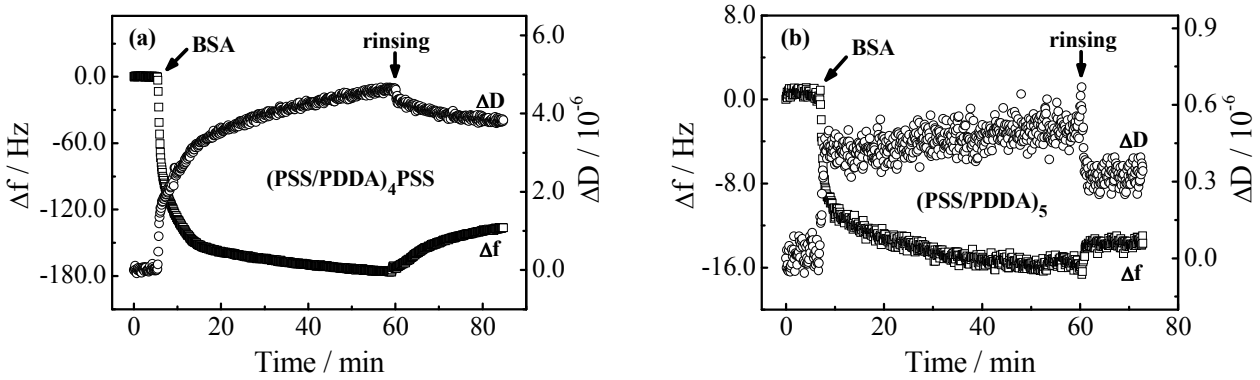


Fig. 5 Changes in frequency (Δf) and dissipation (ΔD) as a function of time for the adsorption of BSA on the PSS/PDDA polyelectrolyte multilayer surface, where the overtone number (n) is 3, for $(PSS/PDDA)_4PSS$, panel (a) and $(PSS/PDDA)_5$, panel (b); PEMs.

Protein adsorption to PEMs consisting of flexible and semi-flexible polyelectrolytes

The polyelectrolyte multilayers described above were formed from polyelectrolytes chains that were either both semi-flexible or both flexible. We now concentrate on two different PEMs that are formed from flexible and semi-flexible polyelectrolyte chains. The SC/PDDA system combines a semi-flexible anionic polyelectrolyte with a flexible cationic polyelectrolyte, whereas the PSS/CGG system combines a flexible anionic polyelectrolyte with a semi-flexible cationic polyelectrolyte.

First we consider the PEM where the cationic polyelectrolyte is flexible — the SC/PDDA system, see Fig. 7, panels (a) and (b). The surface excess of adsorbed protein after rinsing when the PEM was terminated with a cationic chain was 0.32 mg·m^{-2}. When it was terminated with an anionic chain, a surface excess after rinsing of 0.25 mg·m^{-2} was obtained.

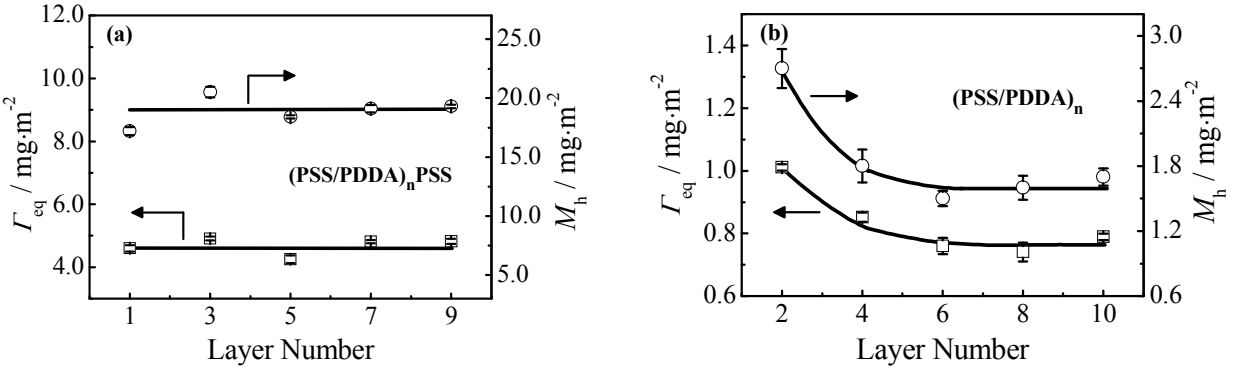


Fig. 6 Changes in surface excess (Γ_{eq}) and hydrodynamic mass (M_h) as a function of layer number for the adsorption of BSA on the PSS/PDDA polyelectrolyte multilayer surface. For $(PSS/PDDA)_nPSS$, panel (a) and $(PSS/PDDA)_n$, panel (b); PEMs. Lines are provided to guide the eye.

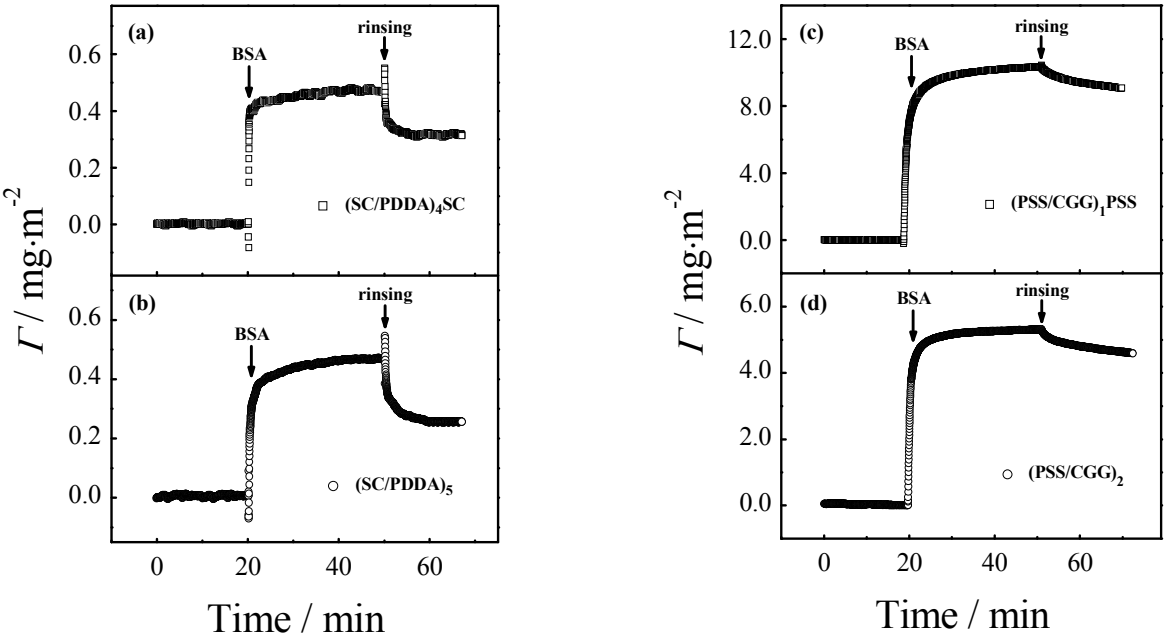


Fig. 7 Change in surface excess (Γ) as a function of time for the adsorption of BSA on the SC/PDDA and PSS/CGG polyelectrolyte multilayer surfaces. For $(SC/PDDA)_4SC$, panel (a); $(SC/PDDA)_5$, panel (b); $(PSS/CGG)_1PSS$, panel (c) and $(PSS/CGG)_2$, panel (d); PEMs.

We now consider the PEM where the anionic polyelectrolyte is flexible — the PSS/CGG system, see Fig. 7, panels (c) and (d). We found that the dissipation during the formation of the PSS/CGG multilayer system dramatically increases with increasing layer number, indicating that the PSS/CGG PEM is highly swollen with water for films of 5 layers or more (Fig. S1, ESI†). OR is not suitable for studying adsorption to very highly swollen films as the requirement that the optical arrangement return a sensitivity parameter that is independent of adsorbed mass is violated.[44] Therefore, the investigation of protein adsorption was conducted on the $(PSS/CGG)_1PSS$ surface instead of the $(PSS/CGG)_4PSS$ surface as for this thinner PEM it is appropriate to use the OR to measure protein adsorption. BSA adsorption on the anionic terminated PEM

produced a very large surface excess of 9.1 mg·m^{-2} after rinsing, as measured by OR. BSA adsorption on the cationic terminated PEM resulted in a large surface excess of 4.6 mg·m^{-2} after rinsing.

QCM-D was also employed to study protein adsorption onto PEMs formed from flexible and semi-flexible polyelectrolyte chains (Fig. 8). As with OR, both anionic terminated and cationic terminated PEMs were investigated. First we consider the PEM with the flexible cationic polyelectrolyte — the SC/PDDA system, see Fig. 8, panels (a) and (b). The frequency change after rinsing due to adsorbed protein when the PEM was terminated with either an anionic or cationic chain was 33 Hz, which corresponds to a Sauerbrey mass of 2.0 mg·m^{-2}. We now consider the PEM with the flexible anionic polyelectrolyte — the PSS/CGG system, see Fig. 8, panels (c) and (d). The frequency change after rinsing due to adsorbed protein when the PEM was terminated with an anionic chain was -400 Hz, which corresponds to a Sauerbrey mass of 23.6 mg·m^{-2}. In comparison the frequency change after rinsing due to adsorbed protein when the PEM was terminated with a cationic chain was only -75 Hz, which corresponds to a Sauerbrey mass of 4.4 mg·m^{-2}.

Protein adsorption on PEMs was also studied as a function of layer number by both OR and QCM-D for the SC/PDDA system. This data is shown in Fig. 9. For both the anionic and the cationic terminated PEM, both Γ_{eq} from OR and M_h from QCM-D decreased as the layer number increased up to 6 layers, and then remained constant with increasing layer number. Note that the layer number dependence of BSA adsorption to the PSS/CGG PEMs is not shown due to the inability of the OR and QCM-D techniques to reliably quantify protein adsorption onto very highly swollen PEMs.

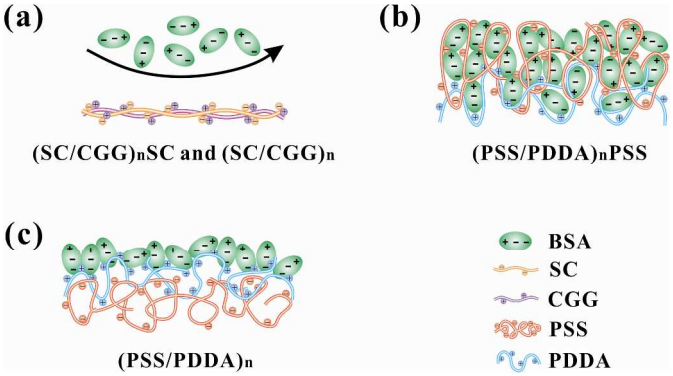
IV Discussion

Here we use BSA as a model protein to study protein adsorption onto PEMs. BSA has a surface that is composed of both negatively and positively charged regions, though the net charge of BSA in PBS buffer is negative.[17,21] BSA is an oblate spheroid and therefore a close packed monolayer adsorption of BSA on a surface is dependent upon the orientation in which it adsorbs. The formation of a monolayer of BSA molecules with a side-on configuration corresponds to a surface excess of 0.6 mg·m^{-2} and with an end-on configuration on the surface, a surface excess of 1.5 mg·m^{-2}.[17] Thus surface excesses of BSA exceeding 1.5 mg·m^{-2} indicate an adsorption process that results in adsorption exceeding that of a monolayer, regardless of the configuration.

Many features of the polymers that make up the PEM can contribute to the level of protein adsorption, including the hydration of the polymer, molecular weight, specific chemical interactions and electrical charge.[18,21,22,45] We wish to consider how the relative conformation of the two polymers that form the PEM influences the level of protein adsorption and how this might be employed to enhance or reduce protein adsorption. As we cannot hope to do enough experiments to control all the other factors we have primarily concerned ourselves with how protein adsorption changes upon changing the pairing of the polymers.

The conformation of a polyelectrolyte chain is influenced by both the intrinsic chain persistence length and the electrostatic chain persistence length.[46,47] PBS buffer used in the present study has an ionic strength of 0.14 M, which gives rise to a Debye length of ~ 0.8 nm.[48] Thus, in this study, the intrinsic chain persistence length determines the stiffness of the polymer chains. The intrinsic chain persistence length of PSS is ~ 0.9 nm.[40] Therefore, the PSS chains are expected to form numerous loops and tails at the PEM interface, especially for the large molecular weight PSS sample employed here. The intrinsic chain persistence length of PDDA is ~ 2.7 nm, which is longer than that of PSS.[41] Therefore, it is expected that the PDDA-terminated multilayer surface would contain a significant number of loops and tails, but fewer than for PSS.

In contrast SC and CGG are considered semi-flexible polyelectrolytes because of an intrinsic chain persistence length of ~ 10 nm.[42,43] Therefore, the SC and CGG chains on the terminating surface of the PEM are expected to lie relatively flat on the multilayer surface. Considering the conformation of the chains and the measurements of protein adsorption reported above, we can propose a description of the protein adsorption mechanism for the PEMs studied. We summarize this in Scheme 1 and discuss the details below.



Scheme 1. Schematic illustration of BSA adsorption on the different types of polyelectrolyte multilayers studied here. (a) Protein resistance can be achieved on the SC/CGG multilayer surface due to the semi-flexible nature of the polyelectrolyte chains. (b) A multilayered structure of adsorbed protein molecules is formed on the $(PSS/PDDA)_nPSS$ surface due to the high flexibility of the PSS terminating layer. (c) A monolayered structure of adsorbed protein molecules is formed on the $(PSS/PDDA)_n$ surface.

Protein adsorption to PEMs consisting of two semi-flexible polyelectrolytes

BSA adsorbs at very low levels on both anionic and cationic terminated SC/CGG PEMs (see Fig. 3). Both Γ_{eq} and M_h decrease as the layer number increases up to 5 and then remain constant. The decrease in the adsorbed amount of protein

up to 5 layers is attributed to the influence of the PEI layer. The multilayer growth proceeds in a patchy growth regime for the initial layers and we see that for these semi-flexible polyelectrolytes several layers are required to fully cover the substrate.[21] Therefore the effect of PEI on the protein adsorption is gradually weakened as the layer number increases to 5 and then disappears. Importantly, Γ_{eq} approaches zero when the layer number exceeds 5. Note that both SC and CGG polymer layers adsorb protein when they alone make up the surface.[49,50] The conclusion is that a sufficiently thick PEM consisting of the two semi-flexible polyelectrolytes SC and CGG exhibits protein resistance properties regardless of which layer is used to terminate the PEM and that 5 or more layers is sufficient to completely cover the substrate and produce a protein resistant film.

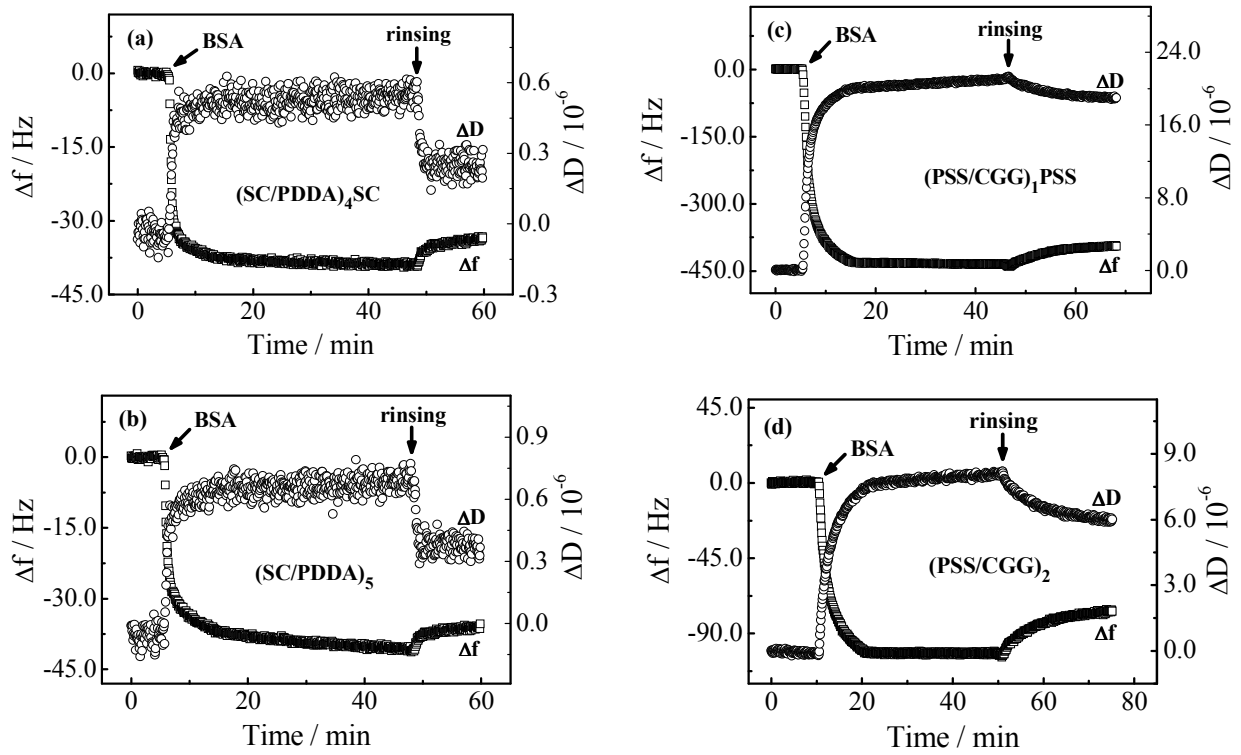


Fig. 8 Changes in frequency (Δf) and dissipation (ΔD) as a function of time for the adsorption of BSA on the SC/PDDA and PSS/CGG polyelectrolyte multilayer surfaces, where the overtone number (n) is 3, for $(SC/PDDA)_4SC$, panel (a); $(SC/PDDA)_5$, panel (b); $(PSS/CGG)_1PSS$, panel (c) and $(PSS/CGG)_2$, panel (d); PEMs.

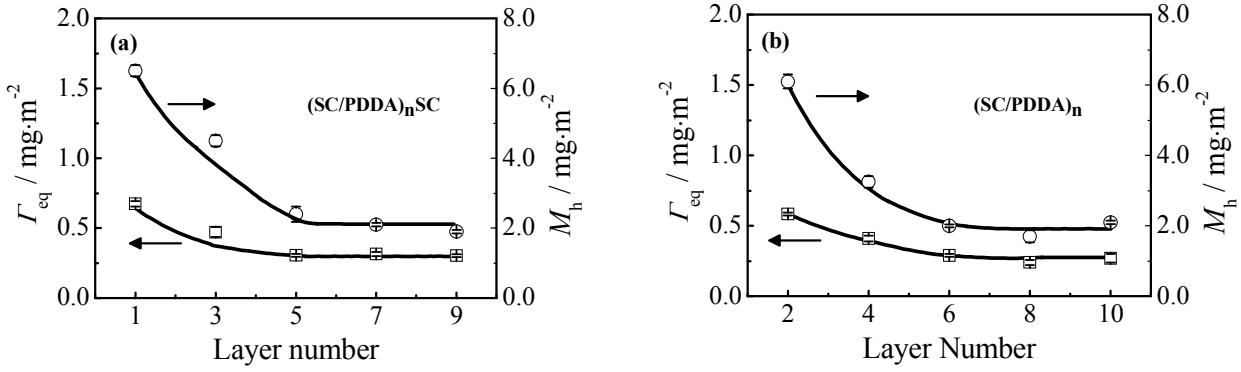


Fig. 9 Changes in surface excess (Γ_{eq}) and hydrodynamic mass (M_h) as a function of layer number for the adsorption of BSA on the SC/PDDA polyelectrolyte multilayer surface. For $(SC/PDDA)_nSC$, panel (a) and $(SC/PDDA)_n$, panel (b); PEMs. Lines are provided to guide the eye.

To investigate the origin of the protein resistant properties of the SC/CGG system we measured the Zeta potential of the surface. The Zeta potential of SC/CGG PEM is close to zero when the layer number is larger than 4 regardless which layer is used to terminate the PEM. (Fig. S2, ESI†). Thus the SC/CGG PEM surface is almost neutral.[27] This is because the interpenetration of polyelectrolyte chains causes the underlying oppositely charged chains to be present in the outermost surface

of the PEM. Thus, the outermost region of the PEM consists of both negatively and positively charged polyelectrolyte chains.[25, 26] Furthermore, the flat interfacial chain conformation of SC and CGG results in a high level of intrinsic charge compensation, a low level of charge overcompensation, and a near neutral surface. Such a surface is similar to a polyzwitterion covered surface, which is known to have protein resistance properties.[14,15] For these reasons the semi-flexible nature of SC and CGG are important in producing a PEM that exhibits good protein resistance. This is depicted in Scheme 1a. Actually, the SC/CGG multilayer film can also resist the adsorption of positively charged protein, e.g., lysozyme (Fig. S3, ESI †). Our previous work demonstrated that the polyzwitterionic brushes (i.e., poly(sulfobetaine methacrylate)) resist the adsorption of BSA at the ionic strength of 0.14 M with ~ 0.17 mg·m^{-2} (Sauerbrey mass) of protein adsorbed.[15] In the present work, the SC/CGG multilayer can also inhibit the BSA adsorption at the ionic strength of 0.14 M with ~ 0.25 mg·m^{-2} (Sauerbrey mass) of protein adsorbed. That is, the SC/CGG multilayer has a similar level of protein resistance with that of the polyzwitterionic brushes.

Protein adsorption to PEMs consisting of two flexible polyelectrolytes

The terminating layer strongly influences protein adsorption on PSS/PDDA PEMs, as shown in Fig. 6. The anionic terminated PEM acquires a large amount of protein that exceeds monolayer coverage by a factor of ~ 3 (Fig. 6a). Also the surface excess is independent of the number of layers in the PEM. The finding that BSA adsorbs more strongly on the anionic terminated surface is counterintuitive as the negatively charged BSA molecules are electrostatically attracted by PDDA chains but are electrostatically repelled by PSS chains. Also the surface excess implies that more than a monolayer of BSA is formed on the anionic terminated surface whereas the BSA forms a monolayer with a mixed side-on/end-on configuration on the cationic terminated surface. So what is the likely surface configuration on the anionic terminated PEM?

The very high surface excess suggests that BSA may be penetrating into the PEM. Penetration has been demonstrated in previous studies when the protein is adsorbing to an oppositely charged multilayer surface.[17,18] In this case protein adsorption increases with the number of layers in the PEM.[18] However, for (PSS/PDDA)$_n$PSS both Γ_{eq} and M_h are independent of the layer number and therefore this is not an appropriate explanation for our system. Therefore, we propose that the adsorbed BSA is encapsulated by the flexible PSS chains that protrude from the surface of the PEM as depicted in Scheme 1b. This process is assisted by electrostatic interactions between the positively charged BSA patches and the PSS chains; and between the negatively charged BSA patches and the PDDA chains. Such a configuration will promote extensive protein adsorption, leading to the formation of a multilayered structure of adsorbed protein molecules consistent with the measured surface excess. Moreover, the trapped BSA molecules should desorb slowly from the multilayer surface upon rinsing with PBS buffer, which is consistent with the observations in Figs. 4 and 5. As this type of protein adsorption is mainly determined by the interfacial chain conformation of the PSS chains, the BSA adsorption should be independent of the layer number, as observed in Fig. 6a. It is known that large dissipation factors are indicative of the formation of swollen protein layers.[51] Thus, the large increase in ΔD induced by the BSA adsorption on the anionic terminated PEM indicates that the adsorbed BSA molecules form a swollen polymer-protein structure (Fig. 5a).

On the cationic terminated PEM both Γ_{eq} and M_h decrease as the layer number is increased up to 6 layers and then remain constant with increasing layer number. As with the SC/CGG system described above, we attribute this to the influence of the exposed PEI layer as the multilayer growth proceeds in a patchy growth regime for the initial layers.[21] As the layer number exceeds 6, the adsorbed PDDA chains are able to cover the entire surface and protein adsorption is only related to the PDDA-BSA interactions. As a result, the surface excess of BSA is independent of the layer number, provided the layer number is 6 or greater. BSA will directly adsorb onto the PDDA surface by electrostatic attraction forming a monolayer. Consequently, the adsorbed amount of BSA on the (PSS/PDDA)$_n$ surface is less than that on the (PSS/PDDA)$_n$PSS surface. The rapid desorption of adsorbed BSA molecules from the (PSS/PDDA)$_5$ surface upon rinsing with PBS buffer (Figs. 4 and 5), is consistent with monolayer adsorption (Scheme 1c).

The small increase in ΔD (~ 0.5 × 10^{-6}) induced by the protein adsorption on the (PSS/PDDA)$_5$ surface, is consistent with the formation of a stiff monolayer on the surface. We conclude that protein adsorption onto PEMs is promoted by using flexible polyelectrolytes, which is also true for the positively charged protein, lysozyme (Fig. S3, ESI†). We have demonstrated that PEM's consisting of stiff chains inhibit and PEM's with flexible chains promote protein adsorption for both negatively and positively charged proteins. We therefore believe that the hypothesis proposed in this study may be generally applied to other proteins.

Protein adsorption to PEMs consisting of flexible and semi-flexible polyelectrolytes

We have seen above that protein adsorption on the (PSS/PDDA)$_n$PSS surface is dominated by the interfacial chain conformation of PSS, whereas protein adsorption on the (PSS/PDDA)$_n$ surface is governed by electrostatic interactions between BSA and PDDA. On the other hand, the PEM resists protein adsorption when it is composed of semi-flexible SC and CGG polyelectrolytes. As protein adsorption on PEMs is strongly influenced by the interfacial chain conformation, it is informative to examine the effect of combinations of flexible and semi-flexible polyelectrolytes on protein adsorption.

First we consider the PEM where the cationic polyelectrolyte is flexible — the SC/PDDA system. On both the anionic and the cationic terminated PEMs, both Γ_{eq} and M_h decrease as the layer number is increased up to 5 layers and then remain

constant (Fig. 9). As with the other systems that exhibit this behavior (see Fig. 3 and Fig. 6b) we attribute this to the influence of the PEI layer, which remains partially exposed when few layers of polyelectrolyte are employed. We therefore consider the SC/PDDA PEMs with 5 layers or more as shown in Fig. 9. This shows that BSA adsorbs to both cationic and anionic terminated SC/PDDA PEMs at sub-monolayer coverages (~ 0.3 mg·m^{-2}). Since BSA is expected to be electrostatically repelled by the negatively charged SC chains, protein adsorption on the $(SC/PDDA)_4SC$ surface may be driven by the hydrogen bonding interactions between SC and BSA as numerous hydrogen bond acceptors and donors exist on SC and BSA surfaces.[42,52,53] We note that both $(SC/PDDA)_4SC$ and $(SC/PDDA)_5$ surfaces have lost the protein resistance properties exhibited by the $(SC/CGG)_4SC$ and $(SC/CGG)_5$ surfaces (Fig. 3). We attribute this to the formation of loops of PDDA in the outer layer of the PEM, due to the flexibility of the PDDA chains.

We now consider the PEM where the anionic polyelectrolyte is flexible — the PSS/CGG system (Fig. 7c and 7d). On both the anionic and the cationic terminated PEMs the protein adsorption is considerable and far exceeds monolayer coverage, suggesting that a multilayered structure of adsorbed protein molecules is formed on the surface, as found for the $(PSS/PDDA)_n$PSS PEM. We attribute the large surface excess of BSA on the $(PSS/CGG)_1$PSS surface to the encapsulation of the protein molecules by the flexible PSS chains. This process is assisted by electrostatic interactions between the positively charged BSA patches and the PSS chains; and between the negatively charged BSA patches and the CGG chains. We note here that due to the very swollen nature of the PEMs formed we were unable to study the systems with more than 4 layers by OR, so we cannot be sure that the BSA is not penetrating into the PEM.

A large, but lesser, amount of protein (Γ_{eq} 4.6 mg·m^{-2}) is also observed on the $(PSS/CGG)_2$ surface, indicating that a multilayered structure of adsorbed BSA molecules is also formed on this surface. The amount of protein on the $(PSS/CGG)_2$ surface is higher than that on the $(SC/CGG)_2$ surface (Fig. 3b), suggesting that the adsorption of BSA on the $(PSS/CGG)_2$ surface is enhanced by the encapsulation of BSA molecules by PSS chains, despite the PSS not being the terminating layer. This interpretation is reasonable because the flexible PSS chains can penetrate the terminating CGG layer and form loops and tails beyond the CGG surface. That is PSS chains are in the outermost surface of the film despite the fact that the CGG is the terminating layer, and they therefore are able to encapsulate protein molecules. The relative stiffness of the cationic CGG chains prevents them from pairing with the anionic PSS loops. We can conclude that PEMs formed from a combination of flexible and semi-flexible polyelectrolytes can be used to produce surfaces that have very different protein adsorbing properties. Therefore when control over the adsorption of proteins is desirable one should consider the influence of chain flexibility on protein adsorption when selecting polyelectrolytes.

As the techniques of OR and QCM-D are complementary we can compare the two measures of surface excess to extract additional information. In Table 2 we present the measured values of surface excess from the two techniques for thick PEM films. What is revealed is that when protein adsorbs to the PEMs, the mass measured by QCM-D increases by a greater amount than the mass measured by OR. The difference (M_h-Γ_{eq}) is the increase in the amount of coupled water associated with the protein adsorption. We can normalize this value by the increase in mass due to protein adsorption and obtain the ratio of the increase in mass due to coupled water to the increase in mass due to adsorbed protein [(M_h-Γ_{eq})/Γ_{eq}]. What is apparent is that when the anionic polymer is PSS this value is considerably lower than when the anionic polymer is SC. This reflects the strong interaction between the protein and PSS compared to SC. The stronger protein-polymer interaction will lead to a lower water mass being coupled into the QCM-D measurement for each protein molecule adsorbed.

Table 2. Comparison of change in mass measured by QCM-D and OR during protein adsorption

Multilayer (n≥5)	M_h (mg·m^{-2})	Γ_{eq} (mg·m^{-2})	M_h-Γ_{eq} (mg·m^{-2})	(M_h-Γ_{eq})/Γ_{eq}
$(PSS/PDDA)_n$PSS	18.8 ± 0.40	4.6 ± 0.20	14.2 ± 0.40	~ 3.1
$(SC/PDDA)_n$SC	2.1 ± 0.30	0.3 ± 0.01	1.8 ± 0.30	~ 6.0

V Conclusions

This study has shown that the stiffness of the polyelectrolyte chains used to produce polyelectrolyte multilayer films have a strong influence on protein adsorption. We confirm our hypothesis that PEMs consisting of relatively stiff chains will be less susceptible to fouling by protein adsorption for the systems studied here. This resistance to protein adsorption is due to the lack of loops in the terminating layer of the PEM and the resulting zwitterionic nature of the surface. Conversely, flexible polyelectrolytes, which promote the formation of loops in the terminating layer interact strongly with proteins and can be used to promote protein adsorption in a multilayered structure. Thus, protein adsorption on PEMs can be enhanced or reduced by tuning the interfacial chain conformation via the polyelectrolyte chain stiffness. Further work is required to demonstrate that this general concept is applicable to a wide range of systems.

Acknowledgements

The financial support of National Program on Key Basic Research Project (2012CB933800), the National Natural Science Foundation of China (21374110, 91127042, 21234003)

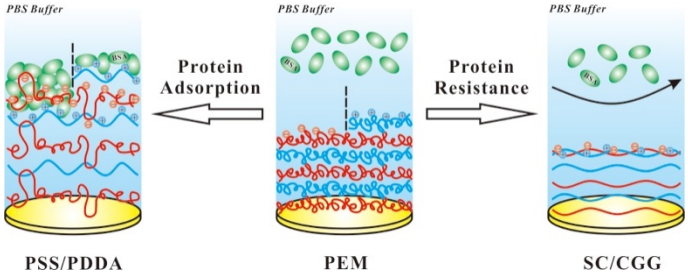
is acknowledged. V.S.J.C. gratefully acknowledges financial support from the Australian Research Council through a Future Fellowship (FT0991933). B. Wu also gratefully acknowledges the financial support from China Scholarship Council as a visiting Ph. D student at The Australian National University.

References

[a] *Hefei National Laboratory for Physical Sciences at the Microscale, Department of Chemical Physics, University of Science and Technology of China, Hefei, 230026, P. R. China.*
[b] *Department of Applied Mathematics, Research School of Physics and Engineering, The Australian National University, Canberra, ACT 0200, Australia.*
[c] *Faculty of Materials Science and Engineering, South China University of Technology, Guangzhou, 510640, P. R. China.*
*To whom correspondence should be addressed. E-mail: gml@ustc.edu.cn (G.M.L); vince.craig@anu.edu.au (V.S.J.C).
† Electronic Supplementary Information (ESI) available: The dissipation change for the growth of PSS/CGG multilayer, the surface Zeta potential of the SC/CGG multilayer, and the adsorption of lysozyme on the SC/CGG and PSS/PDDA polyelectrolyte multilayer surfaces. See DOI: 10.1039/b000000x/

1. G. L. Kenausis, J. Voros, D. L. Elbert, N. P. Huang, R. Hofer, L. Ruiz-Taylor, M. Textor, J. A. Hubbell and N. D. Spencer, *J. Phys. Chem. B*, 2000, **104**, 3298-3309.
2. T. Merian and J. M. Goddard, *J. Agr. Food Chem.*, 2012, **60**, 2943-2957.
3. R. Gref, M. Luck, P. Quellec, M. Marchand, E. Dellacherie, S. Harnisch, T. Blunk and R. H. Muller, *Colloid Surf. B-Biointerfaces*, 2000, **18**, 301-313.
4. M. Beyer, T. Felgenhauer, F. R. Bischoff, F. Breitling and V. Stadler, *Biomaterials*, 2006, **27**, 3505-3514.
5. R. R. Seigel, P. Harder, R. Dahint, M. Grunze, F. Josse, M. Mrksich and G. M. Whitesides, *Anal. Chem*, 1997, **69**, 3321-3328.
6. B. D. Ratner, A. S. Hoffman, F. J. Schoen and J. E. Lemons, *Biomaterials Science an Introduction to Materials in Medicine*, Elsevier, Boston, 2004.
7. D. Dell'Orco, M. Müller and K. W. Koch, *Chem. Commun.*, 2010, **46**, 7316-7318.
8. L. B. John and A. H. Thomas, *Proteins at Interfaces: an Overview*, American Chemical Society, 1995, vol. 602, ch. 1, pp. 1-23.
9. Y. H. An and R. J. Friedman, *J. Biomed. Mater. Res.*, 1998, **43**, 338-348.
10. G. Y. Zhou, C. F. Ma and G. Z. Zhang, *Polym. Chem.*, 2011, **2**, 1409-1414.
11. A. Rosenhahn, S. Schilp, H. J. Kreuzer and M. Grunze, *Phys. Chem. Chem. Phys.*, 2010, **12**, 4275-4286.
12. C. F. Ma, Y. Hou, S. Liu and G. Z. Zhang, *Langmuir*, 2009, **25**, 9467-9472.
13. C. F. Ma, H. Zhou, B. Wu and G. Z. Zhang, *ACS Appl. Mater. Interfaces*, 2011, **3**, 455-461.
14. S. Y. Jiang and Z. Q. Cao, *Adv. Mater.*, 2010, **22**, 920-932.
15. T. Wang, X. W. Wang, Y. C. Long, G. M. Liu and G. Z. Zhang, *Langmuir*, 2013, **29**, 6588-6596.
16. M. Müller, T. Rieser, P. L. Dubin and K. Lunkwitz, *Macromol. Rapid. Comm.*, 2001, **22**, 390-395.
17. G. Ladam, C. Gergely, B. Senger, G. Decher, J. C. Voegel, P. Schaaf and F. J. G. Cuisinier, *Biomacromolecules*, 2000, **1**, 674-687.
18. D. S. Salloum and J. B. Schlenoff, *Biomacromolecules*, 2004, **5**, 1089-1096.
19. G. Jackler, C. Czeslik, R. Steitz and C. A. Royer, *Phys. Rev. E*, 2005, **71**, 041912.
20. C. Gergely, S. Bahi, B. Szalontai, H. Flores, P. Schaaf, J. C. Voegel and F. J. G. Cuisinier, *Langmuir*, 2004, **20**, 5575-5582.
21. R. A. Silva, M. D. Urzua, D. F. S. Petri and P. L. Dubin, *Langmuir*, 2010, **26**, 14032-14038.
22. S. Y. Wong, L. Han, K. Timachova, J. Veselinovic, M. N. Hyder, C. Ortiz, A. M. Klibanov and P. T. Hammond, *Biomacromolecules*, 2012, **13**, 719-726.
23. R. Heuberger, G. Sukhorukov, J. Vörös, M. Textor and H. Möhwald, *Adv. Funct. Mater.*, 2005, **15**, 357-366.
24. B. Schoeler, G. Kumaraswamy and F. Caruso, *Macromolecules*, 2002, **35**, 889-897.
25. J. B. Schlenoff and S. T. Dubas, *Macromolecules*, 2001, **34**, 592-598.
26. J. B. Schlenoff, H. Ly and M. Li, *J. Am. Chem. Soc.*, 1998, **120**, 7626-7634.
27. B. Wu, C. L. Li, H. Y. Yang, G. M. Liu and G. Z. Zhang, *J. Phys. Chem. B*, 2012, **116**, 3106-3114.
28. G. M. Liu, S. R. Zou, L. Fu and G. Z. Zhang, *J. Phys. Chem. B*, 2008, **112**, 4167-4171.
29. E. Poptoshev, B. Schoeler and F. Caruso, *Langmuir*, 2004, **20**, 829-834.
30. S. C. Howard and V. S. J. Craig, *Soft Matter*, 2009, **5**, 3061-3069.
31. J. C. Dijt, M. A. C. Stuart and G. J. Fleer, *Colloids and surf.*, 1990, **51**, 141-158.
32. J. C. Dijt, M. A. C. Stuart and G. J. Fleer, *Adv. Colloid Interface Sci.*, 1994, **50**, 79-101.
33. R. M. Azzam and N. M. Bashara, *Ellipsometry and polarized light*, North-Holland. sole distributors for the USA and Canada, Elsevier Science Publishing Co., Inc., 1987.
34. J. Wen and T. Arakawa, *Anal. Biochem.*, 2000, **280**, 327-329.
35. M. Rodahl, F. Höök, A. Krozer, P. Brzezinski and B. Kasemo, *Rev. Sci. Instrum.*, 1995, **66**, 3924-3930.
36. N.-J. Cho, C. W. Frank, B. Kasemo and F. Höök, *Nat. Protoc.*, 2010, **5**, 1096-1106.
37. G. Z. Sauerbrey, *Z. Phys.* 1959, **155**, 206-222.
38. M. V. Voinova, M. Rodahl, M. Jonson and B. Kasemo, *Phys. Scr.*, 1999, **59**, 391-396.
39. V. S. J. Craig and M. Plunkett, *J. Colloid. Interface Sci.*, 2003, **262**, 126-129.
40. A. Brûlet, F. Boué and J. Cotton, *J. Phys. II*, 1996, **6**, 885-891.
41. K. W. Mattison, P. L. Dubin and I. J. Brittain, *J. Phys. Chem. B*, 1998, **102**, 3830-3836.
42. M. Rinaudo, *Macromol. Biosci.*, 2006, **6**, 590-610.
43. G. A. Morris, T. R. Patel, D. R. Picout, S. B. Ross-Murphy, A. Ortega, J. Garcia de la Torre and S. E. Harding, *Carbohyd. Polym.*, 2008, **72**, 356-360.
44. S. C. Howard, V. S. J. Craig, P. A. FitzGerald and E. J. Wanless, *Langmuir*, 2010, **26**, 14615-14623.
45. V. Gribova, R. Auzely-Velty and C. Picart, *Chem. Mater.*, 2012, **24**, 854-869.
46. A. V. Dobrynin, *Macromolecules*, 2005, **38**, 9304-9314.
47. M. Schönhoff, *J. Phys.: Condens. Matter.*, 2003, **15**, R1781-R1808.
48. R. A. McAloney, M. Sinyor, V. Dudnik and M. C. Goh, *Langmuir*, 2001, **17**, 6655-6663.
49. Y. P. Jiao, Z. H. Liu, X. H. Shao and C. R. Zhou, *J. Appl. Polym. Sci.*, 2012, **125**, E501-E510.
50. J. L. Doublier, C. Garnier, D. Renard and C. Sanchez, *Curr. Opin. Colloid Interface Sci.*, 2000, **5**, 202-214.
51. F. Höök, M. Rodahl, P. Brzezinski and B. Kasemo, *Langmuir*, 1998, **14**, 729-734.
52. X. W. Wang, G. M. Liu and G. Z. Zhang, *Langmuir*, 2012, **28**, 14642-14653.
53. E. A. Vogler, *Biomaterials*, 2012, **33**, 1201-1237.

Graphical abstract



The relative stiffness of polymers used to form polyelectrolyte multilayers can be used to control protein adsorption. Flexible chains promote polymer adsorption whereas inflexible chains can produce antifouling surfaces, even if the constituent chains have no antifouling properties on their own.