Effect of Protein Adsorption and Ionic Strength on the Equilibrium Partition Coefficient of Ionizable Macromolecules in Charged Nanopores

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Recent experimental data on the diffusive transport of globular protein molecules through nanopores ($D \sim 20$ nm), covered with chemisorbed self-assembled monolayers (SAMs) of carboxylic acid functional groups (Ku, J.-R.; Stroeve, P. *Langmuir* **2004**, *20*, 2030), show a strong increase in flux around the isoelectric point of the protein molecule at 10 mM ionic strength (on−off behavior), but not at 100 mM. To understand these observations we calculated the equilibrium partition coefficient (distribution coefficient) of a spherical protein molecule in a nanopore consisting of ionizable carboxylic acid groups as a function of ionic strength, pH, charge and size of the protein and the pore wall. When transport is diffusion-controlled, the flux of macromolecules through porous media is directly proportional to the equilibrium partition coefficient. To calculate the charge on the pore wall, the ionizable character of the carboxylic acid groups is incorporated as well as protein adsorption. At values sufficiently above the isoelectric point of the protein, pI, protein adsorption is zero. However, with decreasing pH, adsorption strongly increases. Therefore, the pore is negatively charged at sufficiently high pH (just as the protein molecule) and positively charged at sufficiently low pH (just as the protein molecule). Consequently, the equilibrium partition coefficient of additional, thus nonadsorbing, protein molecules is strongly pH-dependent with a maximum at pH around pI. The predicted influence of ionic strength on the peak width of the equilibrium partition coefficient is in qualitative agreement with the experiments, suggesting that electrostatic interactions and especially protein adsorption are important determinants of protein transport through charged nanopores.

Introduction

The transport of charged macromolecules through charged nanopores is important for protein purification, controlled release of proteins, and the detection and identification of DNA. Functionalized membranes consisting of charged nanopores can be obtained from self-assembling mercaptoundecanoic acid on gold-coated track-etched polycarbonate[1−3] and have been used for transport studies using, for instance, bovine serum albumin (BSA), a globular protein molecule. As a function of pH and ionic strength (10 and 100 mM), the flux was measured in the absence of driving forces other than a concentration gradient (diffusive protein flux). The flux of protein molecules was at a maximum around the isoelectric point, pI, and decreased strongly at both lower and higher pH values at 10 mM ionic strength (on−off behavior).[2] At 100 mM the decrease in flux was much less pronounced.[1]

To understand these observations, we theoretically describe the equilibrium partition coefficient ϕ (distribution coefficient) of BSA in cylindrical nanopores grafted with carboxylic acid functional groups. The equilibrium partition coefficient ϕ describes the ratio between the concentration of a certain molecule in a membrane pore and that in the adjacent bulk phase at thermodynamic equilibrium. Calculation of ϕ is important because the transport rate of protein through a membrane is considered to be proportional to ϕ, for both convective and diffusive transport.[4,5] The partition coefficient depends on the various interactions such as van der Waals forces, hydrophobic interactions, steric interactions and electrostatics. The short-range van der Waals forces may be relatively weak in our problem, because the self-assembled monolayer shields the protein from the polycarbonate membrane. Because of the very strong electrostatic forces between BSA and the carboxylic charges of the monolayer, exemplified by the strong pH dependence of protein flux found in the experiments, we focus in this report on the electrostatic contribution to the partition coefficient. Steric interactions are automatically accounted for, while hydrophobic interactions can be built into the protein adsorption model that will be discussed later on.

Important in the calculation of the electrostatic contribution to ϕ are the charges on the pore wall and on the molecule. For the pore wall, the ionizable character of the carboxylic charges is incorporated in the present work, as well as the modulation of surface charge due to protein adsorption, especially below the isoelectric point of the protein. Protein adsorption is described using theory that determines the equilibrium adsorbed amount of protein from the minimum in the free energy.[6] Such a thermodynamic equilibrium model is most applicable when protein adsorption is reversible, and this is reported to be the case for surfaces consisting of self-assembled thiols.[2] We assume that there is sufficient time for the protein adsorption equilibrium to establish (or else a time-dependent flux would be observed) and therefore incorporate the regulation of the charge of protein and SAM interface upon protein adsorption. When additional protein is brought into the pore it will not adsorb on the pore wall but will diffuse through the pore volume. In the calculation

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of the partition coefficient ϕ we assume that the nonadsorbing molecules do not regulate their charge upon entry into the pore (and neither the surface). As argued by Overbeek,[7] Lyklema,[8] and Weaver and Feke[9] surface charge adjustment can be very slow at the time scale of colloidal interaction, while in ref 4 it is argued that a protein molecule may only reside in the pore for ~10 ms. Thus, though a protein molecule will regulate its charge after being in the pore for some time, the free energy change upon entry (that determines ϕ) must be based on constant-charge interaction. For the (protein-covered) internal pore surface, regulation due to the nonadsorbing molecule is also unlikely because (for a low enough concentration of protein in the pore) each surface element only briefly experiences a passing molecule. Note that when a protein concentration gradient is the only driving force applied across the membrane, small ions have a much higher diffusion velocity than the protein molecules and can be assumed to be at equilibrium with the bulk solution. In that case the Poisson−Boltzmann equation applies.

In the present work we use a recently developed model for protein adsorption to planar oxidic interfaces and apply it to the adsorption of protein to a SAM-coated nanopore. Subsequently, we use the Smith and Deen[10] model (summarized in ref 5) to calculate the distribution coefficient, ϕ. Smith and Deen assume a fixed charge on the (spherical) protein molecule as well as on the pore wall, use the Debye−Hückel linearization and present expressions for the energy change of a protein molecule entering the pore and arriving at an arbitrary (off-center) position. Around pI, where we will find the maximum in ϕ, electrostatic potentials are not very high after protein has adsorbed on the wall and the Debye−Hückel approximation underlying the model[10] is appropriate.

Above pI, where both pore and protein are negatively charged, repulsion of the protein from the pore seems obvious. With decreasing pH (but still >pI), the charge on both the protein molecule and the pore wall decreases and ϕ will increase (as observed experimentally). With further decreasing pH to below pI, the protein becomes positively charged, and if the pore wall remains negatively charged, ϕ theoretically should increase without limit (in striking contradistinction with the experimental observation of a decrease of ϕ). One could explain the decrease of ϕ as being due to the repulsion of the counterions of the protein molecule (below pI being anions) by the negative pore wall.[1,2] Such an explanation has similarities to the description of pressure-driven nanofiltration of aqueous solutions in which both coions and counterions are retained, the coions because of their interaction with the like-charged membrane and the counterions because of a net zero electric current.[11,12] However, pressure-driven transport of ions is different from diffusive flow of (much larger and slower) protein molecules (thus in the absence of driving forces other than a protein concentration gradient), in which case it can be assumed that the small ions in the pore are always at thermodynamic equilibrium with those in bulk solution. Using the analogy with nanofiltration for the equilibrium interaction of charged interfaces would also imply that electrostatic interaction is always repulsive, either because of the charge on the interface, or because of the charge of the counterions. However, in theory and experiment of the equilibrium interaction of oppositely charged interfaces, attraction is a general phenomenon.

In the present work we will quantitatively investigate another explanation for the observed influence of pH on the diffusive transport of protein molecules through charged nanopores, namely that the decrease of ϕ with pH (<pI) is due to protein adsorption.[1,4,5,13−16] Thereby (at sufficiently low pH) the charge sign of the pore wall is reversed, making it positive and of the same sign as additional incoming protein molecules.[13] Adsorption of charged molecules to oppositely charged interfaces is a general phenomenon and has been invoked to understand the influence of pH on the flux of protein through charged pores. In this report we will attempt to formalize these elements in a self-consistent theory for protein adsorption on and transport through charged nanopores as function of pH and ionic strength.

Theory

The equilibrium partition coefficient, ϕ, relates the equilibrium concentration of a molecule (or particle) in a pore, c_{pore}, with the concentration in bulk solution, c_{bulk}, as function of the interaction energy (in units of kT), V_r, when the molecule enters the pore and arrives at a radial coordinate r by[4,5,13,17]

$$\phi = \frac{c_{pore}}{c_{bulk}} = 2\int_0^{1-\lambda} \exp(-V_r)\beta \, d\beta \qquad (1)$$

where $\beta = r/r_p$, $\lambda = r_s/r_p$, r_p is the pore radius and r_s the radius of the molecule. In the centerline approximation V_r is assumed to be independent of r (and typically calculated for the molecule being at the centerline) which results in[4]

$$\phi = \frac{c_{pore}}{c_{bulk}} = (1-\lambda)^2 \exp(-V) \qquad (2)$$

In the absence of van der Waals, hydrophobic, and electrostatic interactions, which requires for the latter that both pore wall and molecule are discharged, $V_r = 0$ and

$$\phi = (1-\lambda)^2 \qquad (3)$$

At stationary conditions, in the absence of driving forces except for a protein concentration gradient, and for low enough protein concentrations, integration of Fick's law results for the diffusive protein flux J in

$$J = \phi \epsilon D l^{-1} \Delta c \qquad (4)$$

with ϵ porosity, l pore length, Δc the concentration difference between the two bulk compartments and D an effective diffusion coefficient [m^2/s] for the molecule in the pore. The proportionality of ϕ and J expressed in eq 4 is at the basis of the use of the present theory for ϕ to describe experimental observations of the diffusive flux of protein J through charged nanopores. In general, V required in eqs 1 and 2 to obtain ϕ is the difference between the free energy of the situation in which a protein molecule is located in the pore, and the free energy of the situation in which the protein molecule is in solution and the pore is empty. We will use the Smith and Deen[10] model for the interaction energy of a spherical particle at coordinate β in a cylindrical pore, both of fixed charge, and integrate over β according to eq 1 to obtain ϕ.

To calculate the charge on the protein molecule, Z, a model for the ionization of the protein surface is required. Here we use the classical titration model for BSA as explained in Pujar and Zydney.[4] According to the model, Cl^- adsorption is important and results in the isoelectric point, pI, being dependent on ionic strength (when Cl^- is the anion of the background salt). Here, we assume a constant Cl^- adsorption irrespective of ionic strength (namely 12.4 adsorbed anions per molecule, which corresponds to an ionic strength of 0.11 M and leads to pI = 4.7). We use a simplified model in which we assume that

all arginine and lysine units have a fixed positive charge (22 + 58) from which we subtract the adsorbed Cl^- anions, resulting in a net fixed positive charge of $Z_+ = 67.6$. Besides, there are $Z_A = 100$ ionizable anionic charges (aspartic acid plus glutamic acid, $pK_A = 4$) and $Z_B = 16$ positive histidine charges ($pK_B = 6.9$) (we neglect the weakly acidic tyrosine residues) resulting in

$$Z = Z_+ + \sum_i Z_i z_i \alpha_i \tag{5}$$

with i either A or B (for the anionic and cationic ionizable charges), z_i the charge sign ($z_B = 1$; $z_A = -1$) and the ionization degree, α_i, given by

$$\alpha_i = \frac{1}{1 + 10^{z_i(pH - pK_i)} \exp(z_i y_s)} \tag{6}$$

with y_s the electrostatic potential at the surface of the isolated protein molecule. For a spherical molecule in solution and under the Debye−Hückel assumption, y_s and Z are related as well by

$$Z = \frac{\kappa}{\lambda_B} r_s^2 \left(1 + \frac{1}{\kappa r_s}\right) y_s \tag{7}$$

where $1/\kappa$ is the Debye length, $\kappa^2 = 2e^2 n_\infty/(\epsilon kT)$, λ_B Bjerrum length, $\lambda_B = e^2/(4\pi\epsilon kT)$, e electronic charge, n_∞ ionic strength (m^{-3}), ϵ the dielectric constant, k the Boltzmann constant and T temperature. Equations 5−7 can be solved self-consistently to obtain the electrostatic potential y_s as well as the surface charge Z.

The net charge density of the pore wall, N_p (m^{-2}), is a function of the ionization degree of the carboxylic charges and of the amount of protein adsorption. The model underlying protein adsorption is discussed in detail in ref 6 and is summarized in Appendix A. The dimensionless charge on the pore wall, σ_p, and on the protein sphere, σ_p, are given by

$$\sigma_p = \frac{e^2 r_p N_p}{\epsilon kT}$$

$$\sigma_s = \frac{e^2 Z}{4\pi\epsilon kT} \frac{r_p}{r_s^2} = Z \frac{r_p \lambda_B}{r_s^2} \tag{8}$$

after which the Smith and Deen[10] model can be applied. The model is concisely summarized in Table A1 of Burns and Zydney[5] (Note that a plus sign in the numerator of the entry for A_p should be minus).

Results and Discussion

Calculations were performed for a pore of open diameter of 22 nm,[2] thus radius $r_p = 11$ nm. When protein adsorbs, the radius decreases by 4 nm, the shortest diameter of the BSA molecule,[1] resulting in an open radius of $r^* = 7$ nm.

The surface has a density of carboxylic acid groups $N = 3$ nm^{-2} (∼33 Å^2 per group) with pK of the carboxylic surface groups $pK_{SAM} = 4.5$. For the calculation of the molecule in free solution and in the pore (but not adsorbed) we approximate the cigar-shaped BSA molecule by a sphere of radius $r_s = 3.4$ nm (based on volume $\upsilon = 163$ nm^3)[18] and assume a homogeneous distribution of charges over the surface. The concentration of BSA in solution is $\phi_\infty = 0.15$ vol % (1 g/l BSA).[2] For water, the relative permittivity is $\epsilon^w = 78$ and for protein we use $\epsilon^p = 20$. Calculation results are presented for $3 < pH < 6$ and $10 < c_\infty < 100$ (mM).

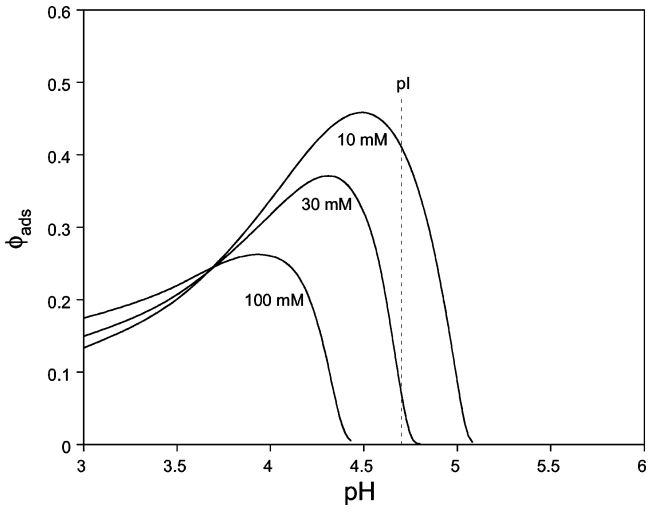

Figure 1. Influence of ionic strength and pH on the adsorbed amount of BSA, ϕ_{ads}, in a charged carboxylic nanopore of radius $r_p = 11$ nm ($\phi_{ads} = 1$ corresponds to an adsorbed mass of 2.7 mg/m^2).

The calculations are performed using the software package *Maple*, in which the differential equations are discretized (up to order 6), and the resulting set of equations is solved using a Newton routine. A typical number of grid points is 30 for the adsorption layer, as well as for the open part of the pore.

Figure 1 shows for these conditions the adsorbed amount of protein, ϕ_{ads}, using the adsorption model of ref 6. ϕ_{ads} is the fractional coverage, and $\phi_{ads} = 1$ corresponds to a fully packed protein layer, when the adsorbed amount is ∼2.7 mg/m^2 (based on a protein volume of $\upsilon = 163$ nm^3 and a protein mass of 67 kDa). Adsorption mainly occurs below pI, though at 10 mM the protein is able to reverse its charge and adsorb.[4,6] Above a critical pH*, adsorption is almost zero. Below pH* the adsorbed amount increases quite rapidly, reaches a maximum and decreases slowly with decreasing pH. With decreasing ionic strength both the adsorbed amount and pH* increase.

Figure 2A and B show calculation results of the partition coefficient ϕ as function of pH and ionic strength c_∞. In Figure 2A, the adsorption model is used, and the effective charge N_p is extracted at radius $r^* = 7$ nm. This radius is subsequently used in the calculation for the partition coefficient ϕ. Figure 2B is based on the following modification. If protein adsorption according to the model is below ∼1 vol %, protein adsorption is set to zero and the distribution coefficient is calculated on the basis of an empty pore of radius $r_p = 11$ nm. In that case, the pore surface charge is obtained from the analytical Debye−Hückel expression for a cylindrical pore[17]

$$y_p = \frac{\sigma_p}{r_p \kappa} \frac{I_0(r_p\kappa)}{I_1(r_p\kappa)} \tag{9}$$

in combination with eqs 8 and 13.

For an ionic strength of 10 mM, protein adsorbs up to pH ∼ 5, and thus, protein adsorption influences the entire ϕ−pH curve. For $c_\infty = 30$ mM protein adsorption is close to zero beyond pH ∼ 4.7 (just beyond the maximum in ϕ) and the downward curve at higher pH is purely a consequence of the increasing repulsion between protein and uncovered pore wall. At 100 mM the transition occurs at an even lower pH, ∼4.4.

The predicted sharp increase in ϕ in a small pH window for $c_\infty = 10$ mM ionic strength (about one-half of a pH point) is in

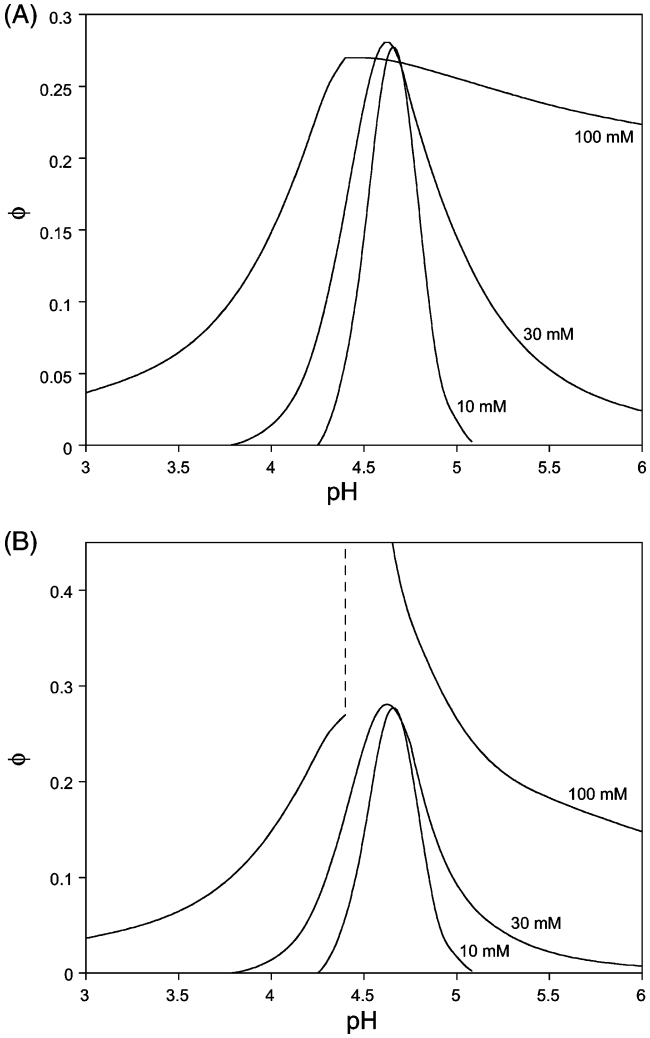

Figure 2. Equilibrium partition coefficient ϕ as function of pH and ionic strength. (a) N_p at r^* from adsorption model irrespective of adsorbed amount of protein. (b) Same as (a) but for adsorbed amount <1 vol %, ϕ based on empty pore, r_p.

agreement with experiments of the flux of BSA (and bovine hemoglobin, BHb) through carboxylic-acid-coated nanopores (on−off behavior).[2] For $c_\infty = 100$ mM a much weaker dependence of flux on the pH is predicted, also in agreement with experiment.[1] The difference in peak width between 100 and 10 mM is mainly due to the higher Debye length in the latter case (∼3 nm for 10 mM vs ∼1 nm for 100 mM). As a result, the free energy change upon entry of a protein molecule is much higher for 10 mM than for 100 mM (for the same charge on surface and protein) and the effect of wall and protein charge on ϕ is much more pronounced for an ionic strength of 10 mM.

In Figure 2A, the calculated maximum in the distribution coefficient ϕ is rather independent of ionic strength, whereas in the experiment a higher flux was observed at the higher ionic strength condition (∼3 times higher) even though the pore size was somewhat smaller (with the number of pores per cm^2 membrane area the same).[1,2] An important reason may be the following. At the condition of the maximum in ϕ, protein adsorption is zero for 100 mM but very high for 10 mM (see Figure 1). Thus, the open pore space is much reduced for 10 mM because of protein adsorption but not for 100 mM (a reduction in pore area by a factor of ∼2.5). To investigate the influence of pore size more quantitatively, Figure 2B was constructed in which, dependent on the amount of protein adsorption, the pore radius is set to either r^* or r_p. For 10 mM there is no difference, as protein adsorption only reduces to below 1% at a pH sufficiently beyond the maximum in ϕ. For 30 mM the transition occurs just after the maximum which results in the right tail of the curve decreasing more steeply. Though the pore area increases in the modified calculation which would make ϕ increase, the Debye−Hückel approximation is now used for $r^* < r < r_p$, which results in a slower decrease of the electrostatic potential y away from the pore surface, thereby decreasing ϕ.

For 100 mM ionic strength the situation becomes more complicated and the discussion rather technical. The point is that around the transition pH of 4.4 the modified model (based on the empty pore radius of $r_p = 11$ nm) predicts a much higher ϕ than the original model that includes protein adsorption and is based on the open radius of $r^* = 7$ nm. This seems not surprising, as the pore area increases by a factor of ∼2.5. However, the question then becomes why this sudden jump is not observed, for instance, in the 30 mM calculation. This is due to the fact that for 30 mM at the pH where we change over to the modified calculation, pore and protein repel each other strongly, and ϕ is determined mainly by radial positions near the center of the pore (low β). It hardly makes a difference whether the charge is fixed at r_p or r^* (in the latter case the charge contains all charges behind r^* and is thus appropriately reduced compared to the pore wall charge at r_p). However, for 100 mM and approximately at pH 4.4, the interaction energy is close to 0 at all values of β and thus, the size of the pore makes a significant difference resulting in the ∼2.5 increase in ϕ. In reality ϕ probably increases more gradually (starting from a somewhat lower pH).

In the modified procedure ϕ_{max} is close to 1 for 100 mM and thus ∼4 times higher than ϕ_{max} for 10 and 30 mM, which corresponds well to the difference in maximum protein flux for 100 mM compared to 10 mM.[1,2] Figure 2B suggests for 100 mM an increase in protein flux around pI, as reported in ref 1, but certainly not the on−off behavior in protein flux observed and predicted for an ionic strength of 10 mM.

Conclusion

We describe the equilibrium distribution coefficient of an ionizable macromolecule inside a charged nanopore incorporating charge adjustment of the nanopore by protein adsorption. Calculations show a sharp increase in the equilibrium partition coefficient in a small pH window around the isoelectric point at low enough ionic strength (30 mM and below) but not at 100 mM. These results are in agreement with the experimental observation of on−off behavior of protein flux around its isoelectric point for sufficiently low ionic strength.

Appendix A. Protein Adsorption on Carboxylic Acid Coated Nanopore

Here we briefly summarize the model from ref 6 for protein adsorption to charged interfaces. A crucial element of that model is the regulation of surface and protein charge during adsorption. Related to charge regulation is a chemical contribution to the free energy besides an electric contribution related to the formation of the double layer. Typically, when protein, oppositely charged to the surface, adsorbs, both the protein and the surface are able to increase their charge, which is a driving force for adsorption (chemical contribution). The release of small ions from the diffuse layer next to the surface and around the protein molecule is the electric contribution to the driving force of adsorption. With increasing protein adsorption these effects become less important and the mutual protein−protein repulsion

increases. Consequently, an equilibrium adsorption is reached. Two additional elements of the model are the following: A nonelectrostatic energy of adsorption per protein molecule can be included that can be due to, for instance, hydrophobic interaction or protein unfolding. This term is set to zero in the present report. Second, when protein adsorbs, an amount of solution phase equal to the volume of the protein molecule is expelled from the surface. This leads to a force that opposes adsorption especially at high ionic strength or when the protein molecule has a low charge. For instance, in Figure 1 we see that at high ionic strength and for pH below pI, the adsorption of protein already decreases strongly near pI because of this competition with the small ions, even though the protein still has a charge opposite that of the surface.

Protein molecules are assumed to adsorb in a layer of a certain thickness and distribute their ionizable groups (charges) evenly over this layer (and take out volume homogeneously as well). Thus, the layer is assumed to be structureless, or gel-like. With increasing adsorption the concentration of protein charges gradually increases and the volume available to solvent decreases. At monolayer coverage ($\phi_{ads} = 1$) the entire layer is filled with protein (for close-packed spheres on a plane the theoretical maximum $\phi_{ads,max} = 0.605$). In each plane parallel to the surface the electrostatic potential is assumed to be constant. This assumption is valid when the spacing between molecules is small compared to the Debye length, thus at a high coverage and/or a low ionic strength.

In the calculation the chemical potential of the protein molecules is fixed (grand canonical), which is valid for the situation in which the surface is in contact with a large reservoir of protein of given concentration. The free energy per protein molecule in solution can be based on a calculation using a spherical double layer such as that discussed in the main text. Because charge regulation is important, the free energy per protein molecule in solution, F_I, follows from

$$F_I = \frac{1}{2} Z y_s + \sum_i Z_i (\ln(1 - \alpha_i) - z_i \alpha_i y_s) \qquad (10)$$

where Z is the total charge and Z_i the number of each of the i types of ionizable groups. The Debye−Hückel low-potential limit used in eqs 7 and 10 can be made because of the large size of BSA and the relatively low number of charges. The chemical potential of a protein molecule in a sufficiently dilute solution is given by $\mu_I = \ln(\phi_\infty) + F_I$, where ϕ_∞ is the protein volume concentration in solution.

Now, in ref 6 a planar adsorption layer containing protein was assumed as well as a planar diffuse layer extending from that layer. In the present report we use a completely cylindrical approach in which the pore of radius r_p is divided in an inner open core, $0 < r < r^*$, and an outer annular space, the adsorption layer, $r^* < r < r_p$, in which protein adsorbs.

For a fractional coverage of protein, ϕ_{ads}, the electrostatic potentials and ionization degrees are calculated at the surface, through the adsorption layer, and into the open pore. In the adsorption layer, a modified Poisson−Boltzmann equation is used

$$\frac{\epsilon^{red}}{r} \frac{d}{dr} r \frac{dy}{dr} = \kappa^2 \left[(1 - \phi_{ads}) \sinh y - \frac{\phi_{ads}}{2 n_\infty \upsilon} Z \right] \qquad (11)$$

where υ is the volume of a protein molecule and ϵ^{red} the reduced permittivity of the adsorption layer due to protein adsorption. On the basis of the permittivity being a linear function of the composition, ϵ^{red} is given by $(1 - \phi_{ads} + \phi_{ads} \cdot \epsilon^p/\epsilon^w)$ with ϵ^p the relative permittivity of protein and ϵ^w that of water (κ is based on ϵ^w). In the open pore, eq 11 simplifies to the Poisson−Boltzmann (PB) equation

$$\frac{1}{r} \frac{d}{dr} r \frac{dy}{dr} = \kappa^2 \sinh y \qquad (12)$$

Note that Z in eq 11 is a function of location within the adsorption layer, because it is a self-consistent function of the local electrostatic potential y via eqs 5 and 6 (with y_s in eq 6 replaced by y). We solve the PB equation in the pore and in the partly filled adsorption layer with boundary conditions $dy/dr = 0$ at $r = 0$, a continuous y and dy/dr at $r = r^*$, and for $r = r_p$ at the SAM surface

$$\alpha_p = \frac{1}{1 + 10^{pK_{SAM} - pH} \exp(-y_p)} \qquad (13)$$

together with

$$-N\alpha_p = \frac{\epsilon kT}{e^2} \left. \frac{dy}{dr} \right|_{r_p} \qquad (14)$$

where N is the density of ionizable carboxylic charges of the monolayer (m^{-2}). Subsequently, we obtain the electrostatic contribution to the free energy of the partially protein-covered pore (per unit area of pore surface)

$$F_{II} = -\frac{n_\infty}{r_p} \int_0^{r^*} \left[2(\cosh y - 1) + \frac{1}{\kappa^2} \left(\frac{dy}{dr} \right)^2 \right] r \, dr - \frac{n_\infty}{r_p} \int_{r^*}^{r_p} \left\{ 2(1 - \phi_{ads})(\cosh y - 1) + \frac{\epsilon^{red}}{\kappa^2} \left(\frac{dy}{dx} \right)^2 - \frac{\phi_{ads}}{n_\infty \upsilon} \left[Z_+ y + \sum_i Z_i \ln(1 - \alpha_i) \right] \right\} r \, dr + N \ln(1 - \alpha_p) \qquad (15)$$

where α_i is the ionization degree of the two types of ionizable amino acids, α_p the ionization degree of the carboxylic pore surface groups given by eq 13, and N the density of these groups.

Now, when the protein free energy in solution is independent of the adsorption process, at equilibrium the chemical potential of protein in solution, μ_I, equals the chemical potential of a protein molecule in the adsorption layer, μ_{II}, which relates to f_{II}, the (volumetric) free energy density of the adsorption layer, and F_{II}, the (surface) free energy density (scaled to the empty pore surface) according to

$$\mu_{II} = \ln \phi_{ads} + \upsilon \frac{df_{II}}{d\phi_{ads}} = \ln \phi_{ads} + \upsilon \frac{2 r_p}{r_p^2 - r^{*2}} \frac{dF_{II}}{d\phi_{ads}} \qquad (16)$$

At equilibrium $\mu_I = \mu_{II}$, which corresponds to the minimum in the function

$$\Delta F = \phi_{ads}(\ln \phi_{ads} - 1) + \upsilon \frac{2 r_p}{r_p^2 - r^{*2}} F_{II} - \phi_{ads} \mu_I \qquad (17)$$

Compared to eq 26 in ref 6, we have taken account of the cylindrical geometry and left out a nonelectrostatic adsorption energy, $\Delta\mu^{ads}$. Furthermore, a Stern capacity at the SAM surface

is not implemented, and the electrostatic potential at the surface of the molecule in solution is not set to zero but obtained from eq 7.

Having calculated the equilibrium adsorption of protein from the minimum in free energy given by eq 17, we extract from the calculation the net surface charge N_p (via eq 14 related to dy/dr at $r = r^*$), which is the total charge behind the dividing plane of the open pore and the adsorption layer (thus "behind" $r = r^*$). This is the surface charge which we will fix in the subsequent calculation of the partition coefficient ϕ as discussed in the main text. It implies that we add all charges behind $r = r^*$ to the surface charge which then includes carboxylic surface charges, adsorbed protein charges, and the small ions between the adsorbed protein molecules.

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