A New Approach to Study Adsorbed Proteins on Biomaterials: Immunogold Staining[1]

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Immunogold staining techniques were applied to study the adsorption of fibrinogen and albumin on polymer surfaces. Colloidal gold particles were coated with antibodies (immunogold) and used to stain protein molecules adsorbed on surfaces. The two-dimensional distribution of immunogold particles was visualized by scanning electron microscopy. Since immunogold staining relies on antigen–antibody reactions, the distribution of fibrinogen and albumin on polymer surfaces could be observed even when both proteins were adsorbed simultaneously. The specific reaction also allows investigation of the change in orientation or conformation of one protein in the presence of the others.

The surface distribution of fibrinogen and albumin was also examined on polymer surfaces which were exposed to blood in a canine *ex vivo* experiment. It was found that the sites where thrombi had embolized contained much less fibrinogen and albumin on them compared to portions of the surface where thrombi had not yet formed.

INTRODUCTION

The study of protein adsorption at solid–liquid interfaces has been stimulated by the importance of protein adsorption on materials used for biomedical applications. Protein adsorption onto blood contacting biomaterials and its role in surface-induced thrombosis has been investigated extensively. It has been shown that the adsorption of blood proteins occurs immediately upon exposure of a biomaterial to blood (1–3) which suggests that the protein film largely influences thrombogenicity (4). Indeed, it has been shown that platelet deposition on polymer shunts can be significantly influenced by the nature of preadsorbed proteins (5).

Protein adsorption onto biomaterials has been investigated using various techniques, such as radiolabeling (6), infrared internal reflection spectroscopy (7), total internal reflection fluorescence (8), and ellipsometry (9). Most techniques, however, measure only the average surface concentrations and provide little information as to distribution and conformation of adsorbed protein (2). The data on adsorbed protein layer thickness measured by ellipsometry can complement the information on surface protein concentration to give an assessment of protein conformation (10). Conformational changes of adsorbed proteins have been examined by transmission circular dichroism (11), hydrodynamic thickness measurements (12), quasielastic light scattering (13), scanning and transmission electron microscopy (14), and proton magnetic relaxation (15). In addition to these methods, the conformational change of adsorbed proteins has also been indirectly studied by measuring changes in their functional properties. These techniques involve measurements of electrophoretic mobility (16), enzyme activity (7), antigenicity (17), cell adhesion and spreading behavior (18) and potentiometric titration (19).

Despite the extensive research on protein adsorption and adsorbed protein conforma-

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tional changes, there is little published information on the two-dimensional distribution of adsorbed proteins. Protein surface distribution has been investigated by a few techniques, such as X-ray photoelectron spectroscopy (XPS) (20), partial gold decoration transmission electron microscopy (21), immunoferritin transmission electron microscopy (21), and modified negative-staining electron microscopy (7). The last two techniques require surface replication procedures which may introduce several artifacts in the final image produced in the electron microscope (7). Although XPS is a powerful technique, direct observation of specific proteins is not achieved.

In this report, we describe immunogold staining which allows visualization of the two-dimensional distribution of adsorbed protein molecules. The technique is simple and does not require a surface replication procedure.

BACKGROUND

Colloidal gold is an electron dense, negatively charged hydrophobic sol whose stability is maintained by electrostatic repulsion (22). Colloidal gold particles absorb visible light displaying a single peak of absorption (λ_{max}) between 520 and 550 nm. The shape and position of the peak is influenced by the mean size and shape of the marker population. In general, the λ_{max} moves to a longer wavelength with increasing mean particle diameter (23).

At high salt concentration, colloidal gold particles aggregate due to reduced charge repulsion. Colloidal sols can be stabilized even at high salt concentrations by the adsorption of macromolecules on the surfaces of the particles. The effectiveness of various polymers as stabilizers is determined by the gold number (24). Colloidal gold granules have been labeled with a variety of substances of biological interest, such as staphylococcal protein A (25), fibrinogen (26), lectins (27), or purified antibodies (28, 29), for cytochemical uses. The carrier molecules are adsorbed onto colloidal gold particles by noncovalent electrostatic adsorption, and the proteins immobilized on gold particles have been shown to maintain their specific biological functions. Although colloidal gold has been used as a tracer for electron microscopy for more than 20 years (30), it was only recently that the basic method was established for adsorbing proteins to colloidal gold (23, 28, 31–34). The adsorption of proteins to colloidal gold is known to be influenced by parameters such as particle size, ionic strength, pH, and protein concentration (31, 34). Colloidal gold when coated with antibodies is referred to as immunogold. The antibodies are essentially irreversibly adsorbed on gold sols and maintain their ability to interact with antigens (27). Thus, immunogold particles can bind to protein molecules adsorbed on a surface, if the gold sol carries antibodies specific to the particular protein on the surface. This immunogold staining method has been used for the detection and localization of surface target molecules by light microscopy or both transmission and scanning electron microscopy (32). The size of colloidal gold can be easily controlled and different sizes of immunogold particles with different antibodies can be used simultaneously.

Factors Affecting Immunogold Staining

The size of colloidal gold. The size of the gold particles has several important implications. The accessibility of gold markers to receptors is often dependent upon the size of the probe. Small particles are favorable due to a higher yield of label, although large particles can be more easily observed at lower magnifications. For example, the density of the labeling with a 20-nm colloidal gold particle is much higher than that obtained with a 45-nm marker (35). This finding is interpreted as being due to decreased steric hindrance when markers of smaller size are used (35). Although the binding of an immunogold particle to the target protein is largely irreversible (23), the possibility of dissociation of the probe from the antigen, or the dissociation of the target protein from the surface must always be considered.

Antibody binding to colloidal gold. The orientation and conformation of adsorbed antibody is an important factor in determining the staining efficiency, since the antigen–antibody interaction depends largely on the physical conformation of the two reaction sites (36). A good three-dimensional fit between antigenic determinant and the antigen-binding site is necessary for the maximum binding strength. For such binding, outward extension of F_{ab} portion of an antibody molecule may be a prerequisite. Currently no information is available on the orientation, conformational change, and the extent of denaturation, if any, of antibody molecules adsorbed on gold particles. We can only speculate that presumably half of the protein molecules are adsorbed in a sterically favorable position necessary for the antigen–antibody reaction. Since colloidal gold particles of 20-nm size can adsorb 20–30 antibodies (28), there may be enough antibody present with the proper conformation to react with the antigen.

It is a more difficult problem to determine whether different antibodies adsorb in different orientations and conformations and thus result in different labeling efficiencies. It is expected that different antibodies adsorb to gold particles with approximately the same conformation, since the structures of different antibodies are not so different since they are differentiated only by small changes in amino acid sequence in specialized domains of the F_{ab} regions (37).

Conformation of target protein. The immunogold staining is also affected by factors such as access to the antigenic site and surface charge on the target proteins. Thus, the number of immunogold particles adhering to a protein-coated surface varies depending on the orientation and conformation of the protein, in addition to protein surface concentration. Another factor which is expected to affect immunogold staining efficiency might be the interaction (mostly repulsion) between immunogold particles. Since gold sols are covered with antibodies and polyethylene glycol (see Experimental section), flocculation due to direct contact of bare patches does not occur. When immunogold particles approach and contact, the free energy of repulsion becomes appreciable at about the point of adsorbed layer contact (38). This will increase gradually during interpenetration or compression of the adsorbed antibodies. For this reason, close contact of immunogold particles is not expected, unless under unusual circumstances the repulsion between immunogold particles is overcome.

EXPERIMENTAL

Protein Preparation

Human fibrinogen from Calbiochem–Behring was further purified by the method of Laki (39) and canine fibrinogen was prepared from fresh citrated plasma by the β-alanine precipitation procedure (40). Fibronectin was removed from the purified fibrinogen using a gelatin–Sephadex column (Bio–Rad). The clottability of the purified fibrinogen was at least 97%. Human and canine albumin (Sigma) was used as received. Protein concentrations were measured spectrophotometrically. Absorptivities used for a 0.1% solution at 280 nm were 1.506 and 0.58 for fibrinogen and albumin, respectively. The proteins were dissolved in phosphate buffered saline (PBS, pH 7.4) solution.

Preparation of Colloidal Gold Particles

Colloidal gold particles with an average diameter of 5 nm (Au_5) were prepared as described by Horisberger (32). Three milliliters of 1% tetrachloroauric acid ($HAuCl_4$, Fisher) solution was added to 240 ml of distilled water and the pH was adjusted to 7.0 with 0.2 *N* K_2CO_3. The pH was measured using a gel-filled pH electrode (semimicro comb., Orion). The milliliters of phosphorous saturated white ether solution was added and the mixture was gently shaken for 15 min. The solution was heated under refluxing conditions for 5 min. Ether was evaporated by heating the mixture below boiling after removing the condenser.

Colloidal gold granules having an average diameter of 18 or 50 nm (Au_{18} or Au_{50}) were prepared as described by Loftus and Albrecht (26). One-half ml of 4% $HAuCl_4$ solution was added to 200 ml of deionized distilled water and brought to a boil. Then, a freshly prepared solution of 1% trisodium citrate (4 ml for Au_{18}, and 1 ml for Au_{50}) was rapidly mixed into the boiling solution and the mixture refluxed for 30 min. The formation of the monodisperse colloidal gold particles was indicated by a color change from dark blue to red. The colloidal solution was cooled and stored at 4°C. Before use the pH was adjusted to 7.4 by adding 0.2 N K_2CO_3 and the solution was filtered through a microporous filter (Millipore Millex-HA, 0.45 μm) and stored at 4°C.

Preparation of Antibody-Gold Complex

Rabbit antiserum against human albumin was obtained from Calbiochem–Behring and goat antiserum against human fibrinogen was obtained from Sigma (dialyzed, fractionated serum). Antibody was separated from other serum proteins using a diethylaminoethyl cellulose (DEAE-Sephacel, Sigma) column equilibrated with 0.05 M Tris buffer (pH 8.5). Antiserum was added to the column and antibody was eluted with the same buffer. The concentration of the purified antibody was measured using a protein assay solution (Bio–Rad) and aliquots of antibody were stored at −70°C. The concentration of the purified antibody was adjusted to 150 μg/ml with deionized distilled water (DDW) and was dialyzed against DDW for 2 h. The dialyzed antibody was filtered through a 0.2-μm-pore filter (Nucleopore).

The minimum amount of protein necessary to stabilize the colloidal gold particles was determined from adsorption isotherms (31, 41). A series of protein solutions of increasing concentration was made and 1 ml of colloidal gold was added to 0.1 ml of the protein solution. After 5 min, 1 ml of 10% NaCl was added and rapidly mixed. If the protein was not adsorbed and the gold particles were not stabilized, aggregation of the gold granules was indicated by a color change from red to light blue. The minimum amount of protein which prevented this color change was used. One ml of antibody solution (150 μg/ml) was added to 10 ml of filtered gold solution (pH 7.4). After 5 min, 0.5 ml of freshly made and prefiltered (0.45-μm Millipore) 1% polyethylene glycol (M.W. 20,000) was added to prevent aggregation of protein coated gold particles (32).

The antibody-labeled gold particles were centrifuged in polycarbonate tubes in an angle rotor (Beckman) at 10,000 rpm (average centrifugal force of 8700g) for 30 min. The supernate was discarded and the concentrated labeled gold granules were resuspended in 2 ml of 0.1 M phosphate buffer (pH 7.4). The immunogold solution was stored at 4°C for up to 1 week.

The specificity of the immunogold particles was verified for every preparation. A small but significant number of antialbumin gold particles occasionally bound to fibrinogen adsorbed on polymer surfaces. Those batches of immunogold particles were not used further.

Protein Adsorption on Polymer Surfaces

The polymer tubings used were plasticized polyvinyl chloride (PVC, Tygon, Norton Plastics, 0.125-in. i.d.), polyethylene (Intramedic, 0.125-in. i.d.), and silicone rubber (Silastic, Dow Corning, 0.132-in. i.d.). Polyethylene and silicone rubber were washed with running deionized distilled water for 2 h. The PVC tubing was first washed with 0.1% Ivory detergent before rinsing with running deionized distilled water. Polymer shunts were filled with PBS and kept at 4°C until use. For protein adsorption, the PBS was replaced with the desired protein solution.

Albumin or fibrinogen was adsorbed from single protein solutions (0.3 mg/ml) or from an albumin–fibrinogen mixture (0.3 mg/ml for both proteins). The protein adsorption time was varied from 1 min to 2 h. Albumin and fibrinogen were also sequentially adsorbed onto the polymer shunts. The first protein, ei-

ther albumin or fibrinogen, was adsorbed onto the polymer shunts at room temperature. After a given time period ranging from 1 min to 1 h, unadsorbed protein was flushed out using PBS and then the second protein was introduced into the same polymer shunt. The adsorption time of the second protein was also varied from 1 min to 1 h. Polymer shunts which were coated with protein *in vitro* were labeled with immunogold beads as described below.

The protein-coated polymer shunts were also implanted into a dog and exposed to flowing blood for a predetermined time period before labeling with immunogold beads. The canine *ex vivo* arteriovenous shunt model has been described previously (5). Samples from the *ex vivo* experiment were fixed with 0.1% glutaraldehyde in PBS for 10 min at room temperature before labeling. Residual glutaraldehyde on the sample was neutralized by incubating with 10% glycine in PBS for 10 min. Fixation was necessary to preserve the platelet shape while immunogold labeling.

Labeling the Protein Layer with Immunogold Beads

Three different methods were developed for immunogold labeling as described in Fig. 1.

Method 1. Three different polymer shunts, PVC, polyethylene, and silicone rubber, were connected and preadsorbed with protein as described above. Protein adsorbed shunts were flushed with excess PBS which, in turn, was replaced by the immunogold solution. After 30 min at room temperature, the polymer shunts were flushed with PBS and filled with 2% glutaraldehyde in PBS for 30 min at room temperature. Small segments of the polymer shunts were removed and kept in 2% glutaraldehyde solution overnight at 4°C. This procedure requires a large quantity of immunogold solution and this limits the number of

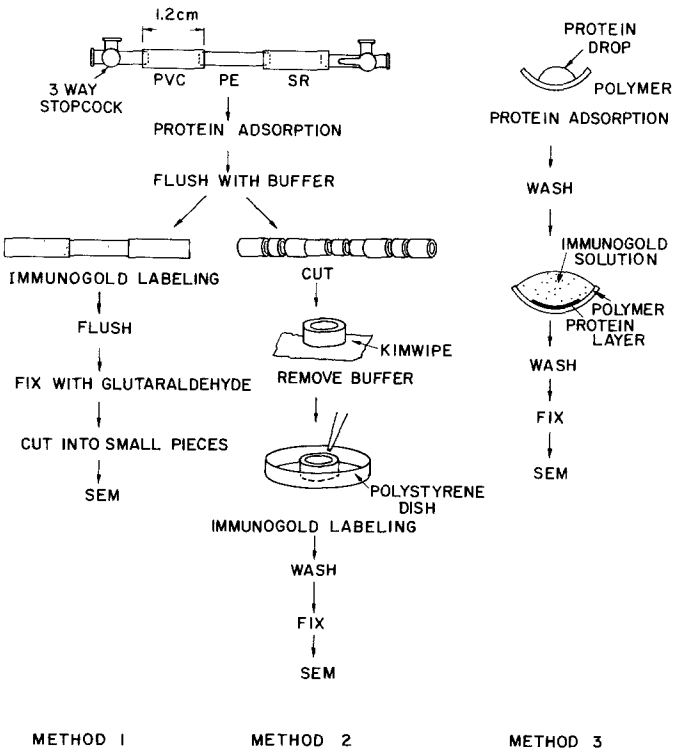

FIG. 1. Procedures for immunogold staining.

samples that can be tested. To overcome this problem, the second method was developed.

Method 2. The polymer shunts were preadsorbed with proteins as in Method 1. After polymer shunts were flushed with PBS, small segments (3 mm in length) were separated from each polymer shunt. The PBS in the polymer segments was removed by touching the bottom of the segment to tissue paper (Kimwipe). The polymer segment was then immediately placed on a polystyrene petri dish and the immunogold solution pipetted into the tubing section. After 30 min of immunogold labeling, the segment was rinsed in PBS and fixed in 2% glutaraldehyde solution. This procedure requires only 20–30 μl of immunogold solution and a large number of samples can be tested.

Method 3. A drop of protein solution was placed on a small piece of a shunt section which was not preexposed to a buffer solution. After a given time period, the protein solution was removed by a pipet, and the surface was washed in buffer solution and labeled with immunogold particles. The segment was then washed with PBS and fixed in a 2% glutaraldehyde solution. Samples from Method 3 were further treated with osmium and thiocarbohydrazide following the Malick and Wilson procedure as described by Murphy (42). This step was carried out to distinguish the protein-coated polymer surface from the bare surface. Method 3 was used to investigate protein adsorption at the air–water–surface interface.

Glutaraldehyde-fixed samples were further treated for scanning electron microscopy (SEM).

Scanning Electron Microscopy

Samples were dehydrated in a graded ethanol series and were dried by the critical point method using molecular sieve-dried CO_2 as the transitional fluid. Samples were sputter-coated with 100 Å of gold or gold–palladium and examined on a JEOL JSM 35C scanning electron microscope (SEM) at 20 kV accelerating voltage.

RESULTS

Conditions for Immunogold Labeling

Size. Immunogold particles of three different sizes were tested for their ability to label the adsorbed protein molecules. Gold markers with an average diameter of 50 nm (Au_{50}) were easy to observe, but the staining efficiency was far lower than that of gold markers of smaller sizes. Au_5 and Au_{18} resulted in the same labeling efficiency, but clear resolution was not achieved with Au_5 due to the small size of the gold particles. Au_{18} was large enough to be observed easily and distinguished from other particles on a surface, especially on the polymer surfaces exposed to blood. Thus, Au_{18} was used for subsequent experiments because of its superior sensitivity and resolution. Au_{50}, however, provided valuable information which Au_{18} did not, as described below.

Labeling time. The staining efficiency of gold markers is a function of the labeling time and the concentration of immunogold particles (Fig. 2). The concentration of gold markers is expressed as the absorbance at 525 nm (A_{525}). The staining efficiency of antifibrinogen gold particles increased until 30 min of immunogold incubation at high concentration (A_{525} = 4.2). Labeling with immunogold par-

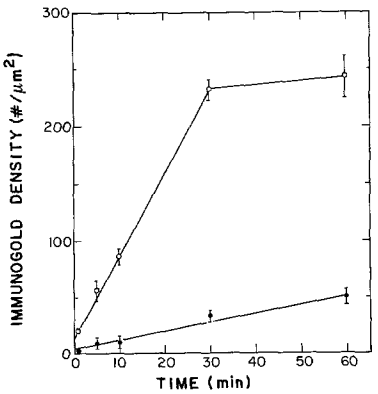

FIG. 2. The density of antifibrinogen-gold particles (Au_{18}) on fibrinogen-coated polyethylene as a function of labeling time. Staining was carried out using Method 2 at gold marker concentrations of A_{525} = 4.2 (○) and A_{525} = 0.42 (●).

ticles at low concentration (A_{525} = 0.42) did not result in the same efficiency over the same time period. The same time dependence of staining efficiency was observed on all three polymer surfaces. Thus immunogold labeling for subsequent experiments was conducted at room temperature for 30 min using the high concentration of gold markers (A_{525} ranged from 3.5 to 4.3).

Two-Dimensional Distribution of Adsorbed Protein Molecules

Topographical distributions of fibrinogen and albumin on polymer surfaces are shown in Fig. 3. It was found that antibodies against human fibrinogen and albumin react with canine proteins with staining efficiencies the same as for human proteins. Because one of the objectives in this study was to observe the protein distribution on polymer shunts used in the canine *ex vivo* system, canine proteins have been used. The distribution of gold markers appears to depend on the type of protein preadsorbed. The two-dimensional distribution of antifibrinogen gold markers on PE appears random and covers the whole surface in a macroscopically homogeneous fashion, although the surface coverage is not complete. The distribution pattern observed for antialbumin gold markers on PE is quite different from the expected random adsorption of protein molecules. Antialbumin gold markers form streaks of beads which are closely attached to each other and cover only a small portion of the surface. The albumin adsorption pattern is not an artifact of the preparation for electron microscopic observation, since fibrinogen-coated samples, treated the same way, did not show this pattern. The distribution of fibrinogen on PVC and SR is the same as on PE. Figure 3C shows a portion of the fibrinogen layer which has peeled from the SR surface. The presence of folds on which antifibrinogen gold markers attach suggests that fibrinogen molecules form a network on the surface. Figure 3D shows the immunogold staining at the edge of a protein drop (Method 3). While the immunogold staining inside the drop is the same as that shown in Fig. 3A, the air liquid interface region is more heavily covered with individual and aggregates of gold markers compared to the inside region. Since the colloidal gold solution covered the entire surface including the protein layer when staining (Method 3 in Fig. 1), the edge of the gold markers coincides with the edge of the protein layer.

Relation between Surface Protein Concentration and the Immunogold Staining Efficiency

Since immunogold staining depends on the protein conformation and protein adhesiveness to a surface, it was of interest to compare the staining efficiency to the amount of protein on a surface. The density of gold markers on protein-coated polyethylene was compared to the surface protein concentration which was measured using radiolabeled protein. Surface protein concentrations were varied by changing the protein adsorption time and bulk protein concentration. Figure 4 shows the density of immunogold beads as a function of surface protein concentrations. As shown in the figure, the density of antifibrinogen gold increases until the fibrinogen surface concentration reaches approximately 0.30 $\mu g/cm^2$. Above this concentration, the staining efficiency does not increase. The same trend was observed with antialbumin gold markers, although their staining efficiency is much lower than that of antifibrinogen gold markers. It remains to be determined whether this lower staining efficiency of antialbumin gold markers is due to a lower antigen–antibody affinity or due to the albumin conformation which results in a lower staining efficiency. It is significant to note that the efficiency of immunogold staining does not increase when the surface protein concentration is above approximately 0.30 $\mu g/cm^2$, even though the amount of protein on the surface is still increasing. This result suggests that only

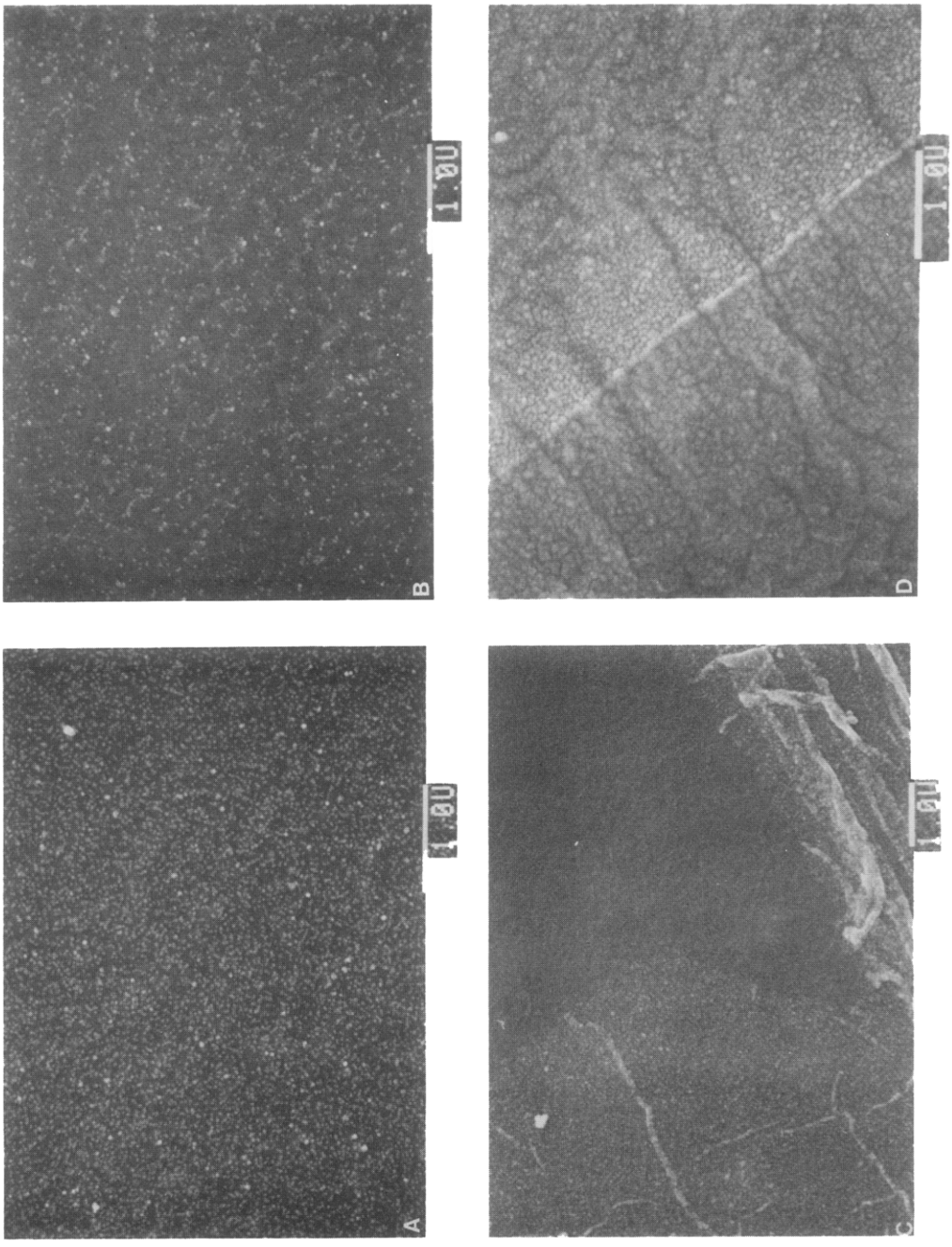

FIG. 3. Two-dimensional distributions of immunogold particles (Au_{18}) on various polymer surfaces. (A) Antifibrinogen gold markers on fibrinogen-coated polyethylene at a surface protein concentration of 0.35 $\mu g/cm^2$. (B) Antialbumin gold markers on albumin-coated polyethylene at a surface protein concentration of 0.27 $\mu g/cm^2$. (C) Antifibrinogen gold markers on fibrinogen-coated silicone rubber at a surface protein concentration of 0.50 $\mu g/cm^2$. A portion of the protein layer has peeled from the surface. (D) Antifibrinogen gold markers on PVC coated with a drop of fibrinogen solution. The edge of the drop is shown. (A), (B), and (C) were stained by staining Method 1, while (D) was stained by Method 3.

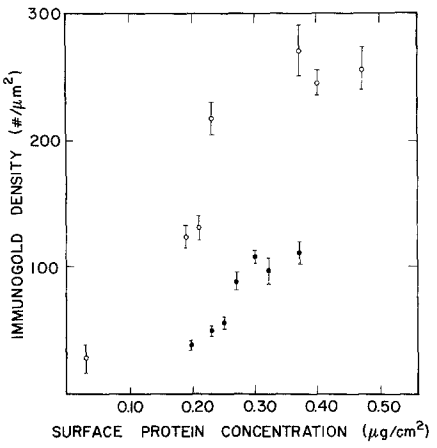

FIG. 4. The density of immunogold particles (Au_{18}) as a function of surface protein concentration. Surface protein concentration was varied by changing protein adsorption time and bulk protein concentration, with staining done by Method 2. Antifibrinogen gold markers on fibrinogen-coated polyethylene (○) and antialbumin gold markers on albumin-coated polyethylene (●).

part of the adsorbed protein is immunologically active or that weakly bound protein molecules are removed after binding to the immunogold particles.

The removal of loosely bound protein molecules seems to occur more readily with Au_{50} at higher surface protein concentration. The staining efficiency of Au_{50} on PVC was reduced when the surface concentration of fibrinogen was increased from 0.79 to 1.5 $\mu g/cm^2$ (Fig. 5). On all surfaces tested, the number of Au_{50} gold markers was reduced to about 30% of its maximum value (ranges from 19 to 35%) as the surface protein concentration was doubled. This reduced staining efficiency with increased protein concentration was not observed with Au_{18}. Because the weight of Au_{50} is 20 times that of Au_{18}, the reduced labeling of Au_{50} may be related to the removal of weakly bound protein molecules after immunogold staining due to the rinsing procedure. In Fig. 5, fibrous type microstructures underlying the gold markers are part of polymer surfaces and do not represent adsorbed fibrinogen, since the same structures were seen on polymer surfaces without precoated fibrinogen.

Results of Competitive and Sequential Protein Adsorption

Since immunogold beads can identify specific proteins, the distribution of fibrinogen or albumin can be visualized even when they are adsorbed competitively. Figure 6 shows distribution of antifibrinogen and antialbumin gold markers when albumin and fibrinogen were competitively adsorbed at the same bulk concentration of 0.30 mg/ml. Surface concentrations of fibrinogen and albumin were 0.21 $\mu g/cm^2$ and 0.10 $\mu g/cm^2$, respectively. The distribution of antifibrinogen gold markers in competitive adsorption (Fig. 6A) did not change appreciably from that in single protein adsorption (Fig. 3A), when the albumin bulk concentration was the same as that of fibrinogen. The same staining efficiency is surprising, since the staining efficiency of fibrinogen was concentration dependent up to about 0.30 $\mu g/cm^2$ (Fig. 4). When the albumin concentration was increased to 1.0 mg/ml, while the fibrinogen concentration was maintained at 0.30 mg/ml, the staining efficiency of the antifibrinogen gold markers was reduced.

In contrast to the small improvement in staining efficiency of fibrinogen, the staining efficiency of albumin increased by an order of magnitude when adsorbed competitively with fibrinogen (compare Figs. 3B and 6B). The albumin surface concentration was reduced from 0.27 ± 0.02 $\mu g/cm^2$ (for single protein adsorption) to 0.10 ± 0.01 $\mu g/cm^2$ when adsorbed competitively with fibrinogen. Since no cross reaction was confirmed in this particular experiment, the only way that the increase in staining could have occurred with lesser amounts of albumin or fibrinogen on the surface was for the albumin to expose more of its antigenic sites. It thus appears that a change in conformation or orientation of a protein on the surface is promoted in the presence of the other protein. The increase in immunogold

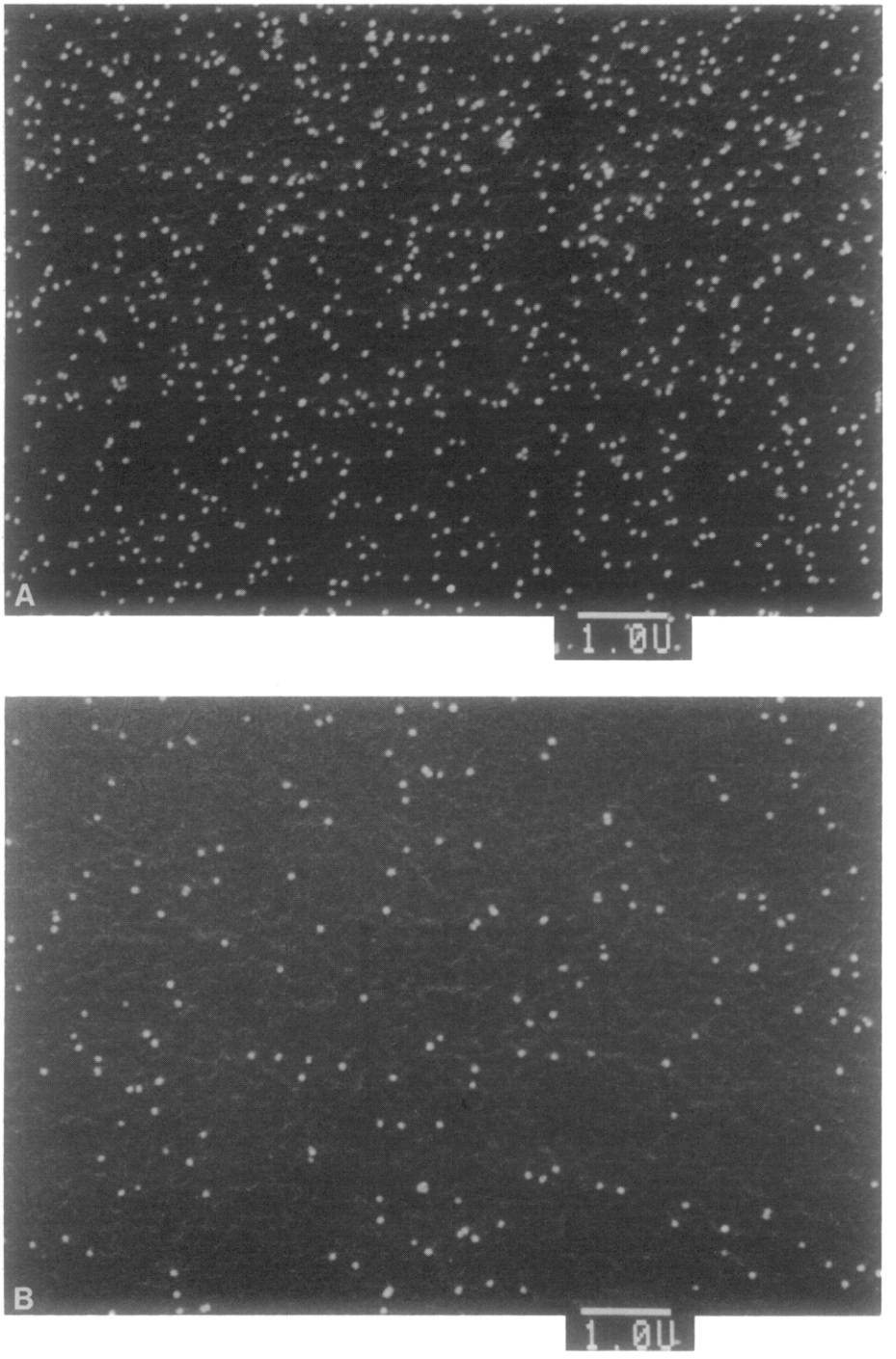

FIG. 5. Two-dimensional distribution of immunogold particles (Au_{50}) on PVC. Antifibrinogen gold markers on PVC coated with fibrinogen at a surface concentration of 0.79 $\mu g/cm^2$ (A) and 1.5 $\mu g/cm^2$ (B). Staining was carried out using Method 1.

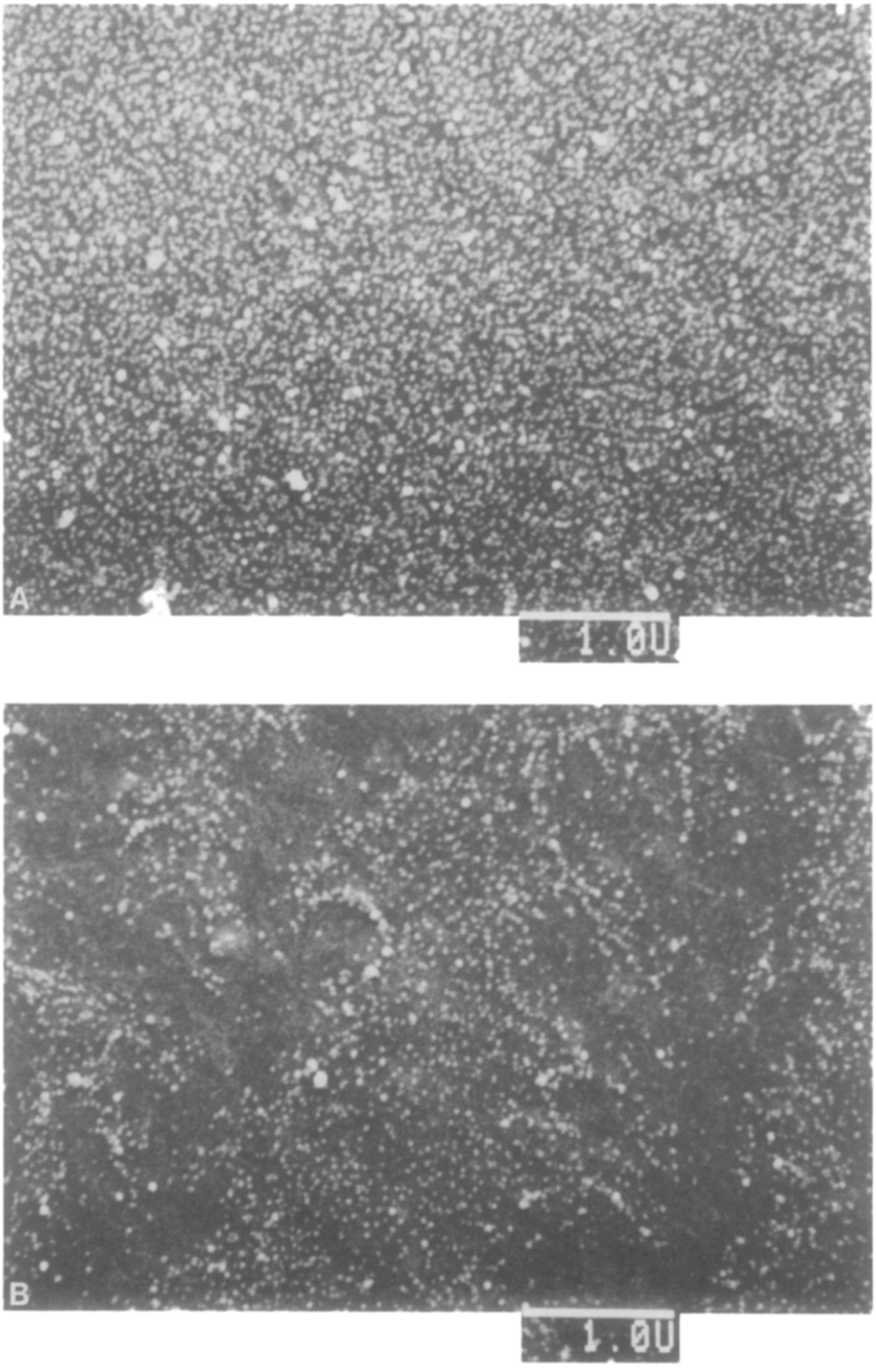

FIG. 6. Two-dimensional distribution of antifibrinogen gold markers (A) and antialbumin gold markers (B) on polyethylene coated with fibrinogen and albumin simultaneously for 10 min at a bulk concentration of 0.3 mg/ml each. Surface concentrations of fibrinogen and albumin were 0.21 and 0.10 $\mu g/cm^2$, respectively. Staining was carried out using Method 1.

staining was also observed when the proteins were sequentially adsorbed. Whether albumin was first adsorbed (5 min) on the PE surface and then followed by fibrinogen adsorption (115 min) or vice versa (fibrinogen 1 min followed by 119 min of albumin adsorption), the staining efficiency of both proteins was increased. Thus, it is assumed that the copresence of albumin and fibrinogen changes the conformation of each protein such that binding of immunogold particles is increased. As the albumin concentration was increased in the albumin–fibrinogen mixture, the albumin staining increased, while the fibrinogen staining decreased.

Immunogold Labeling of ex vivo Shunt

Albumin and fibrinogen distributions on surfaces which were exposed to blood were measured. Polyethylene was precoated with fibrinogen and albumin sequentially (for 10 and 110 min, respectively) and exposed to flowing blood for up to 2 h in the canine *ex vivo* system. The surface was precoated with fibrinogen first to make the surface very thrombogenic. As shown in Fig. 7A, antifibrinogen gold markers covered the surface between fully spread platelets. Although it is not clear whether the stained fibrinogen molecules are the remainder of the precoated fibrinogen or are newly adsorbed from the blood, the presence of fibrinogen suggests a latent thrombogenicity and the potential for platelet adherence and spreading. Figure 7B shows the distribution of antialbumin gold markers on a different portion of the same polymer surface. The density of antialbumin gold markers was less than that of fibrinogen. Because the surface segment shown in Fig. 7B was labeled with only antialbumin gold markers, the presence and distribution of fibrinogen is not known. Simultaneous labeling of antialbumin and antifibrinogen gold markers using two different sizes of gold particles is currently in progress.

From a previous study, it was found that thrombi did not reform on sites where thrombi had embolized, at least over 2 h of blood contact (43). Understanding this short-term surface passivation is important in understanding and preventing long-term surface-induced thrombosis. Since numerous thrombi form and are embolized from PE precoated with fibrinogen, it was possible to stain the sites of embolization with antialbumin and antifibrinogen gold markers. As shown in Figs. 7C and D, the staining efficiency of the embolized site by immunogold particles is far less than that observed in Figs. 7A and B. In fact, only a very small number of gold markers are observed on the embolization sites. Since immunogold particles are seen, it is not likely that the thrombus was detached while the sample was being prepared for SEM observation. The lack of protein, especially fibrinogen, can partially explain why thrombi do not immediately form again on sites where thrombi previously existed in the canine *ex vivo* experiment (43).

DISCUSSION

Recently, the extent and mode of surface coverage by adsorbed protein molecules has been investigated extensively. Rudee and Price (44) have shown that albumin and fibrinogen form a protein layer film on carbon, polystyrene, and polycarbonate after only a few seconds of protein exposure, while other investigators reported incomplete, nonhomogeneous surface coverage. Paynter *et al.* (20) reported an XPS study which showed that fibronectin and hemoglobin form a multilayer which fails to cover the entire surface of three fluoropolymers. From partial gold decoration transmission electron microscopy, Riccitelli (21) observed polymer- and protein-dependent variations in protein distribution. Feder and Giaever (45) suggested that protein adsorption can be best described by the random sequential filling model and showed experimentally that

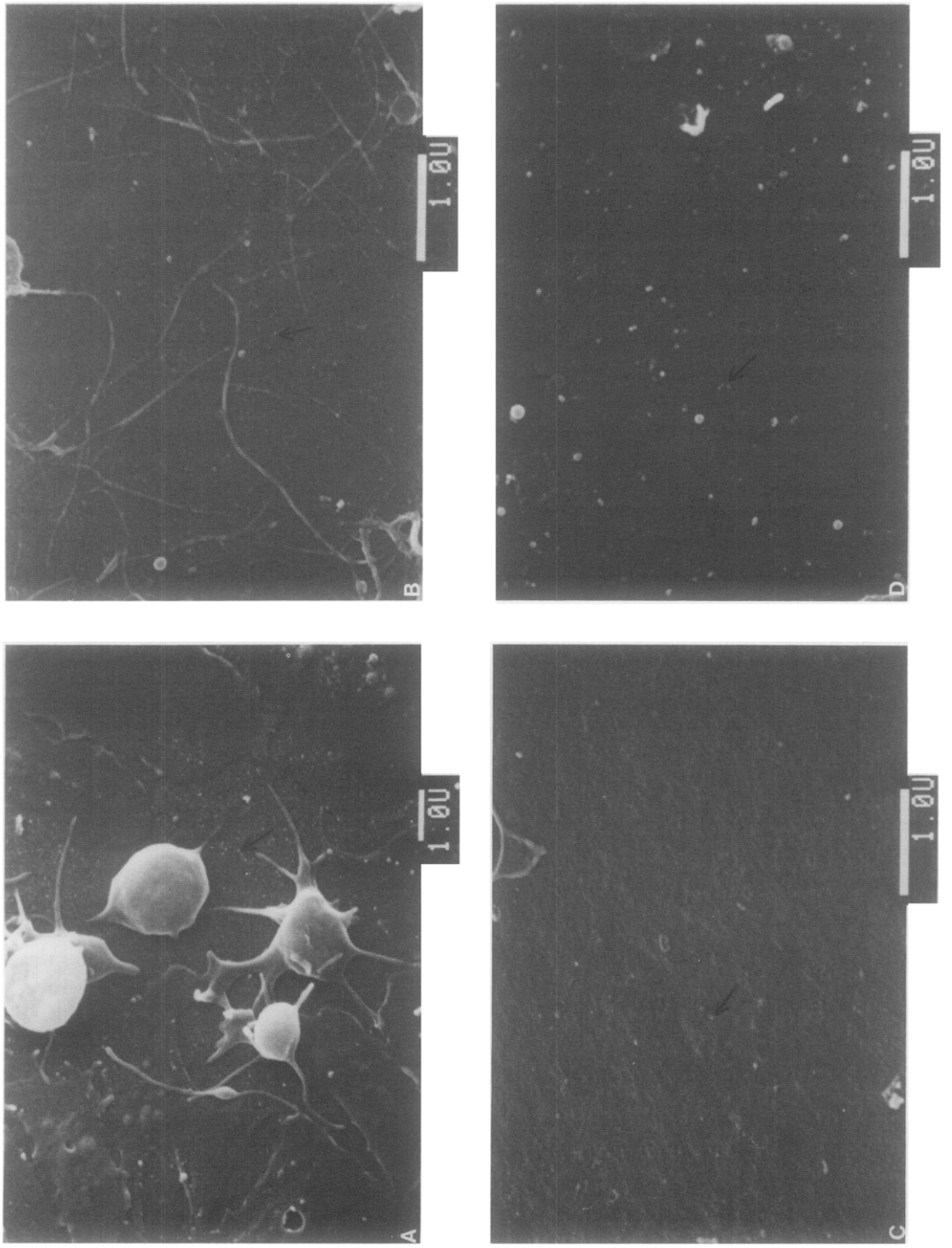

FIG. 7. Distribution of antifibrinogen gold markers (A and C) and antialbumin gold markers (B and D) on polyethylene exposed to flowing blood in the canine *ex vivo* system for 90 min. The polyethylene was preadsorbed with fibrinogen for 10 min followed by albumin for 110 min. (C) and (D) are micrographs of gold markers on embolization sites. Arrows indicate immunogold particles.

ferritin adsorption on carbon indeed occurred in such a way that only about 55% of the surface was covered by protein. It is important to note that 45% of the surface area accounts for the empty space between closely packed molecules. Unless protein molecules change their conformation significantly after adsorption, 100% surface coverage may not be achieved. Since the protein adsorption depends on many factors, such as the nature of the adsorbing protein, its concentration in solution, solvent condition, and the particular substrate material, it is difficult to generalize about the nature of the distribution of adsorbed protein molecules.

In this study, the immunogold staining technique was used to visualize the distribution of protein molecules adsorbed on polymer surfaces *in vitro* and *ex vivo*. The incomplete surface coverage by gold markers is possibly due to one or more of the following factors. First, it is possible that surface coverage by protein molecules is not complete. Although Fig. 3C shows a continuous layer of adsorbed fibrinogen molecules, this does not prove 100% surface coverage and further evidence is required. The protein surface concentration of fibrinogen and albumin on polyethylene (Figs. 3A and B) were 0.39 ± 0.05 and 0.30 ± 0.03 $\mu g/cm^2$, respectively. These concentrations are greater than those required for a side-on packed monomolecular layer, but less than needed for an end-on packed monomolecular layer (46). Thus, complete surface coverage is not impossible, if different conformations on the same surface are assumed. The second possibility is that some protein molecules on a surface possess conformations which do not allow the adhesion of immunogold particles. Since the labeling is based on an antigen–antibody reaction, the exposure of antigenic sites, i.e., protein orientation and conformation, is critical. The third explanation for incomplete coverage by gold markers could be that some protein molecules, to which gold markers adhere, may be weakly attached to the surface and are removed from the surface during rinsing. As mentioned before, we still cannot exclude the possibility that the immunogold bead is dissociated from the target protein. It is also conceivable that the steric stabilization of the gold markers inhibits complete surface coverage. However, the observation that some immunogold particles are closely attached to form linear arrays even in the presence of empty surface area (Figs. 3A and B) suggests that repulsion is not the dominating factor for the incomplete surface coverage of gold markers. Since the flocculation of colloidal gold particles is prevented when they are coated with antibody and polyethylene glycol (27), it is not likely that aggregates of gold markers formed in the bulk adhere to target proteins on the surface. A more complete exploration of these factors is a topic for future study in our laboratory.

The distribution patterns of fibrinogen and albumin resemble those described by Riccitelli *et al.* (21). Antifibrinogen gold markers on PVC, PE, and SR are homogeneously distributed over the entire surface, but incompletely cover the surface. The distribution pattern does not change as the surface fibrinogen concentration increases (Fig. 4). Antialbumin gold markers cover much less of the surface than the antifibrinogen gold markers. In addition, a secondary structure in the antialbumin gold markers was apparent. Brash studied protein exchange between a solid surface and solution and suggested heterogeneity existed in the binding energies of different sites (47). Since it can be assumed that the adsorbed protein molecules are oriented and/or distorted in various ways due to the heterogeneity of binding energies, nonuniform coverage of immunogold particles may well be due to different protein conformations or orientation. This nonuniformity appears to be small for fibrinogen, but rather large for albumin molecules as shown in Figs. 3A and B.

The mere presence of protein alone does not indicate that the protein present is biolog-

ically active. Proper orientation and conformation are essential for full biological activity (17). If fibrinogen molecules are adsorbed in such a way that the fibrinogen does not bind to immunogold particles, the adhesion of gold markers will be reduced regardless of the surface coverage by the protein. The reduced staining efficiency of Au_{50} compared to Au_{18} deserves further discussion. Horisberger (48) also has noted reduced staining efficiency of immunogold particles on cell surfaces as the particle size becomes larger. It may be that large gold markers require multiple binding sites to securely anchor onto a surface. The observation that the staining efficiency of Au_{50} decreased as the surface concentration increased to more than monolayer coverage suggests that protein molecules have to tightly bind to a surface to resist removal when bound to immunogold particles which undergo the rinsing process which removes the bulk of the staining solution from the polymer surface.

It may be significant that not much fibrinogen or albumin is seen on sites where thrombi have embolized. Further experiments are required to clarify what is causing reduced albumin and fibrinogen concentration at these sites and why these sites are relatively thromboresistant.

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