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SURFACE CHARACTERIZATION OF BIOMATERIALS BY IMMUNOGOLD STAINING -QUANTITATIVE ANALYSIS

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Abstract

The labeling of target proteins by immuno-gold particles has been analyzed based on Einstein's law of Brownian motion. The theory was confirmed from the experiments which employed antifibrinogen gold markers to label fibrinogen molecules adsorbed on the polyethylene surface. The theory predicts that the degree of labeling depends on the concentration of gold markers, temperature, medium viscosity, size of gold markers, and staining time. Of these factors most important is the concentration of immunogold particles. Small change in the marker concentration results in a significant variation in the staining efficiency when other variables are kept constant. The effect of temperature is always accompanied with that of the medium viscosity. There is a linear relationship between the degree of labeling and the temperature when the viscosity effect is combined. The staining of fibrinogen molecules adsorbed on the polyethylene surface at three different temperatures shows a temperature dependence which is in close agreement with the theory. The degree of labeling is inversely related to a square root of the size of gold markers.

This analysis makes it possible to maximize the staining sensitivity and to improve the reproducibility of the labeling. Thus, the immunogold staining under a well defined condition allows quantification as well as positive identification and localization of target proteins. This technique has been used to study protein adsorption on biomaterials.

KEY WORDS: Immunogold staining, scanning electron microscopy, colloidal gold, antibody, biomaterials, protein adsorption, fibrinogen, albumin, Brownian motion, quantification of colloidal gold staining.

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Introduction

The occurrence of thrombosis at the polymer-blood interface presents major difficulties in the development of blood compatible materials. It has been well established that protein adsorption on biomaterials precedes platelet adhesion [3] and the ultimate blood compatibility is determined by the protein interfacial behavior [29,30]. Traditionally, emphasis was given to find the correlation between the quantitative composition of an adsorbed protein layer and the surface thrombogenicity. Recent studies, however, have shown important roles of two-dimensional distribution and conformational change of adsorbed protein molecules in influencing the intensity of thrombus formation [43,47]. It is necessary to understand how the proteins of interest are oriented on the surface and how they are grouped in a two-dimensional plane. Only a few techniques are available to study spatial arrangement of protein molecules adsorbed on biomaterials. These includes immunoferritin transmission electron microscopy [50], modified negative-staining electron microscopy [12], partial gold decoration transmission electron microscopy [50], and X-ray photoelectron spectrosocpy [48]. The surface replication procedure which has been used in the first two techniques is a potential source of artifacts in the final image obtained [12] and the last technique cannot provide direct observation of protein distribution. Recently, immunogold staining techniques have been introduced to visualize protein molecules adsorbed on biomaterials [46].

Immunogold staining methods have been used as a reliable method for assessing topographical arrangement of target molecules on various substrates including cell membranes [2,17,19,26, 63], tissues [53,54,59], plant cell wall [55], nitrocellulose membranes [11,34], fibrin clot [40], and polymer surfaces [46]. This technique, however, requires a certain experience to choose the right marker system suitable for a given problem [31]. In addition, the application of this technique has been limited to the positive identification of target molecules, although the quantification of the target components is potentially possible. The lack of quantification

may be in part due to the lack of reproducibility of the staining sensitivity. Evidently, this relatively new technique requires further evaluation and characterization. In this paper, we will analyze immunogold staining using Einstein's law of Brownian motion and examine various factors which influence the staining efficiency.

Materials and Methods

Protein Preparation

Human fibrinogen (Calbiochem-Behring, San Diego, CA) was purified by the method of Laki [38]. Fibronectin was removed from the purified fibrinogen using a gelatin-Sephadex column (Bio-Rad, Rockville Centre, NY). The clottability of the purified fibrinogen was at least 97% when measured as described by Coller [13]. Human albumin (Sigma) was used as received. Proteins were dissolved in phosphate buffered saline (PBS, pH 7.4) solution and their 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 [46].

Preparation of Colloidal Gold Particles

Colloidal gold granules with an average diameter of 18 nm (Au_{18}) or 50 nm (Au_{50}) were prepared following the procedure described previously [23,31,46]. 0.5 ml of 4% $HAuCl_4 \cdot 3H_2O$ solution (Fisher Scientific, Itasca, IL) was added to 200 ml of deionized distilled water at room temperature and brought to a boil. An appropriate amount of freshly prepared 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 concentration of gold particles was calculated from the size of gold particles, the amount of gold added and the density of gold which is 19.32. The concentrations of 18 nm and 50 nm colloidal gold particles were 8.30×10^{11}/ml and 3.94×10^{10}/ml, respectively. The absorbance values of 0.975 and 0.984 were observed when freshly prepared solutions of 18 nm and 50 nm gold particles were measured at 525 nm.

The colloidal solution was cooled and stored at 4°C. The pH was adjusted to 7.4 before use by adding 0.2 N K_2CO_3 and the solution was filtered through a 0.45 µm Millex-HA filter (Millipore, Bedford, MA). The pH was measured using a gel-filled combination electrode (Orion, Cambridge, MA).

Preparation of Antibody-Gold Complex

Goat antiserum against human fibrinogen was purchased from Sigma (dialyzed, fractionated serum) and rabbit antiserum against human albumin was obtained from Calbiochem-Behring. 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 eluted antibody fraction contains both specific and other naturally occurring antibodies. The antibody concentration 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 0.22 µm Millex-GV filter (Millipore).

The minimum amount of protein necessary to stabilize the colloidal gold particles was determined from adsorption isotherms [24]. 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 protein amount 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 (Millipore Millex-HA, 0.45 µm) 1% polyethylene glycol (M.W. 20,000) was added to prevent aggregation of protein coated gold particles [31].

The antibody-labeled gold particles were centrifuged in polycarbonate tubes in an angle rotor (Beckman, Palo Alto, CA) 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 0.1 M phosphate buffer (pH 7.4). The volumes of immunogold particles and the phosphate buffer were adjusted to make desired concentrations of gold markers. The concentrations of gold particles were calculated from the absorbance at 525 nm. The immunogold solution was stored at 4°C for up to 1 week.

Protein Adsorption on Polymer Surfaces

The polyethylene tubing (0.125 in. i.d., Intramedic, Parsippany, NJ) was washed with running deionized distilled water for 2 h and subsequently filled with PBS. 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) and the surface protein concentration was measured using ^{125}I-labeled proteins [47]. Polymer shunts which were coated with protein were labeled with immunogold beads as described before [46].

Immunogold Staining of Adsorbed Protein Molecules

The protein adsorbed polyethylene tubing was flushed with excess PBS and small segments (3 mm in length) were separated from the shunt. The PBS in the polymer segments was blotted by touching the bottom of the segment to tissue paper (Kimwipe). The polymer segment was then put on a polystyrene petri dish (Corning, Corning, NY) and filled with the immunogold solution. The concentration of gold markers, the staining temperature, and the staining time were varied. After predetermined time period 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.
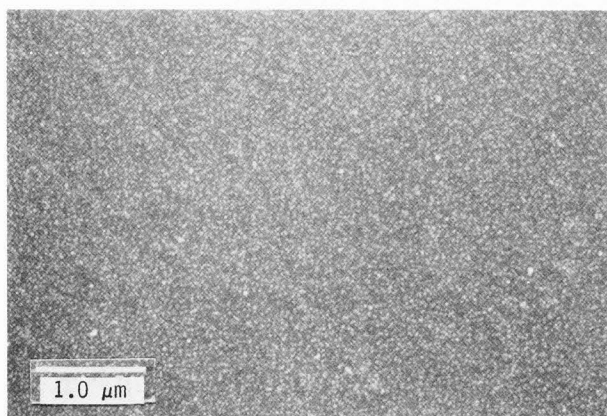
Figure 1. Two-dimensional distribution of immuno-gold particles (Au_{18}) on polyethylene surfaces precoated with albumin. Antialbumin gold markers (4.28×10^{12}/ml) were used to stain at $24^\circ C$ for 30 min. The surface albumin concentration was 0.32 ± 0.05 $\mu g/cm^2$.

Glutaraldehyde fixed 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. Tubing samples were cut into three pieces and placed on sample mounts with inside exposed. The exposed surfaces were sputter-coated with 100Å of gold or gold-palladium and examined on a JEOL JSM 35C scanning electron microscope (SEM) at 20 kV accelerating voltage. The immunogold staining was quantified by directly counting the number of gold markers from SEM pictures [6,7].

Factors Affecting Immunogold Staining

It is important to find the staining condition which results in the maximum degree of staining for quantitative as well as for qualitative studies. Fig 1 shows distribution profiles of immunogold particles stained on the polyethylene surface precoated with albumin. It is easily observed from Fig 1 that the surface coverage by gold markers is not complete and the space between gold markers is large enough to hold more gold markers. The immediate question from Fig 1 is whether there are no target protein molecules between gold markers. Is it safe to conclude that the surface coverage by target molecules is not complete? The staining under a different condition may have resulted in more immunogold staining. Thus, it is difficult to distinguish the lack of the target molecules from the incomplete immunogold staining, unless the staining condition is carefully controlled. It is often observed that the labeling sensitivity depends on various factors, such as temperature, the size of gold markers and the incubation time [31,46,55,58]. The exact relationship between those factors and the staining efficiency, however, has not been examined. We have found that the labeling of target components by immunogold particles can be described by the Einstein's law of Brownian motion.

For the staining to occur, the colloidal gold markers must reach target components on a surface and form antigen-antibody interactions, or other ligand-receptor interactions. Thus, the degree of staining directly depends on the number of colloidal gold particles reaching the surface for a given time period. If a significant portion of the surface is covered with target protein molecules and the gold markers reaching the surface are captured by the target protein, then more gold markers have to migrate to the surface. Because of the random character of the Brownian motion, the number of colloidal particles reaching a unit surface area in time t is given by:

$$q = (1/2)\ nl \tag{1}$$

where n is the particle concentration in the bulk solution, l is the average distance travelled by each particle in time t [44]. Using Einstein's law of Brownian motion, $l^2 = 2Dt$ [22,39], Equation 1 becomes:

$$q = n\ (Dt/2)^{1/2} \tag{2}$$

where D is a diffusion coefficient of the particle. Since $D = kt/6\pi yr$ for colloidal gold particles [57], Equation 2 becomes:

$$q = n(kTt/12\pi yr)^{1/2} \tag{3}$$

where k is Boltzmann's constant, T is temperature in degrees kelvin ($^\circ K$), π is 3.14, y is the viscosity of the dispersed medium and r is the radius of colloidal particles. The necessary values and units for the calculation of q are listed in Table 1. Since it is not expected that all particles arriving to the surface can react with surface protein (see Other Factors Important to Immunogold Staining), the probability factor should be incorporated into the equation. Thus,

$$Q = pq = pn(kTt/12\pi yr)^{1/2} \tag{4}$$

Table 1. Definitions and units of variables in Equation 3

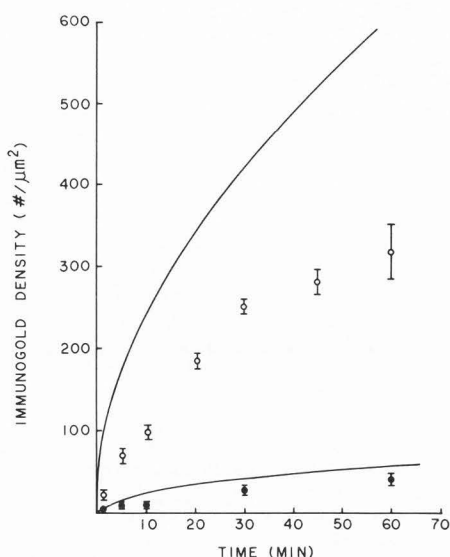| Variable	Definition and Unit
q	The number of particles reaching a unit surface area in time t (particles/cm^2)
n	Bulk concentration of gold markers (particles/cm^3)
k	Boltzmann's constant (1.38×10^{-16} $gcm^2sec^{-2}K^{-1}$)
T	Temperature ($^\circ K = 273.15 + {}^\circ C$)
t	Time (sec)
y	Viscosity of the medium (poise = $gcm^{-1}sec^{-1}$)
r	Radius of gold markers (cm)

Figure 2. The density of antifibrinogen-gold particles (Au_{18}) on fibrinogen-coated polyethylene as a function of labeling time. Surface fibrinogen concentration was 0.43 ± 0.04 $\mu g/cm^2$ and the staining temperature was $26.5^\circ C$. The concentrations of gold markers were $3.91\times10^{12}/ml$ (o) and $3.66\times10^{11}/ml$ (●). Solid lines indicate the theoretical q values.

where p is the probability that antibodies on colloidal gold particles react with target proteins on the surface and Q is the number of particles that actually form stable complexes with the target proteins. The p cannot be greater than unity and is not necessarily a constant. The p value can be calculated by comparing q and Q values, since the Q value can be obtained by direct counting of the number of particles in a given area. Equation 4 describes 5 factors, except the probability factor, which influence the efficiency of the immunogold staining. Each factor has been examined by staining fibrinogen molecules adsorbed on the polyethylene surface with antifibrinogen-coated gold markers.

Concentration of Immunogold Particles

The number of colloidal gold particles can be easily calculated by measuring absorbance of the immunogold solution at 525 nm (see Preparation of Colloidal Gold Particles). A relationship between the number of gold particles and the absorbance at visible wavelengths which was presented by Horisberger and Rosset [32] was slightly different from ours. The radius of immunogold particles should include the thickness of the antibody layer, since it is a part of a colloidal particle which determines the friction factor. Assuming the average thickness of antibody monolayer on colloidal gold particles is 10 nm [45], the radius of antibody coated Au_{18} becomes 19 nm. From these values, we can calculate q as a function of time. In Fig 2, the theoretical q values at two different particle concentrations are shown as well as experimental Q values. It is noted that p is significantly

Table 2. Temperature effect on the staining efficiency

Temperature $^\circ C$	$^\circ K$	Viscosity $(y)^a$	$(K/y)^{1/2}$	Relative value
0	273.15	1.787	12.36	0.72
5	278.15	1.516	13.55	0.79
10	283.15	1.306	14.72	0.86
15	288.15	1.138	15.91	0.93
20	293.15	1.002	17.10	1.00
25	298.15	0.8903	18.30	1.07
30	303.15	0.7975	19.50	1.14
35	308.15	0.7194	20.70	1.21
40	313.15	0.6531	21.90	1.28

[a] Viscosity of water in centipoise (10^{-2} g/cmsec) from reference 37.

Table 3. Immunogold densities at three different temperatures[a]

Temp.	Immunogold density ($\#/\mu m^2$)			
	Theory	Relative value	Experiment	Relative value
$4.0^\circ C$	176.7	(1.00)	83.4 ± 8.0	(1.00)
$26.5^\circ C$	247.9	(1.40)	114.6 ± 7.5	(1.37)
$37.0^\circ C$	281.4	(1.59)	153.1 ± 7.8	(1.84)

[a] Experimental condition: Fibrinogen molecules adsorbed on polyethylene surface (surface concentration of 0.43 ± 0.04 $\mu g/cm^2$) was stained with antifibrinogen gold markers (Au_{18}) for 10 min. The concentration of gold markers was $3.91 \times 10^{12}/ml$.

lower than unity at higher particle concentration ($n = 3.91\times10^{12}/ml$). The p value observed at lower particle concentration ($n = 3.66\times10^{11}/ml$) is larger than that observed at higher particle concentration. Caution is necessary, however, to interpret the data, since only a small number of gold markers were counted. At the higher particle concentration, it takes about an hour to reach the plateau value of about 300 gold markers/μm^2. Further staining up to 3 h did not increase the immunogold density. At the lower particle concentration, it will take more than 40 h to reach the immunogold density of $300/\mu m^2$. Since the incubation time of overnight appears to be the longest time in practical situations, the concentration of gold markers has to be high enough to obtain the maximum staining efficiency.

Temperature and Viscosity

Since temperature change is always accompanied by the change in solution viscosity, temperature effect on the staining efficiency has to be considered simultaneously with the viscosity effect. The viscosity of the dispersed

system is $y_0(1 + 2.5f)$, where y_0 is the viscosity of the solvent and f is the volume fraction of colloidal particles [28]. Since a volume fraction occupied by gold particles is negligible in most practical situations, viscosity value of the solvent can be used. Since water appears to be the only medium used in the immunogold staining, the viscosities of water at various temperatures are listed in Table 2. The staining efficiency is a linear function of temperature when the viscosity effect is combined. According to the table, change in temperature from 5°C to 35°C is expected to result in more than 50% increase in the staining efficiency. When fibrinogen (0.3 mg/ml) was adsorbed on the polyethylene surface for 2 h at room temperature, and stained with immunogold particles for 10 min at three different temperatures, the staining efficiency was increased as temperature increased. The relative increase in the staining efficiency from 4°C to 26.5°C is exactly the same as predicted by the theory (Table 3). However, the relative increase from 26.5°C to 37°C is much greater than predicted. This may be due to the temperature-dependent nonspecific staining or the increased flexibility of protein molecules at 37°C which may improve antigen-antibody reaction (see Protein Conformational Change upon Adsorption on Biomaterial Surfaces).

Size of Colloidal Gold Particles

In equation 3, the staining efficiency is inversely related to a square root of the particle size. We tested the effect of particle size on the staining efficiency by comparing those of Au_{18} and Au_{50}. The numbers of gold markers used were comparable (3.91×10^{12}/ml and 4.00×10^{12}/ml for Au_{18} and Au_{50}, respectively). As shown in Fig 3, the immunogold density of Au_{50} is close to that of the theoretical value up to 10 min of the staining period. The reduced efficiency after 10 min is simply due to the saturation of the surface with rather large gold markers. The comparison of Fig 2 and Fig 3 suggests that the probability of forming antigen-antibody complexes (p in equation 4) is larger for Au_{50} than Au_{18}. This is probably due to the larger number of antibody molecules present on Au_{50} than on Au_{18} (see Presence of Nonspecific Antibodies). It has been often stated that small particles are favorable for immunocytochemistry because of a higher staining efficiency and, hence, of a more sensitive display of the immunoreaction [31,55,58]. This conclusion, however, is based on experiments which have not controlled the concentration of gold markers. The lower yield of label with larger size gold markers observed in the literature is most likely due to the smaller number of gold markers used in the experiments. Unless the experimental condition is carefully controlled, such as the number of gold markers, temperature, and staining time, the effect of size on immunogold staining cannot be judged. In order to obtain the same staining efficiency using different sizes of gold markers, the effect of size change should be compensated by adjusting other variables in equation 3. This is critical when multiple labeling is attempted using gold markers of different sizes. Since quantitative measurements
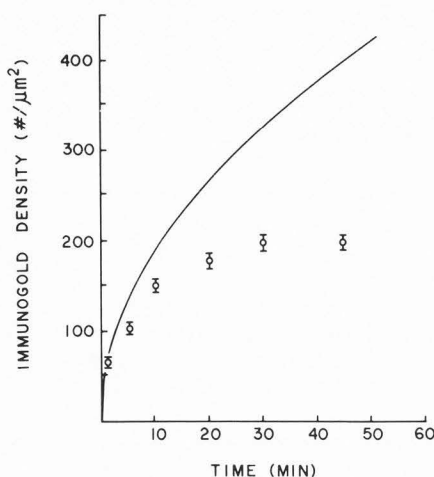

Figure 3. The density of antifibrinogen-gold particles (Au_{50}) on fibrinogen-coated polyethylene as a function of labeling time. Surface fibrinogen concentration was 0.43 ± 0.04 μg/cm^2 and the staining temperature was 26.5°C. The concentration of gold markers was 4.00×10^{12}/ml. Solid line indicates the theoretical q values.

have shown that the Brownian movement is observed as long as the particle diameter is less than 5 μm [36], a wide range of different sizes of colloidal gold particles can be used for multiple labeling.

Staining Time

At a given condition, it is possible to calculate the time required to reach the maximum staining efficiency, assuming p is equal to 1. In so doing, other variables, such as the concentration of gold markers, the staining temperature, and the size of gold markers, should be known. At any labeling condition, the maximum staining can be obtained if the staining time is long enough. As mentioned above, however, the longest labeling time appears to be overnight, and it is necessary to adjust other parameters to complete labeling in a reasonably short period of time. It goes without saying that the total number of particles in the applied staining solution is larger than the number of particles necessary to stain the whole surface area.

Some literature examples of immunogold staining conditions are listed in Table 4. Unfortunately, the concentration of gold markers was not described in most of the papers (#1-10 in Table 4). The staining was done mostly at room temperature for a period of 0.5 h to 2 h. Sometimes overnight staining was done at 4°C [34]. Only examples 17 and 18 satisfy the optimum staining condition. Examples 11-16 require longer staining time for the maximum staining of target molecules. For the accurate interpretation of the marker distribution patterns and quantitation (although in a relative sense), the immunogold staining has to be carried out under the optimum condition which can be predetermined using equation 4.

Table 4. Examples of conditions for colloidal gold staining

| # | Colloidal gold | | | Staining | | |
	Concentration (Particles/ml)	Size (nm)	Coated protein	Temp. (^{o}C)	Time (h)	Ref.
1	N.D. [a]	20	antibody	20	N.D.	15
2	N.D.	5,17	enzyme	N.D.	1/2	55
3	N.D.	12,19	protein-A	20	1/2	5
4	N.D.	5,16	protein-A	20	1/2	25
5	N.D.	20,45	protein-A	20	1/2	16
6	N.D.	5,20	antibody	22	1	14
7	N.D.	N.D.	protein-A	20	1	62
8	N.D.	5,15	protein-A	20	1	53
9	N.D.	16	antibody	20	2	27
10	N.D.	10-20	α_2-M	0	4	20
11	6.75×10^{10}	50	lectin	25	2-6	32
12	3.40×10^{11}	50	lectin	25	1	32
13	3.75×10^{11} [b]	18	protein-A	20	1	11
14	4.26×10^{11} [b]	18	antibody	4	overnight	34
15	4.26×10^{11} [b]	18	antibody	20	1/2	34
16	about 10^{12} [b]	40	antibody	20	1	18
17	3.66×10^{12}	18	antibody	20	2	46
18	5.00×10^{12}	18	lectin	25	2	32

[a] Not described in the original paper.

[b] The number of colloidal gold particles is calculated from the absorbance described in the paper assuming the size of 18 nm except for #16.

Other Factors Important to Immunogold Staining

In addition to the parameters examined in Factors Affecting Immunogold Staining, the following factors are expected to influence the staining efficiency through changing the probability of antigen-antibody complex formation in equation 4.

Antibody Orientation on Colloidal Gold Particles

Colloidal gold granules have been labeled with various proteins and most immobilized proteins are known to maintain their specific biological functions. Although the biological reactivity of antibodies appears to be preserved, this does not mean that all antibody molecules adsorbed on colloidal gold particles have the same orientation. According to the electron microscopic study of Bauer et al. [4], all colloidal gold particles are covered by antibodies, but not necessarily with the same orientation. It will be reasonable to speculate that presumably half of the adsorbed antibodies are in a sterically favorable orientation necessary for the antibody-antigen reaction [26]. Further studies, such as functional analysis of F_{ab} and F_c portions of antibody, are necessary to determine the dominant antibody orientation on gold particles. Colloidal gold particles of 18 nm size are known to adsorb about 20 antibodies [17]. If these antibodies have different orientations on a colloidal particle, the interaction of the particle with antigenic protein will be influenced by the position of the particle facing the target protein.

Presence of Nonspecific Antibodies

Except in a few cases where monoclonal antibodies were used for the immunogold staining [18], antisera have been used in most experiments. In our study, we have used antibodies which were purified using an anion exchange column (see Preparation of Antibody-Gold Complex). Since antibodies were not affinity purified, there may be other nonspecific antibodies on colloidal gold particles. The previous observation that nonspecific staining of immunogold particles did not occur on the polyethylene surface [46] suggests that the actual staining is a result of antigen-antibody interactions. The presence of nonspecific antibodies on colloidal gold particles may influence the degree of staining. The p value much lower than 1 which was observed with Au_{18} at the high concentration (Fig 2) may be in part due to the use of antibodies which were not affinity purified. The p value of Au_{18} particles at the lower concentration (Fig 2) is difficult to accurately measure, since the number of particles counted is so small. The presence of nonspecific antibodies, however, does not appear to reduce specific labeling when the particle size becomes 50 nm (Fig 3). This may be ascribed to a few factors. First, since the size of antibodies is known to be about 8.5 nm x 12 nm [45], the number of antibodies adsorbed on Au_{50} can be more than 70. Thus, there can be enough specific antibodies that interact with fibrinogen molecules on the surface. Second, the size of antibody coated Au_{50} is larger than the size of fibrinogen molecule (9 nm x 45 nm) [61], so that specific antibodies on a colloidal gold particle can interact with adjacent fibrinogen molecules, even though nonspecific antibodies are facing the fibrinogen molecule which is directly beneath the gold marker. This implies that the larger size immunogold particles in fact have a higher chance to label target protein molecules on the surface.

Antibody Release from Gold Particles

The binding of an immunogold particle to the target protein is known to be largely irreversible [32]. This may be a safe assumption considering the fact that the antigen-antibody affinity (or association constant) is very large which ranges from 10^5 M^{-1} to 10^{12} M^{-1} [49,60]. Thus, antibody-gold conjugates are not expected to be released leaving antigenic proteins on the surface. In addition, it is generally assumed that proteins are irreversibly adsorbed onto colloidal gold particles [33]. The labeling activity of protein A-gold complex was maintained up to one year when stored at 4°C [53]. This implies that desorption of proteins from the gold particles does not occur or occur to a negligible extent. It has been observed occasionally that considerable amounts of proteins are released with time in the presence of competing proteins [13]. It is possible that there exists a population of protein molecules that are not very tightly bound and they can be exchanged with bulk protein molecules. This protein exchange can be eliminated by removing excess competing protein. Thus, washing is necessary to obtain

stable protein-gold conjugates [17]. As long as immunogold particles are used within a short period of time after preparation or centrifuged again before use, the staining efficiency should remain the same [54].

Protein Conformational Change upon Adsorption on Biomaterial Surfaces

Since the immunogold staining employs the principle of antigen-antibody interaction, the staining efficiency is expected to be influenced by various factors critical for the interaction. Antibodies elicited in response to immunization with protein antigens are reactive with various antigenic determinants and may be directed against one or more of the structural aspects of the proteins [56]. In theory, the antigenic determinants can be divided into two structural categories according to whether their specificity is due only to continuous segment of amino acid sequences in the protein (sequential or segmental site) or to other structural features such as folding in three dimensions (conformational site) [8]. In practice, however, all antigenic determinants are conformational in a sense that the three-dimensional conformation of the antibody combining site defines an antigen conformation most complementary to itself [8,9,10,35,56]. The protein structure in solution is flexible enough to fit itself into an antibody combining site. In many instances, subtle conformational alterations in a protein are also accompanied by a change in antigenic reactivity [1]. Thus, the efficiency of immunogold staining for protein adsorbed on biomaterials is expected to depend on the nature of biomaterial surfaces, since the interaction of protein with different surfaces may result in different degrees of conformational change. The small change in protein conformation on different surfaces may not be detected if polyclonal antibodies are used, since polyclonal antibodies can react with more than one antigenic site of a particular protein. The dependence of staining efficiency on protein conformation may be recognized more prominantly, if monoclonal antibodies are used. In this paper, we have used polyclonal antibodies, since it is desirable to stain all protein molecules for the quantitation and visualization of the spatial arrangement of protein molecules. Even so, the effect of protein conformation on the staining efficiency is expected to occur to a certain extent.

Type of Antigenic Proteins on the Biomaterial Surface

As mentioned above, the sensitivity of immunocytochemical techniques is predominantly determined by the accessibility of antibodies to antigenic sites [9,59]. Since different proteins may adsorb in different orientation and may experience different extents of conformational change, the staining efficiency may not be the same for different types of proteins at a given condition. In addition, the affinity of antigen-antibody and the number of antigenic sites are not the same for all proteins.

Surface Affinity of Adsorbed Protein Molecules

In the immunogold staining, antibodies are attached to colloidal gold particles which are rather bulky and heavy. As discussed in Antibody

Release from Gold Particles, the dissociation of gold markers from the antigenic protein is not likely to happen. We have to consider, however, the possibility of the dissociation of antigenic protein from the surface along with the gold markers.

To date, the effects of the above mentioned factors on the degree of staining have not been discussed much in the literature. It should be possible to study these factors, since we now know how to optimize the staining efficiency by controlling the parameters in Equation 4. Once the optimum staining condition is set up, the change in protein conformation and the surface affinity of protein on different biomaterials can be studied by comparing the staining efficiencies. As mentioned above, monoclonal antibodies are preferred for this purpose.

Discussion

Mode of Protein Adsorption on Biomaterial

One of the advantages of using immunogold staining techniques is that nonspecific staining of gold markers is absent [46] or negligible [52]. Thus, this technique provides information on two-dimensional protein distribution and the fractional surface coverage by protein molecules. Caution should be exercised, however, in determining the actual protein distribution, since the immunogold staining is expected to depend on many factors as described above.

The two-dimensional distribution of immunogold particles appears random and covers the whole surface in a macroscopically homogeneous fashion, although the surface coverage is not complete. One or more of the following factors are expected to be responsible for the incomplete surface coverage. First, the protein adsorption from a single protein solution does not result in a compact monolayer. The incomplete surface coverage may be due to competition for adsorption sites with solvent molecules and lateral repulsion between adsorbed protein molecules [21]. Second, some protein molecules on a surface may possess conformations which do not allow the interaction with immunogold particles, although polyclonal antibodies were used in this study. Third, some protein molecules, to which gold markers adhere, may be weakly attached to the surface and are removed from the surface during the rinsing procedure. Fourth, the steric stabilization of the gold markers may inhibit complete surface coverage. The fourth possibility, however, cannot explain the observation that the size of some empty spots is larger than that of individual gold markers.

Quantification of Protein Molecules using Immunogold Staining

In general, it is considered as a successful labeling if the nonspecific labeling as measured by control experiments is at least an order of magnitude less than the labeling observed in actual test experiments [42]. It has been observed that protein-gold complexes show negligible degrees of nonspecific labeling on thin sections from resin embedded tissues or frozen materials and even on biological samples

with the exception of nuclei and mitochondria which sometimes show some background [32,59]. The extent of nonspecific binding will depend on the surface characteristics, i.e. charge, polarity, molecular groups, etc. of both the markers and the cells [42]. Unless there is a strong electrostatic interaction due to the presence of highly charged functional groups on the surface, nonspecific labeling is usually negligible. This makes it possible to use the immunogold labeling to quantify the amount of protein adsorbed on the surface.

When the density of gold markers on polyethylene was compared to the surface concentration of either fibrinogen or albumin, a linear correlation was observed [46]. Although this linear relationship is limited up to a certain surface concentration (approximately 0.4 $\mu g/cm^2$ for both fibrinogen and albumin), this implies that the immunogold staining can be used to quantify the amount of protein on biomaterials. It should be noted that the calibration curve should be generated for each protein because of the reason discussed in Type of Antigenic Proteins on the Biomaterial Surface.

Advantages of the Immunogold Staining Technique

The analysis of the immunogold staining based on Einstein's Law of Brownian motion allows: 1) optimization of the staining condition, 2) improved staining sensitivity and reproducibility, 3) correct interpretation of the distribution patterns of the target components, 4) quantification of the target protein on a microscopic area, and 5) correct comparison of dual labeling using gold markers of different sizes.

Probably the most significant advantage of the immunogold staining technique is that we can label and examine the surface at the SEM level. Most techniques require a large surface area to calculate the average surface concentration of proteins. For example, the surface protein concentration measured using radiolabeled protein is an average value obtained from a relatively large surface area. However, the immunogold staining technique allows examination of a microscopic area. Using this technique, it was found that not much fibrinogen or albumin was present on sites where thrombi have embolized when biomaterials were exposed to flowing blood [46]. This kind of information cannot be obtained using other techniques. Since immunogold particles can identify specific proteins, the information on the presence of a particular type of protein, their distribution, and their quantity can be easily obtained in the presence of other types of proteins. This is an extremely useful technique especially when a biomaterial is exposed to the whole blood.

The improved staining sensitivity as a result of this analysis may be useful for the detection of protein in a very small quantity. For example, the sensitivity of "golden blot" [11,34,41,51] is expected to be increased when the staining condition is carefully controlled. From now on, it is encouraged to control the concentration of immunogold particles, temperature, and the size of gold markers in order to obtain the correct interpretation of the

localization and distribution patterns of target molecules.

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Discussion with Reviewers

J.W. Slot: In the system used in this study, so many binding sites occur at the surface, so that gold particles are efficiently cleared from the solution upon their first arrival at the surface or soon thereafter. However, if only a few percent of the surface is covered with antigen molecules, most particles are not captured in immunocomplex at the surface, move back into the solution, and will approach again. In this case, diffusion of particles from a deeper layer of solution may not be a rate limiting factor in the labeling. We usually work with smaller gold particles (5-10 nm) in concentrations of approximately 10^{12} particles/ml and 30-60 min incubation times in quantitation studies.

Authors: If only a few percent of the surface is covered with antigenic proteins, most colloidal gold particles are not consumed on the surface and bounce back to the solution. Then, we may assume that the number of gold particles reaching the surface in the first second will be maintained throughout the experiment. Using equation 3, we can calculate the number assuming room temperature and 10 nm gold particles with 10 nm of protein thickness (total diameter is then 30 nm).

$$q = (10^{12})\{ \frac{(1.38 \times 10^{-16})(293.15)(1)}{(12 \times 3.14)(1.002 \times 10^{-2})(15 \times 10^{-7})} \}^{1/2}$$

$q = 2.67 \times 10^8$ particles/cm^2 = 2.67 particles/μm^2. If each particle contacts 706.5 nm^2 (3.14x15^2 nm^2) of the surface, 1415 particles (1 μm^2 ÷ 706.5 nm^2/particle) should reach the surface to complete the staining. Since only 2.67 particles reach the surface every second, 530 sec (8.8 min) is required for a good staining. Thus, staining for 30 min with the gold particle concentration of 10^{12}/ml is enough, if only a minor fraction of the surface is covered with target proteins. If 10^{11} gold particles were used, however, the staining time has to be increased 10 times to 5300 sec (88 min). This simple calculation again demonstrates the importance of the concentration of colloidal gold particles.

J.W. Slot: Did you try to help diffusion, simply by agitating the solution during incubation, or by changing the solution each few minutes?

Authors: We have tried staining polymer particles by constant stirring or agitation. Agitation or stirring increased diffusion and resulted in much faster staining. This method will be especially useful for the staining of cell suspensions. For the comparison of different samples or the same sample stained at different times, the same stirring rate should be used. We have not tried the frequent changing of the

solution. However, we expect faster staining, since this is the same as maintaining the initial gold particle flux throughout the experiment. This method may be inconvenient and uneconomical, especially when the labeling protein is expensive.

W.D. Geoghegan: No one has published the calculation for the number of gold particles/volume. Perhaps the authors should include the calculation.

Reviewer IV: The method to calculate the number of gold particles from the absorbance at visible wavelength used by Horisberger and Rosset (32) has not been proven to be true by another method.

Authors: The number of colloidal gold particles was calculated as described below. The following example is for 18 nm gold particles and gold particles of other sizes can be calculated by exactly the same way.

We have used $HAuCl_4.3H_2O$ (M.W.=393.83) from Fisher Scientific. 0.5 ml of 4% $HAuCl_4.3H_2O$ contains 10 mg of Au (M.W.=196.97). Since the total volume of the freshly prepared colloidal gold solution is 204.5 ml (200 ml of water + 4 ml of citrate + 0.5 ml of $HAuCl_4.3H_2O$), the concentration of Au is 10mg/204.5ml, or 4.89×10^{-5} g/ml. The volume of a 18 nm gold particle is $(4/3)(3.14)(9\ nm)^3 = 3.05\times10^3\ nm^3 = 3.05\times10^{-18}$ ml. The density of Au is 19.32 g/ml and the weight of each gold particle is $3.05\times10^{-18}\times19.32 = 5.89\times10^{-17}$ g. Because the concentration of Au is 4.89×10^{-5} g/ml, the total number of gold particles in 1 ml is $(4.89\times10^{-5}\ g/ml) \div (5.89\times10^{-17}g) = 8.30\times10^{11}$ /ml. The absorbance of freshly made 18 nm gold particles at 525 nm was 0.975. Thus, absorbance of 0.975 corresponds to 8.30×10^{11} gold particles/ml. As we checked this result with that of Horisberger and Rosset (32) (absorbance of 1.45 at 525 nm corresponds to 1.12×10^{12} particles/ml in their calculation), we found that there is a 10 % difference in the particle concentration for the same absorbance value. It is important to realize that the relationship between absorbance and the particle concentration suggested by Horisberger and Rosset (32) is similar to ours, although the absolute values are different. This shows that the data of Horisberger and Rosset (32) may be used as a first approximation to calculate the particle concentration from the absorbance value."

Reviewer IV: The concentration n in equation 3 is constant. Depletion by loss due to binding is not accounted for.

Authors: The concentration of immunogold particles in equation 3 is the initial concentration. In order to understand how equation 3 accounts for the depletion of gold particles, it is necessary to remember that the equation is derived based on the Brownian movement of colloidal particles. Since the gold markers adjacent to the surface are preferably labeled on the surface in the early staining period, particles present at a longer distance from the surface must reach the surface by Brownian movement for the further staining to occur. As described by Einstein, the displacement of a colloidal particle diffusing away from the origin is not proportional to time but proportional to the square root of time (22,39). Since the arrival of colloidal particles to the surface is dependent on the square root of time, the frequency of particles reaching the surface decreases as times goes on. This is clearly shown by theoretical curves in Figs 2 and 3.

Reviewer IV: The q values exhibit a considerable error compared to the Q values in Figure 2 at the higher concentration.

Authors: From Fig 2 the p value can be estimated to be about 0.5. The data can be interpreted in at least three ways. First, it can be said that only about 50% of the surface is covered with target protein molecules. Second, only about 50% of immunogold particles reaching the surface may be successful in interacting with the target protein. Third, although all the particles reaching the surface can interact with target protein, some of them may be removed from the surface while sample preparation, such as washing. At this moment, the three possibilities are not distinguished.

Reviewer IV: Are the authors excluding mass as a determining factor in Brownian motion?

Authors: It may seem strange that shape and size of the particle affects the Brownian movement, whereas its mass and density do not. For particles of same size and shape, if the density increases, the average velocity decreases: at the same time, however, the velocity and direction of motion is less affected by each collision because of greater inertia. As a result, the motion of a denser particle is slower but less random, and the two effects cancel. In terms of random walk, there are fewer steps per unit time, but each is longer. (p115 in Reference 44).

Reviewer IV: I suggest the authors include a sample calculation of the time required to each maximum staining efficacy. This should be done in a non-ambiguous manner such that other investigators might make the same calculation using their own variable, viz. particle size.

Authors: From the known particle size, the particle concentration can be calculated as shown above. The viscosity of water can be easily obtained from Table 2, if temperature is measured. The radius of the gold-protein conjugates will be determined by the type of protein used. If staining is done at $20^{o}C$ using 18 nm (diameter) gold particles with 10 nm thickness of an antibody layer, the necessary values for equation 3 will be T=273.15+20=293.15, $y=1.002\times10^{-2}$, $r=19\times10^{-7}$. To obtain the maximum staining, each spot on the sample surface has to be exposed to gold markers. Thus, the total surface area should be divided by the area that one gold marker can cover and this calculation will provide the number of gold markers which should reach the surface for the maximum staining (this number corresponds to q in equation 3). From this information, t can be calculated.

Reviewer IV: The fibrinogen molecule has different dimensions than albumin. differences in binding could also be due to differences a) in the quantity of bound albumin versus fibrinogen; b) in the number of antigenic sites available; and c) in the area each molecule occupies.

Authors: The Reviewer is absolutely right. In addition to those factors, protein conformational change upon adsorption on biomaterial surfaces and the surface affinity of adsorbed protein molecules are important factors which affect the staining efficiency. These factors are discussed in Other Factors Important to Immunogold Staining section.

Reviewer IV: The authors have not tested viscosity effects alone. Certainly, viscosity can be adjusted separately without changing the temperature.

Authors: We believe that the change in viscosity due to the changing temperature is sufficient to prove the effect of viscosity. The addition of other components not only changes the viscosity of the medium, but also can affect the antigen-antibody interactions. In fact, the change in viscosity by adding other component can be used to examine the interaction between antigen and antibody. We are planning to continue this work.

Reviewer IV: What does the orientation of the antibodies have to do with the effect of particle position in antigen-antibody interaction?

Authors: Since the orientation of the antibodies on colloidal gold particles is not the same, if F_C portion of antibody is exposed, the immunogold particles will not be able to stain the target protein.

Reviewer IV: According to Horisberger, the reported values of adsorbed IgG range very widely for 18-20nm particles.

Authors: The statement that "Colloidal gold particles of 18 nm size are known to adsorb about 20 antibodies" was made not to show that the exact number of antibodies on the 18 nm gold particles is 20. It simply implies that the number of antibodies on 18 nm gold particles is significant and thus, the orientation of antibodies on the surface would affect the interaction with antigenic proteins on the surface.