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Effect of surface wettability on protein adsorption and lateral diffusion. Analysis of data and a statistical model

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Abstract

The initial adsorption of two proteins, ferritin and fibrinogen, from buffer solution onto hydrophilic and hydrophobic silicon surfaces was measured at room temperature with off null ellipsometry *in situ* at a time resolution of 0.1 second under non-diffusion limited conditions. The kinetics of the initial fibrinogen adsorption showed a lag-phase followed by a non-linear accelerated binding. The kinetics of ferritin adsorption at the hydrophobic surface showed a lag phase and an initial non-linear accelerated rate of adsorption whereas adsorption at the hydrophilic surface was proportional to $t^{0.77}$, after an extended lag-phase. The binding rate decreased abruptly and the rate of adsorption became proportional to the logarithm of time. Saturation levels of binding were lower at the hydrophilic surface. Surface diffusion of adsorbed proteins was measured by fluorescence recovery after photobleaching of fluorescein-labelled proteins. Diffusion of ferritin was seen on both surfaces with a diffusion constant a factor of 1000 lower than in bulk solution. Surface diffusion of fibrinogen was not detectable on either of the surfaces within a time scale of 0–100 seconds. The spatial distribution of ferritin was examined by transmission EM. The number of sites of initial adsorption of ferritin was 3×10^9 per cm^2 at the hydrophilic and 2×10^{10} per cm^2 at the hydrophobic surface. The final distribution of adsorbed ferritin was close to random at the hydrophobic surface whereas scattered large clusters and small clusters were seen at the hydrophilic surface. A statistical model is presented that explains: (i) the lag-phase and the non-linear rate of adsorption, (ii) the sudden decrease in adsorption rate and (iii) the logarithmic rate of adsoption.

Key words: Protein adsorption; Wettability; Kinetics; Photobleaching; Surface diffusion

1. Introduction

The adsorption of proteins at liquid–solid interfaces is a primary event in several practically important processes e.g. fouling, blood coagulation, cell adhesion and bacterial infection. The importance of the phenomenon of protein adsorption has attracted many researchers to the field and there is a lot of experimental data available [1–3]. A rapid adsorption and a slow or non measurable desorption are common findings

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irrespective of protein or surface used. In spite of the apparently irreversible adsorption, the amount of bound protein is concentration-dependent and can often be fitted to a simple Langmuir type of isotherm [4,5]. Experimental isotherms which are not in accord with Langmuir type are often explained as the result of heterogeneity in binding [6].

Adsorption of proteins from mixtures in solution often shows a selection towards proteins firstly adsorbed [7]. In complex protein mixtures like blood plasma the possibility of exchange reactions will decide the final composition of the organic film [8]. The surface wettability of the substrate has been shown to be an important factor for the final outcome of exposure of foreign materials to blood [9,10].

The aim of the present study was to investigate the relation between macroscopic contact angle of water (surface wettability; surface energy) and the initial phase of adsorption of two plasma proteins, ferritin being easily soluble in water and fibrinogen being less soluble in water in order to illuminate basic mechanisms of protein adsorption at liquid–solid interfaces.

2. Material and methods

Substrates: Silicon wafers were oxidized to obtain a glass-like surface. The wafers and microscope quartz cover glasses were cleaned and methylised as described by others [11]. Oxidised silicon was used for preparation of supporting grids for electron microscopy as described previously [12]. The contact angle of water was 20° at the hydrophilic and 80° at the hydrophobic surface, which can be used as a measure of the surface energy of the two surfaces.

Chemicals: Ferritin crystallized ×3 (Fluka Chemie AG, Buchs, Switzerland) was dissolved in 0.02 M phosphate buffer pH 7.2 containing 0.15 M NaCl (PBS). Human fibrinogen (Kabi, Stockholm, Sweden) was dissolved in 0.15 M NaCl to a concentration of 10 mg/ml and was kept frozen in aliquots. For the experiments, fibrinogen stock solution was diluted in PBS.

Kinetics of protein adsorption: A null ellipsometer (Rudolph Research model 436) was used with a He–Ne laser (632.8 nm) as the light source. The angle of incidence was 70°. A 50 μl flow cuvette (Hellma, Müllheim, Germany) was used with the back wall comprising the exchangeable silicon substrate. Buffer solution (PBS) was pumped through the cuvette at a rate of 3 ml/min. The instrument was used both as an ordinary null ellipsometer and an off-null ellipsometer. The general procedure followed was to first find the polarizer and analyser positions resulting in a minimum of light transmission through the instrument (null ellipsometry). These settings were used as starting position for the off-null ellipsometry measurements. Any change of the thickness or refractive index of the sample surface will result in an increased reflected light intensity, I, reading of the photomultiplier [13–15]. The relation can be written [15] as:

$$I = k'(n, d_0)d^2, \tag{1}$$

where k' is a constant for a given sample and can be seen as a sensitivity constant since it is a function of all optical parameters involved. In the present study the refractive index of the oxide layer n and its thickness d_0 are the parameters which determine the sensitivity.

Protein solution was then pumped through the cuvette and the change in the reflected light intensity was recorded.

Due to stray light and imperfections there is always a small residual intensity I_0 at null settings. This value is simply subtracted from the measured intensity. The film thickness during adsorption is then given by

$$d = k\sqrt{I - I_0}, \tag{2}$$

where $k = 1/\sqrt{k'}$.

Fluorescence recovery after photobleaching: Ferritin and fibrinogen were labelled with fluorescein isothiocyanate (FITC) by adding a ten-fold molar excess of FITC dissolved in 0.1 M carbonate buffer pH 9.5. The excess of free FITC was removed by dialysis against PBS. Microscope glasses, treated as described above, were incubated with protein at a concentration of 100 μg/ml for 1 min and rinsed with PBS. The glass

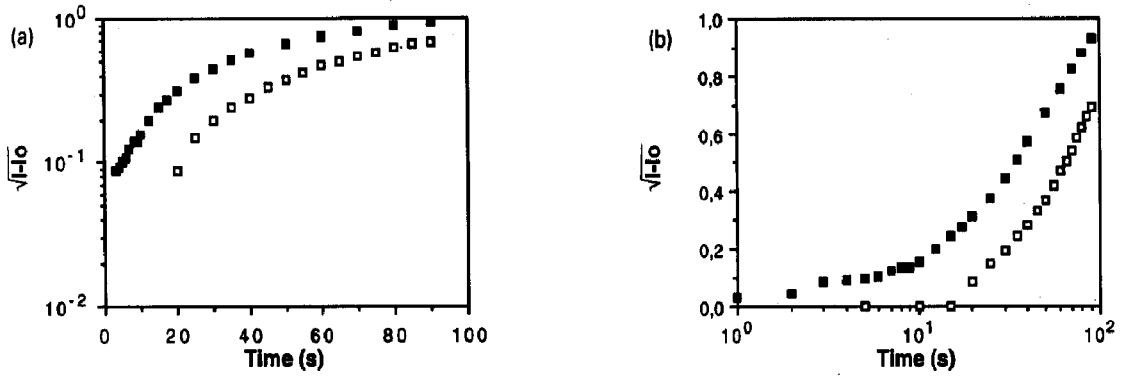


Fig. 1. Adsorption of fibrinogen from a 0.05 M phosphate buffered saline (PBS) solution pH 7.2 containing 10 μg/ml of fibrinogen, onto a hydrophilic silicon dioxide surface (□) and a hydrophobic, methylized silicon dioxide surface (■). Measured with in situ off-null ellipsometry and expressed as $V^{0.5}$ where V is the signal (volts) from the photomultiplier. (a) Lin–log diagram. (b) Log–lin diagram.

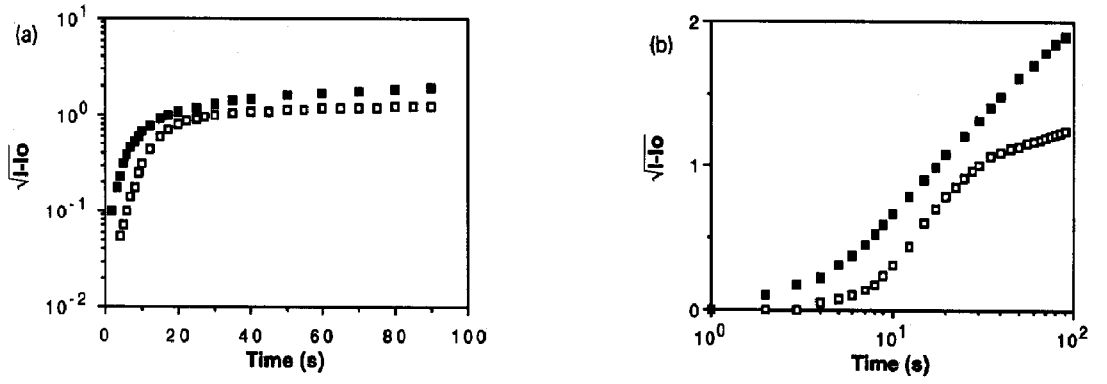


Fig. 2. Adsorption of fibrinogen from a 0.05 M phosphate buffered saline (PBS) solution pH 7.2 containing 100 μg/ml of fibrinogen, onto a hydrophilic silicon dioxide surface (□) and a hydrophobic, methylized silicon dioxide surface (■). Measured with in situ off-null ellipsometry and expressed as $V^{0.5}$ where V is the signal (volts) from the photomultiplier. (a) Lin–log diagram. (b) Log–lin diagram.

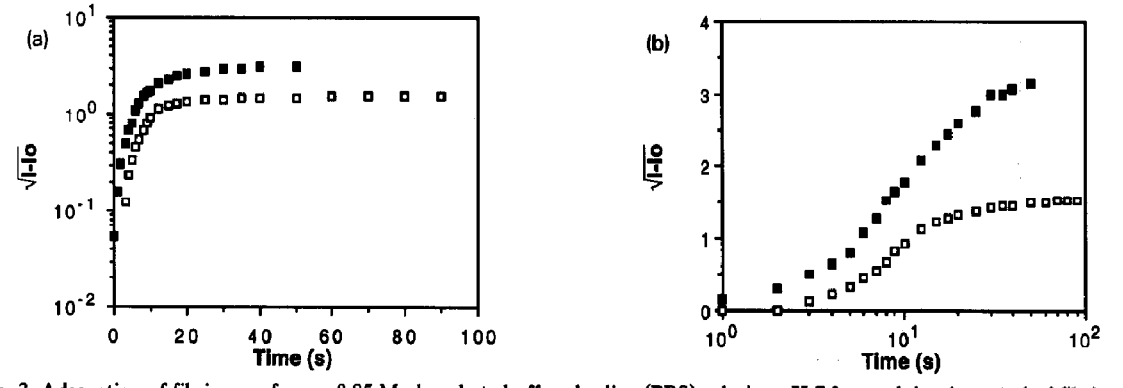


Fig. 3. Adsorption of fibrinogen from a 0.05 M phosphate buffered saline (PBS) solution pH 7.2 containing 1 mg/ml of fibrinogen, onto a hydrophilic silicon dioxide surface (□) and a hydrophobic, methylized silicon dioxide surface (■). Measured with in situ off-null ellipsometry and expressed as $V^{0.5}$, where V is the signal (volts) from the photomultiplier. (a) Lin–log diagram. (b) Log–lin diagram.

slides were placed under a fluorescence microscope and focused, using a tungsten lamp as light source. Photobleaching was performed during 500 ms with a 5 W argon ion laser (488 nm). The fluorescence recovery was measured on a 1.8 μm diameter spot with a photomultiplier and recorded by a ABC 800 computer. The diffusion constant was calculated as described by Axelrod et al. [16].

Electron microscopy: The sample grids were placed in a moist chamber and exposed to ferritin by floating on a drop of solution. The samples were rinsed, dried with a dry air current and examined with a Philips 400 electron microscope. Micrographs were taken at a primary magnification of ×20000 and were further magnified as copies.

The surface concentration of adsorbed ferritin and the number of sites for initial binding of protein molecules was obtained by direct counting.

3. Results

The kinetics of protein adsorption to hydrophilic and hydrophobic silicon dioxide surfaces is shown in Figs. 1–4. The adsorption of fibrinogen is characterized by an initial concentration-dependent lag-phase followed by an accelerated binding rate (Figs. 1a–3b).

At the lowest bulk concentration (10 μg/ml) the length of the lag-phase is 20 s at the hydrophilic surface and 2 s at the hydrophobic surface (Fig. 1). The time dependence of the intitial adsorption is linear in a lin–log diagram, indicating an exponential acceleration of adsorption during 5 s at the hydrophilic surface and 20 s at the hydrophobic surface (Fig. 1a). The binding rate then decreases abruptly and becomes proportional to the logarithm of time (Fig. 1b).

At a higher bulk concentration (100 μg/ml) the length of the lag-phase is 5 s at the hydrophilic surface and 2 s at the hydrophobic surface (Fig. 2). The time dependence of the intitial adsorption is linear in a lin–log diagram, indicating an exponential acceleration of adsorption during 10 s at the hydrophilic surface and 8 s at the hydrophobic surface (Fig. 2a). The binding rate then decreases abruptly and becomes proportional to the logarithm of time (Fig. 2b) with two different slopes at the hydrophilic surface.

At a bulk concentration of 1 mg/ml, the length of the lag-phase is 2 s at the hydrophilic surface and 1 s at the hydrophobic surface (Fig. 3). The time dependence of the intitial adsorption is linear in a lin–log diagram, indicating an exponential acceleration of adsorption during 5 s at the hydrophilic surface and 5 s at the hydrophobic surface (Fig. 3a). The binding rate then decreases abruptly and becomes proportional to the logarithm of time (Fig. 3b). The rate of binding then

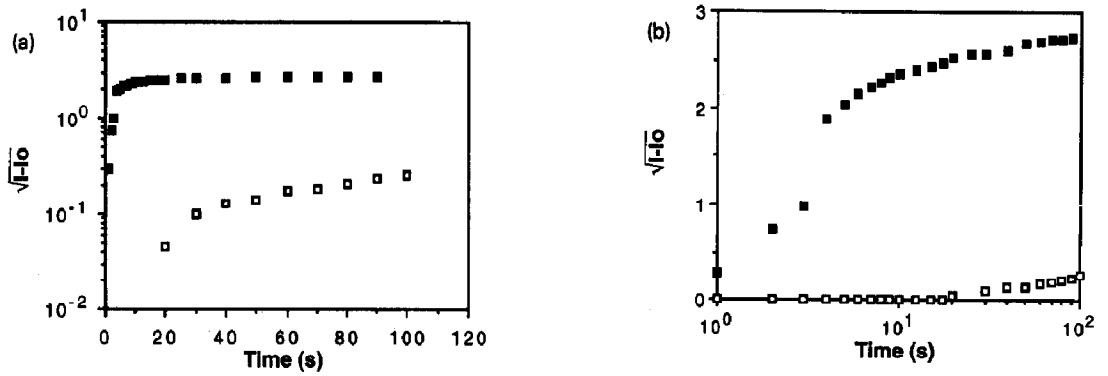


Fig. 4. Adsorption of ferritin from a 0.05 M phosphate buffered saline (PBS) solution pH 7.2 containing 1 mg/ml of ferritin, onto a hydrophilic silicon dioxide surface (□) and a hydrophobic, methylized silicon dioxide surface (■). Measured with in situ off-null ellipsometry and expressed as $V^{0.5}$ where V is the signal (volts) from the photomultiplier. (a) Lin–log diagram. (b) Log–lin diagram.

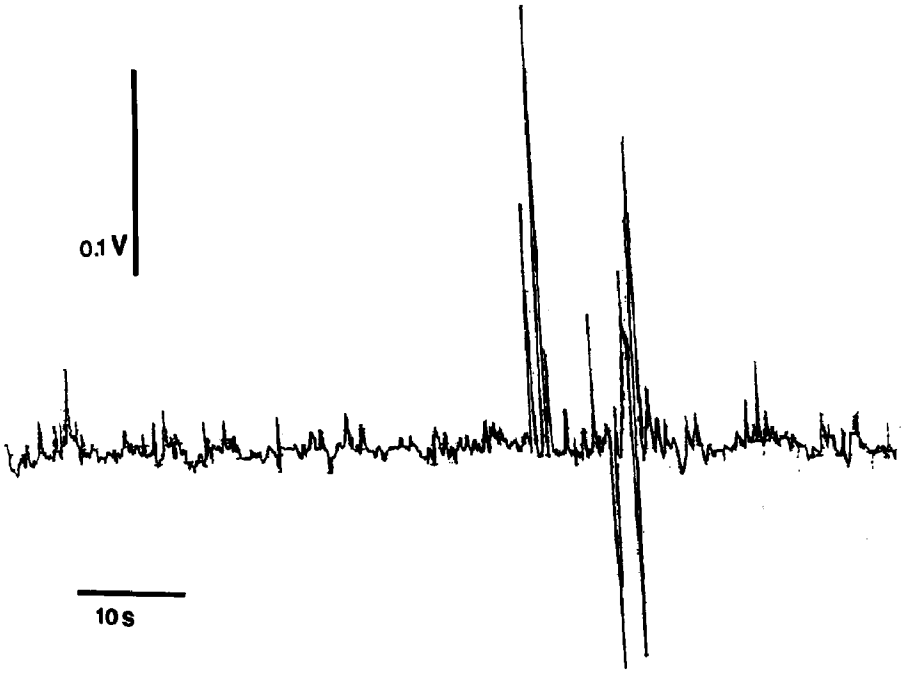


Fig. 5. A computer graph of the recording taken from off null ellipsometry at the saturation level of adsorption of ferritin at a hydrophilic surface.

decreases and the surface concentration of adsorbed protein reaches a stable level at lower surface concentration at the hydrophilic surface $1.5V^{0.5}$ than at the hydrophobic surface $3.0V^{0.5}$ (Fig. 3). V is the change in signal (volts) from the photomultiplier according to Eq. (2).

The adsorption of ferritin from a bulk concentration of 1 mg/ml is strongly dependent on the wettability of the substrate surface. At a hydrophobic surface, an initial acceleration is seen with a time dependence of $t^{1.5}$ during the first seconds, followed by a slower adsorption which is linear with the logarithm of time (Fig. 4). The adsorption of ferritin at a hydrophilic surface shows a lag-phase of 20 s and a time dependence of $t^{0.77}$ during 20–100 s (Fig. 4).

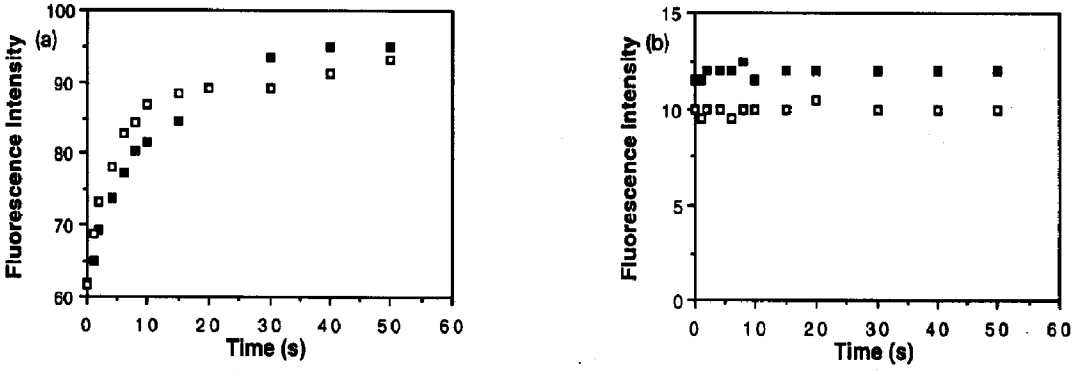


Fig. 6. Fluorescence recovery (fluorescence intensity) as a function of time after photobleaching of adsorbed ferritin (6a) and fibrinogen (6b).

The ellipsometer recording of the adsorption of ferritin to a hydrophilic silicon dioxide surface shows rapid transient fluctuations (spikes) of the thickness of the adsorbed film (Fig. 5).

Fluorescence recovery after photobleaching is shown in Fig. 6. Lateral diffusion of ferritin can be detected with a halftime of recovery of 3.9 s at the hydrophilic surface and 7.1 s at the hydrophobic surface, indicating a diffusion constant of 6×10^{-10} cm^2/s at the hydrophilic surface and 3×10^{-10} cm^2/s at the hydrophobic surface (Fig. 6a). No fluorescence recovery was detectable after photobleaching of FITC-labelled fibrinogen (Fig. 6b).

Electron micrographs of the spatial distribution of ferritin at hydrophilic and hydrophobic surfaces are shown in Fig. 7. The number of sites of initial adsorption was investigated by adsorption from dilute solutions during extended adsorption time (Figs. 7a and 7b). Ferritin adsorbs at 3×10^9 sites per cm^2 at the hydrophilic surface and 2×10^{10} sites per cm^2 at the hydrophobic surface. The distribution of ferritin at saturation was investigated by adsorption over night with high bulk concentrations of ferritin (Figs. 7c and 7d). Micrographs of the hydropbobic surface shows a percolated network of ferritin evenly distributed over the surface whereas micrographs of the hydrophilic surface show scattered distribution of single molecules, pairs and lower oligomers together with large clusters.

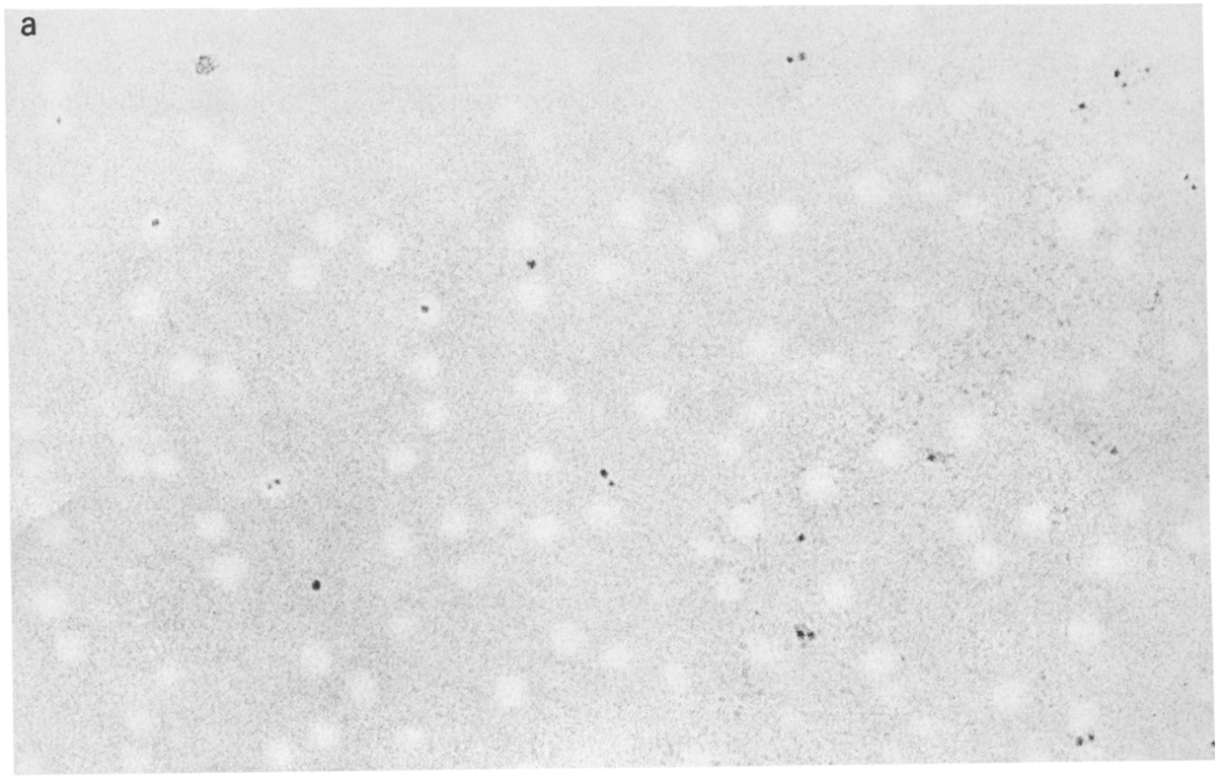
4. Discussion

The qualitative differences of protein adsorption onto surfaces with different surface energies (as defined by the contact angle with water) found in the present study are as follows: (1) the duration of the lag-phase of adsorption; (2) different accelerated kinetics of adsorption; (3) a lower number of initial binding (nucleation) sites at the hydrophilic surface; (4) different slopes of the logarithmich phase of adsorption, and (5) lower saturation levels of adsorption at the hydrophilic surface. We also found that lateral diffusion of proteins at a surface is more related to the properties of the protein molecule than to the wettability of the substrate.

For ferritin a lag-phase of adsorption has been reported earlier [15,17] during adsorption at a methylised quartz surface at lower bulk concentrations than those used in the present study. In the present study this finding is extended to another protein, different surfaces and the concentration-dependence of the lag-phase. Part of the apparent lag-phase is due to mixing and diffusion delay which appears to be of the order of 1–2 s. The experimental conditions were designed to reduce these sources of error. The lag-phase exceeding 2 s is regarded as a part of the adsorption kinetics.

The lag-phase is followed by accelerated adsorption kinetics and cluster growth at the surface (Fig. 7). The growth of clusters means that there must be interaction between adsorbed molecules and/or molecules in solution. Computer simulations of adsorption of ferritin have shown that the initial kinetics may be explained by diffusion-limited adsorption and surface diffusion with $D_{surface}$ being a factor of 1000 smaller than D_{bulk} [18]. These assumption are verified experimentally in the present study (Fig. 6). However, an interaction between adsorbed molecules only would not give the accelerated adsorption kinetics observed. Moreover, fibrinogen which shows no lateral diffusion (Fig. 6) is also adsorbed with accelerated kinetics. Thus the accelerated kinetics must be due to interactions between adsorbed molecules and molecules in the bulk solution. The relative importance of the phenomenon is related to the size of the poten-

Fig. 7. Electron micrographs of the spatial distribution of adsorbed ferritin. Each dark spot represents the iron nucleus of a molecule (diameter = 5 nm). Adsorption was made over night. (a) Initial adsorption on hydrophilic silicon dioxide. Bulk concentration 1 μg/ml. (b) Initial adsorption on hydrophobic silicon dioxide. Bulk concentration 0.1 μg/ml. (c) Saturated adsorption on hydrophilic silicon dioxide. Bulk concentration 10 mg/ml. (d) Saturated adsorption on hydrophobic silicon dioxide. Bulk concentration 100 μg/ml.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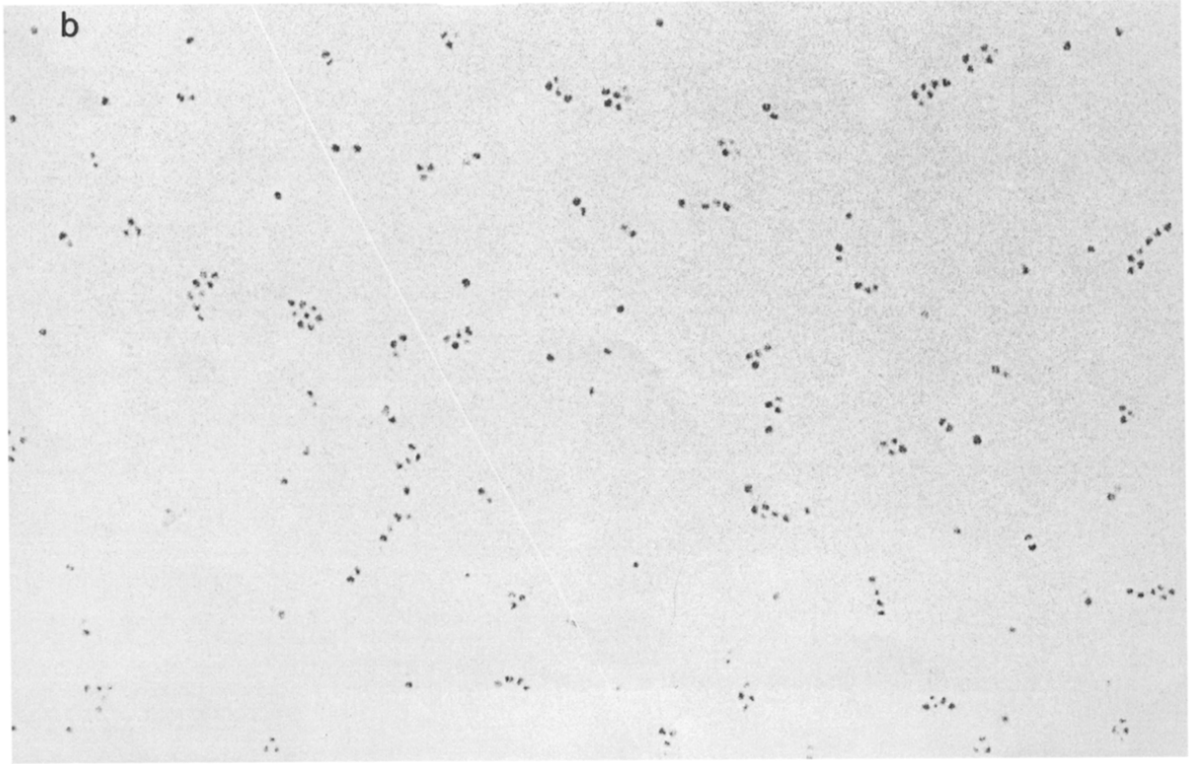
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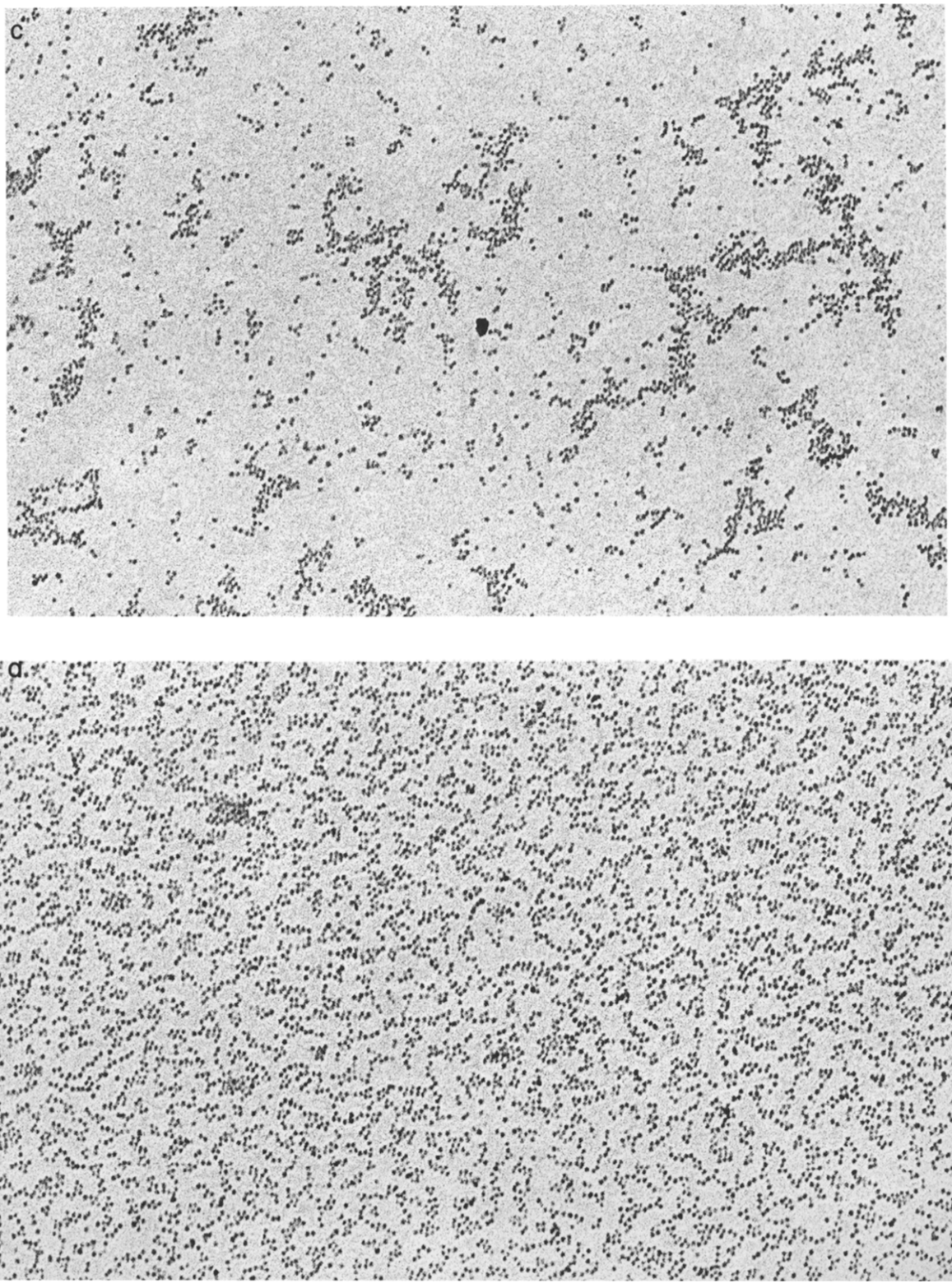

Fig. 7 (continued).

tial energy barrier for adsorption and the interaction energy respectively.

In a simple physical model one would assume that the initial rate of adsorption is linear with concentration and time and that there is a favourable interaction between an adsorbed molecule and molecules in solution. This leads to an equation for the initial adsorption of the form

$$dS/dt = F\left[k_0(N_m - N) + (1 - e^{-aS})\right], \quad (3)$$

where S is the number of surface-bound molecules; N_m and N are the maximum number of nucleation sites and the number of occupied nuclearion sites; k_0 is the probability of binding to the naked surface; F is the flow of protein towards the surface; a relates to the probability of favourable interaction between an adsorbed molecule and molecules in solution; and t is time.

The result of Eq. (3) is thus based on a statistical model related to cooperativity described by an increased probability of binding as a neighbour to previously adsorbed molecules [18].

Mass transport may limit the binding at the largest rate of change of the adsorbed amount of protein [17] as predicted by the second term in Eq. (3). This equation also predicts an initially low adsorption and an accelerated adsorption after a lag-time that decreases with increasing flow. The maximum rate of adsorption and the lag-phase are strongly dependent on the size of the parameter a and differences in this parameter may well describe the behaviour of proteins at different surfaces. Differences in the density of nucleation sites, as seen in experiments will thus have a dramatic effect on the duration of the lag-phase and the rate of binding.

Left to explain is the logarithmic time dependence of the later phase of adsorption which is also seen during the whole process of adsorption of ferritin at a hydrophilic surface.

The time dependence of adsorption above the detection limit showed an apparent $t^{0.77}$ dependence. Spikes in the ellipsometer signal indicates that a desorption process may occur by dissolution of aggregates. Instability of aggregates at the surface has been reported previously [19]. The high bulk concentration of protein may also contribute to the dissociation, [20] which is not seen during rinsing with buffer. Rather, the ellipsometry signal is stabilised by rinsing.

We propose that the logarithmic kinetics of adsorption is due to combined adsorption and desorption in the presence of protein in the solution. This can be described by adding a desorption term $(-k_{-0}S)$ and a term for associative desorption [21] that is suggested by computer simulation of the experimental system [18,20]. From Ref. [21] we get

$$-k_{-1}(F)S^2, \quad (4)$$

which gives the full expression describing adsorption,

$$dS/dt = F\left[k_0(N_m - N) + (1 - e^{-aS})\right] - (k_{-0}S) - k_{-1}(F)S^2, \quad (5)$$

where k_{-0} is the probability of spontaneous desorption of adsorbed molecules and k_{-1} is the flow-dependent probability of desorption as a result of collisions with molecules from the bulk.

A simulation of this equation with Mathematica (Wolfram Res. Inc.) is shown in Fig. 8. As can be seen the logarithmic growth of the surface concentration can be described by Eq. (5).

The lower saturation level of adsorption of both proteins seen on the hydrophilic surface is in agreement with results reported previously [6,7,22], and the phenomenon may be related to the same theoretical modelling.

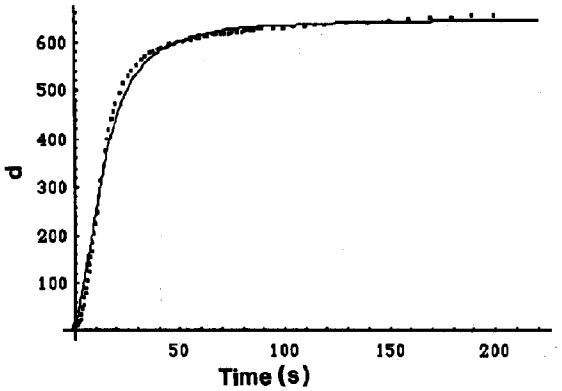

Fig. 8. Simulation of Eq. (5) with Mathematica 2.1 (Wolfram Research Inc). The experimental data are well described with a heavy weight put on the term $1\text{-}e^{-aS}$ and low weight on other terms.

The equilibrium-like saturation level of protein adsorption is thus not in a simple way related to the kinetics of adsorption and desorption as suggested in the Langmuir model. A more realistic model of protein adsorption would be to regard the surface water as one phase and the bulk solution as a separate phase. According to Brönsted partition proteins will be distributed in the two phases according to their molecular size and surface properties as $C_1/C_2 = e^{-lA/kT}$, where A is the surface area and l denotes molecular properties e.g. hydropathy and charge. This formalism has been used in order to explain the partition of proteins in macromolecular two-phase systems [23].

5. Conclusions

For the proteins studied, adsorption at a hydrophilic surface is initially less probable than adsorption of the same protein on a hydrophobic surface.

Intermolecular cooperativity is an important event during protein adsorption.

Desorption of protein clusters and interaction between adsorbed proteins and protein in solution may be important mechanisms behind the observed kinetics and formation of saturation levels.

The saturation levels are in accord with the number of initial binding sites i.e. they are lower at the hydrophilic surface.

The differences between ferritin and fibrinogen adsorption and diffusion on surfaces are in agreement with their different solubilities in water.

6. Acknowledgement

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