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Surface-induced aggregation of ferritin

Concentration dependence of adsorption onto a hydrophobic surface

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The isotherm of ferritin adsorption onto a hydrophobic surface was studied by transmission electron microscopy. Adsorbed ferritin was found to be distributed in molecular clusters. The adsorption process was diffusion-rate-limited after 20 h adsorption time at bulk concentrations below 1 mg/l. The clusters formed during the diffusion-rate-limited adsorption had a fractal dimension $D \approx 1.0$ when averaged over all clusters. The pair distribution function $g(r)$ showed an increased probability of finding nearest neighbours at distances less than 30 nm. The surface concentration of adsorbed ferritin was weakly dependent on the bulk concentration of ferritin in the range 10 mg/l–10 g/l and the average number of nearest neighbour molecules was constant in this concentration range. The mass distribution of adsorbed ferritin $c(r)$ had a fractal dimension $D = 1.8$ at a bulk concentration of 10 g/l and a surface concentration corresponding to $\theta = 0.45 \pm 0.05$. The pair correlation function $g(r)$ showed decreasing probability of finding nearest neighbour molecules over long distances as in percolating clusters. The results indicate that ferritin adsorbs strongly to the surface at low surface concentrations and weakly at high surface concentrations. The stability of ferritin adsorption was correlated to the average number of nearest neighbour molecules, indicating a possibility that desorption is a critical supramolecular phenomenon.

1. Introduction

Adsorption of proteins at a liquid-solid interface is an initially rapid, often diffusion-rate-limited process. The adsorbed protein film is stable during rinsing with buffered salt solutions. These characteristics are valid for a plethora of different proteins [1–3] and for adsorption to many different surfaces, leading to conclusions that surface adsorption is so energetically favourable that the process is practically irreversible [3].

Nevertheless, the amount of adsorbed protein reaches a plateau level and the surface concentration of bound protein at this stable level depends on the bulk concentration of protein [3,4]. Adsorbed protein layers can be rapidly exchanged in the presence of protein in the bulk [5] and this process can be facilitated by shearing flow [6]. These experimental findings indicate the equilibrium properties of the adsorbed film and are paradoxical to the apparent irreversibility of the adsorption process.

In studies of protein adsorption by transmission electron microscopy using ferritin, an electron-dense plasma protein, as a model system, we have shown that the kinetics of ferritin adsorption to a hydrophobic surface is strongly dependent on the supramolecular structure of the adsorbed protein layer, and that a stable plateau level of adsorption is formed below a monolayer [7]. The present study was undertaken in order to elucidate the concentration dependence of the plateau level after a long period of adsorption.

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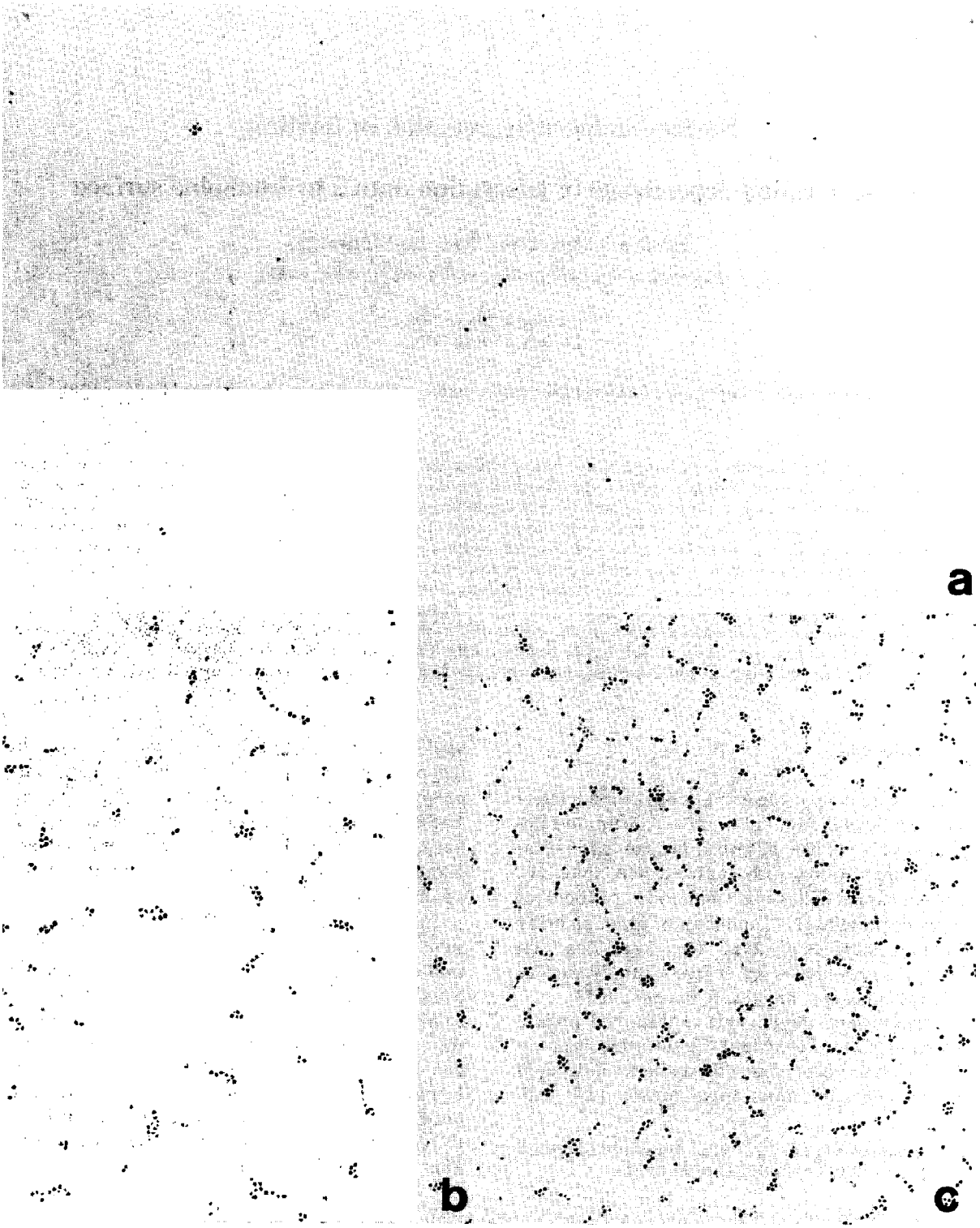
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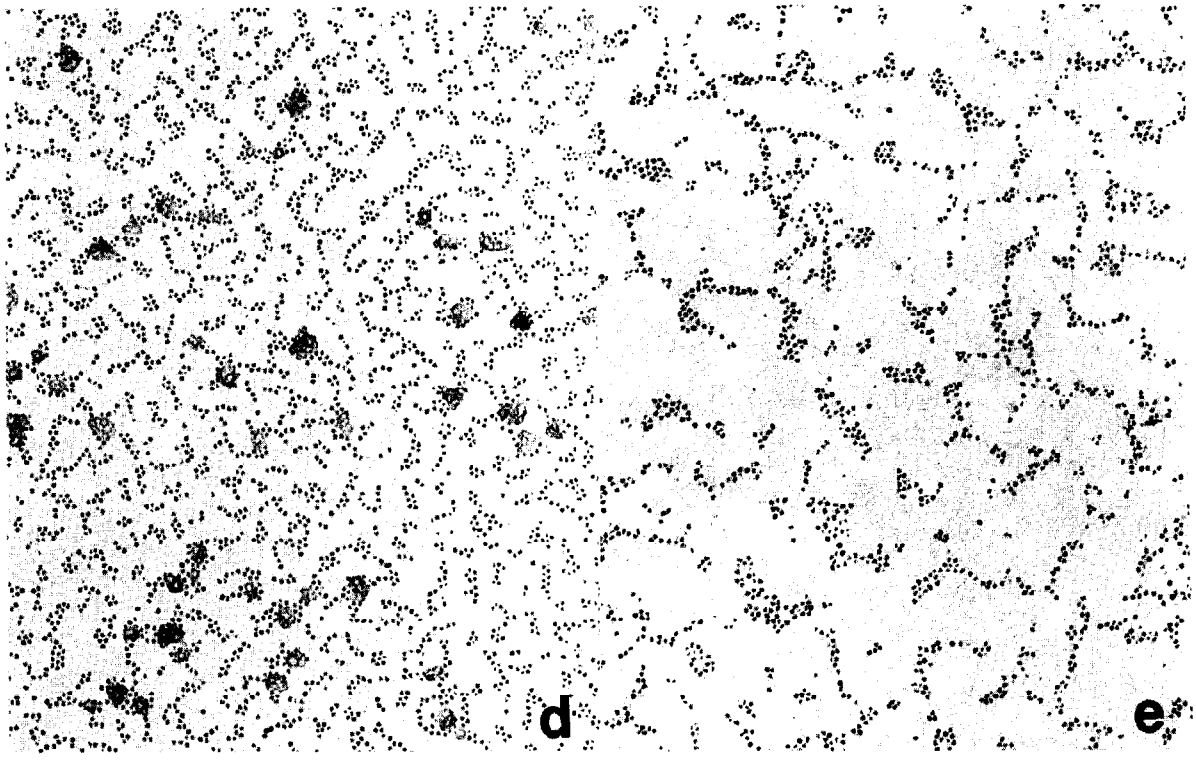


Fig. 1. Electron micrographs of ferritin molecules adsorbed on a methylised quartz grid for 20 h from bulk concentrations of 10 g/l–0.01 mg/l of ferritin dissolved in phosphate-buffered saline. Electron dense particles = 5 nm. (a) Concentration of ferritin in solution: 10^{-5} g/l. Rinsed for 10–15 s without fixation. (b) Concentration of ferritin in solution: 10^{-4} g/l. Rinsed for 10–15 s without fixation. (c) Concentration of ferritin in solution: 10^{-3} g/l. Rinsed for 10–15 s without fixation. (d) Concentration of ferritin in solution: 10^{-2} g/l. Rinsed for 5 s without fixation. (e) Concentration of ferritin in solution: 10^{-2} g/l. Rinsed for 10–15 s without fixation. (f) Concentration of ferritin in solution: 10^{-1} g/l. Fixation with glutaraldehyde. (g) Concentration of ferritin in solution: 10^{-1} g/l. Rinsed for 10–15 s without fixation. (h) Concentration of ferritin in solution: 10^{-1} g/l. Rinsed for 5 s without fixation. (i) Concentration of ferritin in solution: 10 g/l. Fixation with glutaraldehyde. (k) Concentration of ferritin in solution: 10 g/l. Rinsed for 10–15 s without fixation. (l) Concentration of ferritin in solution: 1 g/l. Fixation with glutaraldehyde. (m) Concentration of ferritin in solution: 1 g/l. Rinsed for 10–15 s without fixation.

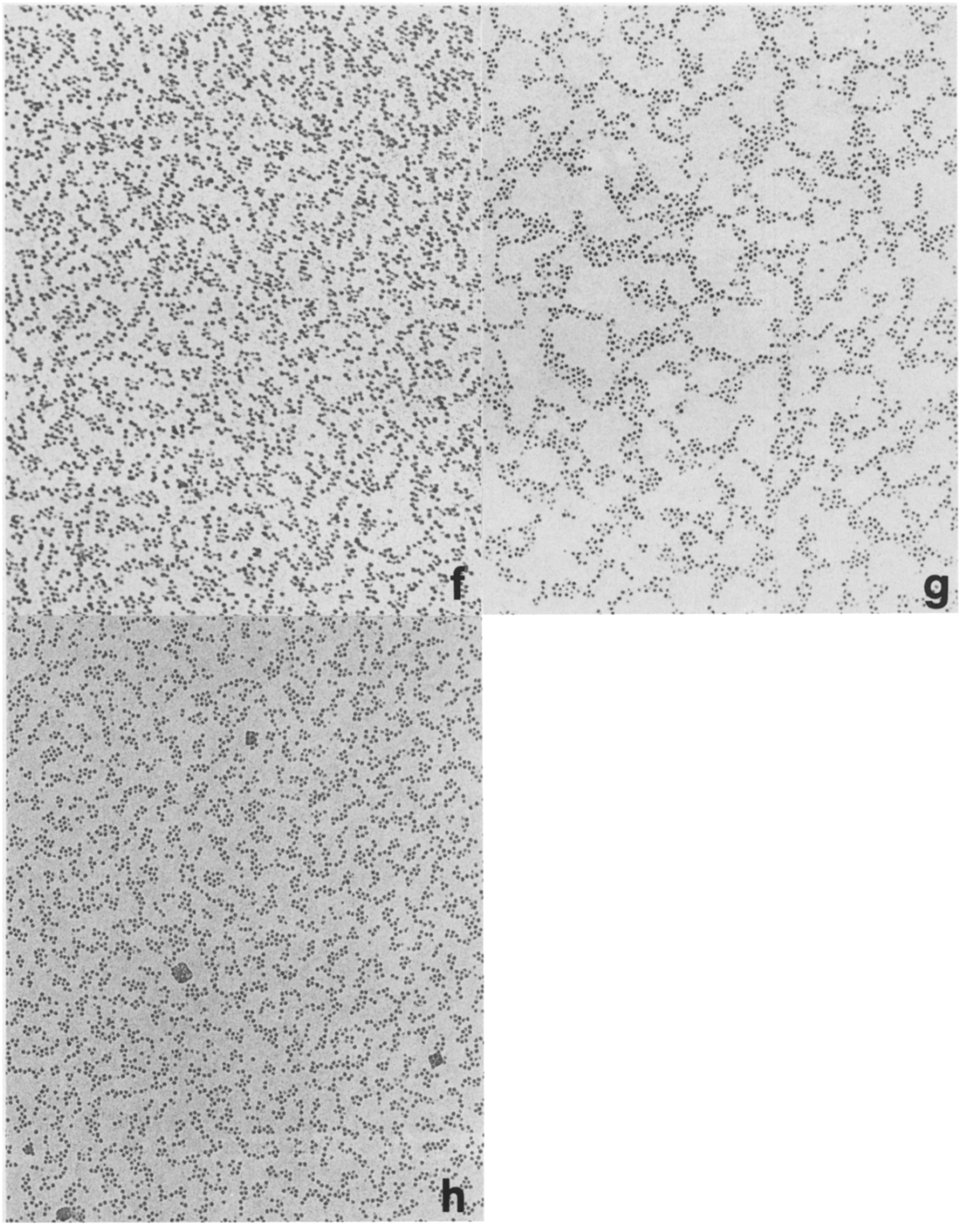
2. Material and methods

Sample preparation

Hydrophobic, methyl silanised quartz grids were made as described previously [8]. Horse spleen ferritin (Fluka, Switzerland; crystallised three times) was dissolved in phosphate-buffered saline (0.02 M phosphate buffer, pH 7.2; 0.15 M sodium chloride). Quartz grids were placed inverted on drops of phosphate-buffered saline in a humified chamber and ferritin solution was injected into the drops to the final concentrations as indicated in the figures. The adsorption was terminated after 20 h by rinsing with phosphate-buffered saline for 5–15 s. In some experiments, the adsorbed protein layer was fixed by addition of glutaraldehyde to the drops at a final concentration of 1% during the last 2 h of adsorption. The grids were finally dried in a dry air current.

Electron microscopy

The grids were examined in an electron microscope at 60 kV accelerating voltage. Electron micrographs were taken at ×20000–×33000 and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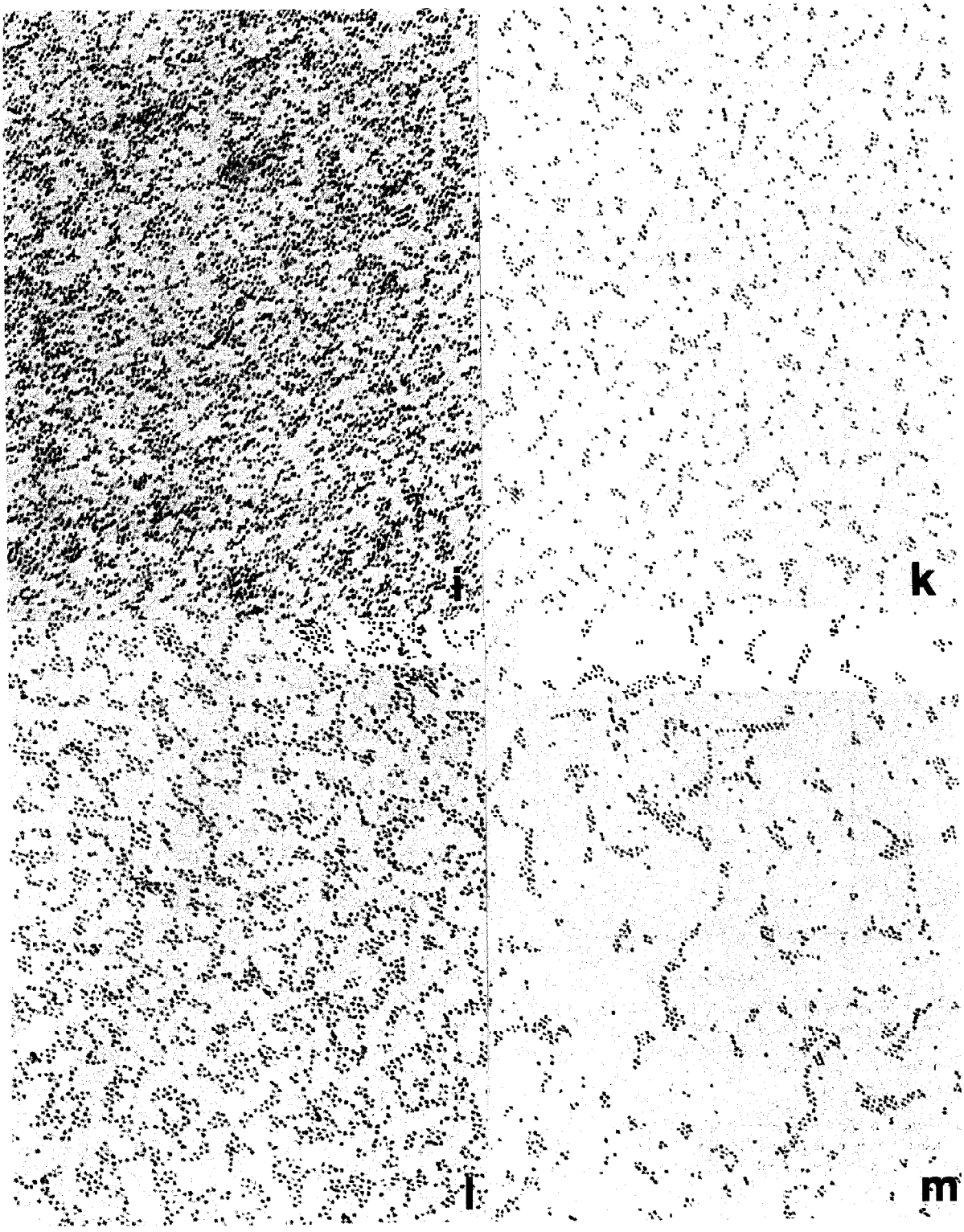
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copies were made at higher magnification. The number of adsorbed molecules/cm^2 and the number of neighbours of each molecule was counted on two to four image fields (9–100 cm^2) at each picture.

Computer-aided image analysis

The micrographs were scanned, digitized and analysed in a computer by a technique similar to that used for analysing computer-simulated aggregates [9,10]. Whole pictures were scanned and digitised. A fast-Fourier-transform was used to obtain the power spectrum and an inverse transform to give the autocorrelation function. The inverse transform was averaged over all directions to yield the radial correlation functions. For analysing clusters, an estimate of the pair correlation function $g(r)$ and the density-density correlation function can be evaluated as described previously [11]. The pair correlation function $g(r)$ describes the probability of finding a nearest neighbour as a function of distance r. An estimate of the density-density correlation function $c(r)$ was evaluated as shown in fig. 3. The correlation function $c(r)$ 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 [12]:

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

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

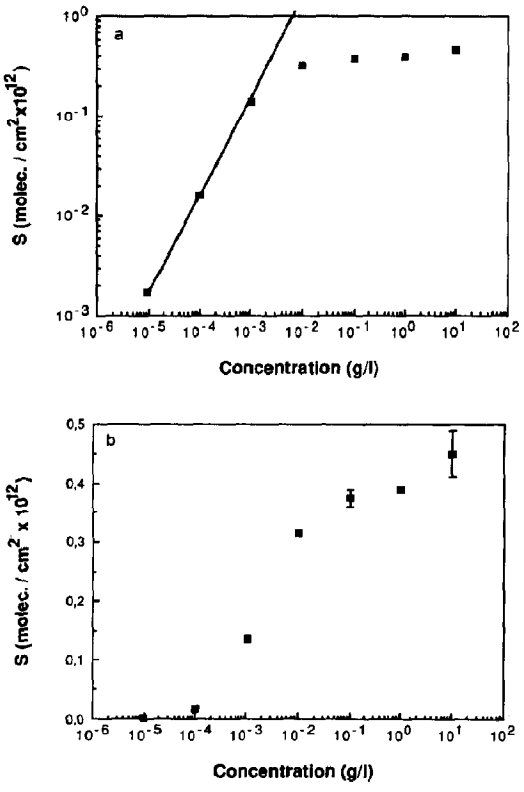

Fig. 2. A plot of the surface concentration of ferritin (molecules/cm^2) vs concentration in solution (g/l). (a) The solid line represents the maximum amount of ferritin that can reach the surface by diffusion, calculated from [11]:

$$S = (2/\sqrt{\pi})C_0\sqrt{D\,t} \quad (1)$$

where S is the surface concentration of ferritin, C_0 the bulk concentration of ferritin, D the diffusion constant of ferritin (3.61×10^{-7} cm^2/s) and t time. (b) Error bars = ±S.D.

3. Results

Electron micrographs of ferritin adsorbed to a hydrophobic quartz surface from different bulk concentrations for 20 h are shown in fig. 1. Ferritin adsorbed from bulk concentrations of less than or equal to 1 mg/l is found distributed as single molecules, pairs or small clusters of 3–10 molecules. These small clusters are often linear polymers of ferritin molecules, but dense clusters with hexagonal close packing are also seen (fig. 1a–c).

Ferritin adsorbed from a bulk concentration greater than or equal to 10 mg/l is found distributed in larger clusters. These large clusters are often stringy and ramified at a few points. The clusters formed at a bulk concentration of 10–100 mg/l may vary in density due to small differences in rinsing conditions between experiments (fig. 1d–h). Fixation with glutaraldehyde shows that the small stringy clusters observed after very short rinsing time represent native clusters and that these clusters are condensed during slightly prolonged rinsing (fig. 1f–h).

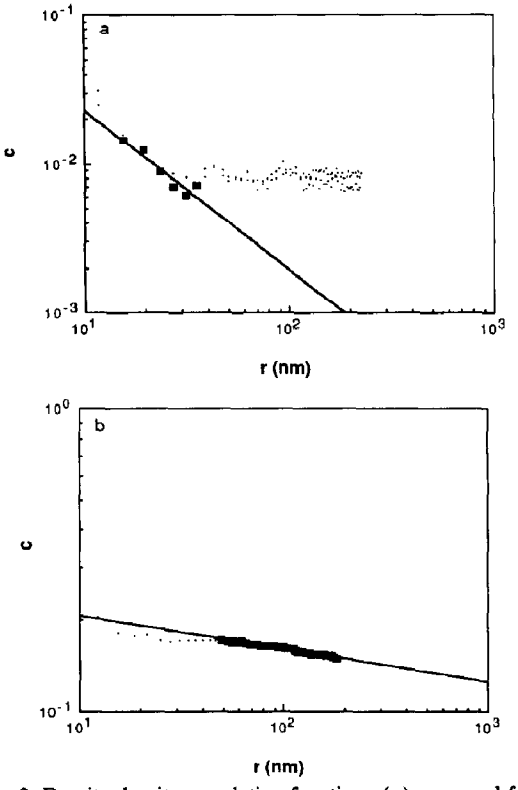

Fig. 3. Density-density correlation function $c(r)$ measured for clusters appearing in fig. 1. Experimental values (dots) and slope of the plot (line) are shown. (a) Mass distribution measured on fig. 1c. (b) Mass distribution measured on fig. 1i.

The clusters formed during adsorption from high bulk concentrations (≥ 1 g/l) become dense and confluent and are sensitive to rinsing. These clusters could be seen reproducibly only when glutaraldehyde fixation was used to preserve the native structure of the adsorbed ferritin layer (fig. 1i–m).

The surface concentration of adsorbed ferritin in relation to bulk concentration is shown in fig. 2, together with a line indicating the maximum amount of ferritin that can reach the surface by diffusion. As can be seen, the isotherm of ferritin adsorption follows diffusion limitation for bulk concentrations less than or equal to 1 mg/l after an adsorption time of 20 h (fig. 2a). At higher bulk concentration, the amount of adsorbed ferritin is weakly concentration-dependent (fig. 2b)

Table 1

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

Bulk concentration of ferritin (g/l)	cn
10	2.64 [a]
1	2.58 [a]
0.1	2.55 [b]
0.01	2.46
0.001	2.0
0.0001	1.84
0.00001	0.6

[a] Measured after fixation with glutaraldehyde.
[b] Measured after fixation with glutaraldehyde and short time of rinsing (5 s).

with a plateau level in the range of bulk concentration of 0.1–1 g/l. At a bulk concentration of 10 g/l, the whole layer of adsorbed ferritin

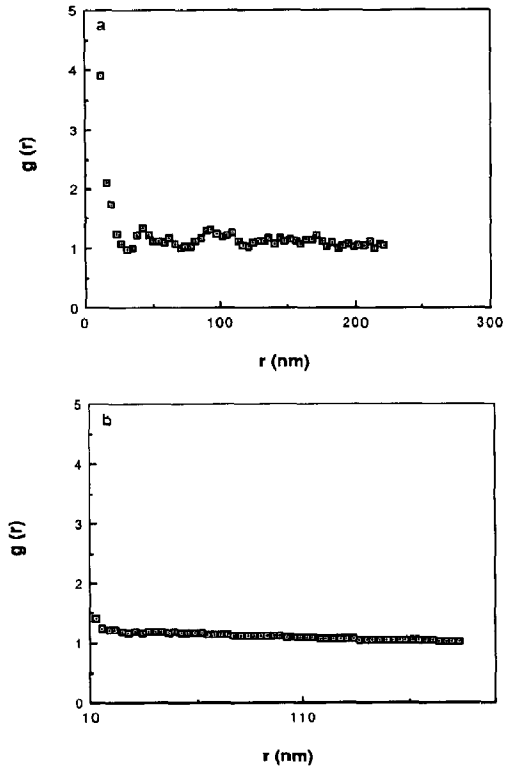

Fig. 4. Pair correlation function $g(r)$ measured for clusters appearing in fig. 1. (a) Pair correlation function $g(r)$ measured on fig. 1c. (b) Pair correlation function $g(r)$ measured on fig. 1i.

appeared as a confluent percolated structure. The surface concentration of adsorbed ferritin at percolation, $0.45 \pm 0.05 \times 10^{12}$ molecules/cm^2, corresponds to $\Theta = 0.45 \pm 0.05$, since a monolayer of dense packed ferritin has a surface concentration of 1.0×10^{12} molecules/cm^2.

The mass distribution of adsorbed ferritin molecules $c(r)$ was determined after adsorption from bulk concentrations of 1 mg/l and 10 g/l (fig. 3). The clusters formed during adsorption of ferritin from a bulk concentration of 1 mg/l have a fractal dimension $D = 0.9 \pm 0.1$ when averaged over all clusters (fig. 3a). The corresponding value for the dense layer of adsorbed ferritin was $D = 1.8$ (fig. 3b).

The pair distribution function $g(r)$ for ferritin molecules adsorbed from bulk concentrations of 1 mg/l and 10 g/l is shown in fig. 4a and b, respectively. In fig. 4a, an increased probability of finding nearest neighbour molecules compared to a random distribution can be seen at distances less than 30 nm, indicating aggregation of molecules. The distribution of molecules over longer distances is close to random.

The pair distribution of ferritin adsorbed from the highest bulk concentration shows a decreasing probability of finding nearest neighbours over a long distance (fig. 4b).

The coordination numbers, cn, of the clusters are listed in table 1. The average number of nearest neighbour molecules is low initially, when adsorbed ferritin appears as single molecules and pairs, but an increase in cn is observed at higher surface concentrations in the diffusion-rate-limited aggregation. The coordination number reaches a stable level at cn = 2.6 and does not increase with increasing bulk concentration over four decades.

4. Discussion

The results of the present study show that the adsorption of ferritin is a transient process after 20 h adsorption time from bulk concentrations less than 1 mg/l. The rate of adsorption is limited by the mass transport of ferritin to the surface which implies that the concentration of ferritin in the solution close to the surface is zero. In spite of this fact, adsorbed molecules are stably bound for hours, indicating that ferritin like other proteins is strongly adsorbed to the surface at low surface concentrations. The clusters formed during the diffusion-limited aggregation have low fractal dimensions in accord with previous findings for the initial adsorption from higher bulk concentrations after short incubation periods [7,13]. The lateral aggregation of molecules at the surface at low surface concentrations is seen in the pair correlation function $g(r)$ which reveals an overrepresentation of nearest neighbours within a distance of 30 nm. It can be noted that no evidence of repulsive interaction is found.

At higher surface concentrations, the adsorbed protein layer is sensitive to rinsing during preparation of the specimen. This finding is consistent with results reported by others [14], and with our findings regarding the kinetics of ferritin adsorption [7] and indicates that the dissociation of bound ferritin is dependent on the surface concentration of bound protein. In the present study, fixation with glutaraldehyde was used in order to enable studies of the properties of the adsorbed ferritin layer at the equilibrium established during adsorption at high bulk concentrations of ferritin. It was thus possible to measure the mass distribution $c(r)$ and the pair correlation function $g(r)$. For the dense layer, the collection of all clusters was characterised by a fractal dimension $D = 1.8$ and the layer was found to be inhomogeneous over long distances. This is typical for percolating clusters [15] and the adsorbed ferritin layer can be regarded as a percolated layer at a surface concentration corresponding to $\Theta = 0.45 \pm 0.05$. It is worth noting that the coordination number, cn, did not change during percolation. This finding is in accord with the result of a previous study showing that clusters with coordination numbers greater than 2.6 were not stable, but underwent reorganization. The result of the present study indicates that such reorganization may be important for the concentration dependence of ferritin adsorption. The adsorption isotherm presented in this study can be explained by assuming that dissociation of adsorbed ferritin is a critical phenomenon which demands high coordination numbers. According to this assumption, dissociation of

ferritin at low surface concentrations and low bulk concentrations during the diffusion-rate-limited adsorption can only occur via lateral diffusion and aggregation. Lateral diffusion of ferritin has been estimated by computer simulation of aggregation [16] and was found to be a factor 1000 slower than diffusion in the bulk. This explains the irreversible binding of the initially adsorbed protein and the apparently slow dissociation rate during rinsing with buffer and the sudden dissociation from large clusters reported earlier [7]. At higher bulk concentrations, where the adsorption process is reaction-rate-limited, protein is present in the bulk close to the surface, increasing the rate of aggregation at the surface. The dissociation rate may then increase with the larger coordination number of the aggregates, thus creating a structure-dependent dynamic equilibrium. The suggested mechanism of critical dissociation is consistent with other experimental findings showing that there is a measurable exchange of protein between surface and bulk while these remain in contact [17–19], where adsorption is apparently irreversible in salt solutions.

Acknowledgements

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