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Immobilization of Binding Proteins on Nonporous Supports

Comparison of Protein Loading, Activity, and Stability

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ABSTRACT

Four different nonporous particulate materials, nylon, polystyrene, soda-lime silicate glass, and fused silica glass, have been evaluated for their appropriateness as immobilization supports for immunoglobulins. A method of protein quantitation that is usually applied to solutions, the bicinchoninic acid (BCA) assay, was used successfully to directly measure ng amounts of protein immobilized on the supports. Two proteins, a monoclonal antibody to theophylline and the biotin binding protein avidin, were studied. Radioactive theophylline and radioactive biotin were used to measure the activity of the immobilized protein. Ligand binding capacity per mm^2 of support was measured as a function of amount of protein immobilized. By measuring both the amount of protein immobilized and its ligand binding capacity, we have determined that antitheophylline antibody adsorbed on polystyrene balls loses almost 90% of its binding activity after 65 h, although little protein is lost from the balls over this time. Avidin retains nearly full activity for biotin on polystyrene. The binding activity of biotinyl–antibody conjugate immobilized on avidin-adsorbed polystyrene is stable, even when stored for over 22 wk. Antibody covalently immobilized on soda-lime silicate glass beads retains its binding

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activity over long-term storage, although only 0.1 mol of ^{3}H-theophylline bind per mol of immobilized antibody. Using fused silica glass particles as the solid support, the same antibody binds approx 0.6 mol of ligand per mol of immobilized antibody protein. The structural "softness" of the immunoglobulin requires that interaction with the surface be prevented in order to maintain activity.

Index Entries: Antibody immobilization; protein immobilization; binding activity; protein determination; glass; nylon; polystyrene.

INTRODUCTION

Immobilized immunoglobulins have a role in biosensors, analytical systems, affinity chromatography, and therapeutic devices. Nonporous particles are of interest as supports in these types of applications, because they minimize some of the problems associated with porous supports, such as large surface areas for nonspecific protein adsorption, size exclusion, and unfavorable mass transport and kinetics (*1–3*).

The immobilization of biologically active proteins onto solid supports involves consideration of several features that are critical for achieving desired functional characteristics. A sufficient amount and density of protein must be immobilized on the support, the protein immobilized must be reactive to its ligand, and the binding capacity of immobilized protein for ligand must not change over time. Finally, the amount of undesired interaction between the support and other molecules must be minimal.

A number of studies have carefully examined the physical chemistry of the interaction of proteins with surfaces. Protein adsorption to, and desorption from, solid supports is a function of the nature of both the protein and the support, and can be dependent on time, temperature, ionic strength, pH, protein concentration, and surface tension (reviewed in *4*). Frequently, conformational changes appear to accompany the interaction of proteins with surfaces (*4*). Changes in conformation of functional proteins can compromise their reactivity. Reactivity in immunoassays has been a criteria for comparing methods of antibody immobilization on various nonporous supports (*1,5–9*). In general, these studies indicate a preference for covalent immobilization over adsorption because of the increased amount of protein activity that can be immobilized, but unfortunately these studies do not measure both the amount of protein immobilized and the numbers of active binding sites. As a result, it is difficult to distinguish between presence or loss of protein and presence or loss of binding activity.

Our goal in this study was to quantitatively compare some nonporous particulate materials on which immunoglobulins could be immobilized with respect to both the amount of protein immobilized and the stability of immunospecific binding activity. Quantitation of adsorbed protein is

frequently performed by UV absorbance differences or by radiolabeling the protein to be adsorbed (*4*). The former method requires that large surface areas be examined in order to provide sufficiently large differences in protein concentrations before and after adsorption (*4*), and the latter method introduces ambiguity because the presence of the label may alter the protein's characteristics (*10*). Also, the presence of ^{125}I on the immobilized protein usually precludes measuring binding activity with another radiolabel on the same preparation. Using the bicinchoninic acid (BCA) assay for protein quantitation (*11*), we quantitated both the protein and the binding capacity of samples from the same preparation of protein-derivatized support. Protein determinations were performed in batch, by monitoring the reaction between Cu^{2+} and peptide bonds of proteins immobilized on the support particles. This provided a sensitive and precise measurement of immobilized protein even when surface area was low, and allowed us to determine the immunospecific binding activity by the amount of ^{3}H-ligand that the immobilized protein could bind. The data were normalized with respect to surface area, so that direct comparison of mol of binding sites per mol of protein per mm^2 surface area of the different supports was possible. The reversibility of immobilization, and the stability of the ligand binding activity, was determined after subjecting the derivatized particles to flowing buffer for 65 h, and after long-term storage.

For this study we used a mouse monoclonal IgG with the capacity to bind 2 mol of the drug, theophylline, per mol of protein. For comparison with a nonimmunoglobulin protein with specific binding activity to a small ligand, the binding protein avidin was studied. Each mol of avidin has approximately 4 mol of binding sites for the vitamin biotin. These proteins were both immobilized onto polystyrene by simple adsorption, and antitheophylline IgG was also immobilized, after derivatization with biotin, onto avidin-coated polystyrene. Both covalent and noncovalent immobilization onto nylon was studied. In addition, antitheophylline IgG was covalently immobilized onto soda-lime silicate glass beads and fused silica glass particles. Observed differences in the behavior of the two proteins will be discussed with respect to their structural characteristics.

METHODS

Antitheophylline was purchased as ascites fluid (American Qualex Corp., La Mirada, CA)[1], and was purified by Protein A affinity chromatography (*12*) using reagents purchased from BioRad (Richmond, CA).

[1]Certain commercial products are identified in order to adequately specify the experimental procedure. This does not imply endorsement or recommendation by the National Institute of Standards and Technology.

Biotinyl-anti-goat antibody, chromatographically purified avidin, bovine gamma globulin, and biotin were purchased from Sigma Chemical Co. (St. Louis, MO).

Polystyrene balls (Precision Plastic Ball Co., Chicago, IL) were 1 mm in diameter. The same conditions were used for noncovalent immobilization of both avidin and antitheophylline. Balls were incubated on a rocker with 0.1 mg mL^{-1} solutions of protein in 0.01*M* HEPES buffer, pH 8.0, with 0.01% thimerosal (a bacteriostat) for 15 h at room temperature. These conditions were chosen to be optimal for immunoglobulin immobilization, including the low ionic strength of the solution (*2*) and the pH at the isoelectric point of this antibody (reviewed in *4*), and the moderate concentration of antibody in solution, which has been shown to result in better activity than highly concentrated protein solutions (*9,13*). The balls were then washed 3 times with the same buffer. Some polystyrene balls to which avidin was adsorbed were further derivatized with antitheophylline, which was covalently modified with biotin. Biotinylation of antitheophylline was achieved by incubation of purified antibody on 0.02*M* HEPES buffer, pH 8.2, with a fivefold molar excess of sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce, Rockford, IL) at room temperature for 2.5 h. The reaction mixture was dialyzed extensively against 0.01*M* phosphate-buffered saline (PBS), pH 7.4. Commercially prepared biotinyl-anti-goat antibody was used with other balls to provide a control sample. Biotinyl-antibody was added to avidin-derivatized balls at a twofold molar excess of biotin binding sites, as determined with ^{3}H-biotin. Balls were incubated while rocking with biotinyl-antibody for 30 min at room temperature, and then washed 3 times with PBS.

Nylon balls (3/64 in) (Precision Plastic Ball Co., Chicago, IL) were derivatized both covalently and noncovalently with antitheophylline antibody or with bovine gamma globulin. For noncovalent immobilization, balls were incubated while rocking at a 4°C overnight with 0.05 mg mL^{-1} of antibody in PBS and rinsed 4 times in PBS. Nylon balls were also covalently derivatized by first hydrolyzing them with 3.5*M* HCl for 24 h to expose free amino groups (*1*). The balls were then reacted with antibody amino groups using the bifunctional crosslinking reagent, bis(sulfosuccinimidyl)suberate (BS^3) (Pierce) at a concentration of 4 m*M* in dimethylsulfoxide (DMSO) for 4 h at room temperature. After DMSO was removed, balls were washed and then incubated with 0.5 mg mL^{-1} antibody in 0.025*M* HEPES buffer, pH 8.2 at 4°C, while rotating, for 16 h. Derivatized balls were washed 4 times in PBS, pH 7.4

Soda lime-silica glass beads (60–80 mesh, 210–250 μm diameter) (Ferro Corporation, Cataphote Division, Jackson, MS) were covalently modified with 3-(glycidoxypropyl)trimethoxysilane (GOPS) (Petrarch, Bristol, PA) as described by Sportsman and Wilson (*14*), with modifications. Beads (50 g) were first refluxed in 5% nitric acid for 45 min, rinsed in water, and

then reacted with 250 mL of 10% aqueous (v/v) solution of GOPS at 90°C for 1 h. The pH of the reaction mixture was adjusted to 3 by additions of 1*N* H_2SO_4. Beads were rinsed in approximately 200 mL water and cured at 110°C overnight. The glycidoxy groups were oxidized to aldehydes with periodic acid. Beads were incubated for 1.5 h with twice their volume of 0.5% periodic acid (w/v) in 80% acetic acid. Beads were then coupled to purified antibody by incubation in an equal volume of 0.04 mg mL^{-1} protein in 0.1*M* carbonate buffer, pH 9.5, at 4°C for 20 h. $NaBH_4$ (0.5 mg/g beads) was then added and allowed to react for 1 h at room temperature. Derivatized beads were washed 5 times with 4 volumes of PBS, pH 7.4, alternated with 4 volumes of a citrate buffer containing NaCl, pH 3, and stored in PBS, 7.4.

Fused silica glass particles (100–200 mesh, 74–147 μm in diameter) were prepared from General Electric type 206 Fused Quartz Glass by crushing and screening through copper screens, followed by a 24 h wash in concentrated HNO_3 at 100°C, to remove any organic matter from the surface. The acid treatment was followed by extensive water rinses to remove metal traces. Surface area of silica was estimated by assuming a spherical shape and an average diameter of 111 μm. These particles were covalently derivatized with antitheophylline and with bovine gamma globulin using the same method as was used for the glass beads.

Determination of protein on solid supports was achieved using the bicinchoninic acid (BCA) assay (*11*). This assay is based on the reduction of soluble Cu^{2+} to Cu^{1+} by the immobilized protein at alkaline pH. Soluble Cu^{1+} then chelates with two molecules of another soluble reagent, bicinchoninic acid, which results in a color change. Nylon or polystyrene balls (usually 20 balls), or 0.05 mL of packed soda-lime silicate glass beads or fused silica glass particles, plus 10 or 100 μL of water, were incubated in test tubes with 200 or 100 μL, respectively, of BCA reagent for 2 h at 60°C. Aliquots of 100 μL were transferred to a microtiter plate and the optical density was measured on a microtiter plate reader using a 560 nm long pass filter. A standard curve was generated with antitheophylline, or with avidin when appropriate. Usually the 200 μL assay, which can measure between 0.05 and 3 μg protein, was used, but occasionally the 210 μL volume assay was used to improve the sensitivity to less than 0.03 μg of protein. Untreated beads or balls were always assayed also, and their values were subtracted from those of the samples.

The binding activity of immobilized antitheophylline or avidin was measured by incubating typically 50 balls or 0.1 mL of packed beads or particles with 0.5 mL of PBS, pH 7.4, containing 3×10^{-8} *M* ^{3}H-theophylline at a specific activity of 4×10^{16} dpm mol^{-1} (custom synthesized by Amersham Corp., Arlington Hts., IL), or 8×10^{-7} *M* ^{3}H-biotin at a specific activity of 2.4×10^{15} dpm mol^{-1} (New England Nuclear, Boston, MA). Nonspecific binding of the radiolabels was tested on underivatized supports,

on polystyrene derivatized with biotinyl-anti-goat antibody, and on the nylon and glass substrates derivatized with bovine gamma globulin. The samples were rocked for 1 h at room temperature, washed with 5 volumes of PBS, and transferred to scintillation vials, where water and Universol scintillation fluid (ICN Biomedicals, Inc., Irvine, CA) were added to produce an emulsion in which the particles were suspended during counting. Preliminary experiments indicated that 60 min was sufficient incubation time for the system to come to equilibrium. Radioactivity associated with underivatized supports or with supports derivatized with nonspecific antibody was never above background, except for nylon. In the tables, radioactivity not above background counts is reported as "0" binding activity. The amount of theophylline or biotin bound to immobilized protein was always less than 2% of the total ligand added.

RESULTS AND DISCUSSION

We have evaluated protein immobilization by two criteria: the amount of protein present on the support as determined by direct measurement using the BCA assay, and the mol of active sites present as determined by the binding capacity for tritiated ligand. All data are presented normalized per mm^2. The ratio of mol of ligand bound to mol of protein present on the support per mm^2 of surface area is referred to in the Tables as the binding activity.

The purpose of this study was to establish that both types of information, protein quantity and protein activity, as can be obtained by the techniques described, are required to permit complete evaluation of the adequacy of immobilization methods and supports for active proteins. We chose four supports with different physical characteristics: polystyrene (hydrophobic), nylon (polar, net neutral), and GOPS-derivatized soda-lime silicate glass and fused silica glass (polar, uncharged surface covering a negatively charged substrate).

The advantage of polystyrene as a noncovalent immobilization support is the ease with which immobilization of proteins can be achieved. Data for polystyrene are summarized in Table 1. No radioactivity was associated with underivatized balls. By simple adsorption, approximately 1×10^{-13} mol of antibody and 4×10^{-14} mol of avidin were immobilized per mm^2 of polystyrene. This is very similar to the data of Sorensen and Brodbeck (*15*) who used the BCA assay to determine the amount of protein immobilized on microtiter plates under various incubation conditions. Their data were similar to ours with polystyrene, in that they showed that different amounts of IgG and BSA adsorbed, and approximately 1×10^{-14} to 1×10^{-13} mol of protein were immobilized per mm^2 of polystyrene surface. Bale et al. (*16*) also recently measured approximately 3×10^{-14} to 5×10^{-14} mol of ^{125}I-labeled protein adsorbed per mm^2 of polystyrene. The

Table 1
Immobilization of Protein on Polystyrene Balls

AGENT	PROTEIN (moles/mm^2)	BINDING CAPACITY (moles/mm^2)	ACTIVITY (moles bound /mole protein)
αtheophylline	1.0×10^{-13} ($\pm$ 5×10^{-15})	1.0×10^{-14} ($\pm$ 3×10^{-15})	0.1
Avidin	4.3×10^{-14} ($\pm$ 5×10^{-15})	1.2×10^{-13} ($\pm$ 1×10^{-15})	2.8
αtheophylline[a]	8.2×10^{-14} ($\pm$ 1×10^{-14})	1.3×10^{-15} ($\pm$ $3X10^{-16}$)	0.02
Avidin[a]	7.9×10^{-14} ($\pm$ 3×10^{-14})	1.2×10^{-13} ($\pm$ 2×10^{-15})	1.5
B-αtheoph. 1	4.7×10^{-14} ($\pm$ 7×10^{-15})	2.1×10^{-15} ($\pm$ 3×10^{-16})	0.05
B-αtheoph. 2	1.1×10^{-14} ($\pm$ 3×10^{-15})	2.0×10^{-15} ($\pm$ 2×10^{-15})	0.18
B-αGoat	3.6×10^{-14} ($\pm$ 2×10^{-14})	0	0.0
B-αtheoph. 1[b]	n.d.	1.9×10^{-15} ($\pm$ 4×10^{-16})	n.d.
B-αtheoph. 2[b]	n.d.	2.2×10^{-15} ($\pm$ 1×10^{-16})	n.d.

Preparation, immobilization, and quantitation are as described in Materials and Methods. Sequential numbers indicated replicate preparations. Numbers in parentheses are standard deviations. [a] = after 65 h in continuously flowing buffer; [b] = after storage at 4°C for 22 wk; n.d. = not determined.

correlation between amount of immobilized ^{125}I-labeled protein and immobilized protein measurements by the BCA assay supports the validity of the BCA assay for solid-phase determinations.

Less avidin adsorbed to polystyrene than did immunoglobulin, even though the balls were exposed to the same concentration of both proteins in 0.01*M* HEPES buffer, pH 8.0, and avidin is also a basic protein (*17*). Comparison of binding capacity of immobilized avidin for ^{3}H-biotin and immobilized antitheophylline for ^{3}H-theophylline indicates a significant difference between the two proteins. A fully active avidin molecule in solution can bind a maximum of 4 biotin molecules. As seen in Table 1, immobilization did not alter avidin's binding activity greatly. More than 2 mol of biotin bound per mol of avidin immobilized.

Studies of avidin's biotin-binding activity under various conditions have indicated that avidin is a very stable protein over a wide pH range, and its activity is insensitive to a variety of group specific reagents (*17*). The small amount of binding activity lost on immobilization of avidin is easily explained by steric hindrance of sites that may be oriented toward the surface. The activity of immobilized antitheophylline was quite different. This preparation of antibody is of the IgG class, and therefore has 2 binding sites for ligand per mol of antibody. Immobilized antitheophylline, however, bound only 0.1 mol of ^{3}H-theophylline per mol of antibody, indicating a substantial loss of native antibody activity.

With time, structural differences between the two proteins on polystyrene became more apparent. The tendency for desorption and the stability of activity were examined by packing the derivatized polystyrene balls in glass columns and subjecting them to 65 h of continuously flowing PBS, pH 7.4, at room temperature. Balls were then tested with the BCA assay and for ^{3}H-ligand binding. The results of this experiment are shown in Table 1. Avidin proved to be extremely hardy. The amount of avidin protein attached to the balls as measured by the BCA assay was not significantly affected, and the binding activity was comparable (within experimental error) to the original activity measured. Antitheophylline adsorbed onto polystyrene balls was not stable under these conditions. Although only a small amount of protein was lost from the balls, the binding capacity decreased by a factor of 50. Loss of activity of proteins immobilized on hydrophobic surfaces such as polystyrene has been noted frequently (*18;* reviewed in *4*) and is probably the result of changes in protein conformation such as has been inferred by a number of groups (reviewed in *19,20*). A recent study of immobilized enzymes, which involved measurement of both active and inactive enzyme on polystyrene, confirmed the inactivation of protein as a result of immobilization (*21*). The fact that a loss of theophylline binding activity was seen to be a time-dependent event is not totally unexpected. This was predicted from desorption experiments with BSA (*22*) and from a dynamic model for protein adsorption based on elipsometry data (*23*).

Because of the instability of antitheophylline on polystyrene, we examined the possibility that immobilizing biotinyl-antibody onto the avidin-adsorbed surface may have an advantage over adsorbing antibody protein directly. These data are also shown in Table 1. Biotinyl-antitheophylline or biotinyl-anti-goat antibody were incubated with avidin-adsorbed balls for 30 min, and rinsed twice with buffer before analysis. The amount of protein on avidin-adsorbed balls that were not treated with biotinyl-antibody was subtracted from the total protein quantitated. The amount of ligand that bound per mm^2 of polystyrene to antitheophylline immobilized in this way was somewhat less than that for antibody immobilized by direct adsorption onto bare polystyrene, and binding activity relative to amount of protein immobilized was not significantly different. Control balls that were derivatized with biotinyl-anti-goat antibody showed no radioactivity above background, and therefore, nonspecific binding of radiolabel was absent. This is indicated in the Table as "0" binding activity for the biotinyl-anti-goat control balls. Activity was again measured on balls after storage for 22 wk at 4°C in PBS. After 22 wk of storage, binding capacity per mm^2 was not significantly different from the binding capacity measured immediately after derivatization. Apparently, the presence of avidin effectively shielded antitheophylline from the hydrophobic polystyrene surface and prevented the activity loss that was observed when the antibody was adsorbed directly to polystyrene. This activity loss is presumably the result of time-dependent conformational rearrangements of the protein during interaction with the hydrophobic surface. Thus, it appears that for antibodies there may be a significant advantage to this method of immobilization over adsorption of antibody directly onto polystyrene.

With nylon balls, two immobilization approaches were attempted: noncovalent adsorption and covalent derivatization. For covalent derivatization, amino groups exposed on the nylon by treatment with HCl were reacted with the primary amines of antitheophylline using the bifuncional crosslinking reagent, BS^3. BCA assays on nylon without added protein produced a detectable response, which was subtracted from the results of protein-derivatized nylon samples. As seen in Table 2, the amount of protein immobilized on nylon, as determined with the BCA assay, was approximately 1×10^{-13} mol per mm^2, regardless of whether the protein was allowed to noncovalently adsorb or whether a covalent coupling agent was present. This is equivalent to the amount of antitheophylline noncovalently adsorbed to polystyrene, and is also approximately that reported by Hendry and Herrmann (*1*) for ^{125}I-IgG on underivatized nylon. We have previously used the BS^3 reagent to successfully couple protein to amino groups on glass beads derivatized with aminosilane (*24*). However, in the present study with nylon, we found that as much protein was associated with nylon balls noncovalently as was associated with balls that were reacted with HCl and BS^3.

Table 2
Immobilization of Antibody on Nylon Balls

AGENT	PROTEIN (moles/mm^2)	THEOPHYLLINE BOUND (moles/mm^2)
Covalent		
αtheophylline	1.3x10-13 (± 1x10-14)	2.9x10-13 (± 4x10-14)
after 64hr flow	8.0x10-14 (± 6x10-15)	1.9x10-13 (± 2x10-14)
bovine globulin	1.3x10-13 (± 7x10-15)	3.2x10-13 (± 1x10-14)
after 64hr flow	7.7x10-14 (±3x10-15)	2.4x10-13 (± 2x10-15)
Noncovalent		
αtheophylline	1.2x10-13 (± 1x10-14)	8.9x10-14 (± 8x10-15)
after 64hr flow	9.7x10-14 (± 4x10-15)	9.0x10-14 (± 4x10-15)
bovine globulin	9.2x10-14 (± 4x10-15)	8.6x10-14 (± 4x10-15)
after 64hr flow	6.8x10-14 (±4x10-15)	9.0x10-14 (± 4x10-15)
no protein	0	8.9x10-14 (± 1x10-14)

Procedures for derivatization are described in Materials and Methods. Numbers in parentheses are standard deviations.
standard deviations.

The most noteworthy observation with nylon was the tremendous amount of nonspecific binding of radioactive theophylline. Bovine gamma globulin was used in place of antitheophylline on some balls to provide a test of the specificity of the interaction of ^{3}H-theophylline with balls to which antitheophylline had been immobilized. In our preparations, the same amount of theophylline binding was observed whether the nylon was treated with antitheophylline or with bovine globulin. Approximately 30% of maximum signal was associated with underivatized nylon, and the binding capacity of balls to which protein was noncovalently adsorbed was not above that for untreated nylon balls. Nylon treated with HCl and BS^3 bound more ^{3}H-theophylline, which may have suggested increased specific activity, but immobilized bovine globulin balls also bound as much as antitheophylline balls. Subjecting these balls to flowing buffer resulted in a loss of about 20% of the protein from nylon, which was allowed to noncovalently adsorb antibody, and about 30% from balls to which protein was presumably covalently bound. Nylon to which antitheophylline was covalently bound lost approximately the same percentage of binding capacity for ^{3}H-theophylline as the amount of protein lost. All other nylon preparations lost a small amount of protein, but did not lose a corresponding amount of binding capacity. Thus, it appeared that the nylon itself was either trapping or adsorbing ^{3}H-theophylline, and that association of ligand with the support was not because of an immunospecific interaction with the protein. Hendry and Herrmann (*1*) reported that more antibody adsorbed to HCl-treated nylon than to untreated nylon, and that in both cases, antibody activity was significantly compromised. They found that the use of large chemical spacers to tether antibody to the nylon, which probably reduced the interaction between the protein and the surface, provided a sufficient amount of active antibody to be useful in immunoassay. However, in our case, if immunospecific activity was present, it was very low with respect to nonspecific background binding.

Covalent immobilization of antibody onto either soda-lime silicate glass beads or fused silica glass particles derivatized with GOPS was compared. The data for these two support materials are shown in Table 3. As with polystyrene, there was no nonspecific interaction between the tritiated ligand and the two glass substrates, and levels of radioactivity associated with these substrates derivatized with bovine globulin were not above background levels. The amount of antibody protein immobilized per mm^2 was almost 100 times less than that immobilized by adsorption onto polystyrene, and the binding activity was also significantly lower. Only 0.004 mol of ^{3}H-theophylline bound per mol of immobilized protein. However, stability of antibody binding capacity was much better on glass. Noncovalently adsorbed antitheophylline on polystyrene bound 1×10^{-14} mol of ^{3}H-theophylline per mm^2 compared to an average of 6×10^{-17} mol of ^{3}H-theophylline bound per mm^2 by antitheophylline covalently immobilized on soda-lime silicate glass, but polystyrene lost 90% of its activity

Table 3
Comparison of Antibody Immobilization on Soda-Lime Silicate Glass and Fused Silica Glass

AGENT	PROTEIN (moles/mm^2)	BINDING CAPACITY (moles/mm^2)	ACTIVITY (moles bound /mole protein)
SODA-LIME GLASS			
αtheophylline	5.6×10^{-15} ($\pm$ 5×10^{-16})	2.1×10^{-17} ($\pm$ 7×10^{-18})	0.004
Bovine globulin	1.0×10^{-14} ($\pm$ 2×10^{-15})	0	0
αtheophylline[a]	n.d.	1.4×10^{-17} ($\pm$ 4×10^{-19})	n.d.
αtheophylline[b]	n.d.	1.2×10^{-17} ($\pm$ 4×10^{-18})	n.d.
FUSED SILICA GLASS			
αtheophylline	3.0×10^{-15} ($\pm$ 5×10^{-16})	1.8×10^{-15} ($\pm$ 7×10^{-17})	0.61
Bovine globulin	1.5×10^{-15} ($\pm$ 2×10^{-16})	0	0

Immobilization chemistry is described in Materials and Methods. Numbers in parentheses are standard deviations. [a] = after storage of derivatized beads for 15 wk at 4°C; [b] = after an additional 65 h in flowing buffer; n.d. = not determined.

after 65 h in flowing buffer. In contrast, measurement of a soda-lime silicate glass preparation after 15 wk of storage at 4°C indicated that antitheophylline on glass beads did not lose a statistically significant amount of binding capacity over this time. Placing these beads under continuously flowing buffer for 65 h resulted in no change in this binding capacity.

Fused silica glass particles proved to be even better for immobilization of antitheophylline than soda-lime silicate glass. Slightly less protein was immobilized on the silica glass particles than on the soda-lime glass beads, but the binding capacity of antitheophylline on the fused silica glass was almost two orders of magnitude higher. The net result is a very high binding activity for fused silica glass-immobilized antitheophylline. Approximately 0.6 mol of theophylline bound per mol of immobilized protein. This is an increase of 150-fold in relative activity. Sportsman and Wilson (*14*) measured both protein immobilized and total antibody binding capacity on porous silica derivatized with GOPS and calculated relative antibody activity similar to what we saw, but which varied with protein immobilized. In their case, a polyclonal antibody retained about 11% of its activity, and a monoclonal retained about 7%. Bhatia et al. (*25*) reported the relative activity of antibody immobilized on glass slides by preparing some slides with ^{125}I-labeled antibody, and binding radioactive ligand to other slides. Using polyclonal anti-IgG and a thiol-terminal silane, they found binding activities of 0.37 and 0.55 mol of ligand bound per mol of protein. This compares very favorably with our measured binding activity on fused silica glass of 0.6.

The difference in activity of antibody immobilized on pure silica versus soda-lime glass is probably a result of the chemistry and relative reactivity of the two surfaces. Soda-lime glass has a lower concentration of silanol groups, and therefore is less reactive to silanizing agents. Immunoglobulins and other proteins are known to adsorb strongly to negatively charged groups, such as glass, and blocking these groups by silanization reduces adsorption (*26*). The use of silanizing agents to inhibit close contact between the glass surface and proteins has been shown to be important for preventing the inactivation of immobilized enzymes (*27*). Since pure silica is more reactive to silanization, it appears that a more protective coating of GOPS is responsible for the greater stability of antibody on silica. The improvement we see in activity of antibody immobilized on silica compared to glass may be caused by better coverage of the silica surface by GOPS, thus protecting antibody from direct contact with the surface. In addition, the effect of possible efflux of sodium ions from the soda-lime silicate glass cannot be discounted. Efflux of sodium ions from the glass could increase the surface pH, resulting in a reduced efficiency of silanization or a destabilization of silane bonds. This, of course, would not occur in fused silica glass.

Table 4
Reproducibility of Protein Immobilization by Noncovalent and Covalent Methods

AGENT	PROTEIN (moles/mm^2)	BINDING CAPACITY (moles/mm^2)	ACTIVITY (moles bound/mole protein)
GLASS			
atheophylline 1	9.7×10^{-15} ($\pm$ 8×10^{-16})	1.1×10^{-16} ($\pm$ 2×10^{-17})	0.011
atheophylline 2	8.8×10^{-15} ($\pm$ 5×10^{-16})	5.1×10^{-17} ($\pm$ 6×10^{-18})	0.006
atheophylline 3	5.6×10^{-15} ($\pm$ 5×10^{-16})	2.1×10^{-17} ($\pm$ 7×10^{-18})	0.004
atheophylline 4	7.4×10^{-15} ($\pm$ 5×10^{-16})	6.7×10^{-17} ($\pm$ 2×10^{-17})	0.009
atheophylline 5	5.1×10^{-15} ($\pm$ 7×10^{-17})	7.0×10^{-17} ($\pm$ 2×10^{-17})	0.014
MEAN	7.3×10^{-15} ($\pm$ 2×10^{-15})	6.4×10^{-17} ($\pm$ 3×10^{-17})	0.009 ($\pm$0.004)
POLYSTYRENE			
Avidin 1	4.3×10^{-14} ($\pm$ 5×10^{-15})	1.2×10^{-13} ($\pm$ 1×10^{-15})	2.8
Avidin 2	5.3×10^{-14} ($\pm$ 1×10^{-15})	1.0×10^{-13} ($\pm$ 9×10^{-15})	1.9
Avidin 3	4.8×10^{-14} ($\pm$ 2×10^{-14})	n.d.	n.d.
MEAN	4.8×10^{-14} ($\pm$ 5×10^{-15})	1.1×10^{-13} ($\pm$ 1×10^{-14})	2.4 ($\pm$ 0.6)

Antitheophylline was immobilized covalently onto glass. Avidin was immobilized noncovalently onto polystyrene. Preparation, immobilization, and quantitation are as described in Materials and Methods. Sequential numbers indicated replicate preparations. n.d. = not determined.

Table 4 shows a comparison of the reproducibility of avidin immobilization by noncovalent adsorption onto polystyrene with the reproducibility of covalent glass derivatization with antitheophylline using GOPS. Avidin immobilized in three separate preparations of polystyrene indicates that the reproducibility of the amount of protein immobilized and the binding capacity is quite good, with a coefficient of variation (C.V.) of 10%. Repeatability of the covalent immobilization of antibody was examined with 5 different preparations of soda-lime glass beads. An average of 7×10^{-15} mol of protein per mm^2 was obtained with a standard deviation of 2×10^{-15}, giving a C.V. of almost 30%. A similar variation in the amount of binding activity was observed.

SUMMARY

The measurement techniques presented here have permitted direct observation of certain phenomena associated with protein interaction with surfaces. Many of our observations confirm phenomena that, up to now, have been only inferred from indirect measurements. For example, we have direct evidence in these data for the time-dependent loss of functional activity of a protein as a result of association with a surface. In addition, we have shown that we can quantitatively describe the effect that physical shielding of a protein from a surface can have on its functional stability.

For adequate comparison of immobilization methods for analytical and therapeutic purposes, it is important to have information about both the amount of protein immobilized and its binding capacity, since loss of activity frequently accompanies protein immobilization. What complicates the issue is that, as we have shown here, different solid supports affect proteins differently, and different proteins behave differently even on the same support. Without information of both the amount of protein and the amount of activity, it is difficult to ascertain whether a particular immobilization technique is simply achieving greater surface coverage or if it results in a more active immobilized protein.

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