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TWO DIMENSIONAL, SPATIAL ARRANGEMENT OF FIBRONECTIN ADSORBED TO BIOMATERIALS WITH DIFFERENT WETTABILITIES

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Abstract

The effects of concentration, pH and substratum wettability on the two dimensional, spatial arrangement of adsorbed fibronectin are determined. Substrata with different wettability were exposed to a well defined flow of a fibronectin solution (0.1 or 1 mg/ml) in potassium phosphate buffer (pH 7.0 or 4.8) during 120 minutes at a shear rate of 20 s⁻¹. Rotary shadowed replicas of the surfaces were examined by transmission electron microscopy. Well defined structures, island-like in character on the low wettability substrata and knotted, reticulated on the high wettability substrata, could be seen in case of adsorption from pH 7.0 and the high concentration solution. Structures became more blurred upon lowering the solution pH and fibronectin concentration. Compared with bovine serum albumin, fibronectin shows smaller island-like structures, but the reticulated structure is thicker than for albumin.

Key words: Fibronectin, adsorption, wettability, biomaterials.

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Introduction

It is a generally accepted notion that proteins adsorb patchwise to solid surfaces (Rudee and Price, 1985; Park *et al.*, 1988). The patchwise character of adsorbed protein layers is often neglected however, mainly due to experimental limitations. The use of, for example, radiolabeled proteins, ellipsometry, or X-ray photoelectron spectroscopy does not offer a straightforward means to account for patchwise adsorption and only yields an estimate of the total number of monolayers that can be stacked given a certain amount of adsorbed proteins.

Analysis of the spatial arrangement of adsorbed proteins is extremely important with regard to the biocompatibility of biomaterials: patchwise adsorption of proteins may allow cells or bacteria to directly contact the biomaterials surface, whereas in case of a continuous, homogeneously adsorbed protein film, the biomaterials surface is effectively shielded from cells or bacteria (Busscher *et al.*, 1989; Schakenraad and Busscher, 1989). It has been suggested that patchwise protein adsorption accounts for the fact that substratum properties can be transferred by an adsorbed protein film to the interface with adhering cells (Schakenraad and Busscher, 1989) or bacteria (Busscher *et al.*, 1989).

The tendency of proteins to adsorb patchwise depends on the experimental conditions and on the type of protein involved. Park *et al.* (1988) adsorbed fibrinogen and albumin to glass beads and eluted them from the surface by sonication in the presence of Sodium Dodecyl Sulphate. From the elution patterns they concluded that patchwise adsorption on glass was a unique feature of fibrinogen but not of albumin. This is in agreement with our recent transmission electron microscopy (TEM) observations on replicas of albumin adsorbed on glass, showing adsorption in a homogeneous, well distributed, fine knotted reticulated structure (Uyen *et al.*, 1990). The tendency of albumin to adsorb in patchwise structures increased with decreasing substratum wettability and on polytetrafluoroethylene (PTFE-Teflon) only island-like structures were found. In addition electron microscopy observations by Rudee and Price (1985) showed that albumin on Teflon,

polystyrene and polycarbonate, all low to medium wettable substrata, is less homogeneously arranged than on highly wettable substrata as glass and mica.

The occurrence of island-like structures will most likely be determined by a complicated interplay of protein-substratum interactions, adsorbed protein-adsorbed protein interactions, and the prevailing hydrodynamic conditions.

Whereas in a previous study (Uyen *et al.*, 1990) we have focussed on the influence of substratum wettability, protein concentration, and hydrodynamic conditions on the two dimensional, spatial arrangement of adsorbed albumin, it is the aim of the present work to determine the influence of substratum wettability, protein concentration and pH during adsorption on the spatial arrangement of adsorbed fibronectin. Results will be compared with those previously obtained for albumin.

Materials and Methods

Proteins and solid substrata. Fibronectin was obtained from the Red Cross Blood Transfusion Service, Amsterdam, the Netherlands. This partially purified fibronectin is a byproduct obtained during purification of human Factor VIII. This fibronectin was further purified over a Sephadex G200 column (Pharmacia, Sweden) and checked for purity by gradient SDS-Page.

Purified fibronectin was dissolved in PBS (phosphate buffered saline; 150 mM sodium chloride and 10 mM potassium phosphate) in two concentrations (0.1 mg/ml and 1.0 mg/ml) at either pH 7.0 or pH 4.8.

Three solid substrata were selected for this study on the basis of their different wettabilities and included polytetrafluoroethylene PTFE (Teflon type 701 M Du Pont), polymethylmethacrylate PMMA (Casterpex, ICI) and glass (microscopic slides, Menzel). Samples were cut in pieces of 10x15x1 mm and polished, if necessary, to obtain a stylus surface roughness $R_A < 0.1 \mu\text{m}$. Subsequently samples were washed extensively (as described in detail previously, Uyen *et al.*, 1990) to give water contact angles of 109°, 69° and 15° for PTFE, PMMA and glass, respectively.

Fibronectin adsorption. In this study, a rectangular flow cell was used for protein deposition experiments. The dimensions of the flow chamber were 0.9x4.5 cm, while the separation distance between the plates was fixed by spacers to 0.3 cm. Samples were positioned in the center of the bottom plate of the flow cell. The protein solution flowed under the influence of hydrostatic pressure and was recirculated by a roller pump. The flow rate during the experiments was adjusted to 0.27 ml/s, yielding a shear rate of 20 s⁻¹.

Prior to flowing with the protein solution, air was purged out of the system by flushing with PBS. Using a valve system, as described in detail by Pratt-Terpstra *et al.* (1987), the flow could be switched from PBS to the protein solution without air entering the flow system again. After the adsorption period, which was kept constant at 120 minutes, the system was again switched to PBS (3 minutes at the same shear rate as did the protein solution) in order to remove unbound proteins.

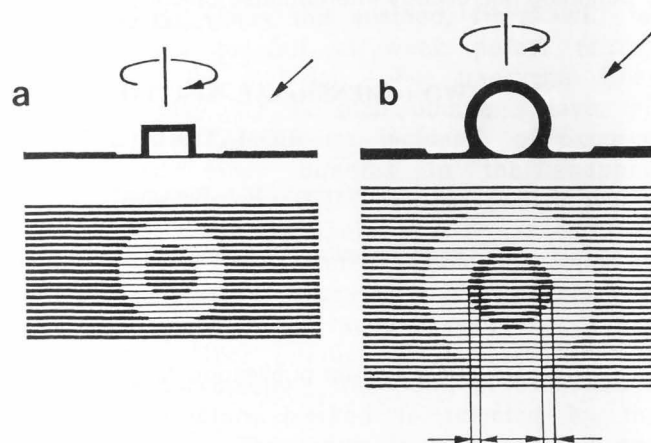


Figure 1. Illustration of the contrast phenomena that can be observed with rotary shadowed replicas in TEM. The thick lines represent electron dense material collected on a rectangular (a) and spherical (b) object during rotary shadowing. Absorption of electrons occurs preferentially on the sides of the objects due to the longer path of the transmitted electrons through electron dense material. Less electron dense material is collected in the shadow zone of an object. Note the complicated contrasts in the images arising from these simple objects indicated schematically below the objects.

For evaluation of the two dimensional, spatial arrangement of deposited fibronectin, adsorbed proteins were fixed with 2% glutaraldehyde for two minutes and subsequently rinsed with water at identical shear rates as during the experiment, prior to removal from the flow cell and drying in air. Since the cell was flushed with buffer and adsorbed proteins were fixed prior to removal of the samples from the flow cell at the same shear rate as operating during the experiments, we believe that beading up of solution droplets during drying does not happen and that therefore the possibility of creating artefacts in the electron micrographs is minimized.

Two dimensional, spatial arrangement of adsorbed fibronectin. The two dimensional, spatial arrangement of adsorbed fibronectin was examined using TEM. Replicas of the adsorbed protein surface were made by rotary shadowing 2 nm of platinum/carbon on the protein layer at an angle of incidence of 10° using an evaporating device (Balzers BAE 120) equipped with an electron beam source (EVM 052 Pt/C) and a quartz crystal monitor (Balzers QSG 201D) giving a distance between surface and source of 150 mm. A 15 nm thick carbon layer was sputtered perpendicularly on top of this layer to increase the strength of the final replica. In Fig. 1 the contrast phenomena that can be observed with this type of replicas in TEM are explained.

Removal of this double layer was easy in case of glass and PMMA. The replica could be stripped off the glass surface after immersion of the sample in 0.5 M potassium hydroxide for several hours, whereas in case of PMMA 100% acetone was employed.

Removal of the double layer was much more

difficult in case of PTFE and a procedure described by Eberhart *et al.* (1977) was adapted. Briefly, a 1% Formvar/chloroform solution was applied on top of the replica. After drying, the complete layer, including the replica, was pulled off the PTFE surface and the Formvar mass was removed by washing in chloroform.

Subsequently, the glass-, PMMA- and PTFE replicas were washed in distilled water and treated with chromic acid for several hours to remove the fibronectin. A final rinsing was done in distilled water prior to mounting the replicas on 300 Mesh EM copper grids for examination in an Akashi 002A-ISI Transmission Electron Microscope.

Results

Fig. 2 is a transmission electron micrograph of replicas from the uncoated substratum surfaces, serving as a control for sampling handling and for the ease of comparison with protein coated substrata.

Transmission electron micrographs of adsorbed fibronectin to the various solid substrata are shown in Figs. 3-5. Fig. 6 presents a comparison of the two dimensional, spatial arrangements of adsorbed fibronectin with those of adsorbed bovine serum albumin. It is clear from these micrographs, that island-like structures appear mostly on the lower surface free energy substrata (PTFE-Teflon and PMMA), while reticulated structures predominate on glass.

The effect of fibronectin concentration on the spatial arrangement of adsorbed proteins is evident from a comparison of Figs. 3a-c and 3d-f: both the island-like structures as well as the reticulated structures occurring look more stalky in case of adsorption from the high concentration solution, while a little blurred in case of the low concentration solution.

The effect of pH is not very clear. Fig. 4, representing fibronectin adsorption from a low concentration solution shows similarly blurred images as in Figs. 3a-c. Blurred island-like structures on PTFE-Teflon turn into stalky, reticulated structures upon lowering pH, but oppositely turn into well-defined island-structures on PMMA. Also on glass upon lowering the pH, blurred dots of proteins come into existence.

The effect of lowering the pH is shown in Fig. 5 for a high concentration fibronectin solution. The effects are essentially the same as observed in Fig. 4 for the low concentration solution.

Fig. 6 compares the spatial arrangement of adsorbed bovine serum albumin (BSA) and fibronectin. For BSA it is more obvious than for fibronectin that the tendency to form island-like structures gradually disappears while going from PTFE-Teflon and PMMA to glass. BSA island-like structures are also larger than for fibronectin (compare Figs. 6a and 6d), although the reticulated structures are thicker for fibronectin than for BSA (compare Figs. 6c and 6f).

Discussion

In this paper we studied the adsorption behaviour of fibronectin on surfaces with different wettability with

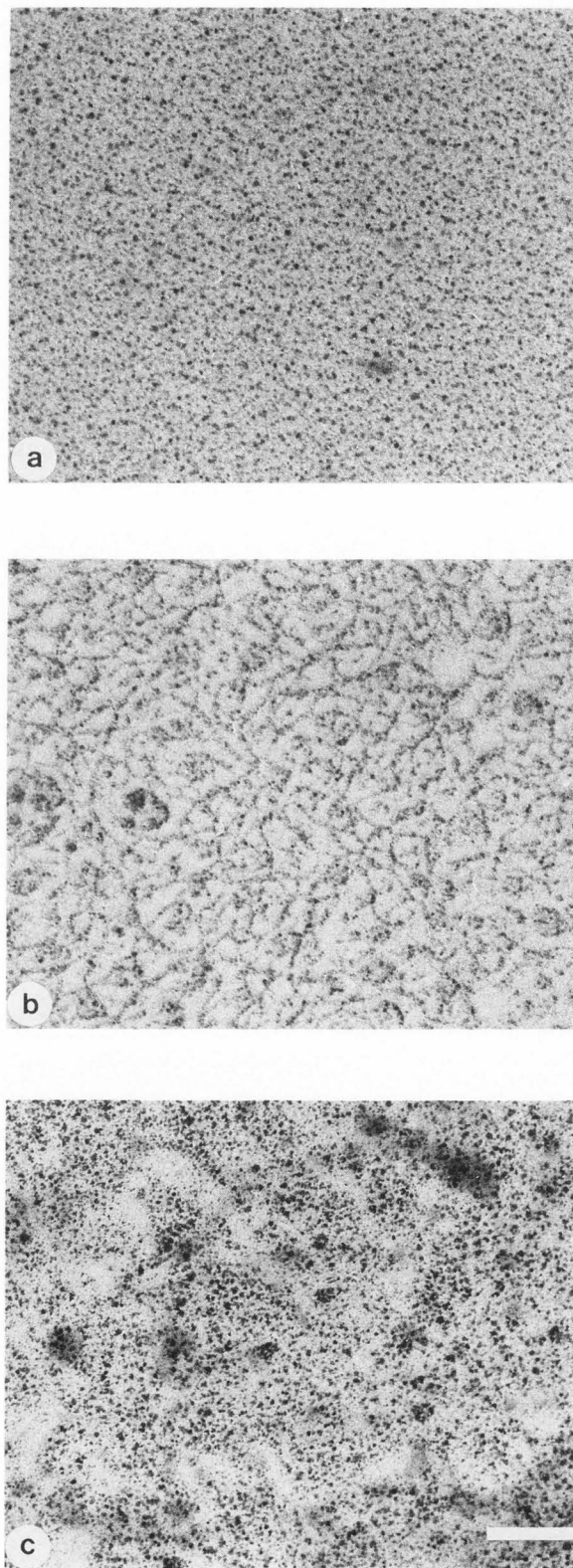


Figure 2. Transmission electron micrographs of rotary shadowed replicas of uncoated substrata: PTFE-Teflon (a), PMMA (b) and glass (c). Bar = 100 nm.

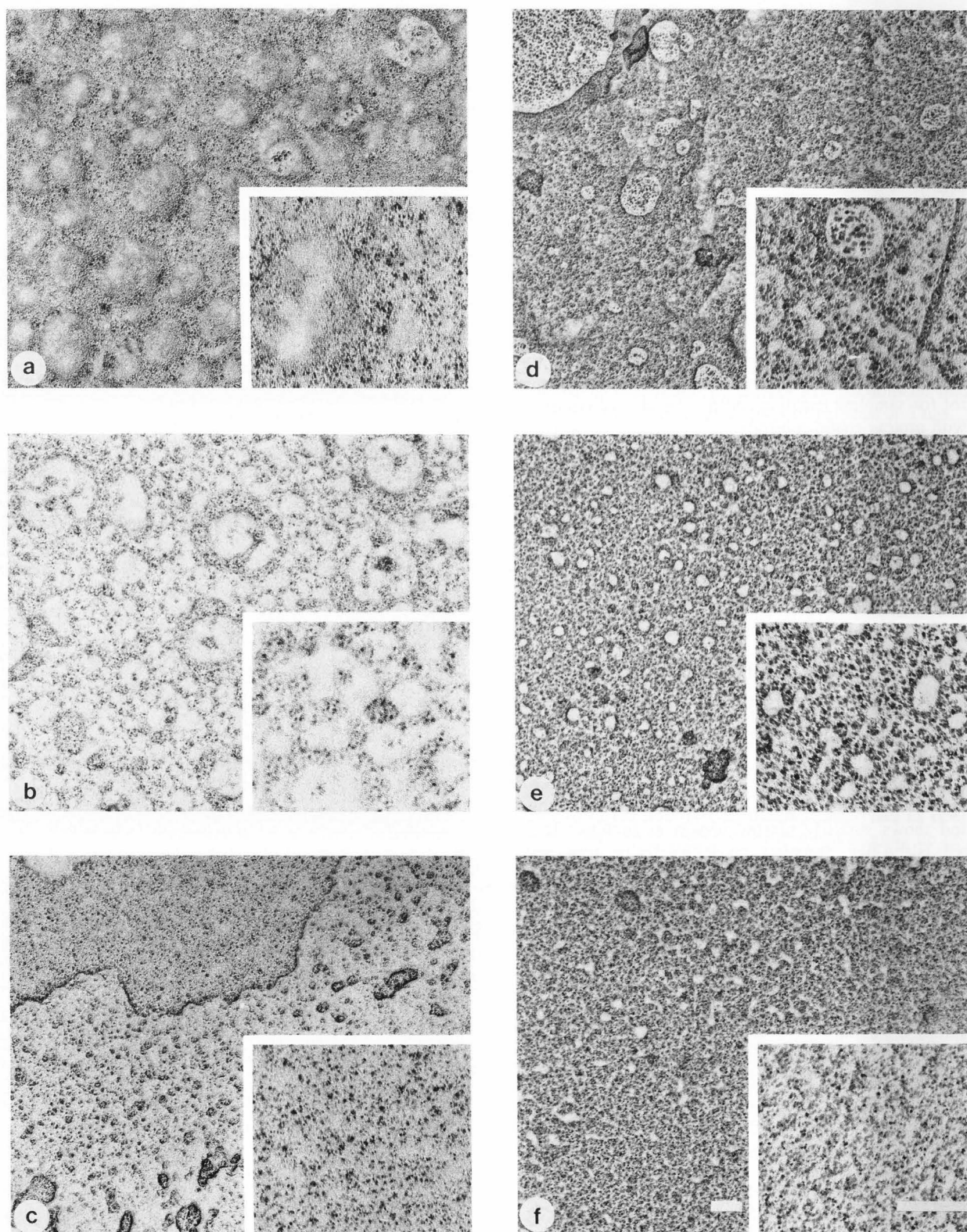


Figure 3. The effect of fibronectin concentration on the two dimensional, spatial arrangement of adsorbed proteins: replicas were made after 120 minute exposure of PTFE-Teflon (a, d), PMMA (b, e) and glass (c, f) to a flowing solution of fibronectin in PBS (pH 7.0) at a shear rate of 20 s^{-1} . a, b, c : 0.1 mg/ml; d, e, f : 1 mg/ml. The insets are higher magnification details of the electron micrographs. The bar markers denote 100 nm.

Fibronectin Adsorption

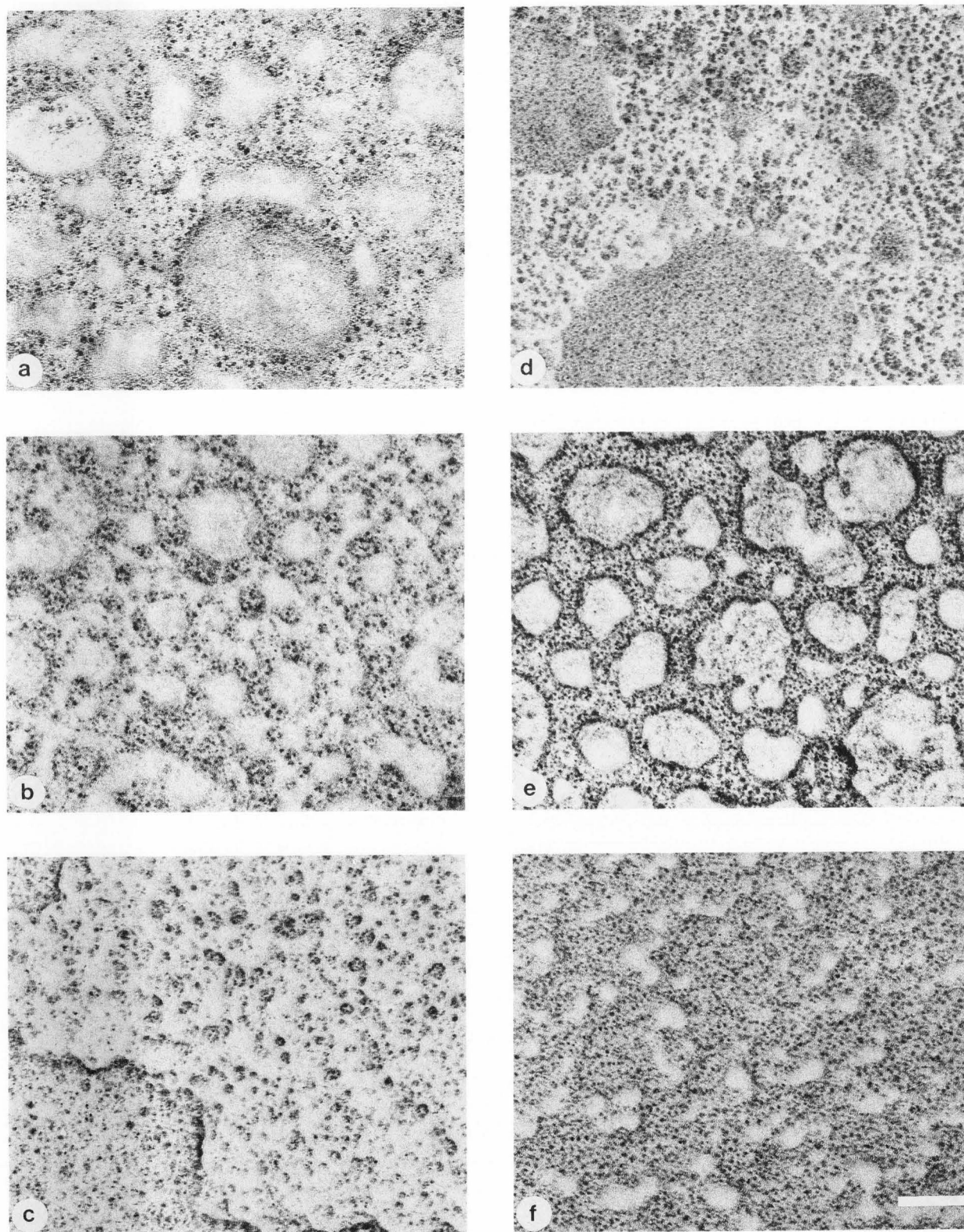


Figure 4. The effect of pH on the two dimensional, spatial arrangement of fibronectin adsorbed from a low concentration (0.1 mg/ml) solution in PBS: replicas were made after 120 minute exposure of PTFE-Teflon (a, d), PMMA (b, e) and glass (c, f) to a flowing solution at a shear rate of 20 s^{-1} . a, b, c : pH 7.0; d, e, f : pH 4.8. The bar marker denotes 100 nm.

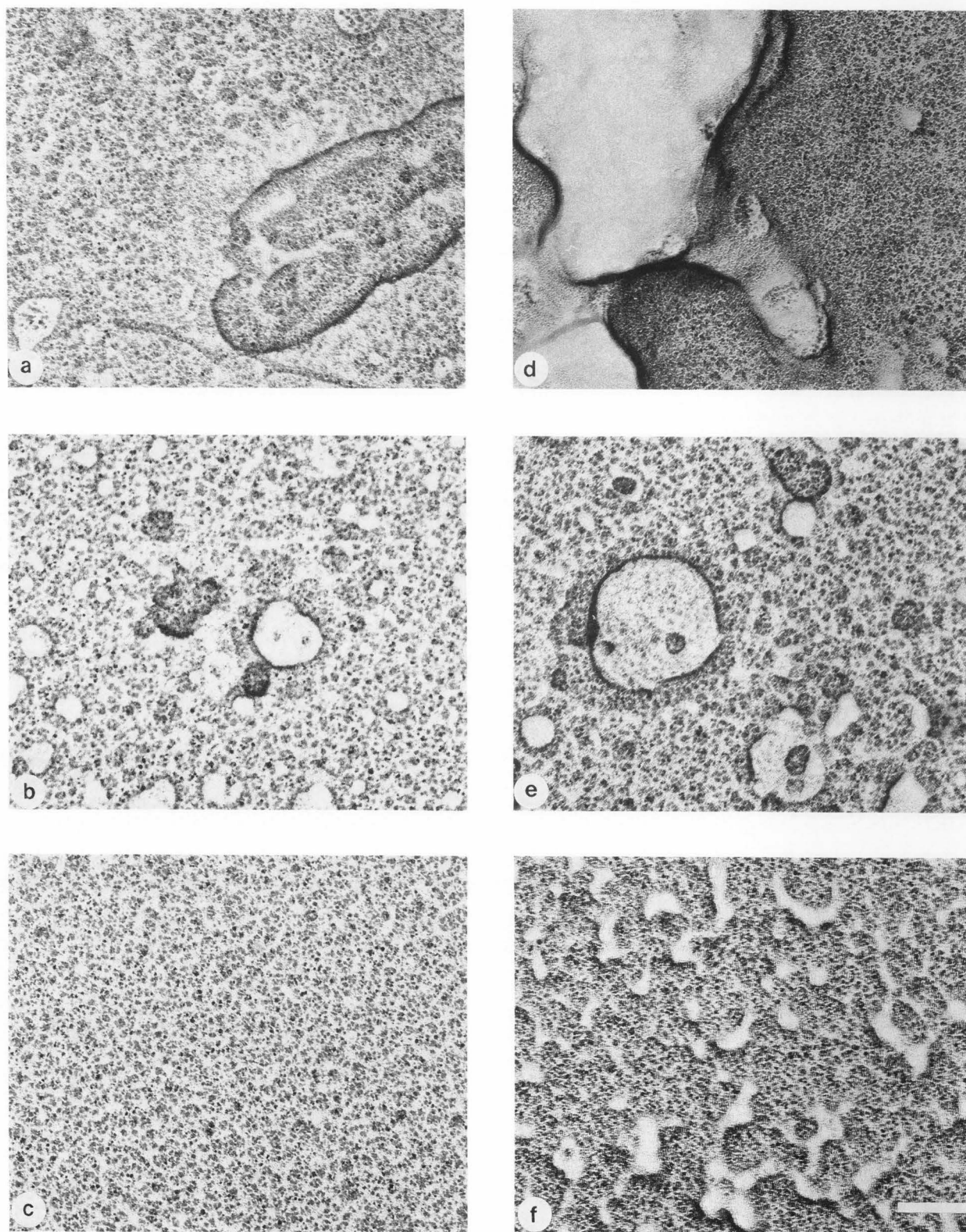


Figure 5. The effect of pH on the two dimensional, spatial arrangement of fibronectin adsorbed from a high concentration (1 mg/ml) solution in PBS: replicas were made after 120 minute exposure of PTFE-Teflon (a, d), PMMA (b, e) and glass (c, f) to a flowing solution at a shear rate of 20 s^{-1} . a, b, c : pH 7.0; d, e, f : pH 4.8. The bar marker denotes 100 nm.

Fibronectin Adsorption

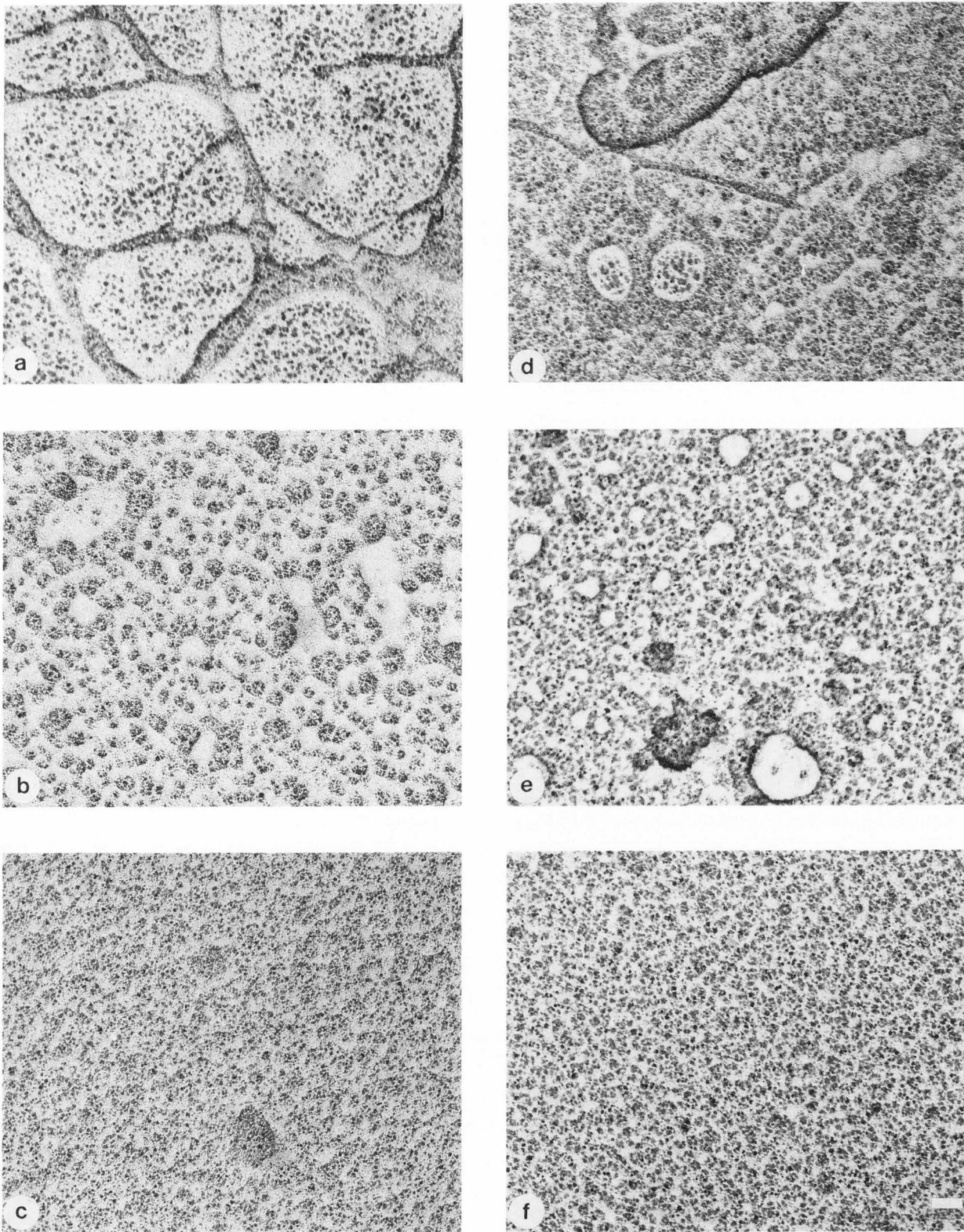


Figure 6. A comparison of the two dimensional, spatial arrangement of adsorbed bovine serum albumin (3 mg/ml) and fibronectin (1 mg/ml): replicas were made after 120 minute exposure of PTFE-Teflon (a, d), PMMA (b, e) and glass (c, f) to a flowing solution of proteins in PBS (pH 7.0) at a shear rate of 20 s^{-1} . a, b, c : bovine serum albumin; d, e, f : fibronectin. The bar marker denotes 100 nm.

respect to its two dimensional, spatial arrangement. The technique employed justifies doubts on whether the spatial arrangement of adsorbed proteins, as existing during the experiment, is indeed preserved during drying. Evidence in support of our point of view that this is the case, has recently been obtained from an analogy observed between the spatial arrangements of adsorbed proteins and adhering bacteria. It was found that the occurrence of island-like structures in protein adsorption on low energy surfaces was concurrent with a high collection of near-neighbour sites in bacterial adhesion (Busscher *et al.*, 1990). Since bacterial adhesion was observed *in situ*, i.e. in the liquid phase without drying, this was taken as an argument to exclude (or minimize) the possibility that island-like structures in protein adsorption are artefacts.

One of the important aspects of protein adsorption to biomaterials is that protein adsorption precedes any other events. The ambivalent nature of the adsorbed protein layer therefore determines the ultimate biocompatibility of biomaterials. Island-like structures in an adsorbed fibronectin layer may act as high affinity sites for cells to adhere. Also patchwise adsorption may enable cells or bacteria to directly contact the biomaterials surface without any intervening influence of the adsorbed protein layer.

Fibronectin concentration in solution did not greatly affect the spatial arrangement of the adsorbed layer. This seems to rule out the possibility that fibronectin aggregates in the bulk solution and adsorbs as aggregates (Park *et al.*, 1988) since aggregate formation is concentration dependent whereas the spatial arrangement of adsorbed fibronectin can hardly be called so. Surface diffusion of adsorbed proteins remains in our opinion a likely explanation for the formation of the spatial arrangements observed.

Lateral interactions between adsorbed fibronectin as well as the interactions between proteins and substratum consist mainly of attractive Lifshitz-van der Waals forces and repulsive electrostatic forces. Lateral interactions can, in addition to surface diffusion (Michaeli *et al.*, 1980), enhance the movement of proteins over the surface. Principally, we would expect more homogeneous spatial arrangements at pH 7.0, due to repulsive electrostatic lateral interactions than at pH 4.8, which is close to the isoelectric point of fibronectin. At the isoelectric point only attractive lateral interactions exist, which may enhance surface aggregation. The electron micrographs taken so far however, neither confirm nor contradict the above expectations. The differences in the micrographs for pH 7.0 and 4.8 point more to an alteration of the protein molecules themselves rather than to a major influence on their spatial arrangement. A more extensive unfolding of the large (molecular weight 220,000) fibronectin molecular at pH 4.8 most likely accounts for the blurred images at this pH.

With the present technique, it is easily possible to discern individual proteins: both the dimensions of albumin (4x14 nm, Peters, 1975) as well as of fibronectin (approximately 8 x 24 nm, as estimated from a comparison of the molecular weights of fibronectin and albumin, whose dimensions are known) are within the resolution limit. The larger dimension of fibronectin may account for the thicker reticulated structures seen for this protein.

Finally image analysis and statistical evaluation of the two dimensional, spatial arrangements of fibronectin from the transmission electron micrographs at the magnifications used in this study are not feasible at present. Maybe the method of radial and angular distribution analyses, as recently developed by Sjollem *et al.* (1990a, b) for the analysis of the spatial arrangement of bacteria adhering to solid substrata, can also be adapted for protein adsorption, although in this case immunogold staining (Park *et al.*, 1986) of adsorbed proteins would probably be a better technique to visualize the proteins.

Acknowledgements

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References

- Busscher HJ, Weerkamp AH, Van der Mei HC, Van Steenberghe D, Quirynen M, Pratt-Terpstra IH, Marechal M, Rouxhet PG (1989). Physico-chemical properties of oral streptococcal cell surfaces and their relation with adhesion to solid substrata *in vitro* and *in vivo*. *Col. Surf.* **42**, 345-353.
- Busscher HJ, Van der Mei HC, Schakenraad JM (1990). Analogies in the two dimensional, spatial arrangements of adsorbed proteins and adhering bacteria: Bovine serum albumin and *Streptococcus sanguis* 12. *J. Biomat. Sci.* in press.
- Eberhart RC, Prokop LD, Wissenger J, Wilkov MA (1977). Observation of albumin deposits on Teflon surfaces. *Trans. Am. Soc. Artif. Intern. Organs.* **XXIII**, 134-139.
- Michaeli I, Absolom DR, Van Oss CJ (1980). Diffusion of adsorbed protein within the plane of adsorption. *J. Col. Interf. Sci.* **77**, 685-687.
- Park K, Albrecht RM, Simmons SR, Cooper SL (1986). A new approach to study adsorbed proteins on biomaterials: Immunogold staining. *J. Col. Interf. Sci.* **111**, 197-212.
- Park K, Gerndt SJ, Park H (1988). Patchwise adsorption of fibrinogen on glass surfaces and its implication in platelet adhesion. *J. Col. Interf. Sci.* **125**, 702-711.
- Peters T Jr. (1975). Serum Albumin. In: "Plasma protein structure, function and genetic control (Putman FW, Ed.), Academic Press, New York, pp. 133-181.
- Pratt-Terpstra IH, Weerkamp AH, Busscher HJ (1987). Adhesion of oral streptococci from a flowing suspension to uncoated and albumin coated surfaces. *J. Gen. Microbiol.* **133**, 3199-3206.
- Rudee ML, Price TM (1985). The initial stages of adsorption of plasma derived proteins on artificial surfaces in a controlled flow environment. *J. Biomed. Mat. Res.* **19**, 57-66.
- Schakenraad JM, Busscher HJ (1989). Cell-polymer interactions: the influence of protein adsorption. *Col. Surf.*

42, 331-343.

Sjollema J, Busscher HJ (1990a). Deposition of polystyrene particles in a parallel plate flow cell: Pair distribution functions between deposited particles. *Col. Surf.* 47, 337-352.

Sjollema J, Van der Mei HC, Uyen HWM, Busscher HJ (1990b). Direct observation of (non-)cooperative effects in oral streptococcal adhesion to glass by analysis of the spatial arrangement of adhering bacteria. *FEMS Microbiol. Lett.* 69, 263-270.

Uyen HMW, Schakenraad JM, Sjollema J, Noordmans J, Jongebloed WL, Stokroos I, Busscher HJ (1990). Amount and surface structure of albumin adsorbed to solid substrata with different wettabilities in a parallel plate flow cell. *J. Biomed. Mat. Res.*, 24, in press.

Discussion with Reviewers

R.C. Eberhart: From kinetics, we know that these proteins both rapidly adsorb (about 1 minute to obtain significant surface coverage). Therefore, by 1 hour, there has been ample time for conformational changes and protein-protein interactions. Can you comment about the surface structure at the early phase?

Authors: From a similar study that we have conducted with salivary proteins and dental enamel [Busscher HJ, Uyen HMW, Stokroos I, Jongebloed WL, (1989) A transmission electron microscopy study of the adsorption patterns of early developing artificial pellicles on human enamel. *Archs. oral Biol.* 34, 803-810] we know that a homogeneous protein film indeed develops within 1 minute. The structural features develop later, approximately within 10 minutes. Whether this is also valid for albumin and fibronectin cannot be said with certainty at present.

K. Park: It is not clear why the pH change alters the island-like structures to reticulated ones on PTFE alone but turns them into more well defined island-like structures on PMMA. Do the authors have any explanation on the effect of pH at all?

Authors: The short, honest answer is "no". Surely the development of island-like structures depends on the relative magnitude of van der Waals forces and electrostatic forces between substratum-protein and protein-protein. Since only electrostatic forces are affected by pH changes, we can envisage different effects on PTFE than on PMMA, dependent on the magnitude of the van der Waals forces.

S.R. Simmons: I have difficulty in visualizing where the protein is in some of the micrographs. Indications on the micrographs would be helpful.

Authors: It is indeed tempting to indicate the protein in the micrographs by, for instance, arrows. Realizing the complexity of image formation by shadowing techniques (see Fig. 1, text) which is, in addition, dependent on the shape of the object, we feel this would be too dangerous.