PROTEIN ADSORPTION ON POLYMER PARTICLES

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INTRODUCTION

Protein adsorption on polymer particles has significant importance in biomedical applications, both in vitro and in vivo. It has, therefore, been studied from all viewpoints for the last two decades, but the results were inconsistent due to its complexity. It is not an exaggeration to say that nobody knows what exactly happens at the interfaces between proteins and particles. Proteins are so large that they cannot be treated as small solutes, and the surface of polymer particles is somewhat hairy, which makes quantification difficult. Furthermore, particles are continuously moving, which makes time-dependent in situ measurement impossible.

Protein adsorption on flat and fixed surfaces is relatively easy to analyze; hence, many techniques have been introduced and tested. Although the whole adsorption mechanism is not yet completely analyzed, what is known can give some clues about protein adsorption on polymer particles.

Biomedical applications of protein adsorption on polymer particles include the following: artificial tissues and organs, drug delivery systems, biosensors, solid-phase immunoassays, immunomagnetic cell separations, and immobilized enzymes (1–5).

FUNDAMENTALS

Definition of Terms

Protein adsorption can be defined as ''adsorption (that is, adhesion or sticking) of protein(s) on a variety of surfaces.'' *Proteins* are generally serum proteins, enzymes, antibodies, and foreign antigens, but genes (that is, polynucleotides) are sometimes included in this category, although they are not proteins in fact. *Surfaces* are generally artificially implanted biomaterials (or simply implants), drug and gene carriers (or therapeutic carriers), polymer particles, and membranes. *Adsorbate* and *adsorbent* are also common terms for adsorption. In the protein adsorption on polymer particles, adsorbate is protein and adsorbent is polymer particles.

Protein adsorption can occur either in vitro or in vivo, depending on its application. In vivo protein adsorption takes place in implants and therapeutic carriers, whereas membranes generally have in vitro protein adsorption, except for artificial kidneys containing polymer membranes (6). Polymer particles have both in vitro and in vivo protein adsorption. Protein adsorption can also be classified depending on the type of surface—whether it is flat and fixed or particulate and moving. Excluding a few exceptions, implants and membranes have flat and fixed surfaces and polymer particles and therapeutic carriers have particulate and moving surfaces. Because we are dealing here with ''protein adsorption on polymer particles,'' protein adsorption on flat and fixed surface will not be covered here. However, these two different types of adsorbents will be compared.

In Vitro and In Vivo

In vitro protein adsorption on polymer particles includes: 1) batchwise and chromatographic protein separation and/or purification with polymer particles; 2) solid-phase (or latex-based) immunoassay, especially latex agglutination testing; and 3) enzyme immobilization on polymer particles (1–5). In vivo protein adsorption on polymer particles takes place in: 1) polymer particles as bearing or filler implants, especially for artificial cartilage and synovial fluid; 2) magnetized polymer carriers for tumor cell separation; 3) hemoperfusion carriers for blood purification; and 4) therapeutic carriers for controlled release and targeting (1–4).

In applications in vitro, particles are generally copolymerized (to get core–shell structure) or their surfaces are modified chemically and/or biologically. Most common biological modification can be done by either enzymes or monoclonal antibodies. Because the properties of envir-

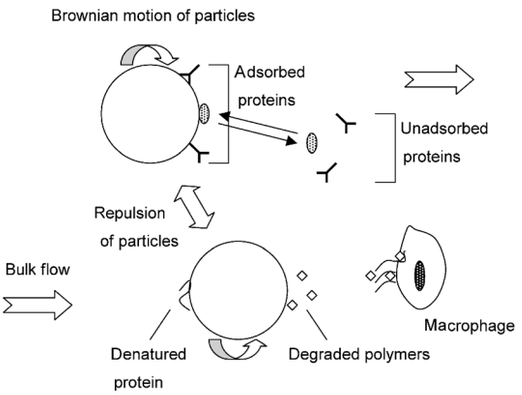

Fig. 1 Schematic illustration of the protein adsorption in vivo.

onmental media are relatively moderate (that is, Newtonian behavior, low ionic strength, and moderate pH) for this type of application, the key factor is only protein itself.

However, in vivo applications are much more complicated than in vitro. Besides protein, blood and lymphatic vessels are also involved, which makes the entire phenomenon much more complicated. Blood clotting triggered by fibrinogen causes another significant problem. The size of cells (normally larger than polymer particles) also makes the phenomenon difficult to analyze. Some cells, such as macrophages, even attack the particles, recognizing them as foreign enemies. Lots of preexisting proteins compete with each other, which causes the so-called Vroman effect. Besides, medium properties are different from those in the conditions in vitro—non-Newtonian behavior and high ionic strength. The most complicated part in the application in vivo is that the surface itself is continuously moving, either by forced blood flow or by intrinsic Brownian motion. Fig. 1 illustrates the protein adsorption in vivo, including cell and tissue responses.

Flat and Fixed Surface vs. Particulate and Moving Surface

As mentioned earlier, protein adsorption can be classified into two categories depending on the nature of the surface—flat and fixed surface, and particulate and moving surface. Protein adsorption on flat and fixed surfaces can be observed in implants and membranes. Common implants include orthopedic implants (bone, cartilage, ligament, and tendon), dental implants and coatings, artificial blood and lymphatic vessels, hard tissue implants (a heart valve, for example), and soft tissue implants (breast implants being the most common example). Both metals and polymers can be used as implants. Due to their flat and fixed nature, protein adsorption is slower and their equilibrium is less dynamic than adsorption on particulate and moving surfaces. This slowness is preferably helpful for analyzing the phenomenon and makes static analysis possible. Lots of experimental methods are currently available for the flat and fixed surface: SPR (surface plasmon resonance, powerful for kinetics study) (7), in situ ellipsometry (measures the adsorbed layer thickness) (8), QCM (quartz crystal microbalance, powerful in quantifying extremely small amounts of adsorption) (9), TIRF (total internal reflection fluorescence spectroscopy, powerful in determining conformational change in adsorbed proteins) (10), and ATR-FTIR (attenuated total reflectance–Fourier transform infrared spectroscopy, powerful in analyzing sequential and competitive adsorption) (11). These experimental techniques produce data on kinetics, conformational change, and competition behavior, as well as on the amount adsorbed.

However, none of these techniques are of any use in studying protein adsorption on polymer particles, where adsorption equilibrium is faster and more dynamic and adsorbents (polymer particles) are continuously moving. Therefore, we must rely on the rather primitive method of separating aqueous phase from polymer particles and quantifying residual protein content. This gives us sufficiently reliable and reproducible data, but kinetic and conformational information cannot be obtained in this way. Advanced methods have therefore been developed, such as in situ monitoring of the adsorbed proteins with fluorescent labeling (radiolabeling can also be used, but is not so common today) (12), kinetic study with a flow cell (13), and adsorbed layer study with DLS (dynamic light scattering) (14). We will discuss details of these in a later section.

Biological Importance

In vitro applications of protein adsorption on polymer particles include protein separation, solid-phase immunoassay, and enzyme immobilization (1–5). Ligand coupling to polymer particles is required for all of these applications, where the ligands have strong affinity for specific biomolecules. Monoclonal antibodies, protein A, affinity dyes, and enzymes are the most common ligands used. These ligands are generally coupled by covalent bonding; hence, protein adsorption is not so important for ligand coupling itself. However, nonspecific adsorption causes lots of problems, because proteins adsorb on almost all surfaces. One might assert that the saturation by ligands could solve this problem, but this saturation causes the so-called prozone phenomenon, which lowers the efficiency of affinity ligands (5, 15). (The general scheme of optimal ligand coupling is shown in Fig. 2.) Therefore, we need a

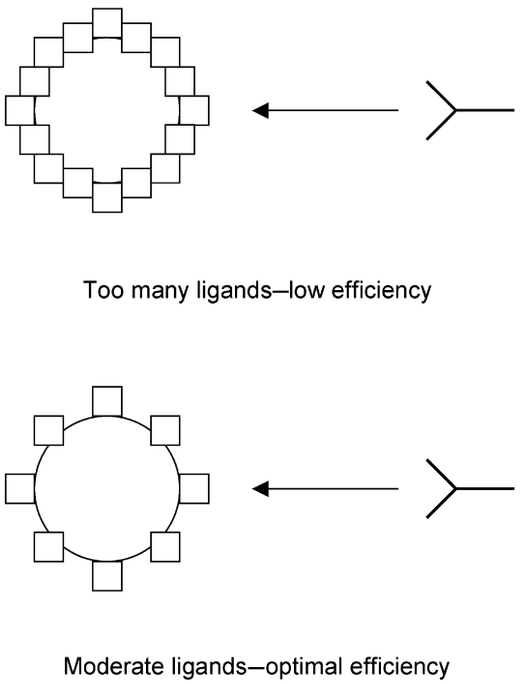

Fig. 2 Optimal ligand coupling prevents prozone phenomenon.

precise control mechanism to prevent this nonspecific adsorption. That is the main motivation for studying protein adsorption on polymer particles, because the exact mechanism of protein adsorption has not yet been established. If the exact adsorption mechanism were known, then hydrogen and ionic bonding (or even hydrophobic interaction) could be used for ligand coupling, which is expected to reduce dramatically the time and effort required for complicated covalent coupling.

Protein adsorption on polymer particles is much more important in the applications in vivo—bearing or filler biomaterials and various therapeutic carriers. Numerous proteins exist in the human body, and their competitive adsorption, denaturation on the surface, and blood clotting are directly involved with overall biocompatibility and performance. Competitive adsorption and denaturation will be covered in the following sections.

Adsorption Isotherms

Generally speaking, proteins adsorb on any surface with only a few exceptions. The fractional coverage is, therefore, strongly dependent on the bulk concentration of proteins. According to basic adsorption theory, *adsorption* can be regarded as a reaction between adsorbate molecules (here, protein) and active sites of adsorbent (here, the surface of polymer particles). The Langmuir isotherm is a famous example for 1:1 matching of adsorbate and active site:

$$\frac{\Gamma}{\Gamma_m} = \frac{KC}{1+KC} \quad (\text{one-parametered}—K)$$

Γ is the adsorbed amount per unit surface area, Γ_m is its maximum value (full coverage of surface, or saturated adsorption), and C is the concentration of protein. K is the adsorption-to-desorption ratio, or equilibrium constant for the adsorption process.

The shapes of experimental isotherms are pretty similar to the Langmuir isotherm, so researchers used this equation to determine the saturated adsorption and kinetic constant. However, the validity of the Langmuir isotherm has been questioned for a long time, because it is so unrealistic that one protein molecule attaches to only one active site. We proposed to use the Langmuir-Freunlich isotherm for protein adsorption, where the second parameter (n) was fixed as a constant that depends on the type of protein (1, 16–18).

$$\frac{\Gamma}{\Gamma_m} = \frac{KC^{1/n}}{1+KC^{1/n}} \quad (\text{two-parametered}—K \text{ and } n)$$

Although there are several mathematical models for the adsorption isotherm of proteins, the universal one-parametered isotherm is not yet established. Considering the different characteristics of each protein, the two-parametered isotherm makes more sense than the one-parametered. Note that K depends on both protein and surface,

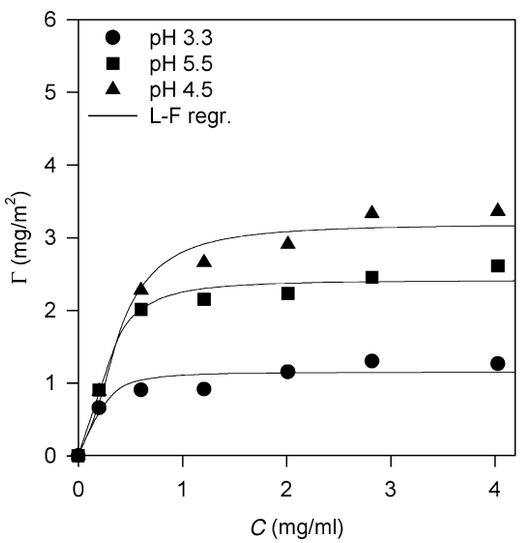

Fig. 3 Adsorption isotherms of BSA on sulfonated polystyrene particles. The Langmuir-Freundlich isotherm equation was used. (From Refs. 1, 36.)

whereas n depends only on protein (16–18). Fig. 3 is one example of experimental data fitted to the Langmuir-Freundlich isotherm (1, 19).

Kinetics, Conformational Change, and Denaturation

In the 1980s, researchers found that protein adsorption was irreversible. They first assumed that this irreversibility was caused by multiple attachment to the surface, that is, an n active site binds to one protein (the Langmuir isotherm assumes 1:1 binding). Later, it was found that the activity of the enzyme was significantly lowered upon adsorption. Now, it is well known that protein adsorption occurs in the following two steps: 1) reversible adsorption and 2) irreversible conformational change (20). The second step would be a simple orientational change on the surface, or even unfolding or uncoiling, which cause denaturation. The famous example is side-on and end-on conformations of BSA (bovine serum albumin), which was first believed to be caused by simple orientational change, but now is known to be by unfolding or uncoiling. Fig. 4 shows the scheme of conformational change of BSA. Due to the second step, protein adsorption turns out to be irreversible. It can also be noticed that the adsorption is reversible if C (protein concentration) is low, which is highly important in applications in vivo.

Competitive Adsorption and the Vroman Effect

We have at least two different proteins in real applications. Because of this, lots of results for competitive adsorption

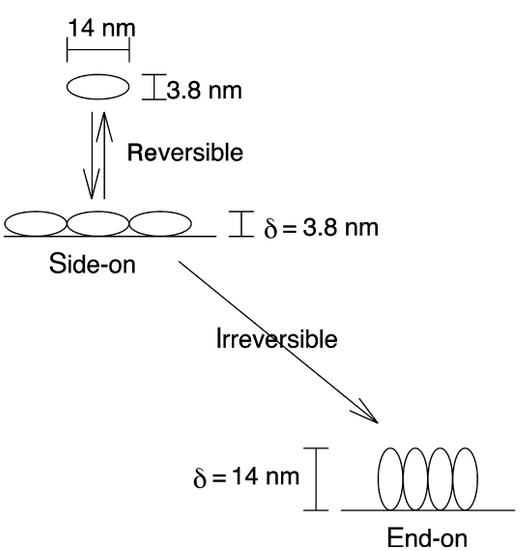

Fig. 4 Conformational change of the adsorbed BSA.

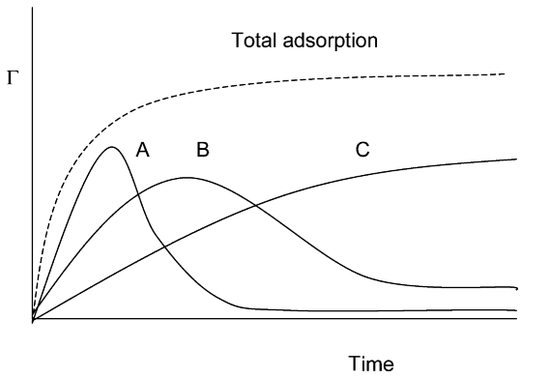

Fig. 5 Vroman effect for three different proteins A, B, and C.

have been published. A simple example of competitive adsorption is as follows: Suppose we have Γ_m values for proteins A and B of 3 and 5 mg m^{-2} respectively, evaluated from one component adsorption experiment. The fractional coverage of the surface is almost 100% for each saturated condition. Suppose that the affinity of protein A is higher than that of protein B (these affinities can be determined from adsorption constant K). If we mix the two equimolar proteins, the fractional coverage of protein A is, surprisingly, much higher, or even near 100% if sufficient time is given. Sequential adsorption and in situ kinetic monitoring enable us to understand the entire phenomenon more easily.

This phenomenon was first observed by Vroman, hence it is called the *Vroman effect*. A definition of the Vroman effect, found in Ref. 21 is: "The Vroman effect is characterized by a decrease of the amount of the initially adsorbed fibrinogen from plasma onto foreign surfaces with increasing contact time, as well as by a maximum in the adsorption of fibrinogen as a function of the plasma dilution." This definition seems to be somewhat narrow, because this effect can also be observed in almost all protein adsorption processes. In general, protein adsorption of more than two components occurs in the following way: First, abundant proteins of low affinity adsorb reversibly, and later, scarcer proteins of high affinity displace preadsorbed proteins. It is also known that the preadsorbed proteins alter the properties of the surface, which facilitates the adsorption of secondary proteins. Fig. 5 illustrates the Vroman effect. Of course, lots of factors affect the Vroman effect—concentrations, relaxation time, and differences in affinities (22, 23).

EXPERIMENTAL METHODS

Bulk Concentration Measurement

This is the most fundamental method for evaluating the adsorption isotherm (1, 16–19). Polymer particles are

dispersed in appropriate media, followed by mixing with protein solution(s). Particles (solid phase) are removed by centrifugation and/or membrane filtration after enough contact time. The protein concentration of the residual solution is measured by spectrophotometry. To increase efficiency, Biuret, Lowry, Bradford, and fluorescent assays can be used. At least six different samples (with varying protein concentrations) are required to obtain a full isotherm. Kinetic information can be obtained from nonlinear regression fitting to isotherm equations (for example, K in the Langmuir-Freundlich isotherm). This method is relatively accurate and reproducible for measuring Γ and Γ_m. Direct kinetic information can also be obtained with varying contact time, but it is not so reproducible.

Surface Concentration Measurement

Bulk concentration measurement can be regarded as an indirect assay. Surface concentration measurement is a direct assay that counts the adsorbed proteins on the surface. Fluorescent labeling is the most frequently used method, but enzyme–substrate colorimetric assay and ELISA (enzyme-linked immunosorbent assay) are also frequently used. These methods are exceptionally useful for quantifying the *active* proteins (that is, adsorbed proteins not denatured), by adopting an intelligent *switch*. The drawbacks are: 1) the results are not reproducible (heavily depends on the medium condition), and 2) they are not good for kinetic study.

Flow Cell Measurement

This involves measuring the continuous adsorption with a flow cell. Polymer particles are trapped in a flow cell, and protein solutions to be adsorbed are continuously fed into it. Typically, *breakthrough curves* are obtained with this method, and Γ, Γ_m, and the kinetic parameter can be calculated (24–27). This method is exceptionally powerful for kinetic study but it has the following defects: 1) Binary or multicomponent adsorption cannot be monitored in real time; 2) because it is hard to give enough contact time, conformational change and/or the Vroman effect cannot be measured accurately. Fig. 6 shows the typical flow cell apparatus for protein adsorption and a breakthrough curve.

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is generally useful for determining the molecular weight of polymers and the size of particles. The basic idea of DLS for protein adsorption is that the particle size increases when proteins adsorb onto polymer particles. These increases are caused either by protein layer formation on the surface (for flat and fixed surfaces, this can be measured by ellipsometry) or by aggregation of particles triggered by protein adsorption. [Choi et al. have demonstrated that the aggregation of particles is triggered by protein adsorption (27).] Thus, it is hard to quantify the protein adsorption itself (the results are valid only when Γ is sufficiently lower than Γ_m), but the method is quite useful for latex-based immunoassay, which is based on the aggregation of particles (more precisely, agglutination).

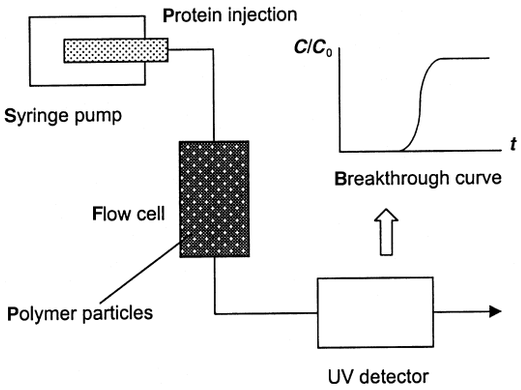

Fig. 6 Typical flow cell apparatus for protein adsorption and its breakthrough curve.

THEORETICAL APPROACHES

Interactions Between Proteins and Polymer Particles

The interaction forces between protein molecules and polymer particles can be classified as hydrophobic interaction, ionic (or electrostatic) bonding, hydrogen bonding, and van der Waals interaction. Fig. 7 shows schematically the interactions at the interfaces between proteins and polymer particles.

Hydrophobic interaction

It is understand that hydrophobic interaction plays a major role in protein adsorption phenomena. The adsorption of proteins on a weakly modified surface occurs by this interaction. Generally, monomers, such as styrene, offer a hydrophobic surface that protein molecules adsorb to. The amount of protein adsorbed by this interaction is maximum in the neighborhood of the isoelectric point of the protein, and the pH at maximum adsorption shifts to a more acidic region, with an increase of ionic strength (1, 16–19). Protein adsorption is greater on a hydrophobic surface than on a hydrophilic one, if there is hydrophobic interaction only.

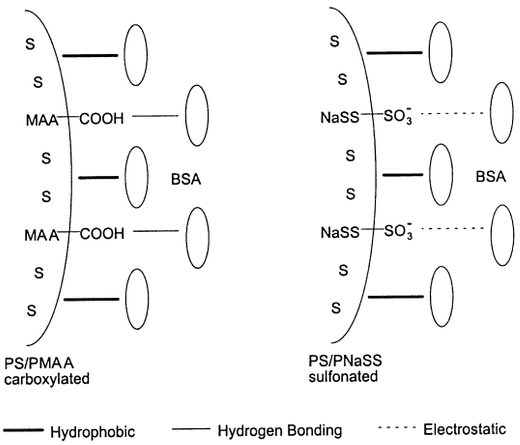

Fig. 7 Interactions at the interface between proteins and polymer particles. PS = polystyrene, PMAA = poly(methacrylic acid), PNaSS = poly(sodium styrene sulfonate).

Ionic (or electrostatic) bonding

Negatively charged polymer particles have anionic functional groups, such as sulfate and carboxyl radicals, on their surfaces. Sulfate groups originate from an initiator, such as potassium persulfate, and carboxyl groups originate from hydrophilic comonomers, such as acrylic acid or methacrylic acid. Ionic bonds are formed between the negative charges of particles and local positive charges of protein molecules. The conventional low-charge polymer particles rarely form these ionic bonds.

Hydrogen bonding

Hydrogen bonds are frequently formed between hydroxyl–carbonyl or amide–carbonyl radicals, and hydroxyl–hydroxyl or amide–hydroxyl bonds are also formed in protein adsorption. Carboxyl radicals are also important in protein adsorption by hydrogen bonding, in low and moderate pH.

van der Waals interaction

This interaction is operative over small distances, and only when water has been excluded and the two nonpolar groups come close to each other. Theoretical calculation showed that the van der Waals interaction is negligible when the other interactions (especially hydrophobic interaction) exist (1, 16–18).

History

Protein adsorption on polymer particles has been extensively studied over the last 20 years, and many experimental results have been published. However, the experimental results are not consistent, and depend on the proteins and media used. Due to this inconsistency, the generalized model for the process is not established and remains under study. There have been diverse explanation of protein adsorption, but only two approaches are now considered. One explanation is taken from the viewpoint of entropy (ΔS), and the other uses the energy term, the Gibbs free energy (ΔG).

Entropic Approach

The entropic approach, or the explanation from the viewpoint of entropy, starts with the question of which surface is more hydrophobic or hydrophilic (in essence, a hydrophobic interaction is an entropic force) (28, 29). This is often insufficient, however, to explain the modified surfaces, especially under nonoptimized conditions, such as deviations in pH from the pI (isoelectric point) of protein, which generally result in the participation of electrostatic attraction or the hindrance of electrostatic repulsion. The electrostatic consideration was therefore included in the entropic (hydrophobic) approach (23, 30). The entropic approach generally does not use mathematical expressions, and consequently it is an empirical interpretation rather than a quantitative prediction.

Energetic Approach

Norde and Lyklema first introduced this thermodynamic explanation (31), which is still being investigated by many research groups (32). This approach is generally accompanied by complicated expressions, headed by Gibbs free energy (G), Helmholtz free energy (F), or chemical potential (μ), which does not appear in entropic approach. In the energetic approach, the estimation of protein adsorption is generally analyzed by electrostatic and van der Waals interactions, both of which contribute to the free energy. These interactions can be estimated by classical principles, such as DLVO (Derjaguin-Landau-Verwey-Overweek) theory. Many researchers extended their models to include these two interactions and tested them for the adsorption isotherms and kinetics. However, this approach could not explain the increase in the amount adsorbed resulting from the conformational change on the surfaces; it could only suggest the adsorption kinetics. Keys to solving this problem are testing diverse kinds of particles and controlling the number density of surface functional groups.

Predicting the Adsorbed Amount

Adsorption kinetics are now well known, and how conformational change occurs is also largely revealed. How-

ever, researchers do not yet know the degree of conformational change; it is believed to be dependent on the strength of interaction forces. In our research, we established a novel generalized mechanism of protein adsorption on polymer particles, especially for the conformational change of proteins with respect to interaction forces (1, 33). Different surface properties were simplified to N (degree of surface modification), and interaction forces were classified into two groups—entropic and bonding interactions. Entropic interaction is exactly the same as hydrophobic interaction, and bonding interaction includes both ionic and hydrogen bonding. Van der Waals interaction was ignored. Entropic contribution is dominant in low N, whereas bonding contribution is dominant in high N. Combining the experimental results and postulates yields the following equation:

$$\Gamma_m = \Gamma_m^E + \Gamma_m^B = \Gamma_{m0} \exp(-aN) + bN$$

$\Gamma_m{}^E$ and $\Gamma_m{}^B$ are the saturated adsorption by entropic and bonding contributions, respectively, Γ_{m0} is the saturated adsorption for an unmodified bare hydrophobic surface, and a and b are experimental parameters. This equation fitted quite well with BSA and BHb (bovine hemoglobin). The prediction of BHb adsorption using the parameters obtained from BSA experiments is successful (no significant differences were found between the prediction and the experiments), although the mechanisms of conformational change are quite different from each other. Part of this result is shown in Fig. 8.

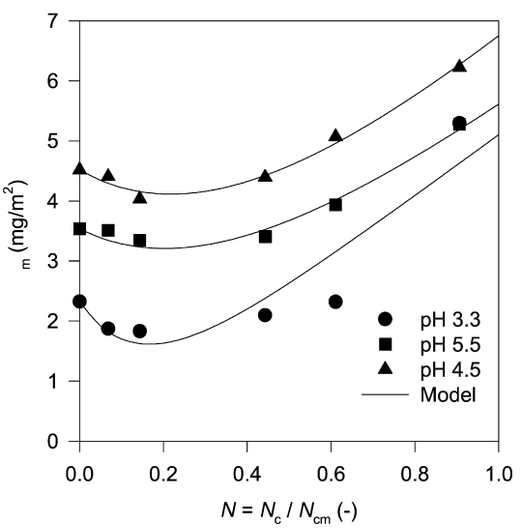

Fig. 8 Γ_m vs. N, BSA adsorption on carboxylated polystyrene particles.

CURRENT PROBLEMS

Discrepancies Between the Different Theories

As mentioned earlier, there exist two different theories for describing protein adsorption on polymer particles. The main problem is that these two theories are not consistent with each other. The prediction of protein adsorption on polymer particles has two different objectives: 1) determining the final amount adsorbed, and 2) describing the time-dependent kinetics. We proposed a unified theory for the first objective that can explain both the entropic and energetic approaches. But we do not have a general theory for the second objective, time-dependent kinetics. For example, the famous Vroman effect is sometimes not observed, depending on the conditions, but a quantified theory for this phenomenon is still unavailable. A generalized time-dependent kinetic theory for protein adsorption on polymer particles still remains as a work for the future.

Experimental Window

The experimental window is the main cause of discrepancies between theories. In the 1980s, some researchers asserted that increasing the hydrophilic comonomer in a polymer particles resulted in a decreased adsorbed amount. Others said the opposite. In fact, both groups are correct—the increase of hydrophilic comonomer results in decreased adsorption in low comonomer content and increased adsorption in high comonomer content (see Fig. 8) (16–18).

In fact, a number of other factors affect the protein adsorption on polymer particles: 1) the size, rigidity, solid content, stability, and hydrophobicity of *particles*; 2) the isoelectric point, solubility, molecular weight, and three-dimensional structure of *protiens*; and 3) the pH, ionic strength, and viscosity of the *media*. Because of this, it is hard to predict protein adsorption on polymer particles. Therefore, we have to limit the experimental window to make the problem easier, but great care should be taken for this limitation to be justified in the application. That is the another reason why in vitro and in vivo studies should be performed simultaneously.

In Vitro to In Vivo

In vivo protein adsorption is much more complicated than in vitro, for the following reasons: 1) So many proteins exist, and they are interrelated with each other. 2) The motion of particles cannot be estimated because fluid flow in the human body is not steady and not laminar (blood flow in an artery is a good example: it has branches, it has both laminar and turbulent eddy, and it is a pulsed flow). 3)

Media are non-Newtonian and generally heterogeneous. Therefore, simple protein adsorption experiments in vitro are not directly applicable to in vivo applications. Much more sophisticated in vitro experiments that mimic the in vivo conditions are required, as well as animal experiments.

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