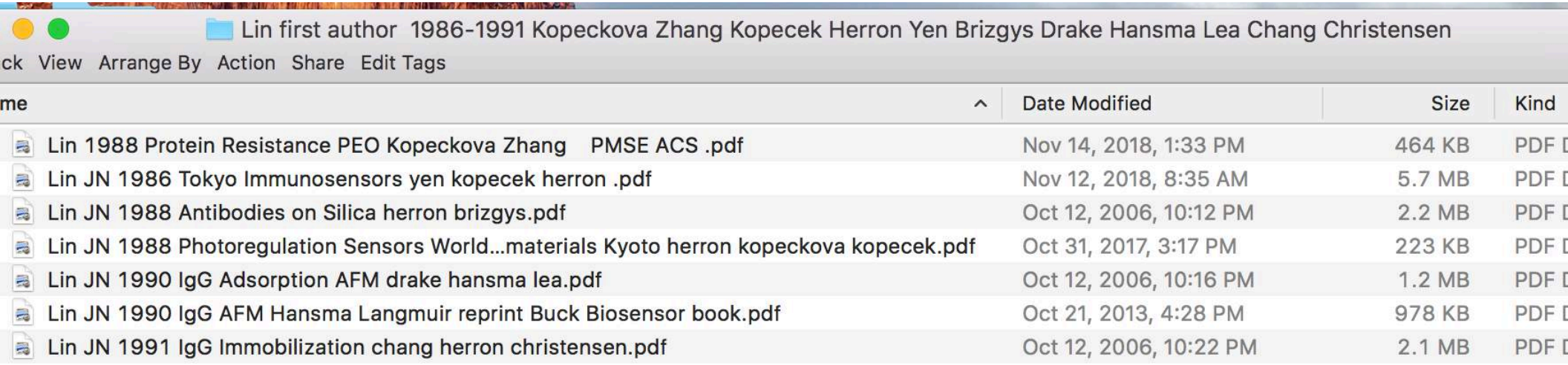
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PROTEIN RESISTANCE OF POLYETHYLENE OXIDE SURFACES

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Abstract : Polyethylene oxide (PEO) surfaces were prepared by a simple coating treatment of a common hydrophobic medical material (low density polyethylene, LDPE) in a solution of random copolymers of alkyl methacrylates and methoxy (polyethylene oxide) methacrylates. The adsorption properties of the polymer on LDPE surface were investigated using X-ray photoelectron spectroscopy (XPS) and ^{125}I-labeled polymers. The protein-resistant character of the prepared PEO surfaces were also evaluated using XPS and ^{125}I-labeled proteins.

INTRODUCTION

PEO is becoming recognized as the most effective polymer for low protein adsorption and low cell adhesion characteristics, due to its high mobility, unique solution properties, molecular conformation in aqueous solution and steric stabilization effects (Refs.1-4). PEO-water interfaces have also very low interfacial free energies, and thus low driving forces for adsorption (Refs.5,6).

In the previous papers (Refs.7-9), we treated hydrophobic surfaces with commercially available PEO-containing block copolymer surfactants in aqueous solution, by physically adsorbing those surfactants onto surfaces. We showed that physical adsorption of surfactants on hydrophobic materials provide a simple and rapid means of producing PEO surfaces as protein-resistant surfaces, if the surfactants can be adsorbed strongly onto the surfaces. From the previous work, however, we found that commercially available block surfactants have two limitations in producing stable PEO surfaces. First, a long hydrophobic block is needed to produce strong adsorption of the surfactant on a hydrophobic surface. However, it easily leads to intramolecular aggregation in aqueous solution, resulting in weak adsorption onto the surface. Second, a long PEO chain is needed to be highly mobile in aqueous solution and thus confer effective protein resistance. It will, however, be less

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strongly adsorbed on the surface than a shorter one, because the PEO-water interactions are stronger than the hydrophobic segment-hydrophobic surface interactions. That is the reason why we synthesized new polymeric surfactants, copolymers of alkyl methacrylates with methoxy (polyethylene oxide) methacrylates discussed in this study.

EXPERIMENTAL

The copolymers were prepared by random polymerization of monomers, monomethoxy poly (ethylene oxide) methacrylates, alkyl methacrylates and methacryloyl tyrosin amide (for ^{125}I-labeling purpose) in toluene for 45 hours at 50 °C in the presence of 2,2'-azobisisobutyronitrile. Monomer compositions to prepare copolymers and the molecular weights determined by GPC are listed in Table 1. As most of polymers were not directly soluble in water, the polymers were dissolved in warm ethanol, then diluted with water and subsequently dialysed against water. Then the solutions were diluted to concentrations needed.

Iodine-125 was used for labeling human albumin (major constituent of plasma) and the copolymers synthesized. Chloramine-T method (Ref.10) was used to prepare ^{125}I-labeled albumin (Ref.9) and ^{125}I-labeled copolymers (Ref.11).

The adsorption properties of the polymers on LDPE (NHLBI DTB Primary Reference Material) surface were investigated using X-ray photoelectron spectroscopy (XPS) and ^{125}I-labeled copolymers. For XPS analysis, the oxygen atomic % on the LDPE surface was used as an indicator of the amount of the polymer adsorbed, as LDPE does not show any oxygen peaks.

The protein-resistant character of the prepared PEO surfaces were also evaluated using XPS and ^{125}I-labeled proteins. For XPS analysis, the nitrogen atomic % was used as an indicator of the adsorbed amount of the protein.

RESULTS AND DISCUSSION

The copolymers synthesized were adsorbed onto LDPE from aqueous solution. The copolymers have two functional units on their polymethyl methacrylate backbone : alkyl side chains as a hydrophobic unit and long PEO side chains as a hydrophilic unit. The long hydrophobic poly methacrylate backbone and alkyl side chains will interact strongly with LDPE surface via hydrophobic interaction and the long hydrophilic PEO chains will be extended relatively freely into the water phase (Fig.1).

After the polymers were adsorbed onto LDPE surface, the amount of adsorption was evaluated by XPS (or ESCA) and ^{125}I-labeled polymers. Adsorbed amount of the polymers with different PEO side chain molecular weight, 1900 and 4000, was compared in Table 2. Polymer No.23 shows interesting feature; this polymer has 50 % hydrophobic portion and shows excellent adsorption properties. All polymers in Table 2 were not directly soluble in water and a special treatment was used to dissolve them in water as discussed before.

The adsorbed amount of human albumin on the surfaces treated with the polymers was compared in Table 3 for both XPS and ^{125}I-labeled protein analysis. We can see that protein adsorption is significantly decreased by the polymer treatment. Polymer No.23-treated surface shows excellent protein resistant properties, probably due to its large adsorption on LDPE surface, even though its PEO portion is only 10 %. Possible explanations for protein resistance of the polymer-treated surfaces include PEO's high mobility, its unique solution properties, its molecular conformation in aqueous solution, and steric effects, as discussed earlier. It appears that PEO surfaces in water are particularly effective in exhibiting rapid motions (Ref.4) and a large excluded volume, thereby actively minimizing the adsorption of proteins.

Now, we have one question about the stability of the copolymers adsorbed on LDPE surface. Protein is a macromolecule. Will the copolymer molecules adsorbed on LDPE surface be not exchanged with the proteins during protein adsorption on the copolymer-treated surfaces? To solve this question, time-dependent protein adsorption was done on the LDPE surfaces treated with the ^{125}I-labeled copolymers. After protein adsorption was done with different time intervals, radioactivity from the polymer remaining on the surface was counted, converted to the values of adsorbed amount of the polymer on the surface, and compared the value of that before protein adsorption (Fig.2). As seen in the figure, there is little evidence of exchange of the polymer molecules with human albumin for upto 100 min.

Work on plasma protein adsorption on the copolymer-treated LDPE surfaces is in progress.

ACKNOWLEDGEMENT

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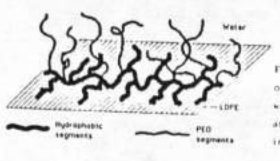

Fig. 1. Expected conformation of methacrylate copolymers with PEO and alkyl side chains at hydrophobic solid(LDPE)/water interface.

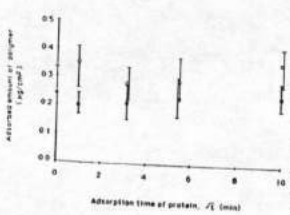

Fig. 2. Amount of polymers remaining on LDPE surface after protein adsorption (protein adsorption, human albumin 1 mg/ml solution; polymer treatment, 30 min desorption in water after 30 min adsorption in 1 mg/ml polymer solution; blank points, polymer No.19; filled points, polymer No.21)

Table 1. Monomer composition and molecular weights of copolymers synthesized[a]

Polymer No.	$MPEO_{1900}MA$[c]	$MPEO_{4000}MA$[d]	MMA[e]	HMA[f]	LMA[g]	MtyrA[h]	Mol.Wt. by GPC[b] Mw	Mw/Mn
16(9)	20	-	19(20)	60	-	1(0)	1,041,000	11.0
17(10)	25	-	19(20)	55	-	1(0)	882,000	10.0
18(11)	25	-	39(40)	35	-	1(0)	540,000	9.0
19(12)	25	-	19(20)	-	55	1(0)	584,000	6.5
20(13)	-	20	19(20)	60	-	1(0)	237,000	2.0
21(14)	-	20	19(20)	-	60	1(0)	294,000	2.2
22(15)	-	15	34(35)	-	50	1(0)	326,000	2.2
23	-	10	19(20)	-	70	1(0)	420,000	2.1

a. Composition, mol %. b. Using PEG calibration samples.
c & d. Monomethoxy poly (ethylene oxide) methacrylates with mol.wt. of PEO, 1900 and 4000 respectively.
e, f & g. Methyl, hexyl and lauryl methacrylate respectively.
h. Methacryloyl tyrosin amide (used for ^{125}I-labeling of the copolymers)

Table 2. Adsorbed amount of the copolymers on LDPE surface[a]

Polymer No.	Mol.Wt.-PEO	Oxygen atomic % (XPS)	$\mu g/cm^2$ (^{125}I-labeling)
16	1900	4.0 ± 0.1	0.25
17	1900	4.0	0.24
18	1900	3.9 ± 0.1	0.21 ± 0.02
19	1900	4.5 ± 2.1	0.33 ± 0.09
20	4000	2.4 ± 0.4	0.20 ± 0.01
21	4000	4.1	0.24
22	4000	4.5	0.35
23	4000	9.0	0.8

a. 30 min desorption in water after 30 min adsorption of LDPE film in 1 mg/ml polymer solution.

Table 3. Adsorbed amount of human albumin[a] on untreated and copolymer-treated LDPE surfaces[b]

Polymer No.	Mol.Wt.-PEO	Nitrogen atomic % (XPS)	$\mu g/cm^2$ (^{125}I-labeling)
Pure LDPE		10.4 ± 3.3[c]	0.45 ± 0.08
9	1900	1.1 ± 0.4	-
10	1900	0.8 ± 0.1	0.10
11	1900	1.5 ± 0.2	0.13
12	1900	1.2 ± 0.7	0.12
13	4000	1.5 ± 0.4	0.15
14	4000	1.3 ± 0.2	0.11
15	4000	1.4 ± 0.6	0.11
23	4000	0.4 ± 0.1	-

a. Protein adsorption: human albumin 1 mg/ml in PBS pH 7.4, 30 min.
b. Polymer treatment: 30 min desorption in water after 30 min adsorption of LDPE film in 1 mg/ml polymer solution.
c. Nitrogen content of human albumin: 15.1 % as determined by XPS analysis

PROTEIN ADSORPTION STUDIES: CLUSTER ANALYSIS OF FT-IR SPECTRA.

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INTRODUCTION

The collection of numerical techniques known as cluster analysis is a vital tool in many areas of science, such as systematic biology[1]. The goal of these techniques is to partition a given set of objects into its inherent classes based on some quantitative measure of similarity. Applications of cluster analysis to several types of spectral data have been reported[2]. In this paper, we apply cluster analysis to a set of protein Fourier transform infrared (FT-IR) spectra. This set consists of spectra of the same protein in two different physical states: in solution and adsorbed to a surface. Definite differences between the spectra of these two states have been observed[3]. We will show how cluster analysis can be used to help quantify this observation.

METHODS

The FT-IR spectra were obtained using attenuated total reflectance (ATR)[4]. In this technique, the IR beam is sent through a crystal, where it undergoes multiple internal reflections. At the points of reflection, a standing wave extends far enough outside the crystal to probe material on and near the crystal face. The FT-IR/ATR technique has been adapted for studying protein adsorption by putting the ATR crystal in contact with a flowing protein solution[5,6].

Experimental. Details of the experimental procedure have been reported elsewhere[3] and so will be reviewed only briefly. The protein solution was composed of bovine serum albumin (BSA) in citrate phosphate buffered saline at 30 mg/ml. This solution was pumped through a special flow cell, one wall of which is a germanium ATR crystal. After an irreversibly adsorbed protein layer was deposited on the germanium surface, a spectrum was collected. Saline without protein was then flowed through the cell, and another spectrum was collected. By subtracting a reference spectrum of saline from the second spectrum, a spectrum of the adsorbed BSA was obtained. By subtracting both the saline reference and the adsorbed BSA spectrum from the first spectrum, a spectrum of the BSA in solution (bulk BSA) was obtained. Six separate runs of this procedure yielded the six adsorbed and six bulk BSA spectra used for cluster analysis.

Spectral Preprocessing. It is often necessary in cluster analysis to preprocess the data to remove significant sources of noise, which might otherwise lead to misleading results. A common source of (low-frequency) noise in biological FT-IR spectroscopy is variation in the spectral baseline. To compensate for this variation, a recently developed filtering method[7] was used. This method (Legendre filtering) involves deriving, from the special functions known as Legendre polynomials, an orthonormal set of polynomials that can serve as the basis for an expansion of any spectrum, within a given wavenumber region. The filtering consists of removing the first few terms of this expansion; these terms contain low-degree polynomial components of the spectrum.

POLYMER SURFACE CHEMISTRY APPLIED TO THE DEVELOPMENT OF IMMUNOSENSORS

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INTRODUCTION

There is considerable interest and activity today in the development of biosensors (1,2) based on a wide range of detection mechanisms, including fluorescence (2,3), changes in refractive index (2,4), change in weight using surface acoustic wave or quartz crystal methods (5), and current voltage detection via semiconductor and/or electrode methods (6). Most biosensors are based on the specific ligand binding characteristics of biochemicals, particularly antibodies (2,3), lectins (7), receptors (8), or enzymes (9).

Our work is based on specific antibody interactions, using fluorescence detection--essentially conventional fluoroimmunoassay (10), but using optical waveguide techniques for signal separation and detection (2,3). The basic concept is given in Figure 1. Basically, specific antibodies (or antigens)

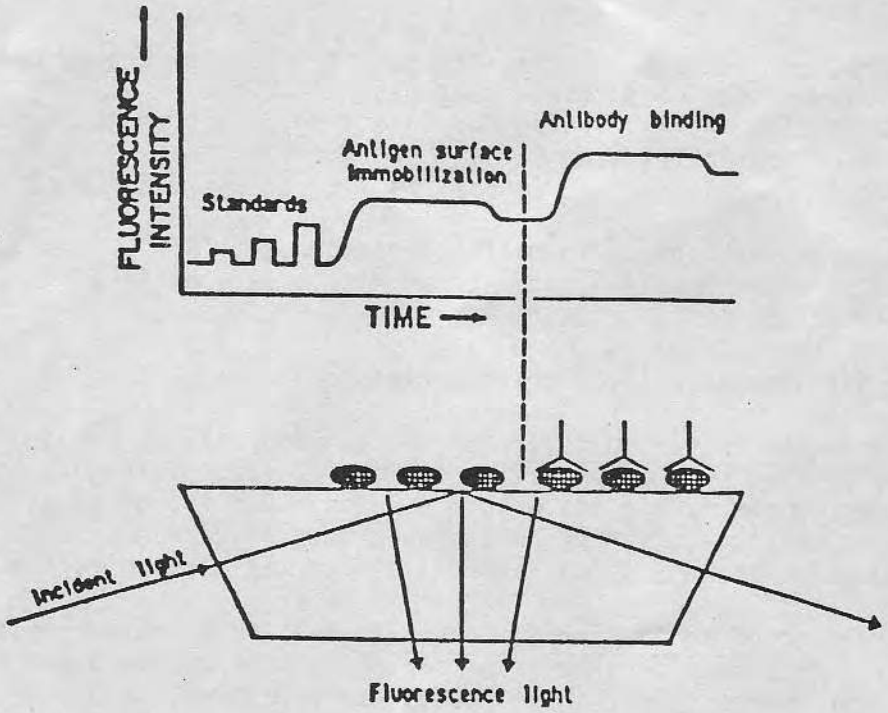

FIGURE 1: Schematic of the optical fluoroimmunosensor concept (see text for details).

are covalently immobilized on the surface of an optic waveguide. The immobilized antibodies bind their specific antigen (or antibody) from the

solution. Generally fluorescent-labelled antigen is introduced locally and competes with endogeneous, unlabelled antigen for the antibody binding sites. Fluorescence of bound molecules at the interface is excited by light coupled into the waveguide via the evanescent field present at the waveguide/solution interface (2,3,11). A major portion of the emitted fluorescence couples efficiently into the same waveguide via optical tunneling (also called near field coupling or optical reciprocity) (not shown in Figure 1) and is readily separated from the exciting wavelength, detected, and quantitated (2,3,11,12).

One can readily measure bound antigen concentration by such methods and several groups are developing this approach for commercial application (13,14,15). Generally, such devices are not sensors, however, for two important reasons:

1. The technique requires a fluorescent-labelled reagent.
2. The Ag-Ab binding constant is so strong that the binding is essentially irreversible (16,17), thus the device is a dosimeter rather than a true sensor (16,17).

POLYMER CHEMISTRY

Polymer surface chemistry must be used in order to develop truly remote, continuous immunosensors. There are five important polymer surface chemistry problems:

1. Fluorescent Reagent Delivery--to permit multiple or even continuous use without the need to add reagents.
2. Modulation of Ag-Ab Binding Constants--to permit short response times and true sensor function (17).
3. Antibody Orientation and Immobilization--to optimize sensitivity (18).
4. Non-specific Binding--to minimize background (19).
5. Bio- or Blood Compatibility--to permit in situ or in vivo applications (19).

Each of these areas will be briefly discussed.

1. Fluorescent Reagent Delivery to the sensing site is being accomplished by the immobilization of fluorescent reagent to a polymeric matrix via photo labile bonds. A pulse of light breaks a number of bonds, thereby releasing a controlled number of fluorescent reagent molecules, which then diffuse to the sensing region.

2. Modulation of Ag-Ab Binding Constants (17) is being accomplished via a four component copolymer containing photosensitive azobenzene pendant groups. The light-induced conformational change of the azobenzene (27) results in expansion of the polymer molecule and perturbation of the solution environment near the antibody active sites.

3. Antibody Orientation and Immobilization (18) is being accomplished by relatively standard means by optimizing tether type, length, and coupling chemistry.

4. Non-specific Binding can be due to at least five causes (Figure 2):

   a. Physical adsorption to areas of the substrate which are not covered by immobilized IgG or Fab.

   b. Non-specific interactions with the chain, tether, or chemistry by which the Ab is immobilized.

   c. Non-specific or weakly specific interaction with the parts of the Ab molecule not involved with the Ag binding site. Such interactions, although occurring for soluble Ab in bulk solution, may be enhanced by the local microenvironment, resulting from immobilization.

   d. "Non specific" interactions with the Ag binding site due to strains, distortions, or microenvironmental effects, resulting from the interfacial immobilization process.

   e. True cross-reactivity or lack of specificity of the intact, native Ag binding site. These effects would also exist in bulk solution and will not be further discussed.

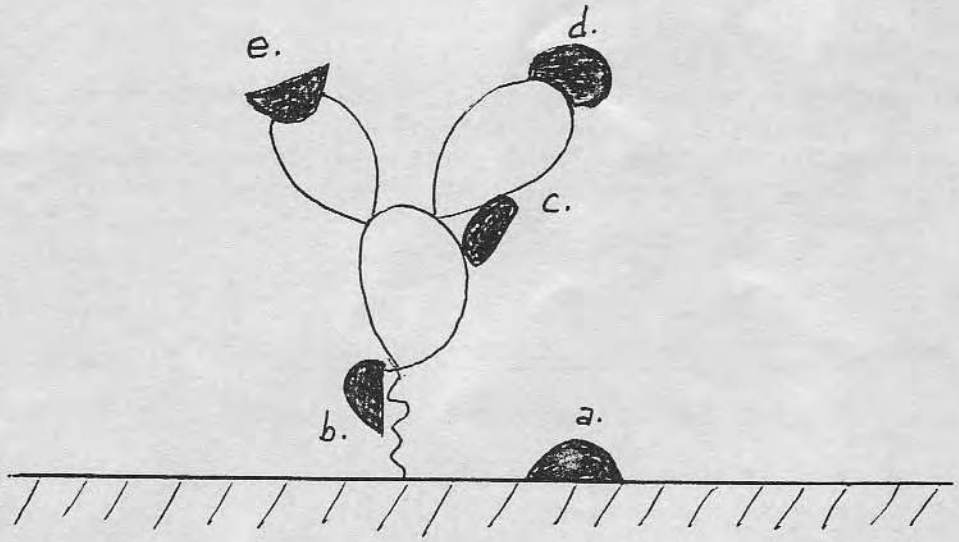

FIGURE 2: Various causes of non-specific binding (see text for details).

Most of these effects can be (and are being) minimized by using the principle of steric exclusion or steric repulsion commonly used for stabilization of colloids (20). We use immobilized polyethylene oxide (PEO) chains to produce protein-resistant surfaces (21). This approach has been discussed and employed by Nagaoka, et al. (23), Merrill, et al. (24), and by our group (21). PEO derivatization of enzymes and antibodies is becoming widely recognized as a means to prevent recognition and interaction of such molecules in biological fluids and environments (25). One can co-immobilize PEO with Ab at the surface, thereby minimizing process #1 in Figure 2. The use of a

PEO tether largely minimizes process #2 in Figure 2. A long hydrophilic tether permits the IgG or Fab to be more flexible and further from the rigid interface, thereby minimizing local microenvironment effects which contribute to process #3 and also minimizes IgG or Fab distortions or strains (process #4).

5. Bio- or Blood Compatibility - Fortunately, the PEO treatments just discussed appear to enhance general blood compatibility (24), perhaps due to the general protein repulsion (steric exclusion) mechanism. The anticoagulant heparin (which functions via specific antithrombin 3 binding) can be coimmobilized or, preferably, released locally by standard polymeric drug delivery means (26).

CONCLUSIONS

Clearly the development of truly remote, continuous, high sensitivity, and specific immunosensors, capable of functioning reliably in blood or in other biological environments, is still in its early stages. There are a number of important technologies which must be developed. As the work progresses, one can envision the development and eventual production of multichannel biosensors based on microintegrated devices incorporating biochemical, optical, and even electronic functions.

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Characterization of Immobilized Antibodies on Silica Surfaces

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Abstract—**There is considerable interest in the immobilization of antibodies on silica surface for fiber optic biosensor applications. The physical and chemical properties of antibodies immobilized on silica surfaces were investigated in this study. Two antibody (Ab)-antigen (Ag) model systems, goat anti-human IgG Ab (polyclonal Ab)/human IgG (multivalent Ag) and mouse anti-digoxin IgG (monoclonal Ab)/digoxin (monovalent Ag), were used. Both physical and covalent immobilization of antibody on silica surfaces were investigated. The covalently immobilized antibody shows better stability and Ag binding capacity. In the case of large Ag molecules, such as human IgG, the maximum antigen binding capacity is probably restricted by steric factors. The results of an antibody specificity study reveal that the positively charged surface shows high nonspecific binding. The binding constants of anti-digoxin IgG and digoxin are similar in solution and on different surfaces, suggesting that the original conformation of Ag binding sites is preserved. However, the binding constants of goat anti-human IgG and human IgG on surfaces are one order higher than that in solution. This suggests that the enhancement in binding constant is a function of both local Ab concentration and size of Ag molecules.**

INTRODUCTION

IMMOBILIZATION of biomolecules to solid phase materials has been widely used for various purposes including purification, analytical assay, synthesis, and sensing. The insoluble supporting materials used currently can be classified as biopolymers, synthetic polymers, and inorganic supports. The hydrophilic biopolymers, such as agarose, dextran, and cellulose, are the most common materials. Enormous amounts of work have been reported on uses of this type of materials. The synthetic polymers, such as polyacrylamide, polyhydroxyalkylmethacrylate, and polystyrene, have been explored as potential supporting materials. Inorganic materials, like silica, are probably the least popular among all supporting materials. The reasons are nonspecific adsorption of proteins on untreated glass surfaces and chemical instability of the silica surface in high pH. However, as pointed out by Weetall, it is possible to use porous glass as a substrate for immobilization of biomolecules if proper surface treatments are conducted [1]. Haller gave a good review on the application of controlled pore glass in solid phase biochemistry [2].

One must admit that silica substrates have not attained the same popularity and versatility as agarose in many aspects of biochemical applications today and thus less work has been done in an attempt to better understand Ab immobilized on silica surfaces [3], [4]. Silica possesses a unique property, that is, it can transmit light. Recently, there has been considerable interest in the use of optical fibers for remote spectroscopic sensing of biological molecules [5]–[7]. The potential application of these biosensors is very broad, including patient bedside monitoring during intensive care, the diagnosis of human disease, biotechnology process control, and therapeutic drug monitoring. In our laboratory, we are interested in developing optical fiber immunosensors [5]. We and others have previously shown that an electromagnetic field generated at a solid–liquid interface by total internal reflection can be used to excite fluorescence of molecules present at the interface [8]. Antibody molecules immobilized at the surface of a silica optical element bind their complementary antigen from solution, permitting analysis of antigen concentration in the bulk solution [9]. This method can also be applied to a cladding-free fiber optic sensor [10].

The main objective of this work was to characterize by isotope labeling the physical and chemical properties of immobilized antibody on three different pretreated silica surfaces. Two antibody–antigen model systems, polyclonal antibody-multivalent antigen and monoclonal antibody-monovalent antigen, were used. Equilibrium binding constants of the antibody–antigen interaction in solution and on surfaces, specificity of immobilized antibodies, maximum antigen binding capacity, surface concentration and stability of immobilized antibody, and effect of immobilization time on antibody activity on different surfaces were studied.

MATERIALS AND METHODS

A. Cleaning of Silica Samples

All silica samples were cut from 1 in × 1 in × 0.1 cm fused silica slides (CO grade, ESCO) followed by polishing the edges very well. The size of the sample is 2.4 cm × 0.955 cm × 0.1 cm and can fit into a 13 × 75 mm culture tube (Fisher) in which all surface reactions took place at room temperature. A beaker containing tubes with silica samples inside was first immersed in hot chromic

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acid at 80° C for 30 min and then thoroughly rinsed in purified deionized water filtered through a Milli-Q® ultrafiltration system (Millipore), without touching anything inside the container. The glassware and samples were dried in a dessicator at 120° C for more than 2 h. After drying, one of the newly cleaned samples was checked by the Wilhelmy plate water contact angle technique to ensure the cleanliness of the surfaces [11].

B. Silanization of Cleaned Silica Samples

Two silane reagents were used in this study: 3-aminopropyltriethoxy silane (APS, Aldrich) and dimethyldichloro silane (DDS, Petrarch). The silica chips were reacted with a fresh ethanol solution of APS (5 percent APS, 5 percent deionized water, and 90 percent absolute ethanol v/v) or toluene solution of DDS (10 percent v/v DDS and 90 percent v/v toluene) for 30 min at room temperature, followed by rinsing with deionized water ten times and absolute ethanol two times for APS surface or rinsing with absolute ethanol three times, deionized water five times, and ethanol again two times for DDS surfaces. The chips were then cured in the vacuum oven (which has been flushed with nitrogen three times) at 80°C for overnight (about 15 h).

C. Antibody (Ab) and Antigen (Ag) Systems

The goat anti-human lgG (anti-H lgG) fraction and human IgG (H-IgG) fraction were purchased from Cappel Laboratories and monoclonal mouse anti-digoxin lgG and unlabeled and ^{3}H labeled digoxin were gifts from Dr. S. Pincus, Department of Pediatrics, University of Utah, Salt Lake City, UT [21].

D. Radiolabeling of Immunoglobulins

Goat anti-H IgG or H-IgG was labeled with carrier-free I-125 (100 mCi/ml, Amersham) by a modification of the chloramine-T method as described by Chuang *et al.* [12]. Centrifugation of 4 cm high Sephadex G-25 coarse grade resin (Pharmacia) minicolumns having 0.3 ml of iodinated protein solution has provided a rapid and simple means to separate the unbound iodide [13]. The final concentration of ^{125}I-protein was measured in a UV-Visible spectrophotometer (Beckman, Model 35) at 280 nm. The constants of 1.35 ml · cm^{-1} · mg^{-1} and 150 000 dalton were used for the absorption extinction coefficient and the molecular weight of IgG, respectively. Labeling efficiency was determined by precipitating IgG molecules with 20 percent trichloroacetic acid (TCA, Sigma) in the presence of bovine serum albumin (Miles). Both supernate and precipitate were counted in a gamma well counter (Beckman).

E. Immobilization of Antibody

Two types of Ab immobilization were performed: 1) physical adsorption of Ab (0.6 mg/ml in 0.15 M phosphate buffered saline, PBS, pH 7.4) onto either DDS or APS treated silica surfaces for various length of time followed by rinsing with PBS, 2) covalent immobilization; the APS samples were reacted with 2.5 percent of glutaraldehyde (Glu, E.M. grade, Polysciences) in PBS for $1\frac{1}{2}$ h followed by rinsing with PBS buffer [14]. The Glu surfaces were then reacted with Ab solution (0.6 mg/ml) in pH 9.2 0.1 M carbonate buffer for various lengths of time. The Ab coupled samples were washed with PBS buffer and the remaining aldehyde groups on the surfaces were deactivated with 0.2 M ethanolamine (Aldrich) for 1 h.

The Ab immobilization "isotherm" for the DDS or Glu surfaces were obtained by varying the length of incubation time in a known ratio of cold and ^{125}I-anti-H IgG solution, and, as a result, the saturation surface concentration of Ab was obtained. The stability of immobilized Ab was studied by soaking the Ab-coated samples in PBS buffer and counting the samples as a function of desorption time.

F. Binding Studies of Immobilized Ab's

Several types of RIA were carried out using radiolabeled Ag. First, we determined inhibition of binding of ^{125}I-H-IgG to Ab-coated surface by adding different amounts of cold H-IgG. This was done to determine if the properties of the IgG molecules remain unchanged after iodination.

Second, the specificity of goat anti-H IgG-coated surfaces (prepared by the three different methods described above) was investigated using a fixed concentration of ^{125}I-H-IgG together with various amounts of cold rabbit IgG (R-IgG) fraction (Cappel). From this experiment, one could see how R-IgG interferes with the interactions of anti-H IgG and H-IgG.

Third, the relative affinity Kr of anti-H IgG and H-IgG in solution was determined according to Minden *et al.* [15]. The second Ab rabbit anti-goat IgG (anti-G IgG, Cappel) was used to precipitate the anti-H IgG and H-IgG complexes. Various amounts of ^{125}I-H-IgG were added to anti-H IgG solution followed by shaking the solutions at room temperature for 1 h. Then a minimum amount of anti-G IgG required for precipitation, which was determined separately, was added to all tubes and stored at 4°C overnight. The binding isotherm was obtained by counting the precipitates which had been washed three times. All microfuge tubes were precoated with human serum albumin (Miles) before use.

Fourth, binding isotherms of the Ag to the immobilized Ab were obtained by adding various amounts of the radiolabeled Ags. Goat anti-H IgG/H-IgG and mouse anti-digoxin-IgG/digoxin on two surfaces, the DDS and Glu, were investigated. Thus, a total of four binding isotherms were obtained.

Fifth, the effect of different Ab immobilization times on the activity of Ab was examined by using anti-digoxin IgG and ^{3}H-digoxin. Since digoxin is a monovalent hapten, it is reasonable to assume that the numbers of bound digoxin molecules can be used as the numbers of Ag-binding sites that still remain active after immobilization. The ^{3}H-digoxin on the silica samples was counted in a liquid scintillation counter (Beckman LS 7500). The ex-

perimental procedures were the same for all: the Ab-coupled samples were incubated for 1 h in Ag solutions consisting of 0.5 percent Tween-20 pH 7.4 PBS buffer.

Results and Discussion

1) Properties of Iodinated Protein Molecules: Since chloramine-T is a strong oxidizing agent it can alter a protein structure by reacting with amino acid side chains. In order to prove the integrity of the iodinated IgG molecules, an experiment was done by adding various amounts of unlabeled H-IgG to inhibit the binding of ^{125}I-H-IgG with anti-H IgG. The results showed that unlabeled and labeled H-IgG's behave identically.

2) Specificity of Antibody Immobilized on Different Surfaces: The three surfaces used to immobilize the Ab were the DDS, APS, and Glu surfaces. The DDS surface is very hydrophobic. The APS surface is more hydrophilic and is positively charged in a pH 7 environment. Since there are no functional groups available on these two surfaces to directly immobilize Ab covalently under the experimental conditions, Ab binding is via physical adsorption. Hydrophobic interactions are primarily involved in the case of the DDS surfaces and electrostatic and/or hydrophobic interactions for the APS surface. The Glu surface permits direct covalent immobilization. The reaction mechanism has been suggested by many groups [14], [16].

Competitive inhibition experiments were conducted to test the specificity of immobilized Ab. The inhibition of binding of ^{125}I-H-IgG to immobilized anti-H IgG by R-IgG on the three surfaces is shown in Fig. 1. The two smooth lines are the theoretical curves. The upper curve indicates no inhibition and the lower curve indicates that the inhibitor has an equal ability to the Ag to react with binding sites. No inhibition was observed for Ab immobilized on the DDS and Glu surfaces within the range of R-IgG concentrations tested; however, the inhibition curve for the case of Ab on the APS surface shows significant nonspecific binding.

Although the exact mechanism of reduction in immunoactivity of Ab on the APS surface is not understood, the protein molecules may have undergone some kind of conformational change or denaturation resulting in the nonspecific binding. Chuang *et al.* [17] observed that when prothrombin was adsorbed to artificial surfaces such as polyvinyl chloride (PVC), the cross reactivity of surface-bound prothrombin with Ab IgG to thrombin was shown to be significantly increased. He also suggested that the increase in cross reaction was due to a conformational change in the adsorbed prothrombin molecules. The DDS and Glu surfaces were used for the remaining experiments because they showed specific binding.

3) Surface Concentration and Stability of Immobilized Anti-H IgG: The anti-H IgG coupling on the DDS and Glu surfaces are shown in Fig. 2. Physical adsorption of Ab on the DDS took a shorter time to reach saturation than did the Glu surface. About 2 h was needed on the DDS and 7–8 h on the Glu surface. The surface concentrations are about 8×10^{-12} mole/cm^2 and 12×10^{-12} mole/cm^2 for the Glu and DDS surfaces, respectively. Fig. 3 shows the stability of bound anti-H IgG on the two surfaces stored in PBS buffer. About 18 percent of the Ab desorbed from the DDS surface into PBS buffer within the first 10 h. However, at the end of the 120 h the loss only increased by another 2 percent or so. This suggests that most of the Ab is irreversibly adsorbed. The Ab immobilized on the Glu surface was stable up to five days incubation in PBS buffer. This result suggests that not only stable bonding was formed between the Ab molecules and Glu layer, but also that the stability of the APS layer was increased by the presence of the Glu layer because the APS surface itself is susceptible to hydrolysis and thus should not be stable for long periods [18]. This result is consistent with Monsan's finding [14] that the attainment of stable products after Glu reaction may involve polymeric forms resulting from aldol condensation of glutaraldehyde molecules to form a Glu polymer.

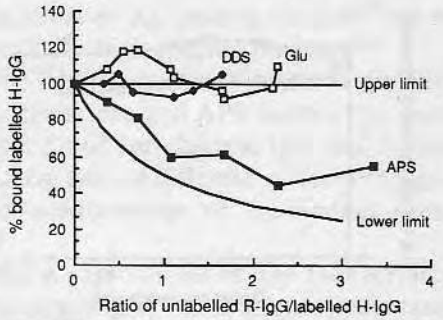

Fig. 1. Binding of labeled human-IgG to goat anti-human-IgG Ab immobilized on the DDS, APS, and glutaraldehyde-activated surfaces in the presence of various amounts of unlabeled rabbit-IgG. Two theoretical lines: upper limit (no inhibition) and lower limit (the rabbit-IgG and human-IgG having equal ability for binding of Ag-binding sites).

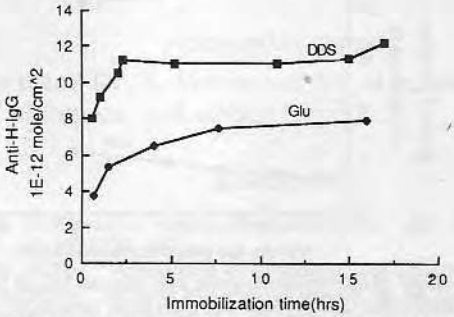

Fig. 2. The kinetics of coupling of goat anti-human-IgG Ab to the DDS and glutaraldehyde-activated surfaces.

4) Ag Binding Capacity: The maximum ^{3}H-digoxin binding capacity as a function of immobilization time of anti-digoxin IgG on the DDS and Glu surfaces is plotted in Fig. 4. Anti-H IgG was frequently used in place of the less available anti-digoxin IgG. It is reasonable to assume that their surface coupling properties are very similar. The anti-digoxin IgG on the Glu surface reaches maximum activity at about 7 h, which is approximately the time required by the anti-H IgG to saturate the surface (see Fig. 2). After about 7 h, the activity of immobilized anti-digoxin IgG started decreasing. This might be due to the

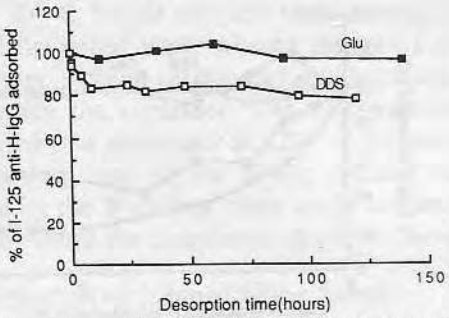

Fig. 3. Stability of immobilized goat anti-human-IgG Ab on the DDS and glutaraldehyde-activated surfaces stored in phosphate buffered saline pH 7.4 for various lengths of time.

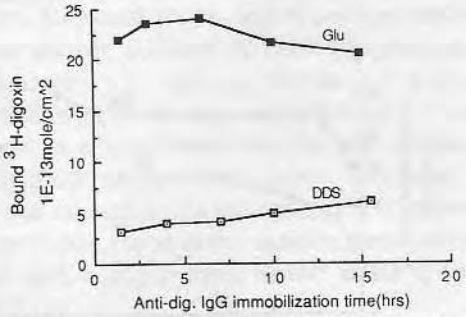

Fig. 4. Activity of immobilized mouse anti-digoxin IgG as function of immobilization time. The digoxin was used as an indication of Ab activity.

fact that more bonds are formed between the Glu layer and IgG molecules. Consequently, the molecule becomes more rigid and results in blockage of the binding site to the Ag molecules. However, the decrease is small compared with the total bound digoxin. The maximum percentage of immobilized anti-dig IgG which remained active can be calculated by taking the highest value of bound digoxin, which is about 24×10^{-13} mole/cm^2, divided by two times the value of maximum surface concentration of anti-H IgG from Fig. 2, which is about 8×10^{-12} mole/cm^2. About 15 percent of immobilized Ab remains active.

The lower curve in Fig. 4 shows the activity of the anti-digoxin IgG on the DDS surface. The bound digoxin increases linearly with increasing Ab adsorption time; from Fig. 2 the Ab saturated the surface at about 2.5 h. This indicates that Ab binding sites continuously increase at a constant amount of adsorbed Ab. The increase is almost doubled at 15 h compared with 2 h. This result is not too surprising because there is much evidence that protein adsorption is a dynamic process [19]. It is believed that the protein molecules continuously reorient themselves and finally reach equilibrium on the surface. The digoxin binding capacity of Ab on the DDS is about 6–4 times lower than on the Glu surface from 1 to 15 h.

The ^{125}I-H-IgG binding isotherms on the anti-H IgG-coated DDS and Glu surfaces with different Ab immobilization times are plotted in Fig. 5. The maximum binding of ^{125}I-H-IgG at plateau region shown in the figure is about 1.5×10^{-13} mole/cm^2 and 3×10^{-13} mole/cm^2 for the DDS and Glu surfaces, respectively. Both numbers are

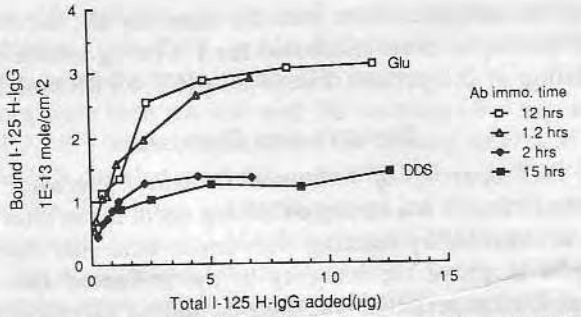

Fig. 5. Binding isotherms of human-IgG and goat anti-human-IgG Ab immobilized on DDS and glutaraldehyde-activated surfaces for different Ab incubation times.

much less than the numbers for the binding of digoxin. The maximum coupling of anti-H IgG for the DDS and Glu surfaces in Fig. 2 are 12×10^{-12} and 8×10^{-12}. Therefore, less than 4 percent immobilized anti-H IgG's remains active. This is because the goat anti-H IgG used was only an IgG fraction and thus the content of specific Ab is low. Another interesting feature shown in Fig. 5 is that the ^{125}I-H-IgG binding capacity shows no dependency on different Ab immobilization times because the two curves plateau at the same region for each surface. The possible explanation might be due to steric hindrance factors of the large H-IgG molecule. In another words, the binding sites that become active with increasing adsorption time are only accessible to small hapten molecules such as digoxin.

5) Equilibrium Constants of Immobilized Antibodies and Antigens: Most discussion of Ab-Ag interactions assume that both of them are in soluble form. They are free to move in the solution and association and/or dissociation of Ag molecules from Ab molecules occurs rapidly to finally reach equilibrium. The main objective of this study was to investigate the properties of immobilized Ab's. Two questions can be asked. First, will the binding constant K change because the conformation of the immobilized Ag-binding site is different from that in solution? Second, will the K increase because the Ab molecules are not free to move, resulting in an increased local Ab concentration?

In order to answer these two questions, the K of two Ab–Ag systems used were determined. The intrinsic association constant Ka for mouse anti-digoxin IgG and digoxin was obtained by using Sip's equation [20]. The highest value of bound digoxin was taken as the concentration of active binding sites on the surfaces from which r (moles Ag bound per mole of Ab) was then computed. The Ka values listed in Table I are close to the value in solution, 3.54×10^8 M^{-1} [21]. Two to three times difference was felt small enough to be attributed to experimental differences. The index of heterogeneity a calculated from Sip's plot and the ratio of bound digoxin to active binding sites on the surfaces are all equal to 1 as expected. Since the Ka directly reflects the free energy of Ab–Ag interaction, this result might suggest that the conformation of Ag-binding sites is preserved, and it is the

TABLE I
THE EFFECT OF DIFFERENT IMMOBILIZATION METHODS AND SURFACES ON THE ASSOCIATION CONSTANT, K

Ab-Ag system	anti-dig IgG/dig.		anti-H IgG/H-IgG	
Surface	Glu	DDS	Glu	DDS
$K(M^{-1})$	1.9×10^8	5.8×10^8	6.1×10^6	6.7×10^8
a	1	1	0.80	0.89
Bound Ag/$(Ab)_{site}$	1	1	0.96	0.99

same on the two different surfaces and in solution. This also suggests that immobilized Ab has no effect either on local concentration or on the positive or negative cooperative binding when monovalent Ag is used. Liu *et al.* [22] made similar observations. He showed that there was no difference in affinity constant between monovalent Ab (Fab) or intact bivalent Ab on surfaces of polyacrylamide beads and in solution with regard to the binding of monovalent hapten.

The determination of relative association constant Kr of anti-H IgG and H-IgG is complicated by the polyvalence of the Ag and the heterogeneity of Ab affinities. Problems caused by the former can be overcome by using proper Ag concentration to prevent the Ag being bound by more than one Ab. Problems caused by the latter, which would have nonlinear binding characteristics, can be surmounted by using the method developed by Steward and Petty to analyze the data [23]. In this method, total Ab active sites were determined graphically from the reciprocal Langmuir equation by extrapolating the plot of $1/[b]$ versus $1/[\mathrm{Ag}]$. Kr listed in Table I for the two surfaces are very close, again suggesting that the conformation of binding sites is the same on the two surfaces. It has been shown that polyclonal Ab exhibits a distribution of binding constants [24]. If certain clones couple preferentially, the experimentally derived Kr will vary for different surfaces. Our data indicated that Kr is essentially unchanged for two surfaces, implying that the coupling of Ab's to these surfaces is a random process. However, Kr in solution is $2 \times 10^7\ M^{-1}$ and is one order lower than that on surfaces (Table 1). Since the ratio of bound H-IgG to Ab active binding sites is close to one for all situations, this implies that the binding enhancement on surfaces can be explained by the factor that the probability of a H-IgG molecule caught by neighboring Ab active sites is higher on surfaces than in solution. As mentioned previously, no binding enhancement was observed in anti-dig IgG and digoxin system. In other words, the magnitude of binding enhancement depends on both local Ab concentration and size of Ag molecules. Lui *et al.* also noticed that the immobilized Ab against multivalent Ag showed binding enhancement [22]. The index of heterogeneity deviates from one as expected.

CONCLUSION

The observations obtained from this study are

1) the Glu surface is a better surface than the DDS surface in terms of Ag binding capacity and stability. Both surfaces have high specific binding.

2) the Ab is denatured when it is directly adsorbed on the positively charged APS surface (no specificity).

3) the Ka of anti-digoxin IgG and digoxin are similar in solution and on different surfaces, suggesting that the original conformation of Ag-binding sites is also preserved.

4) the Kr are similar on the two surfaces, suggesting that the coupling of Ab is a random process.

5) Binding enhancement was not observed in anti-dig IgG and digoxin, whereas it is found in anti-H IgG and H-IgG, suggesting that the enhancement was the consequence of both high local Ab concentration and size of Ag molecules.

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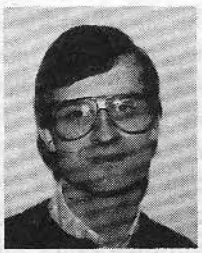

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4C1-33 PHOTOREGULATION OF AB-AG AFFINITIES FOR IMMUNOSENSOR APPLICATIONS

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There has been considerable interest in the use of optical fibers for remote spectroscopic sensing of biomolecules. The basic idea, in short, is to immobilize a receptor (e.g. antibody) on the surface of an optical element to specifically detect the ligand (e.g. antigen) in solution. Various sensing techniques have been developed by many groups. We are developing an immunosensor using an evanescent wave to excite fluorescence of molecules present at the interface. Fiber optic immunosensors should be fast, sensitive, specific, remote, and semicontinuous. The sensor must be designed to be reusable. In order to maximize sensitivity, it is desirable for the Ab-Ag constant to be as high as possible, meaning the off-rate is very slow--hence slow response times. Thus, in this study, we are looking for a means to "zero" the sensor between each measurement without sacrificing the sensitivity.

A panel of anti-fluorescyl monoclonal Abs and fluorescein Ag were used as a model system. Dianionic fluorescein is strongly fluorescent, but the fluorescence of Ab-bound fluorescein is quenched. Therefore, by measuring the fluorescence intensity and polarization, the binding constant can be obtained. The thermodynamic properties of Ab-Ag binding in solutions were investigated. The solutions we have used include pH 8, potassium phosphate buffer (PPB), 2-methyl-2,4-pentanediol (MPD) in PPB, and N-(2-hydroxypropyl) methacrylamide (HPMA) in PPB. The results showed that the affinity of the anti-fluorescyl monoclonal antibodies is strongly dependent on the concentration of the solute (Fig. 1). It is believed that the decreased affinities are due to the decrease in conformational entropy of Ab because negative changes were observed in both ΔS^{o} and ΔCp^{o}. The temperature perturbation experiments also showed that ΔGu increased and the denaturation temperature decreased with increasing MPD concentrations (Fig. 2). By understanding the association and dissociation mechanism of Ab-Ag interaction in solutions, we proposed a method based on the photo-isomerization of copolymers which change the microenvironment of the Ab binding sites, and in turn affects the affinity.

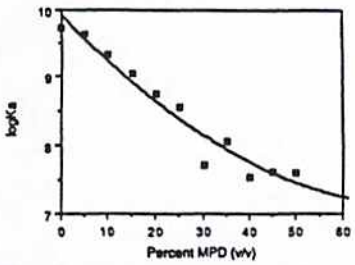

Figure 1: Effect of 2-Methyl-2,4-Pentanediol on the Affinity (Ka) of 4-4-20.

The proposed copolymer has the general structure shown in Fig. 3. The A units provide a hydrophilic, inert polymeric carrier and also can lower the affinity of Ab via their solute effect. The B units provide the photo-isomerizable azo groups. The C units provide the acid polyelectrolyte character needed for coil expansion and contraction. The D units provide a reactive group which can be used to bind the copolymers to Abs. We started with a copolymer containing B and C units to see the effects of azobenzene on carboxyl groups before and after irradiation by the light. A change in pH was observed. Two different methods for introducing the azobenzene side chain were compared. The kinetics of photo-isomerization were also studied. A series of compositions of azobenzene-containing copolymers were synthesized. The conformational changes of copolymers caused by photo-isomerization were investigated by measuring the viscosity and hydrodynamic diameter (light scattering) as a function of pH, temperature, and ionic strength.

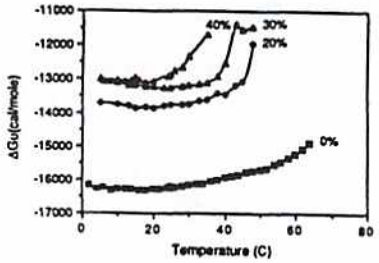

Figure 2: Unitary free energy (ΔGu) for the binding of fluorescein by monoclonal anti-fluorescyl Ab 4-4-20 in various concentrations of MPD.

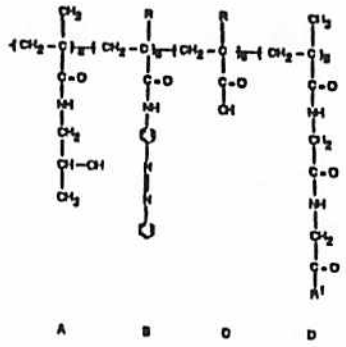

Figure 3: General structure of the proposed copolymer.

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The AFM[2] can be used to obtain atomic-scale images of surfaces.[3-7] The surface to be imaged is mounted onto a (xyz) piezoelectric crystal and is rastered beneath a sharp tip attached to a cantilever. The tip rides across the surface, and the forces between the surface and the tip cause deflection of the cantilever. This deflection can be monitored by using a scanning tunneling tip[2,3] or, more easily, it can be monitored by movement of a laser beam that is reflected off the back of the cantilever.[8,9] Since the cantilever is sensitive to the intermolecular forces between the tip and the surface, the sample need not be a conductor to be imaged. Images have not only been obtained from graphite[3-5] and metals[6] but also from semiconductors[4,7] and insulating polymers.[10-12] Magnetic fields[13] and charged regions in materials[14] have been imaged as well. More detailed reviews of the AFM theory are presented by Marti et al. and Hansma et al.[15,16]

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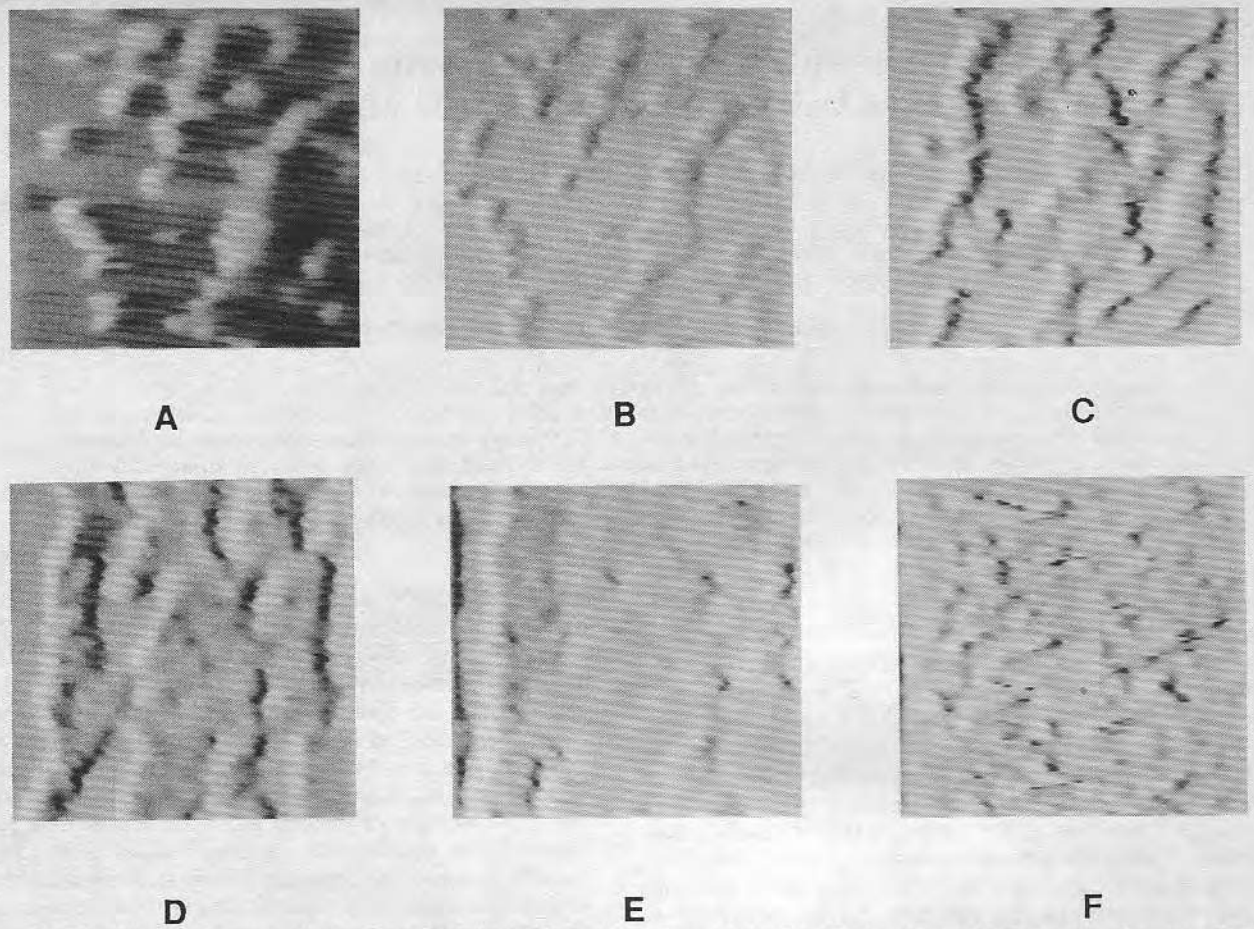

Figure 1. (A) Feedback image of 18 μg/mL IgG 4-4-20 in PBS on clean mica after 5 min. Note the formation of aggregate "ridges". The scan area is 4500 Å × 4500 Å. (B) Variable-force-mode image of 18 μg/mL IgG 4-4-20 in PBS on clean mica after 5.5 min. The ridges are now resolved better. The scan area is 4500 Å × 4500 Å. (C–E) Variable-force mode images of 18 μg/mL IgG 4-4-20 in PBS on clean mica. Times of adsorption are 15, 17.5, and 20 min. Growth of the monolayer centers about the ridges and proceeds until a near monolayer is formed. The scan areas are 4500 Å × 4500 Å. (F) Variable-force-mode image of 18 μg/mL IgG 4-4-20 in PBS on clean mica. Adsorption time is 37 min. After a near complete monolayer is formed, a second layer begins to deposit. The scan area is 4500 Å × 4500 Å.

mica surface might occur, and, if so, this ordering could be imaged with the AFM. We felt that desorption might be observed as well.

Methods

The AFM experimental apparatus has already been described elsewhere.[16] Movement of the microfabricated cantilever[20] is detected by the positioning of a laser light beam that has reflected off the back of the cantilever and is detected by a pair of photodiodes. The AFM images are continuously recorded on video tape for later review. A flow cell has been setup across the surface of the mica and allows rapid exchange of the fluid.

Mica (Asheville-Schoonmaker) was affixed to the piezoelectric crystal stage and cleaved in situ. The flow cell was constructed around the stage, and distilled water was injected onto the mica surface. The microcantilever was advanced until the force between the tip and the surface approximated 10^{-9} N. The mica was then imaged continuously in the feedback mode with a scan area of 1800 Å × 1800 Å and a constant scan speed of 16 ms/line.

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Immediately after the 40-min IgG adsorption, the tip was retracted and the flow cell was flushed with distilled water. The surface was scanned for 10 min at full scale, at 1800 Å × 1800 Å and then at 900 Å × 900 Å.

Results

The image obtained of the mica surface underwater is flat and featureless indicating a pristine surface.

Within the first 2 min after injection of the IgG into the flow cell, a continuously growing aggregate was observed in the lower right-hand corner of the screen. This image was obtained in feedback mode and had dimensions of 1800 Å × 1800 Å. This aggregate appeared on top of the featureless mica background. After 5 min, the scan area was increased to 4500 Å × 4500 Å, and "ridges" appeared (A). The AFM was then switched to variable-force mode (B), and the same image appeared (different contrast), indicating that either mode could be used. As time progressed, it was clear how the adsorption was

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The observations of deposited IgG on the surface appearing only as aggregates and of individual mounds rapidly desorbing from the surface are suggestive of lateral interactions occurring between the adjacent IgG molecules, which appear to be important for formation of a stable protein layer. These lateral interactions are not unexpected, since this protein has some self-aggregating properties. What is interesting is the necessity of lateral interactions for adherence to the surface. Perhaps, an IgG molecule by itself can only get a toehold on the surface at first and can be desorbed easily. Once it has multiple holds with the surface and neighboring molecules, the probability of desorption decreases significantly.[1] This would explain the phenomena we observed here. This argument is also supported by the observation of a second IgG layer arising from many different areas on the monolayer surface. Here, the deposited IgG can arise from any number of places, since interactions can occur from anywhere on the monolayer surface. Prior to this experiment, a protein adsorption isotherm on mica was obtained by using ^{125}I-labeled IgG. The isotherm showed Langmuir-like behavior, and at a concentration of 18 μg/mL, the mica surface was only 40% covered (less than a monolayer).

After the water flush, IgG desorption was not observed. Perhaps, desorption was slower than the observation time (minutes) due to the strength of interaction between the adjacent IgG molecules. Previous data have shown[22] that IgG does desorb from a silica surface, but that study was performed with polyclonal IgG and may behave differently from the present system.

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We emphasize that this experiment is a preliminary one done at one protein concentration. The effect of concentration on the adsorption pattern is yet unknown but is the subject of ongoing studies. Furthermore, the issue of protein adsorption on the probe itself was not addressed here. This issue is important and merits further detailed study and consideration.

Conclusion

By use of an atomic force microscope, real-time imaging of IgG deposition on flat mica was accomplished. While IgG adsorption may occur anywhere on the surface, only those molecules with sufficient lateral interactions had the capability to remain on the surface. Isolated molecules desorb readily. A second layer could be observed after 35 min of adsorption. It was hoped that unique ordering of the IgG on the surface could be visible, but this was not evident. Although, restructuring of the surface had occurred in the desorption experiment, desorption was not conclusive.

While it is exciting that individual molecules could occasionally be seen, it is not clear how much the probe affects molecular conformation. When more sensitive cantilevers and more sophisticated detection systems are developed, it may be possible to operate the AFM with forces of 10^{-10}–10^{-11} N and image biomolecules unperturbed by the probe.

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18

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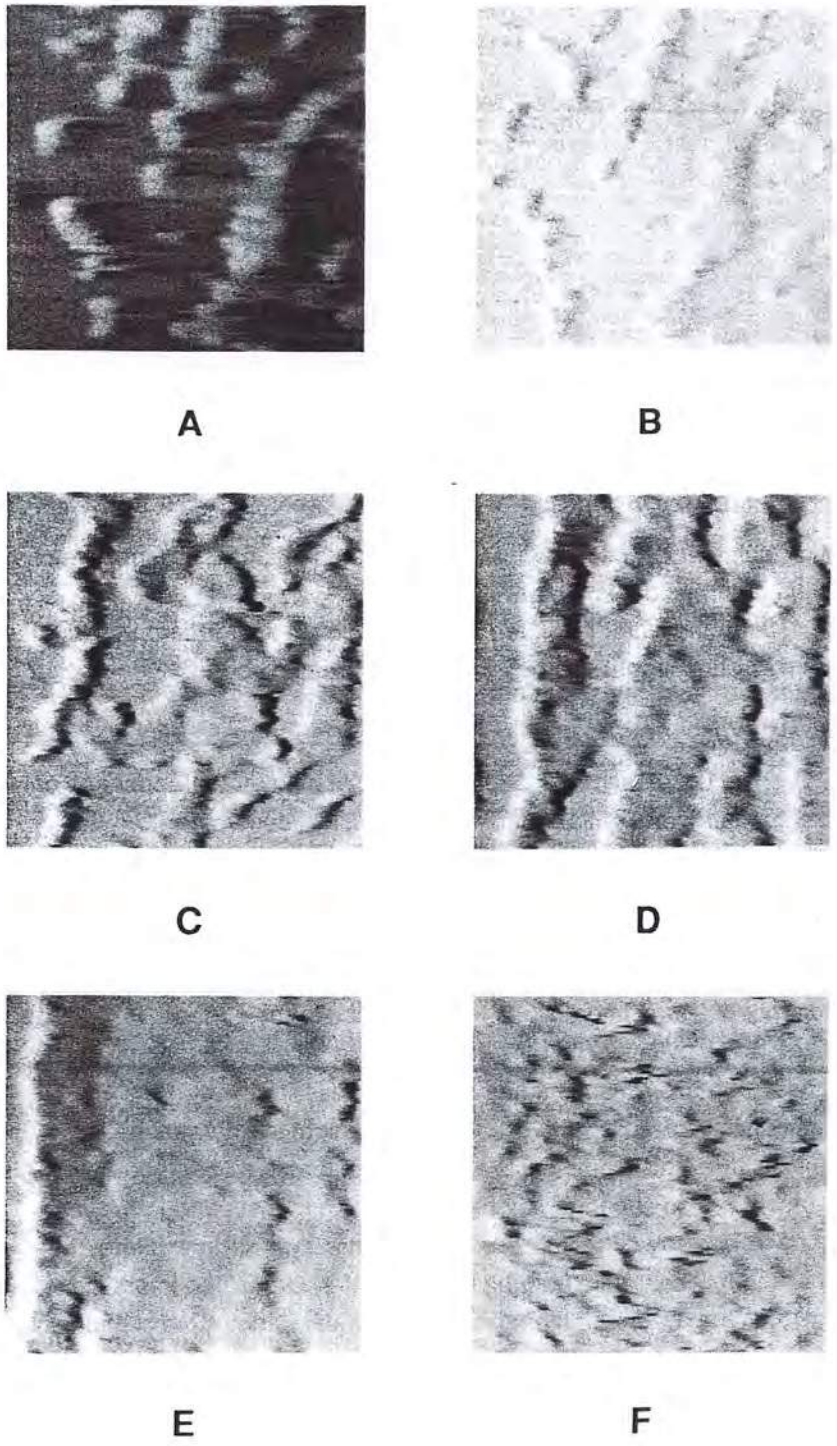

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Comparison of site-specific coupling chemistry for antibody immobilization on different solid supports

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ABSTRACT

Silica is an important chromatographic support for high-performance affinity chromatography due to its high mechanical stability. However, silica is very different from traditional gel chromatographic materials, such as agarose, dextran, and polyacrylamide, with respect to many chemical and physical properties. Thus, it is expected that different immobilization techniques must be used for orienting the immobilized ligand on the surfaces of the two substrates. In this study, a site-specific coupling chemistry for immobilization of antibodies on modified silica surfaces and on agarose gels was investigated. The effects of substrates on the orientations of immobilized native and partially denatured antibodies was deduced. An important conclusion is that non-covalent interactions (physical adsorption) dominate the orientation of immobilized antibodies on silica surfaces.

INTRODUCTION

Due to their high specificity, antibodies immobilized on chromatographic supports have been widely used for purification of molecules since the early 1970s [1]. Numerous coupling chemistries for the immobilization of antibodies have been developed and reviewed [2,3]. Most coupling chemistries rely on the random coupling of antibodies through the amino groups of lysine residues. This often results in a decrease in antigen binding capacity (AgBC) due to improper orientation of the

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where [Ag] and [AL] are the surface concentrations of antigen and antibody, respectively, and the subscripts N and D represent native and denatured antibody cases, respectively. Values greater than 1 indicate that the apparent AgBC of the immobilized antibodies is higher than that of the native antibody. For agarose gels all values are close to 1 as expected, because the orientation of immobilized antibody was controlled by chemical coupling. Thus, it has the least dependence on the conformational changes. However, on silica surfaces all cases exhibit some degree of dependence on the adsorption properties of the antibodies. Positive orientational effects (cases b, c and d) and negative influences (case a) are observed.

In summary, we have demonstrated that exposure of antibodies to a low-pH buffer prior to immobilization results in an increase in AgBC of the immobilized antibody on silica surfaces. This can be a useful method to improve the column capacity in immunoaffinity chromatography. Another very important conclusion is that physical adsorption dominates the orientation of immobilized antibodies on silica surfaces. Although chemical bonds may be useful in providing stable linkages between the antibody and surface, they do not affect orientation of the antibody on the surface. This concept is applicable to all materials that have high non-specific adsorption sites for proteins, including antibodies and enzymes. Therefore, when such materials are chosen as the solid supports for affinity chromatography, one should not approach the problem totally on the basis of trial and error. Instead, one can either modify the antibody molecules in such a way that active sites have a low interaction energy to the surface, or modify the surface to preferentially interact with only certain parts of the antibody.

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antibody molecules on the solid surfaces. To overcome these problems, two approaches for site-specific immobilization of antibodies have been reported recently. In the first approach, antibodies are immobilized to hydrazide-activated supports via their oxidized carbohydrates by forming covalent hydrazone bonds. Immunoglobulins contain carbohydrate moieties linked to the CH_2 domain of the Fc fragments. Under mild conditions, the hydroxyl groups of the carbohydrates can be oxidized to aldehyde groups by sodium periodate without significantly impairing the active sites of the antibody [4-6]. Another method is to immobilize Fab' fragments via thiol groups at the hinge region [6-8]. This can be done by first making $F(ab)'_2$ with pepsin digestion, followed by reduction of the disulfide bond which links the two Fab' fragments by dithiothreitol. The free thiol groups can then be coupled to maleimide-activated supports.

When antibodies are immobilized to agarose gels, both site-specific coupling methods result in an increase in AgBC over non-selective coupling methods. However, agarose gel is not an ideal support for high-performance affinity chromatography (HPAC) because of its low mechanical stability. Recently, it has been suggested that silica is a promising material for HPAC due to its inherent mechanical stability, which provides good flow characteristics even under high pressure [9].

However, silica and agarose exhibit completely different chemical and physical properties. For example, agarose gel is a soft organic material with high water content, while silica is a hard inorganic material. From the point of view of antibody immobilization, there is one important difference between the two materials which has been ignored so far and needs to be examined, protein adsorption or resistance properties. Why are these properties important for site-specific couping of antibodies? Examine Fig. 1.

In the upper part of Fig. 1, materials used for chromatographic supports are roughly classified into three categories, according to their protein resistance properties. The first category includes natural and synthetic uncharged hydrogels, such as agarose, dextran, and polyacrylamide, which exhibit low protein adsorption properties. Although a number of explanations have been offered as to why proteins exhibit weak interactions with these materials[10], the exact mechanism is still unclear. The second category includes materials such as silica, polystyrene, and polyethylene, which exhibit strong protein adsorption. Depending on the nature of the surface, protein, and solution medium, the protein is adsorbed via ionic interactions, hydrophobic effects, polar–polar interactions, Van der Waals forces, or, most probably, a combination of all such interactions [10,11]. An enormous amount of work has been reported in the literature on the protein adsorption to various substrates [11,12]. The third category consists of the materials which have the protein adsorption properties somewhat between the two types of materials discussed above.

Site-specific coupling of a protein to agarose (left) and silica (right) surfaces is schematically shown in the lower part of Fig. 1. Examine the agarose situation first: the open circle on the hydrogel surface represents a functional group which can form a covalent bond with a specific site on the protein (shown by the closed circle on the protein). Our hypothetical protein is a single polypeptide chain and consists of two domains, whose surface includes regions of hydrophobic, charged, or polar character. According to the collision theory of bimolecular reactions, the protein molecule must first approach and collide with the surface before the covalent bond can be formed.

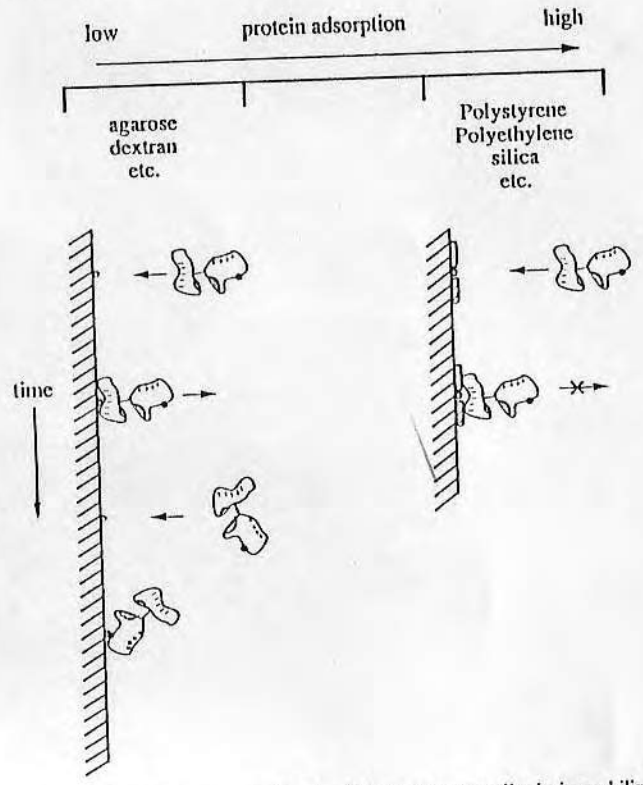

Fig. 1. Schematic representation for the concept of antibody immobilization on different types of solid supports.

The first collision may not be of proper orientation for the formation of a covalent bond. Since agarose has very weak non-covalent interactions with the protein, the protein will then diffuse away from the surface and other proteins will collide with the surface in different orientations. After a number of collisions, protein finally approaches the surface with an orientation suitable for formation of chemical bonds. This surface coupling process is similar to solution reactions except that one of the reactants is a stationary phase.

In the case of silica (right, Fig. 1), non-covalent adsorption sites (represented by the filled squares) are presented on the silica surface, and probably influence the site-specific coupling of the protein. Therefore, in order to understand site-specific coupling to silica surfaces, protein adsorption must be examined. The general concepts of protein adsorption currently accepted have recently been reviewed by Andrade and co-workers [10-12]. In summary, for a one-component system, a protein of given bulk concentration diffuses to and collides with the interface. If the interaction forces and contact area are large enough, the protein stays on the surface for a certain residence time, probably in the range of milliseconds to seconds, while on the surface, the protein may begin surface "denaturation". With increasing contact time, the probability for

desorption decreases. Eventually, the protein may become irreversibly adsorbed on the surface in a completely or partially denatured conformation. Therefore, one could expect that on the silica surfaces the formation of chemical bonds is secondary to physical adsorption.

From the above discussion, it seems that, depending on the types of substrates, different immobilization strategies should be employed for controlling the orientation of the immobilized protein. But, how can we experimentally prove it? We have previously shown that the immobilization of the partially denatured antibodies on silanized silica surfaces resulted in higher AgBC and higher antibody surface concentrations than for the native antibody [13]. The antibody immobilization methods used in the previous study were physical adsorption and non-site-specific chemical coupling. In this study we have immobilized antibodies on different supports to investigate the substrate effect on the site-specific coupling for antibody immobilization. Hydrazide-derivatized agarose and silica were used to immobilize oxidized and non-oxidized native and partially denatured antibodies.

EXPERIMENTAL

Cleaning of silica samples

All silica samples were cut from 2.5 cm × 2.5 cm × 0.1 cm fused-silica slides (CO grade, ESCO) and the edges were finely polished. The size of the sample was 1.1 cm × 0.95 cm × 0.1 cm and fits into a 13 × 75 mm culture tube (Fisher) in which all surface reactions took place at room temperature. Cleaning of the silica samples has been described elsewhere [13].

Silanization of cleaned silica samples

Two silane reagents were used in this study: γ-glycidoxypropyltrimethoxysilane (GOPS, Aldrich) and dimethyldichlorosilane (DDS, Petrarch). The silica chips were reacted with a dry toluene solution of GOPS (2% GOPS, 0.2% triethylamine and 97.8% dry toluene, v/v) [14] or dry toluene solution of DDS (10% DDS and 90% dry toluene, v/v) for 1 h and 30 min at room temperature, respectively. The GOPS samples were rinsed with dry acetone and the DDS samples were rinsed with absolute ethanol. The chips were then cured in a vacuum oven (which has been flushed with nitrogen three times) at 120–130°C for 1 h. The epoxide groups of GOPS on the silica surfaces were then reacted with a 10% hydrazine solution (E. Merck) in methanol for 3.5 h at room temperature, followed by rinsing with deionized water first and then absolute ethanol and dried in a vacuum oven for 1 h at 120–130°C. The hydrazide-treated silica (Si-Hz) surfaces were analyzed by electron spectroscopy for chemical analysis (ESCA) to confirm the occurrence of the surface modification.

Antibody and antigen system

The polyclonal goat anti-human serum albumin (anti-HSA) immunoglobulin G (IgG) fraction was purchased from Cappel Labs. Crystallized human serum albumin (HSA) was purchsed from Miles Diagnostics.

Radiolabelling of proteins

The anti-HSA or HSA was labelled with carrier-free ^{125}I (100 mCi/ml,

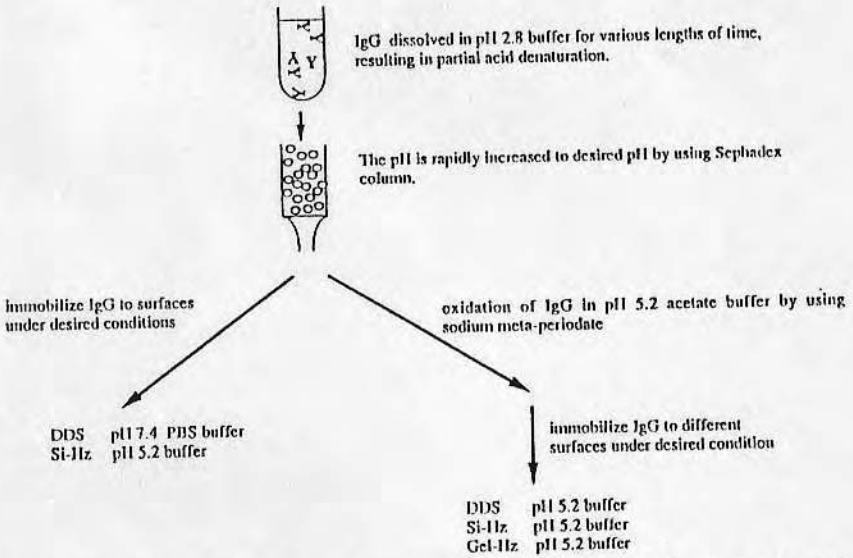

Fig. 2. Schematic representation of the antibody denaturation and immobilization procedures. DDS = dimethyldichlorosilane-treated silica surface; Si-HZ = hydrazide-treated silica surface; Gel-Hz = hydrazide-treated agarose gel.

Amersham) by the chloramine-T method as described by Lin *et al.* [13]. The final concentration of [^{125}I]protein solution was measured in a UV–visible spectrophotometer (Beckman, Model 35) at 280 nm. Values of 0.54 and 66,000 were used for the absorption extinction coefficient ($E^{0.1\%}$) and molecular weight (MW) of HSA, respectively, while for IgG the values of $E^{0.1\%}$ and MW used were 1.35 and 150,000, respectively. Labelling efficiency was determined by precipitating proteins with 20% trichloroacetic acid (TCA, Sigma) in the presence of bovine serum albumin (BSA) as carrier protein. The amount of iodide bound to protein was determined by subtracting the counts in the supernatant from the total counts in solution.

Preparation of the partially denatured antibodies

Antibody was partially denatured by dissolving approximately 5 mg of anti-HSA in 0.5 ml of 0.1 *M* citric acid–phosphate buffer (pH 2.8) for various lengths of time: 1, 20, 60 and 300 min (Fig. 2). The pH of the antibody solution was then increased by using a PD-10 column (Pharmacia) which has been equilibrated with the buffer used for antibody immobilization.

Oxidation antibodies

The native or partially denatured antibodies were oxidized by sodium meta-periodate (J. T. Baker) in 0.1 *M* acetate buffer pH 5.2 (Fig. 2). Sodium meta-periodate stock solution (30 mg/ml) was added to antibody solution (3 mg/ml) at one tenth the final volume. The mixture was gently mixed for various lengths of time (20, 40 and 60 min) at room temperature and the sodium meta-periodate was then removed using

a desalting column which had been washed and equilibrated with diluted coupling buffer, pH 5.2 acetate buffer.

Immobilization antibodies

Silica. Oxidized or non-oxidized samples of native or partially denatured anti-HSA antibodies were immobilized via either physical adsorption or site-specific chemical coupling. For physical adsorption, three experiments were performed: (1) non-oxidized antibodies in 0.15 *M* phosphate-buffered saline (PBS, pH 7.4) adsorbed onto DDS silica surfaces; (2) non-oxidized antibodies in 0.1 *M* acetate buffer (pH 5.2) adsorbed onto S-Hz surfaces; and (3) the oxidized antibodies in 0.1 *M* acetate buffer (pH 5.2) adsorbed onto DDS silica surfaces. For site-specific coupling, the oxidized antibodies were reacted with the Si-Hz surfaces in 0.1 *M* acetate buffer (pH 5.2.). The adsorption or reaction time for all experiments was 3 h at room temperature followed by rinsing with buffer. The concentration of antibody solutions ranged from 0.7 to 0.9 mg/ml. Surface concentrations of the immobilized antibodies were determined by radiolabelling technique.

Agarose gel. The Affi-Gel hydrazide agarose gel (gel-Hz) purchased from Bio-Rad was used to immobilize anti-HSA antibodies. The gel contained hydrazide groups, and coupling procedures have been described elsewhere [15,16]. Solution concentrations of antibodies ranged from 0.8 to 1.1 mg/ml. The amount of antibody immobilized on the gel beads was determined by measuring the depletion of antibody in bulk solution with a UV–VIS spectrophotometer.

Antibody–antigen binding experiments

The anti-HSA-coated silica and hydrogel samples were incubated with an excess amount of iodinated HSA in PBS for 1 and 2 h at room temperature, respectively. The silica samples were held by forceps and rinsed with PBS gently to remove the weakly adsorbed antigen solution layer. The hydrogel samples were rinsed with PBS more than five times until no iodinated HSA was detected in the rinsing solution. In all antigen binding experiments, an excess amount of BSA was added to the HSA solution to minimize non-specific adsorption. The amount of BSA added was 70–100 times higher than that of HSA.

Fluorescence measurement

Conformation changes of the antibodies resulted from acid treatment or oxidation were characterized by 4,4′-bis[8-(phenylamino)naphthalene-1-sulfonate] (bis-ANS, Molecular Probes). Bis-ANS fluoresces strongly with an emission peak at 515 nm when bound to hydrophobic regions of proteins, whereas the unbound dye is virtually non-fluorescent in aqueous buffer [17,18]. For non-oxidized antibodies, the final concentrations of the antibodies and bis-ANS in PBS were $3 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ *M*, respectively. For oxidized antibodies, the fluorescence measurements were performed in pH 5.2 acetate buffer in which cross-linking between antibody molecules was prevented. The final concentrations of the antibodies and bis-ANS were $6 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ *M*, respectively. The fluorescence experiments were performed in a Greg-200 multifrequency fluorometer (ISS, Champaign, IL, U.S.A.). The sample solutions were excited with plane-polarized light at 400 nm from a broadband 200-W xenon high-pressure lamp. The fluorescence at 515 nm was collected at 90° with respect to the incident light.

RESULTS

The AgBC of oxidized and non-oxidized antibodies immobilized on DDS silica surfaces are shown in Fig. 3 as a function of antibody denaturation time. The zero time point is for the native antibody, immobilized without low-pH treatment. Denaturation of antibody in low-pH buffer for 1 min, 20 min, 1 h and 5 h were performed (Fig. 2). Prior to immobilization, the low-pH buffer was exchanged with pH 7.4 PBS buffer for the non-oxidized antibodies or pH 5.2 acetate buffer for the oxidized antibodies.

For non-oxidized antibody, the AgBC after 20 min exposure to low-pH buffer is approximately two times higher than that of the native antibody (0 min) case (Fig. 3a). This increase in antigen binding is probably due to specific interactions because BSA was used to reduce non-specific binding. Furthermore, the AgBC decreases dramatically with increasing denaturation time. After 5 h of exposure to low-pH buffer, the AgBC is slightly lower than that of the native case. Although this set of results has been published previously, it is listed here again for the purpose of comparison [13]. A similar trend was also observed for antibodies which had been oxidized with sodium meta-periodate (Fig. 3b). The AgBC increased with increasing antibody denaturation

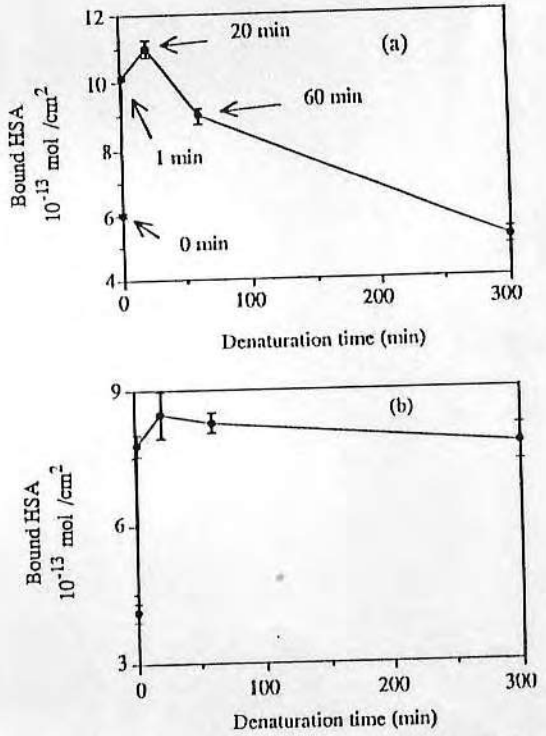

Fig. 3. Antigen binding capacity of immobilized antibody on DDS surfaces as a function of antibody denaturation time: (a) non-oxidized antibody ($n = 3$) (from ref. 13); (b) oxidized antibody ($n > 4$) (n = number of samples). The zero time point is for the native antibody, immobilized without low-pH treatment.

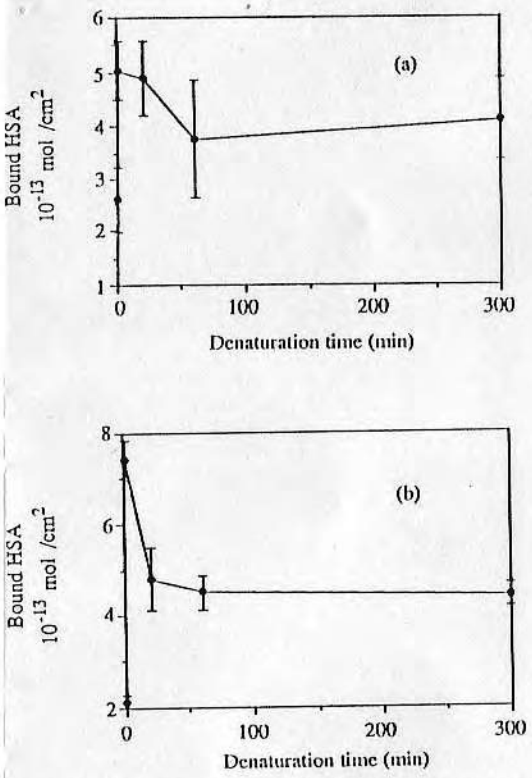

Fig. 4. Antigen binding capacity of immobilized antibody on Si-Hz surfaces as a function of antibody denaturation time: (a) non-oxidized antibody ($n=3$); (b) oxidized antibody ($n=8$).

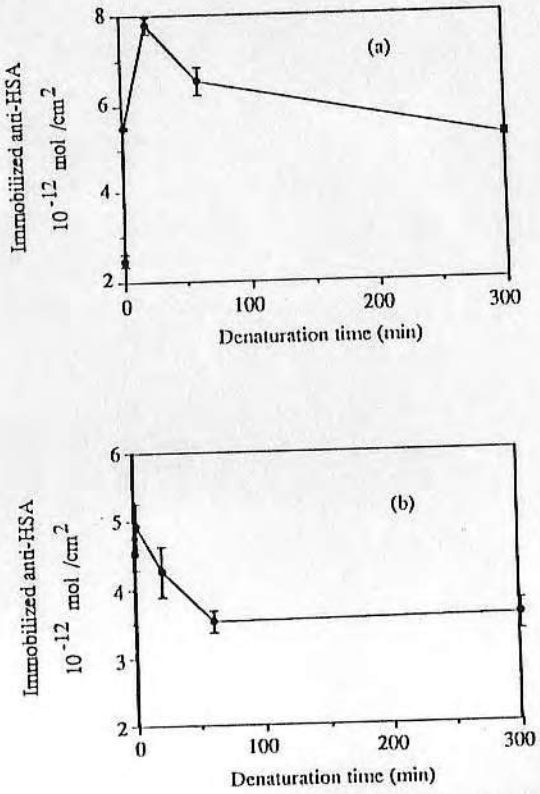

Fig. 5. Surface concentrations of anti-HSA on DDS surfaces as a function of antibody denaturation time: (a) non-oxidized antibody ($n=3$); (b) oxidized antibody ($n>4$).

time and reached a maximum after 20 min. The AgBC was approximately two times higher than that of the native case. At longer times, the AgBC decreased slightly with further denaturation time.

The AgBC of oxidized and non-oxidized antibodies immobilized on Si-Hz surfaces are shown in Fig. 4. The results were similar to those observed on DDS surfaces. The maximum AgBC of both antibodies on Si-HZ surfaces occur after 1 min of low-pH treatment, and they are about two to three times higher than those of the native antibodies. The surface concentrations of non-oxidized and oxidized antibodies immobilized on DDS and Si-Hz surfaces were also determined as a function of antibody denaturation time (Figs. 5 and 6, respectively). In all cases, exposure of antibodies to low-pH solution prior to immobilization dramatically increased antibody surface concentrations.

In contrast to modified silica surfaces, the hydrogel exhibited very good protein resistance [13]. In other words, the antibodies did not adsorb to agarose and can only be immobilized to the beads through covalent bonds. This was demonstrated by incubation of the hydrogel beads with iodinated non-oxidized native and partially denatured antibodies (5-h acid treatment). In principle, formation of covalent bonding is not possible in this case. After incubation and extensive rinsing, the beads were counted for antibody surface concentrations. The results show that only a trace of antibody bound to the beads. Therefore, the hydrogel beads were used as control for a surface which exhibits little or no protein adsorption.

The amount of oxidized antibodies and their AgBC immobilized on the Affi-Gel beads were determined as a function of antibody denaturation time (Fig. 7). The results showed that the AgBC and antibody surface concentrations decreased concomitantly with increasing antibody denaturation time. This suggested that the decrease in AgBC is caused by the decrease in antibody surface concentration, rather than the orientation of the immobilized anibody. Furthermore, the initial increase in the AgBC that was observed with short denaturation time on silica samples (Figs. 2–4) was not observed with hydrogel beads, suggesting that different mechanisms were involved in antibody immobilization on the two different types of materials.

It is well known that proteins in low-pH solution undergo conformational

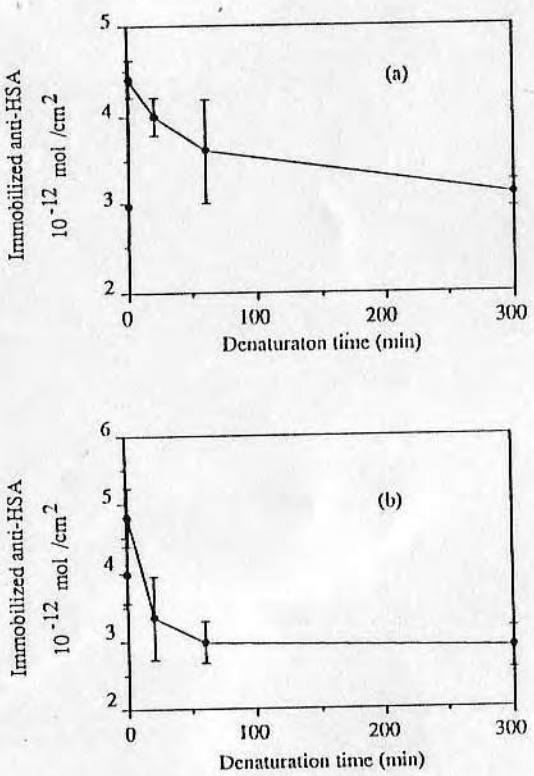

Fig. 6. Surface concentrations of anti-HSA on Si-Hz surfaces as a function of antibody denaturation time: (a) non-oxidized antibody ($n=3$); (b) oxidized antibody ($n=7$).

changes, which in turn affect their adsorption properties. The conformational changes of the partially denatured antibodies were characterized using a fluorescent hydrophobic probe, bis-ANS. The fluorescence spectra of bis-ANS in different antibody solutions are shown in Fig. 8. It can be seen that the fluorescence intensity of bis-ANS increases with increasing antibody denaturation time, indicating conformational changes of the Abs. In Fig. 8, data for the antibody with acid treatment for 5 h are not shown because the fluorescence intensity was too high to measure. The effect of oxidation time on the conformation of antibodies was also characterized by bis-ANS (Fig. 9). The fluorescence spectra for the antibodies oxidized by sodium periodate for 20, 40 and 60 min (curve c and d) overlap each other and are higher than that for the non-oxidized antibody (curve b). This indicates that oxidation processes have changed the conformation of certain parts of the antibody molecule and that the kinetics of the conformational changes are relatively fast (less than 20 min).

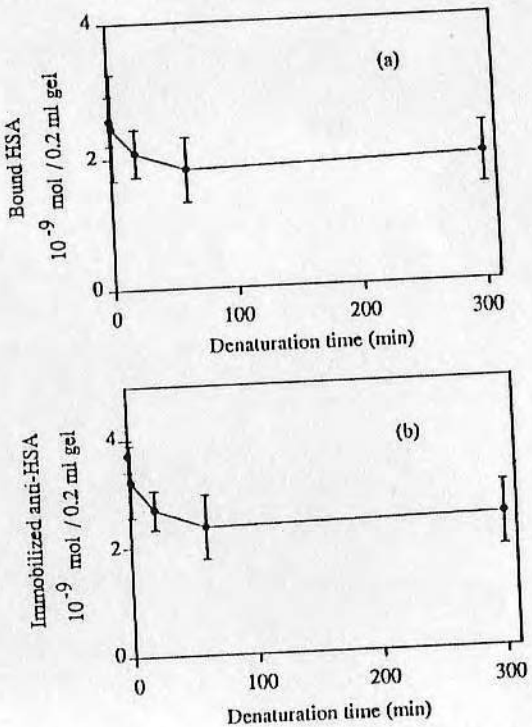

Fig. 7. (a) Antigen binding capacity of immobilized antibody; (b) surface concentrations of anti-HSA on agarose gel as a function of antibody denaturation time; $n=4$ in both experiments.

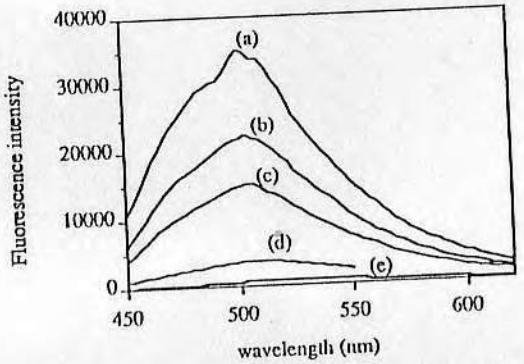

Fig. 8. Fluorescence spectra of bis-ANS binding to the non-oxidized native and partially denatured antibodies in pH 7.4 PBS; bis-ANS in (a) anti-HSA acid treated for 60 min, (b) anti-HSA acid treated for 20 min, (c) anti-HSA acid treated for 1 min, (d) native anti-HSA and (e) PBS.

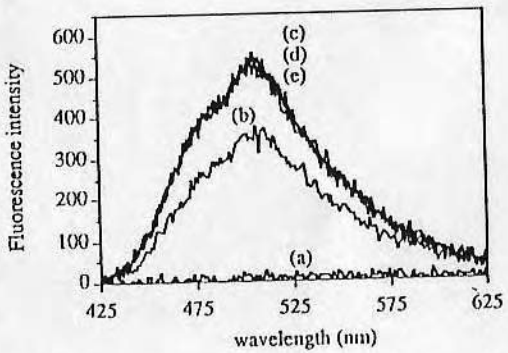

Fig. 9. Fluorescence spectra of bis-ANS binding to anti-HSA as a function of oxidation time in pH 5.2 acetate buffer; bis-ANS in (a) buffer, (b) non-oxidized native anti-HSA and (c–e) anti-HSA oxidized for 20, 40 and 60 min, respectively.

DISCUSSION

Oxidized or non-oxidized native and partially denatured antibodies were immobilized on three different surfaces via physical adsorption or chemical coupling. From the chemistry point of view, the hydrazide on the surfaces can react with aldehyde groups of oxidized carbohydrates in Fc fragments, resulting in site-specific coupling. Our results indicate that this chemistry works very well on the agarose gels. First, since only oxidized antibodies could be immobilized to agarose gels, immobilization was due exclusively to covalent coupling (Fig. 7). Second, the apparent activities of all immobilized antibodies using hydrazide chemistry on the agarose gels were 75 ± 3%, calculated assuming that only half of the IgG is the specific antibody, according to the specification provided by the company (Fig. 7). This value is consistent with other researchers' finding and is generally two to four times higher than that of non-site-specific coupling methods, such as cyanogen bromide-activated and N-hydroxysuccinimide ester-activated agarose gels [4,5].

Does hydrazide chemistry work as well on silica surfaces as on the agarose gels? From the results shown in Fig. 4b and 6b, the apparent activities of the immobilized oxidized antibodies on Si-Hz surfaces exhibited values ranging from 5 to 15%, which are substantially lower than that on the agarose gels. This is because the orientation of the antibodies on Si-Hz surfaces was controlled by the non-covalent interactions between the surfaces and antibodies rather than by site-specific coupling chemistry. This hypothesis is supported by the following experimental observations. First, in the control experiments for Si-Hz surfaces (Fig. 4a and 6a), the non-oxidized antibodies were strongly adsorbed onto the surfaces and showed approximately the same degree of AgBC as oxidized antibodies. This means that even though chemical bonds were formed between the antibody and the surfaces, the rest of the antibody molecule could still interact with non-specific adsorption sites on the surface and may have undergone surface denaturation. As a consequence, there is no advantage of using site-specific coupling with silica surfaces. Furthermore, since antibodies tend to adsorb irreversibly to silica surfaces, the chance of the adsorbed molecule having the right orientation for

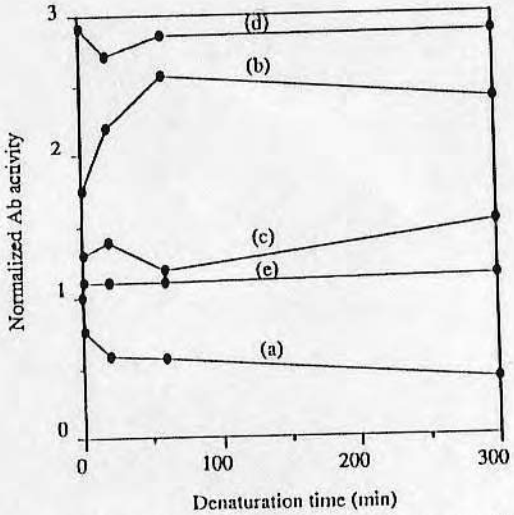

Fig. 10. Normalized antibody (Ab) activity on five different surfaces: (a) non-oxidized anti-HSA immobilized on DDS surfaces; (b) oxidized anti-HSA immobilized on DDS surfaces; (c) non-oxidized anti-HSA immobilized on Si-Hz surfaces; (d) oxidized anti-HSA immobilized on Si-Hz surfaces; (e) oxidized anti-HSA immobilized on Affi-Hz gel.

site-specific coupling is low. Thus, most of the antibodies were immobilized primarily via physical adsorption.

Second, if physical adsorption dominates the orientation of the antibody on silica surfaces, in principle it is possible to manipulate the orientation by altering the adsorption properties of the antibody. This concept is demonstrated by using the partially denatured antibodies. From the fluorescence experiments (Fig. 8), the results show that exposure of hydrophobic regions of the antibody increases with increasing acid denaturation time. As a result, acid treatment prior to immobilization has a significant influence on both antibody surface concentrations and AgBC, as shown in Figs. 2–5. Oxidation can also change the conformation of antibodies (Fig. 9), which in turn affects their adsorption properties, as observed on the non-functionalized DDS surfaces (Figs. 3 and 5). We have previously suggested that the increase in antibody surface concentrations was due to the high affinity of the denatured regions for the surfaces [13]. This phenomenon is analogous to the "Vroman effect" competitive adsorption between proteins [10]. In our case, the competitive adsorption is between the denatured and non-denatured regions of the antibody.

On the agarose gels, antibody surface concentration decreases with increasing denaturation time (Fig. 7). Since non-oxidized antibodies did not adsorb to agarose gels, we believe that the decrease in amount of immobilized antibody is due to the decrease in accessibility of the carbohydrate groups after acid treatment.

Finally, Fig. 10 shows the plot of normalized activity of immobilized antibody as a function of antibody denaturation time for all immobilization experiments. The normalized values were calculated by

$$\text{normalized activity} = \frac{([\text{Ag}]/[\text{Ab}])_{\text{N or D}}}{([\text{Ag}]/[\text{Ab}])_{\text{N}}}$$