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Surface-induced aggregation of ferritin

Kinetics of adsorption to a hydrophobic surface

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The adsorption of ferritin from a water solution to a hydrophobic methylised quartz surface was studied by transmission electron microscopy, allowing direct examination of the iron core of the molecule without further preparation. The initial adsorption was seen to result in small clusters of molecules, the number of sites/cm^2 being concentration dependent. The adsorption process continued via cluster growth. The rate of adsorption increased and the process became mass transport limited. The clusters formed initially had low fractal dimensions ($D \approx 1.0$) and a coordination number, cn of 2.6–2.8, which increased with time. These clusters were abruptly restructured at a coordination number of 3.5, and the apparent rate of adsorption decreased during the reorganisation of the adsorbed layer. Finally, an equilibrium level was reached which was stable for at least 24 h. The distribution of ferritin molecules at equilibrium was in clusters with a fractal dimension of $D = 1.14 \pm 0.16$ and $D = 1.33 \pm 0.08$, respectively, for ferritin concentrations in the bulk of 10 and 100 μg/ml. Rinsing of adsorbed ferritin layers with buffered salt solution resulted in a rapid transient condensation of the clusters, but the net dissociation of protein was slow with the rate of dissociation being proportional to the logarithm of time. The condensed clusters were slowly restructured to linear polymers of ferritin molecules with a coordination number of 1.9 after 24 h of rinsing. The dissociation of protein molecules continued slowly for more than 3 days of rinsing. The results of the present study indicate that the rate of protein adsorption and desorption is strongly related to the supramolecular structure of the adsorbed protein film. Dense clusters of protein are not stable and this phenomenon may explain the formation of a dynamic equilibrium in spite of the fact that protein adsorption to a solid phase may appear to be practically irreversible.

1. Introduction

The kinetics of protein adsorption at a liquid/solid interface is characterised by an initial rapid, often diffusion-rate-limited, reaction followed by a continuous decrease of the forward reaction rate [1]. Adsorbed protein films are stable when rinsed with salt solutions, which has led to conclusions that protein adsorption is an irreversible process [2–4]. Nevertheless, the amount of adsorbed protein is concentration dependent, and equilibrium data may be fitted into a Langmuir isotherm. This kinetics behaviour is similar to that of antigen-antibody reactions at liquid/solid interfaces [5] and may reflect important common properties of heterogeneous macromolecular reactions [6].

Most experimental data on the kinetics of protein adsorption are obtained from integrating measurements with low lateral resolution. However, a few reports present data on protein adsorption at the molecular level studied by transmission electron microscopy. Gorman et al. [7] showed that adsorption of fibrinogen was diffusion rate limited, and a relation has been demonstrated between the isotherm of fibrinogen adsorption and the structure of the adsorbed protein film [8]. Ferritin, a plasma protein of molecular mass 680

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kDa, consisting of 24 similar subunits and a central iron core with a diameter of 5 nm, has been used as a model protein due to its electron density [9]. Adsorbed ferritin was found to be heterogeneously distributed over the surface, showing clustering and large uncovered areas. This distribution was interpreted as being the result of loss of material during sample preparation, since it was possible to obtain a more even distribution of the protein at high bulk concentration and short in-

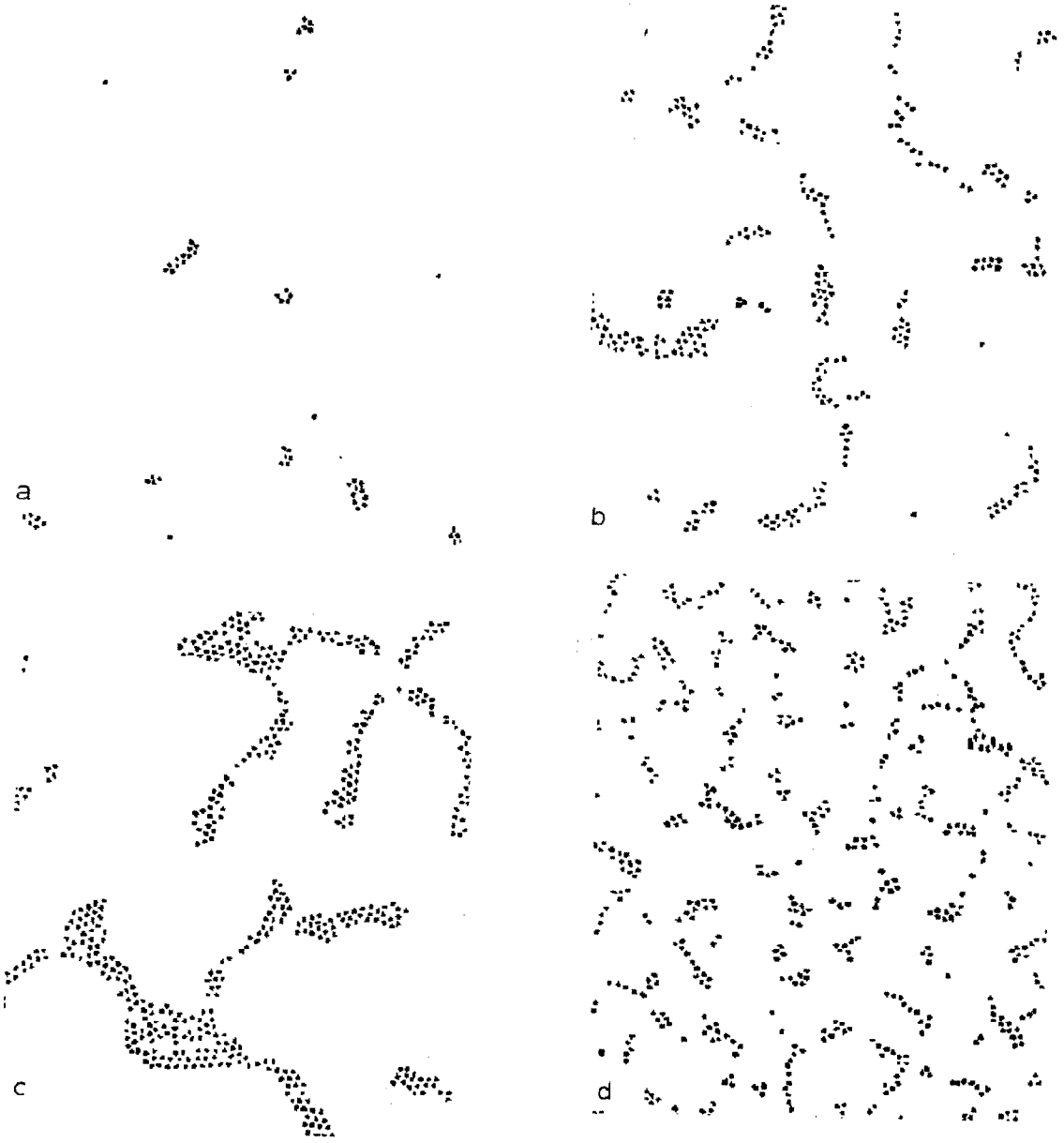

Fig. 1. Electron micrograph of ferritin molecules adsorbed on a methylised quartz grid from a bulk concentration of 10 μg/ml of ferritin dissolved in phosphate-buffered saline. The picture has been digitised and printed on a laser printer. Electron dense particles = 5 nm. Adsorption time: (a) 10 s, (b) 80 s, (c) 280 s, (d) 10^3 s, (e) 8.6×10^4.

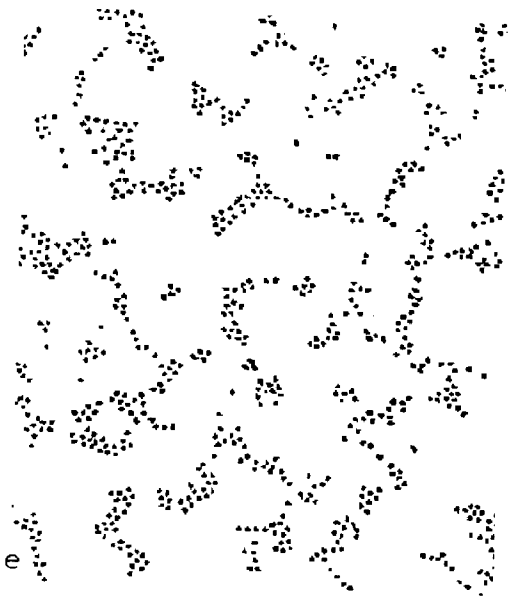

Fig. 1e.

cubation time. Recently, it has been shown that the initial adsorption of ferritin from lower bulk concentration occurs by diffusion-rate-limited, two-dimensional aggregation without any loss of material from the surface during preparation of samples [10,11]. The initial aggregation can be computer-simulated by assuming reversible diffusion limited aggregation [12].

The aim of the present study was to analyse further the kinetics and supramolecular structure of surface-induced aggregation of ferritin.

2. Materials and methods

2.1. Sample preparation

Hydrophobic, methyl silanised quartz grids were made as described previously [13]. Horse spleen ferritin (Fluka, Switzerland; 3 × crystallised) was dissolved in phosphate-buffered saline (PBS: 0.02 M phosphate buffer, pH 7.2; 0.15 M NaCl). The protein solution was applied onto the grids with a pipette for short incubation times (< 100 s). For longer incubations, quartz grids were placed inverted on drops of PBS in a humidified chamber and ferritin solution was injected into the drops to a final concentration of 10 or 100 μg/ml. The adsorption was terminated by rinsing with PBS for 5 s. Studies of the dissociation of adsorbed ferritin were performed by rinsing individual grids in 50 ml of PBS for up to 72 h. The grids were finally dried in a dry air current.

2.2. Electron microscopy

The grids were examined in an electron microscope at 60 kV accelerating voltage. Electron micrographs were taken at ×20000 and copies were made at higher magnification. The number of adsorbed molecules/cm^2 and the number of neighbours of each molecule were counted for two to four image fields (25–100 cm^2) per picture.

2.3. Computer aided image analysis

The micrographs were scanned, digitized and analysed on a computer using a technique similar to that employed for analysis of computer-simulated aggregates [14,15]. Large clusters (12 from each experiment) were chosen, scanned and digitised. Fast Fourier transformation was used to obtain the power spectrum and inverse transformation to yield the autocorrelation function. The inverse transform was averaged over all directions

Table 1

Mean number of neighbour molecules (coordination number, cn) of ferritin adsorbed on a methylised quartz surface

Bulk concentration of ferritin (μg/ml)	Adsorption time (s)	cn
10	10	2.6
	80	2.8
	200	3.5
	1000	2.1
	8.64×10^4	2.5
100	5	2.8
	20	3.5
	40	2.0
	200	2.3
	400	2.8
	8.64×10^4	2.6

to obtain the radial correlation functions. For analysing clusters, an estimate of the density-density correlation function can be evaluated as described previously [16]. An estimate of the density-density correlation function $c(r)$ was evaluated as shown in fig. 4. The correlation func-

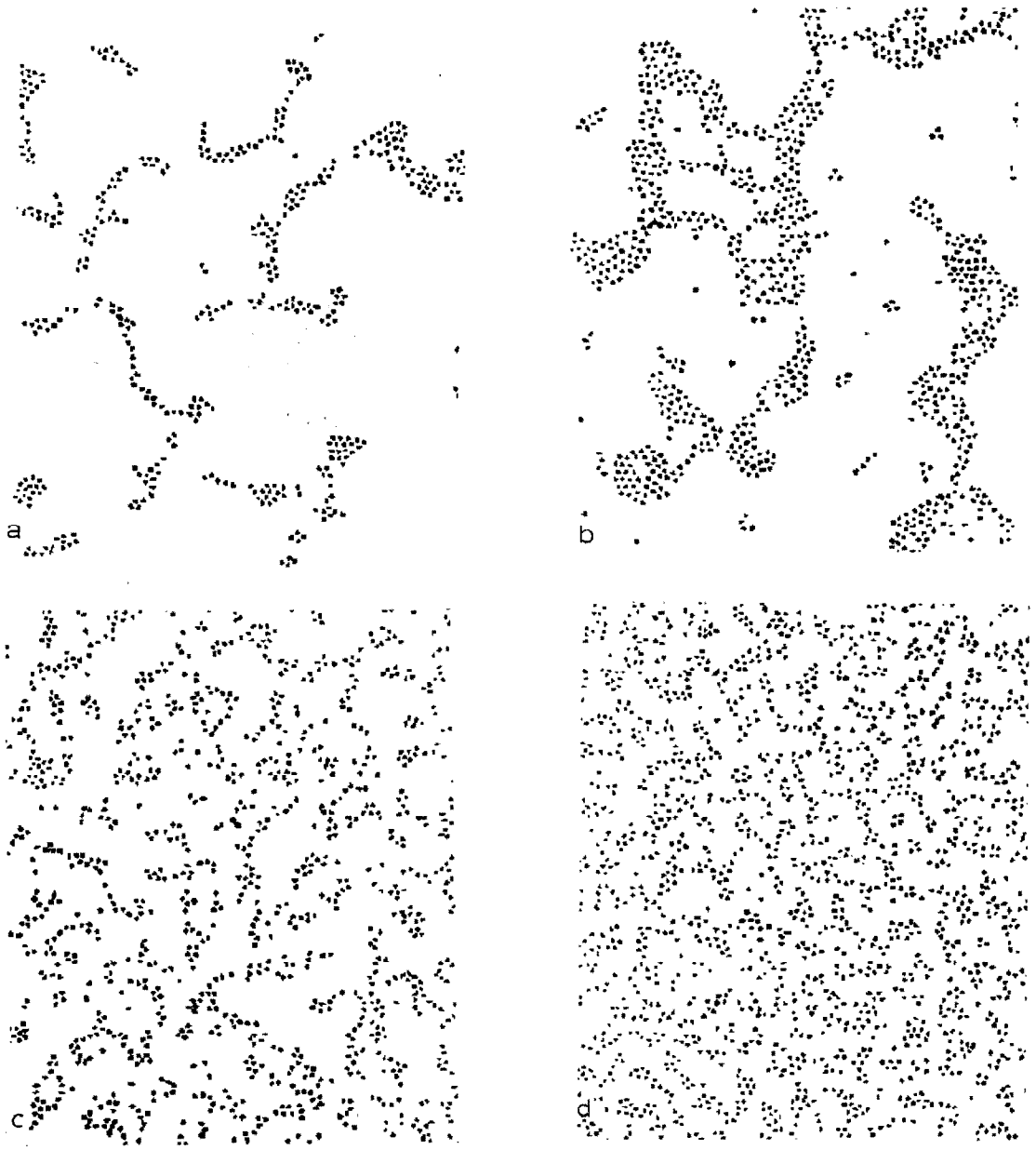

Fig. 2. Electron micrograph of ferritin molecules adsorbed on a methylised quartz grid from a bulk concentration of 100 μg/ml of ferritin dissolved in phosphate-buffered saline. The picture has been digitised and printed on a laser printer. Electron dense particles = 5 nm. Adsorption time: (a) 5 s, (b) 20 s, (c) 40 s, (d) 200 s, (e) 400 s, (f) 8.6×10^4 s.

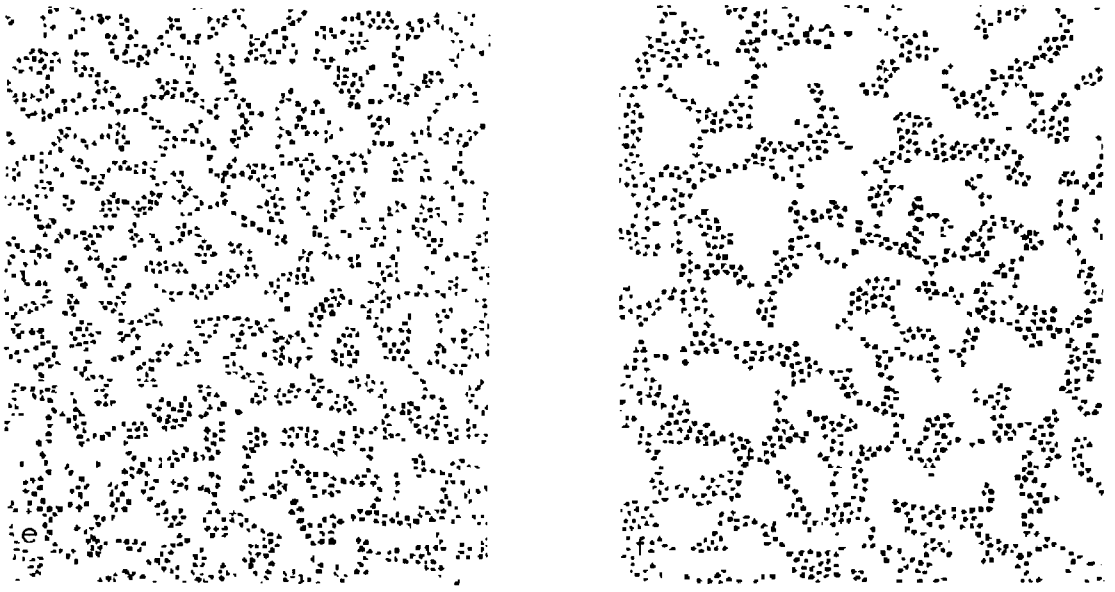

Fig. 2e.f.

tion $c(r)$ describes the probability of finding a second particle at a distance r in the same cluster and is related to the scale invariance of the cluster by [17]:

$$c(r) = k\, r^{-(d-D)}, \quad (1)$$

where k is a constant, d the dimension of space and D a fractal dimension characterising the cluster.

3. Results

Electron micrographs showing the distribution of surface-adsorbed ferritin are depicted in figs 1 and 2. Ferritin adsorbed from bulk concentrations of 10 and 100 μg/ml is initially seen in small clusters at 2.9×10^9 and 7.0×10^9 sites/cm^2, respectively (figs 1a and 2a). The adsorption of new ferritin molecules continues mainly via growth of the clusters (figs 1b and 2b) which become large confluent structures (figs 1c and 2b). The mean number of neighbour molecules increases during the adsorption (table 1).

The rate of adsorption increases during the first seconds, this acceleration being subsequently damped by the mass transport limitation of the adsorption after 10–20 s. The adsorption reaches the diffusion limit after 80 s for a bulk concentration of 10 μg/ml of ferritin. The adsorption from a bulk concentration of 100 μg/ml reaches diffusion rate limitation after an adsorption time of 20 s (fig. 3).

The mass distribution $c(r)$ as a function of the radius r of single clusters is shown in fig. 4. The large clusters formed at 280 s adsorption time from a bulk concentration of 10 μg/ml or 20 s adsorption time from a bulk concentration of 100 μg/ml have fractal dimensions of $D = 0.98 \pm 0.15$ and $D = 1.1 \pm 0.06$, respectively (fig. 4a and b). These large clusters with a coordination number cn = 3.5 are restructured abruptly as shown in figs 1d and 2c. The distribution of ferritin after this restructuring is seen in clusters with a coordination number of 2.0–2.1 (table 1).

The rate of adsorption decreases during the redistribution of adsorbed ferritin (fig. 3). The adsorption of ferritin from a bulk concentration of

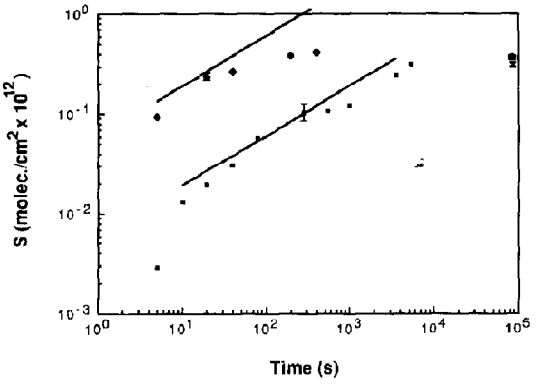

Fig. 3. A plot of the surface concentration of ferritin (molecules/cm^2) vs time (s). The solid line represents the maximum amount of ferritin that can reach the surface by diffusion, calculated from [16]:

$$S = (2/\sqrt{\pi})C_0\sqrt{Dt} \quad (2)$$

where S denotes the surface concentration of ferritin, C_0 the bulk concentration of ferritin, D the diffusion constant of ferritin and t time. Bulk concentration: (■) 10 μg/ml, (◆) 100 μg/ml. Error bars indicate +S.D.

100 μg/ml then reaches a plateau level at a surface concentration of 0.37×10^{12} molecules/cm^2. The rate of adsorption of ferritin from a bulk concentration of 10 μg/ml increases after the establishment of the restructured protein film and again becomes proportional to $t^{0.5}$, although at a level lower than the maximum mass transport limited adsorption (fig. 3). A plateau level is reached abruptly at a surface concentration of 0.31×10^{12} molecules/cm^2.

The spatial distribution of molecules at the plateau level is demonstrated in figs 1e and 2d. The molecules are arranged in clusters with a fractal dimension $D = 1.14 \pm 0.16$ at a bulk concentration of 10 μg/ml and $D = 1.33 \pm 0.08$ at a bulk concentration of 100 μg/ml. The respective coordination numbers, cn = 2.5 and 2.6, are listed in table 1. The effect of rinsing adsorbed ferritin layers with PBS is shown in fig. 5 and table 2. The dissociation of ferritin molecules is a slow process, with an apparent dissociation rate constant of 3.4×10^{-4} s^{-1} during the initial minutes. A transient increase in coordination number can be observed after 10–600 s of rinsing (table 2). The clusters of ferritin molecules break down with time to smaller fragments of linear polymers of ferritin molecules with a coordination number of 1.9 after 24 h. The dissociation process continues and is proportional to the logarithm of time for more than 72 h (fig. 5).

4. Discussion

The experimental procedure used in the present study implies rinsing and drying of the adsorbed protein layer during a period of 5 s. During this preparation artefacts, e.g., loss of material and lateral diffusion of ferritin molecules, can be induced. The fact that we can measure diffusion-rate-limited adsorption in some experiments indicates that there is no significant dissociation of molecules during sample preparation. The diffusion-rate-limited adsorption is achieved only after a delay time of about 10 s which indicates that the ferritin molecules may first be more loosely associated with the surface and that some loss of material may occur for the early samples. The experiments performed with prolonged rinsing show that the net dissociation of molecules is a rather slow process at the surface concentrations attained in these experiments. Experiments performed at higher bulk concentrations of ferritin in order to achieve higher surface concentrations up to monolayers of ferritin are more sensitive to loss of material during preparation [9].

The risk of inducing redistribution of material is more difficult to evaluate. Some experiments have been performed using fixation of the protein layer with glutaraldehyde [11] prior to rinsing and

Table 2

Mean number of neighbour molecules (coordination number, cn) of ferritin adsorbed on a methylised quartz surface after rinsing with phosphate-buffered saline

Dissociation time (s)	cn
0	2.6
10	2.9
600	2.8
3×10^3	2.2
8.64×10^4	1.9
2.59×10^5	1.2

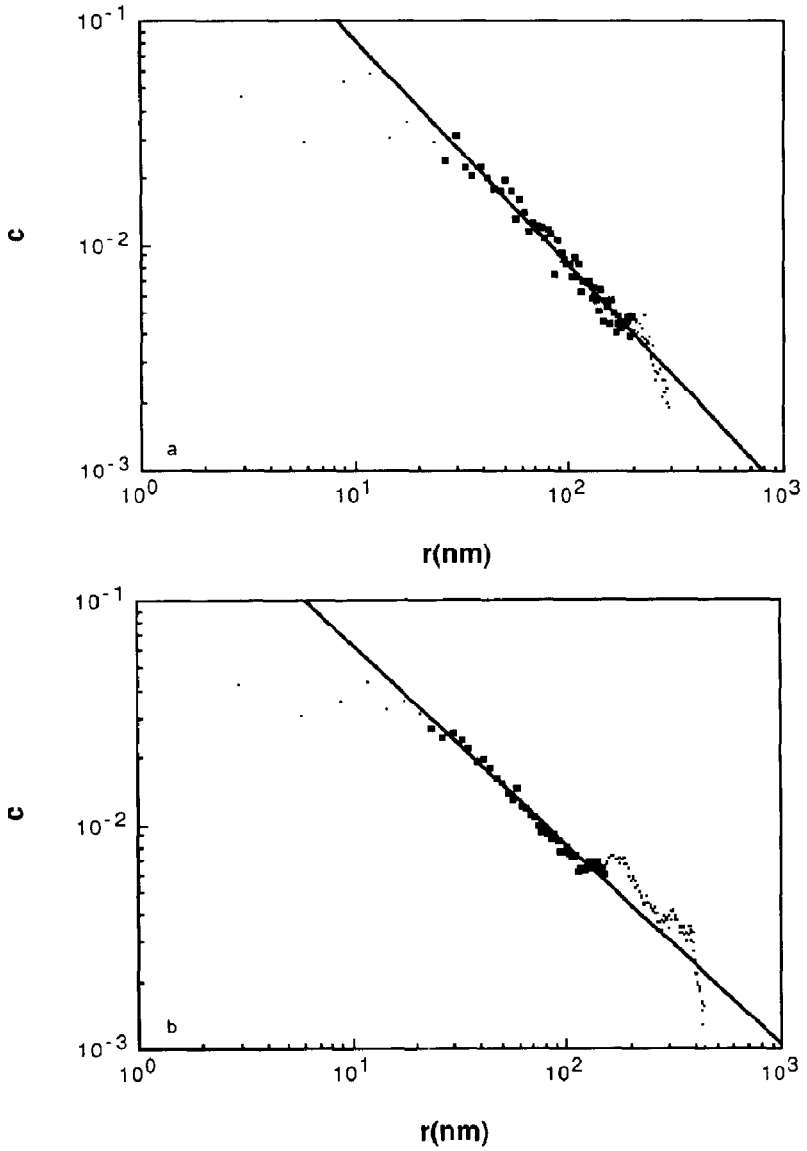

Fig. 4. Density-density correlation function $c(r)$ measured for the clusters appearing in figs 1 and 2. Experimental values (dots) and the slope of the plot (line) are shown. (a) 10 μg/ml, 280 s (fig. 1c); (b) 100 μg/ml, 20 s (fig. 2b).

drying. No significant difference was observed between fixed and unfixed ferritin layers indicating that redistribution during preparation does not provide a satisfactory explanation of the results presented here. We thus conclude that the present experiments yield new and valid data on the relation between the kinetics of ferritin adsorption and the spatial distribution of adsorbed molecules.

The initial adsorption resembles a nucleation and growth process in that there is an increased probability of the adsorption of new molecules as the neighbours of previously adsorbed molecules.

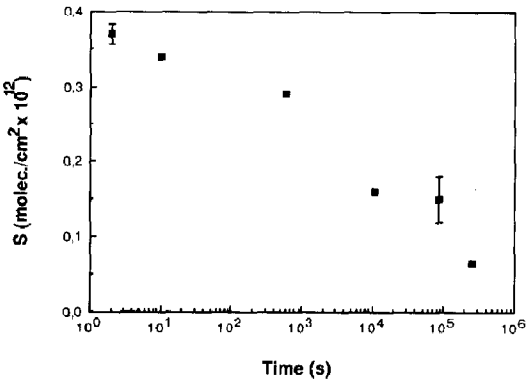

Fig. 5. Plot of the surface concentration of ferritin (molecules/cm^2) vs time (s) of rinsing of an adsorbed layer of ferritin with phosphate-buffered saline. Error bars indicate ± S.D.

The fact that the rate of adsorption increases initially is also in accord with the kinetics of nucleation and growth processes. However, since single molecules are also adsorbed, the process is not dependent on the simultaneous adsorption of a nucleation cluster as in the classical condensation process (for a review, see ref. 18).

The instability of the large clusters (cn = 3.5) seen in the present experiments is consistent with the findings of others [9], showing that monolayers of randomly distributed ferritin are not stable but break up within seconds. This instability of large clusters leads to the sudden dissociation of large amounts of ferritin and the exact dynamics of the process is difficult to study by taking samples at different time intervals. However, the stable association of new molecules can be demonstrated when the adsorbed molecules are distributed in linear clusters with cn = 2 formed after restructuring of the adsorbed protein film.

The adsorption of ferritin reaches a concentration-dependent plateau level far below that of a monolayer of adsorbed protein molecules. This finding is consistent with the observations reported in other studies showing that adsorption of protein is retarded far below monolayer coverage of the surface [1,19,20]. The mechanism behind this phenomenon is not obvious. The possibility of a conformational change of individual protein molecules at the surface which would give rise to surface coverage far beyond that of native molecules has been discussed [20]. This explanation is not valid for ferritin as seen in the present study. Intermolecular repulsion has been suggested as the underlying mechanism for damping of the rate of adsorption with increasing surface concentration of adsorbed protein [1]. The spatial distribution of ferritin observed in our experiments provides no indication of intermolecular repulsion and measurements of the pair-correlation function [21] showed only attractive interaction. In a study on the adsorption of virus particles, repulsive long-range interactions were detected [16], but were too weak (a fraction of kT) to be able to hinder diffusion. The possible mechanisms of attaining the plateau level that are compatible with our experiments are an association rate or a dissociation rate that is dependent on the supramolecular structure of the adsorbed protein layer with the stable adsorption of ferritin at a mean number of neighbours of 2.0–2.5. The clusters formed from an aggregation with this distribution of neighbours have small fractal dimensions.

The fractal dimensions of the clusters observed in the present study are very close to that found in the two-dimensional aggregation of silica particles at an air/water interface ($D = 1.2$ [22]), suggesting possible similarities in the process of aggregation in this system.

5. Conclusions

The results of the current study demonstrate surface-induced aggregation of ferritin at a liquid/solid interface. The distribution of ferritin is in clusters with small fractal dimensions which are restructured at a coordination number of 3.5 to form aggregates with concentration-dependent fractal dimensions and a coordination number of 2.5–2.6. These experimental data may be valuable for development of theoretical models of macromolecular reactions at interfaces.

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