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Nature of immobilized antibody layers linked to thioctic acid treated gold surfaces

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Abstract

Utilization of ^{125}I-labeled IgG enables an investigation of protein immobilized to gold electrodes sputter deposited on microporous nylon membranes, including the precise nature of the surface–protein bond (i.e. covalent or non-specific adsorption), physical location of the immobilized protein (i.e. on the surface of the gold electrode or within the pores of the membrane), and the amount of protein immobilized. This is accomplished by comparing the mass of protein immobilized to gold surfaces that have been treated in several different fashions, as well as, deposition of the gold on nylon membranes that have been treated differently. It is shown that these microporous gold electrodes, proposed previously for conducting novel non-separation electrochemical enzyme immunoassays, consist of multiple protein layers non-specifically adsorbed. Approximately, half of the total adsorbed protein is immobilized to the gold surface with the remaining protein bound within the pores on the nylon membrane.

Keywords: Protein immobilization; Microporous gold electrodes; Nylon membranes; Thioctic acid; ^{125}I-labeled protein; Non-specific adsorption

1. Introduction

Since being pioneered in the mid 1960s, the use of immobilized antibodies in immunoassays has become commonplace, due to their ability to provide a simple and efficient means to separate bound immunoreagents from those remaining unbound at equilibrium (Catt and Tregear, 1967). Today, both covalent attachment (Gregorius et al., 1995; Wink et al., 1997; Rejeb et al., 1998) and non-specific adsorption (Ensing and Paulus, 1996; Fernández-Sánchez and Costa-García, 1997; Wang et al., 1998) are employed to immobilize antibodies. Despite the fact that covalent attachment can increase the stability of the immobilized protein layer under convective conditions, and decrease the extent of denaturation upon immobilization, non-specific adsorption remains the most common immobilization method (Wink et al., 1997; Buijs et al., 1997; Rubtsova et al., 1998).

Recently, thioctic acid has been investigated as a reagent that would allow the covalent attachment of a monolayer of protein to a solid support (Duan and Meyerhoff, 1994; Berggren and Johansson, 1997). One such study, utilized gold electrodes sputter deposited on microporous nylon membranes (0.2 μm pore size) for the immobilization of protein and subsequent development of a non-separation electrochemical enzyme immunoassay (NEEIA) (Duan and Meyerhoff, 1994). Electrodes were modified with thioctic acid, treated with 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (EDAC), and then exposed to monoclonal mouse anti-human chorionic gonadotropin (anti-hCG) or monoclonal mouse anti-prostate specific antigen (anti-PSA). Utilization of a colorimetric protein assay (micro BCA), revealed that 95% of the protein exposed to the microporous gold electrodes remained bound after shaking in Tris buffer, suggesting covalent attachment of the antibodies. However, the amount of protein immobilized corresponded to a surface coverage of 1.3 nmol/cm^2, approximately three orders of magnitude greater than the expected monolayer coverage of bovine IgG (2.2 pmol/cm^2) on polystyrene (Cantarero et al., 1980). A similar monolayer coverage of 1.7 pmol/cm^2

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was reported for immobilized rabbit anti-bovine lutenizing hormone IgG on polystyrene (Walsh and Gosling, 1986). Due to the unexpectedly high protein surface coverage for microporous gold electrodes treated with thioctic acid, this method of immobilization is investigated herein, in greater detail. Several issues are examined, including an explanation for the high surface coverages; the precise nature of protein immobilization (covalent attachment, non-specific adsorption, or protein aggregation); and the physical location of immobilized protein (i.e. gold, nylon). It will be shown via radiotracer experiments that protein immobilization occurs through non-specific adsorption and aggregation, with approximately half of the total protein immobilized on the underlying nylon membrane. These results are consistent with those of similar experiments where attempts at covalent attachment resulted in 75% of the immobilized protein being bound through non-specific adsorption, unless a detergent was included in the immobilization solution (Williams and Blanch, 1994).

2. Experimental

2.1. Apparatus

Amperometric measurements were performed with a BAS LC-3E potentiostat (BAS, West Lafayette, IN) in a three-electrode configuration. All the applied potentials were versus a Ag/AgCl reference electrode with a platinum auxiliary electrode completing the circuit. Gamma (γ) counting was performed using a Packard Auto gamma 5000 gamma counter (Meriden, CT). A Rainin (Woburn, MA) Rabbit peristaltic pump was used to deliver solvent through porous electrodes held in place in a specially designed Teflon flow cell (built in house; Fig. 1). A Plasma Sciences (Lorton, VA) LVC-100 cold sputter-etch unit was employed for sputter deposition of gold. Dynamic light scattering measurements were accomplished with a Nicomp 370/Auto dilute Sub-micron Particle Sizer from Pacific Scientific (Lakewood, NJ). Grazing angle IR experiments were performed with a Nicolet (Madison, WI) 550 Magna IR spectrometer. Gold coated glass slides for these experiments were obtained from Evaporated Films (Ithaca, NY).

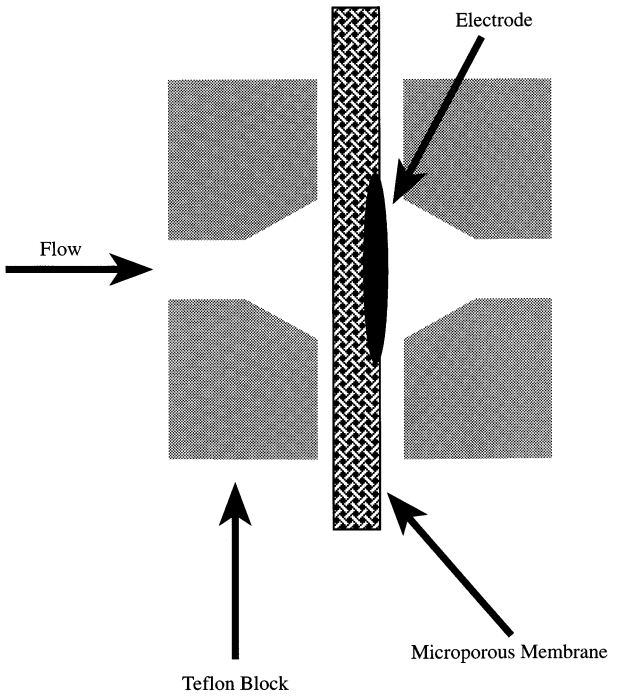

Fig. 1. Teflon flow cell designed to remove non-specifically bound antibody from microporous gold electrodes deposited on nylon membranes. A flow rate of 0.5 ml/min for 20 min was used for electrode washing (unless otherwise indicated). The gold side of the membrane was placed downstream.

2.2. Reagents

Thioctic acid (oxidized form), anhydrous acetonitrile and $K_3Fe(CN)_6$ were obtained from Aldrich (Milwaukee, WI). Sodium dodecylsulfate (SDS) and digoxin were products of Fluka (Milwaukee, WI). Bovine serum albumin (BSA), Tween 20, guanidine-HCl, bovine intestinal alkaline phosphatase (ALP), reagent grade goat IgG (gIgG), and 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (EDAC) were purchased from Sigma (St. Louis, MO). Mouse monoclonal anti-digoxin was obtained from Biodesign International (Kennebunk, ME), while the digoxin-ALP conjugate was purchased from Diatech Research Biochemicals (Boston, MA). Reagent grade sheep IgG (sIgG) was a product of Pierce (Rockford, IL). ^{125}I-labeled sheep anti-rat IgG (50 μCi) was purchased from Amersham (Arlington Heights, IL), while ^{125}I-labeled goat anti-mouse IgG (50 μCi) was obtained from ICN (Costa Mesa, CA). Porous Nytran neutral nylon membranes (0.2 μm pore size) were obtained from Schleicher and Schuell (Keene, NH). Anhydrous ethanol from the McCormick distilling company (Weston, MO) was used in the formation of thioctic acid films, while gold (99.99% purity) targets were obtained from the Kurt Lesker Company (Clairton, PA). Unlabeled IgGs were not raised against a specific species. All the other chemicals were of reagent grade or better purity.

2.3. Preparation of surfaces for immobilization of IgG

Approximately a 600-Å thick layer of gold (based on sputter rate) was sputter deposited onto either micropo-

rous nylon membranes or rough polyurethane chips. A ring of 70% PVC/30% DOS (w/w) in THF was cast around the 7 mm diameter microporous gold electrodes to prevent the penetration of the sample into any other portion of the nylon membrane. Additionally, the plasticized PVC ring functioned as a gasket when the membrane was mounted on the Teflon cell. Microporous and rough gold electrodes were subjected to one of the several treatments prior to antibody exposure. Electrodes were left untreated (BG) or placed into a 1.5% (w/v) solution of thioctic acid in anhydrous ethanol for 24 h (microporous electrodes) or 19 h (rough electrodes). The thioctic acid layer was subsequently left unmodified (TA) or treated with a solution of 1.5% (w/v) EDAC in anhydrous acetonitrile for 5 h (ETA).

An additional set of electrodes were prepared on nylon membranes which had been pre-treated prior to the sputter deposition of gold. This pre-treatment consisted of placing the nylon membranes into 1.5% (w/v) EDAC in anhydrous acetonitrile for 5 h. Membranes were subsequently washed with acetonitrile, dried and placed in a 10% (v/v) solution of ethanolamine in 0.1 M sodium borate, pH 8.75, for 1 h. After extensive washing with doubly distilled and deionized (DDI) water, the membranes were dried for 24 h in vacuum at 40°C. This pre-treatment was designed to block any free carboxylic acid groups endogenous to the nylon membrane, and thus, minimize covalent attachment of protein to the membrane.

2.4. Determination of surface area

The electroactive area of 7 mm diameter gold microporous electrodes (nylon) and 4-mm rough gold electrodes (polyurethane) was used as an estimate of electrode surface area. Electroactive areas were determined by chronoamperometry of ferrocyanide via the Cottrell equation as described earlier (Bard and Faulkner, 1980). Results yielded an electroactive area of 1.2 ± 0.1 cm^2 ($n = 5$), or three times the geometrical area of 0.385 cm^2, for microporous gold electrodes and an area of 0.221 ± 0.009 cm^2 ($n = 5$), or 1.75 times the geometrical area of 0.126 cm^2, for rough gold electrodes. The surface area of the polystyrene tubes employed was calculated based on the geometry of the tube and determined to be 1.188 cm^2.

2.5. Examination of immobilized antibody on microporous gold electrodes with ^{125}I-labeled IgG

Antibody immobilization on microporous gold electrodes was examined using 7 mm diameter gold electrodes, ^{125}I-labeled sheep anti-rat and ^{125}I-labeled goat anti-mouse IgG. Immobilization of antibody was assumed to occur through one of the two possible paths. For BG and TA electrodes on both the untreated and pre-treated nylon membranes, non-specific adsorption is the only means of immobilization possible. However, covalent attachment is believed to account for a portion of immobilized antibody on ETA electrodes prepared with either untreated or pre-treated nylon membranes. Antibody solutions were prepared by spiking 2% (v/v) (0.02 μCi/ml) of the appropriately labeled species into a 2 mg/ml solution of either sheep or goat IgG in 0.1 M sodium borate, pH 8.75, with additional concentrations prepared by serial dilution of the stock solutions. Antibody immobilization was accomplished by placing a 25-μl aliquot of the appropriate solution onto an electrode, and unless otherwise stated, allowing it to dry at 4°C. Electrodes were then washed in either a gentle or stringent manner and subsequently analyzed via γ-counting. Gentle washing consisted of shaking the electrodes three times for 10 min per wash in 20 ml of 0.01 M Tris, pH 7.4, containing 0.15 M NaCl, 1.0 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 0.1% gelatin (TBG). Stringent washes were accomplished by first washing the electrodes gently, then mounting them in a Teflon flow cell (Fig. 1) and pumping 6 M guanidine-HCl with 0.1% SDS through them at the rate of 0.5 ml/min for 20 min. A 7 mm diameter cork borer was utilized to remove the gold electrode from the remainder of the membrane for γ-counting. All the solutions were handled behind a lead-lined glass shield.

Protein aggregation effects on the mass of IgG immobilized was investigated in two separate experiments. The first involved including a surfactant in the antibody immobilization buffer (0.1% (v/v) Tween 20). In the second experiment the electrodes were washed prior to being dry (18 h after antibody exposure at 4°C). In both the experiments, the mass of immobilized protein was determined after stringently washing the electrodes.

The effect of thioctic acid concentration (employed to generate a thioctic acid layer) on the mass of immobilized IgG was also investigated. Electrodes were prepared as described above on untreated nylon membranes. Electrodes were immersed in anhydrous ethanol solutions of thioctic acid ranging in concentration from 0 to 5% (w/v) for 24 h, and subsequently immersed in a 1.5% (w/v) EDAC solution for 5 h. This was followed by exposure to 30 μl of 2 mg/ml ^{125}I-labeled sIgG (0.02 μCi/ml). After allowing the antibody solution to dry at 4°C, electrodes were washed stringently as described above.

2.6. Examination of immobilized antibody on rough gold electrodes with ^{125}I-labeled IgG

Immobilization of antibody on rough gold electrodes was examined by employing 4 mm diameter gold electrodes on a polyurethane film, and ^{125}I-labeled sheep anti-rat or ^{125}I-labeled goat anti-mouse IgG. Immobilization was accomplished by placing a 10-μl aliquot of

an immobilization solution, described above, on the electrode. Electrodes were stored at 4°C for 24 h before being washed three times for 10 min per wash in 20 ml of 6 M guanidine-HCl and 0.1% SDS with shaking. After drying, the electrode leads were cut from the rest of the polyurethane chip so that the radioactive electrode could be analyzed. As before, all the solutions were handled behind a lead-lined glass shield.

2.7. Examination of IgG adsorption on polystyrene

Polystyrene tubes were employed to examine how the surface coverage of antibody changes as a function of IgG concentration. Tubes (6 mm i.d. × 40 mm) were filled with 150 μl ^{125}I-labeled sIgG, ranging in concentration from 1 to 0.002 mg/ml, and allowed to stand for 72 h at 4°C. Individual concentrations were prepared via serial dilutions of a stock 2 mg/ml ^{125}I-labeled sIgG (0.02 μCi/ml) solution employing borate buffer. After 72 h, solutions were removed and the tubes were washed three times with 1 ml of 0.15 M NaCl and 0.05% Tween 20, prior to being assayed for radioactivity.

2.8. Determination of solution phase aggregation of IgG

Solutions of gIgG were prepared from a stock solution of 34 mg/ml gIgG in DDI water, which had been vacuum filtered through a microporous nylon membrane (0.2 μm pore size). Concentrations of gIgG ranged from 0.1 to 20 mg/ml. The mean particle size of these solutions was subsequently determined by dynamic laser light scattering (LLS).

2.9. Investigation of covalent attachment to thioctic acid (size effects)

Glass slides coated with an evaporated gold film were treated with Piranha solution followed by treatment in a 1.5% (w/v) thioctic acid (TA) solution in anhydrous ethanol for 18 h. TA treated slides were subjected to one of the following additional treatments; 5 h in 1.5% (w/v) EDAC and 10% (v/v) butylamine in anhydrous acetonitrile; 5 h in 1.5% (w/v) EDAC in anhydrous acetonitrile, then air dried briefly and placed in 10% butylamine in 0.1 M sodium borate, pH 8.75, for 24 h; or 5 h in 1.5% (w/v) EDAC in anhydrous acetonitrile then air dried for 40 min and placed in 10% butylamine in 0.1 M sodium borate, pH 8.75, for 24 h. All the three types of slides were washed with DDI subsequent to butylamine exposure. Grazing angle IR was performed on the various slides operating the spectrometer in reflection absorption mode with an incident angle of 80° using a multiple reflection adapter. Slides were scanned 512 times and thiophenol treated surfaces were employed as references.

2.10. Investigation of the effect gold treatment with thioctic acid has on the performance of NEEIA

Two sets of microporous gold electrodes (4 mm diameter) were prepared on untreated nylon, as described above. One set of electrodes was subjected to the ETA treatment described earlier while the other set was left untreated (BG). Both the sets were then modified with 10-μl aliquots of 0.05 mg/ml anti-digoxin. These electrodes were then used to perform competitive style NEEIAs (Ducey et al., 1997) on either 0 or 1 μM solutions of digoxin containing 0.05 U/ml digoxin-ALP. These experiments employed 60 min incubations of 2 ml samples in TBG and 2 ml of 5 mg/ml *p*-aminophenyl phosphate (*p*-APP) in 1.0 M carbonate, pH 9.3, with 1.0 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ as the substrate. A + 190 mV potential was applied to the gold electrodes for amperometric detection of the *p*-aminophenol produced.

3. Results and discussion

Immobilization of IgG from several hosts on microporous gold electrodes was examined with respect to both membrane and electrode treatment, as well as the method of antibody immobilization. The goal of the investigation was to determine the nature of the antibody–surface interaction (covalent or non-specific), and to assess whether a monolayer of antibody was forming on the thioctic acid treated gold surfaces.

3.1. Surface coverage of microporous gold electrodes employed in NEEIA

Detection limits for immunoassays, both competitive and non-competitive, are affected significantly by the mass of immobilized antibody (Achord et al., 1991). Competitive assays require that a limited amount of protein be immobilized, while non-competitive assays require a molar excess of protein be immobilized relative to the analyte of interest. Therefore, it is important to know the mass of protein immobilized when implementing a given immobilization scheme, so that a rational approach toward assay optimization can be employed. Further, the nature of the surface–protein bond (covalent vs. non-specific) achieved with a given immobilization scheme is critical since this will impact the conditions under which the assay can be employed (Orthner et al., 1991; Wink et al., 1997).

It was reported earlier that modified microporous gold electrodes used in NEEIA could be prepared by treating the gold with thioctic acid and EDAC prior to antibody exposure (Duan and Meyerhoff, 1995). Here, a similar procedure was used to modify electrodes with ^{125}I-labeled sheep anti-rat IgG, in order to determine

Table 1
Mass of IgG immobilized as a function of both electrode treatment and the concentration of IgG in the immobilization solution[a]

IgG (mg/ml)	Immobilized IgG on BG electrodes (pmol)	Immobilized IgG on TA electrodes (pmol)	Immobilized IgG on ETA electrodes (pmol)
2	355 ± 11	349 ± 10	360 ± 6
0.1	15.3 ± 0.2	14.4 ± 0.4	15 ± 2
0.01	1.15 ± 0.03	0.86 ± 0.06	1.13 ± 0.03
0.001	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.02

[a] Electrodes were washed gently; $n \geq 3$ for all values.

the mass of antibody immobilized as a function of gold modification. The concentration of the IgG solution varied with the specific application and ranged from 1 μg/ml to 2 mg/ml, where the higher concentrations were representative of non-competitive immunoassay conditions. Prior to washing, the solution was dried on the electrode at 4°C for at least 24 h, washed gently, and allowed to dry in air. Three types of antibody modified electrodes were prepared: bare gold (BG); thioctic acid treated gold (TA); and gold treated with thioctic acid and EDAC (ETA). It was found that for all the antibody concentrations tested, regardless of electrode type, the amount of antibody immobilized was essentially the same (Table 1). This table also shows that with the exception from 0.001 to 0.01 mg/ml, an increase in the IgG concentration used for immobilization results in a directly proportional increase in the amount of protein immobilized, over four orders of magnitude. This is probably an indication that a significant amount of non-specific interactions and protein aggregation occurs during the immobilization process.

Results from these preliminary studies also indicated that for both 0.1 and 2 mg/ml solutions, antibody surface coverages were greater than a monolayer (approximately 12 and 218 layers, respectively) (It should be noted that the use of the Cottrell equation to approximate these surface coverages may underestimate the surface area of the electrodes. However, this error should be too small to account completely for the large amount of protein immobilized). The washing method is likely to contribute to these results. First, the wash solution is not chaotropic enough to fully disrupt non-specific interactions occurring between the protein and the electrode surface, the protein and the nylon membrane, and/or protein–protein interactions. Additionally, shaking the electrode in the wash solution would not be sufficient to remove protein bound within the pores of the nylon membrane, since the porous nature of the membrane would have reduced convection effects in this region. Therefore, the non-specific interactions of protein with the electrode surface, and other protein molecules, as well as the location of the immobilized protein (on the electrode surface vs. within the porous nylon membrane) were investigated to determine the origin of such high surface coverages. This latter point was studied since nylon is comprised of repeating amide linkages with the polymer terminating in a carboxylic acid. These terminal carboxylic acid sites may have been activated when the electrode was exposed to EDAC, which would have made it possible for protein to be covalently attached to the nylon membrane.

3.2. Location of immobilized IgG on NEEIA electrodes

To investigate the location of immobilized IgG on microporous gold electrodes, a set of electrodes prepared by depositing gold on nylon membranes pretreated with EDAC and ethanolamine was compared with a set prepared on untreated nylon membranes. Following protein modification of the electrodes, as described above, the electrodes were dried. Then, in order to more effectively wash the pores of the membrane, the electrodes were washed by flowing 2% (w/v) BSA and 1% (v/v) Tween 20 through the membrane at 0.71 ml/min for 20 min, and the mass of immobilized protein was determined. The wash solution in this case was slightly different than that used for the preliminary studies in an effort to more effectively disrupt any non-specific interactions that may occur during the immobilization procedure. A comparison of the amount of protein immobilized to EDAC activated electrodes showed that approximately 200 pmol more IgG was immobilized on electrodes prepared with untreated membranes than with pre-treated ones (419 ± 39 pmol ($n = 6$) and 253 ± 8 pmol ($n = 6$), respectively). This difference is probably the result of covalent attachment of antibody to the nylon on an untreated membrane. This is supported by the fact that there was only a slight statistical difference in the amount of antibody immobilized on TA electrodes utilizing the different membranes (197 ± 20 pmol ($n = 6$) untreated, and 157 ± 14 pmol ($n = 6$) pre-treated) ($P < 0.01$). Additional support was obtained when microporous nylon membranes without gold were treated by one of the three methods. Membranes were used as received, treated with EDAC or EDAC, followed by ethanolamine and subsequently exposed to a 2 mg/ml solution of ^{125}I-labeled sIgG for immobilization. There was no statistical difference observed for membranes

used as received and those treated with both EDAC and ethanolamine (109 ± 12 pmol ($n = 6$) on untreated membranes; 118 ± 6 pmol ($n = 6$) on pre-treated membranes). However, a large increase was observed in the mass immobilized on membranes treated with EDAC prior to antibody exposure (181 ± 11 pmol ($n = 6$)). This represented 72 ± 13 pmol of IgG covalently attached to the nylon membrane.

3.3. The role of protein–protein aggregation in the immobilization of IgG on microporous gold electrodes

To investigate the influence of protein aggregation on antibody immobilization, several methods were employed to minimize protein aggregation, including adding surfactant (Tween-20) to the immobilization solution, washing the electrodes before antibody was allowed to dry on the electrode surface, and decreasing the protein concentration in the immobilization solution. Additionally, extremely chaotropic washing solutions of 6 M guanidine-HCl and 0.1% SDS were employed to remove non-specifically bound material.

When using a 2 mg/ml IgG immobilization solution, it was found that surfactant addition or the use of a chaotropic wash was enough to significantly disrupt non-specific interactions occurring in the immobilization process, independent of electrode type (Table 2). When the concentration of antibody in the immobilization solution was decreased, similar results were obtained for BG electrodes (Table 2). Results for TA and ETA electrodes, however, showed that neither a stringent wash nor the presence of a surfactant significantly affected the mass of protein immobilized. These results again indicate that non-specific interactions were primarily responsible for the protein immobilization. The marked reduction in the mass of protein immobilized on these electrodes from solutions containing Tween-20, even after normal washings, indicate that a large portion of the protein was immobilized to the electrode surface and not in the membrane pores. Although, the observation that stringent washing results in the lowest amount of protein immobilized (high protein concentrations), it appears that all the types of electrodes possess some protein immobilized within the membrane pores. The dramatic effect of the surfactant on the amount of protein immobilized for highly concentrated solutions, compared with the minimal effect at low concentrations, demonstrates that protein–protein aggregation occurs in solutions without surfactant (Table 2). Finally, for both high and low concentrations, there was a significant increase in the amount of protein immobilized on electrodes treated with both EDAC and thioctic acid over those treated only with thioctic acid (55 ± 5 pmol for 2 mg/ml IgG and 0.61 ± 0.16 for 0.01 mg/ml IgG). These results represent a surface coverage of 48 pmol/cm^2, or 24 times the theoretical monolayer coverages, if protein was covalently bound only to the thioctic acid layer. However, since these membranes

Table 2
Surface coverage on various microporous gold electrodes (electroactive area, 1.2 cm^2) as a function of both the washing method and the inclusion of Tween 20 in an IgG immobilization solution[a]

Electrode type	IgG (mg/ml)	Tween 20 (%)	Wash type	Immobilized IgG (pmol)
BG	2	0	Gentle	355 ± 11 ($n = 4$)
BG	2	0.01	Gentle	170 ± 23
BG	2	0	Stringent	119 ± 7
BG	2	0.01	Stringent	-87 ± 7
TA	2	0	Gentle	349 ± 10 ($n = 4$)
TA	2	0.01	Gentle	279 ± 21
TA	2	0	Stringent	96.4 ± 3.3
TA	2	0.01	Stringent	85 ± 4
ETA	2	0	Gentle	360 ± 6 ($n = 4$)
ETA	2	0.01	Gentle	217 ± 31
ETA	2	0	Stringent	136 ± 2
ETA	2	0.01	Stringent	140 ± 3
BG	0.01	0	Gentle	1.15 ± 0.03
BG	0.01	0	Stringent	0.68 ± 0.07
BG	0.01	0.01	Stringent	0.66 ± 0.13
TA	0.01	0	Gentle	0.86 ± 0.06
TA	0.01	0.01	Gentle	0.76 ± 0.21
TA	0.01	0	Stringent	1.02 ± 0.07
TA	0.01	0.01	Stringent	0.64 ± 0.14
ETA	0.01	0	Gentle	1.13 ± 0.03
ETA	0.01	0.01	Gentle	1.26 ± 0.32
ETA	0.01	0	Stringent	1.01 ± 0.06
ETA	0.01	0.01	Stringent	1.25 ± 0.08

[a] For all values, $n \geq 3$.

Table 3
Surface coverages on various microporous gold electrodes (electroactive area, 1.2 cm^2) as a function of both the washing method and if the IgG immobilization solution was allowed to dry[a]

Electrode type	IgG (mg/ml)	Surface dryness	Wash type	Immobilized IgG (pmol)
BG	2	Dry	Gentle	355 ± 11
BG	2	Wet	Gentle	266 ± 4
BG	2	Dry	Stringent	119 ± 7
BG	2	Wet	Stringent	71 ± 2
TA	2	Dry	Gentle	349 ± 10
TA	2	Wet	Gentle	257 ± 6
TA	2	Dry	Stringent	96.4 ± 3.3
TA	2	Wet	Stringent	75.3 ± 0.6
ETA	2	Dry	Gentle	360 ± 6
ETA	2	Wet	Gentle	261 ± 6
ETA	2	Dry	Stringent	136 ± 2
ETA	2	Wet	Stringent	86 ± 4
BG	0.01	Dry	Gentle	1.15 ± 0.03
BG	0.01	Wet	Gentle	1.02 ± 0.04
BG	0.01	Dry	Stringent	0.68 ± 0.07
BG	0.01	Wet	Stringent	0.5 ± 0.02
TA	0.01	Dry	Gentle	0.86 ± 0.06
TA	0.01	Wet	Gentle	0.95 ± 0.02
TA	0.01	Dry	Stringent	1.02 ± 0.07
TA	0.01	Wet	Stringent	0.69 ± 0.05
ETA	0.01	Dry	Gentle	1.13 ± 0.03
ETA	0.01	Wet	Gentle	1.03 ± 0.05
ETA	0.01	Dry	Stringent	1.01 ± 0.06
ETA	0.01	Wet	Stringent	0.67 ± 0.07

[a] For all values, $n \geq 3$.

were not pre-treated prior to gold deposition, a component of this difference is most probably the result of covalent attachment of some antibody to the nylon membrane.

The effect of drying the electrode on the mass of protein immobilized was also examined to determine the role of IgG aggregation during immobilization. The extent of protein aggregation should decrease when protein is not allowed to dry on the surface, as protein aggregation is enhanced by lack of moisture. The mass of immobilized IgG was determined for several IgG concentrations following a gentle washing step with TBG and a stringent washing step with 6 M guanidine-HCl and 0.1% SDS. Washing the microporous electrodes prior to drying of the IgG solution resulted in a large decrease in the mass of antibody immobilized (Table 3). The amount of protein immobilized on ETA electrodes washed stringently before drying, resulted in a lower mass of protein immobilized than for any ETA electrode that was allowed to dry. No statistical difference was observed between the mass of antibody immobilized on TA electrodes compared with ETA electrodes, suggesting that no covalent binding occurs. In fact, these results indicate that almost the whole gig immobilized on the electrodes under these conditions is non-specifically bound, and that protein–protein interactions represent the major source of antibody binding to microprobes gold electrodes. When a 2 mg/ml IgG solution was used for immobilization, approximately 80 pmol of protein remained tightly bound to the electrode surface, even after stringent washing, a result of non-specific binding of protein to both the thioctic acid layer and the nylon membrane. Although protein aggregation plays a large role in the non-specific binding of IgG to microporous gold electrodes, based on these studies, it is unclear whether the mechanism is a solution phase phenomenon or a surface effect.

3.4. The role of IgG aggregation in the solution phase

Upon determining that protein aggregation played a significant role in immobilization, it was necessary to determine whether this was a result of protein aggregation in solution followed by immobilization on the electrode, or protein immobilization followed by aggregation. To elucidate which mechanism was occurring, the mean particle size of various concentrations of sIgG solutions was determined by dynamic laser light scattering (LLS). Results from LLS studies indicate that there is no significant amount of protein–protein aggregation occurs as the concentration of sIgG increases from 0.1 to 20 mg/ml sIgG (0.1, 1, 2, 5, 10 and 20 mg/ml). In fact, for this concentration range the average particle size varied from 12.0 (10 mg/ml) to 17.3 nm (0.1 mg/ml), with an overall average size of 13.9 ± 2 nm. This is slightly smaller than a single IgG molecule (15

nm), and does not represent a significant difference (Esser, 1988).

3.5. Examination of adsorption of IgG on polyurethane

The effect of porosity and substrate type on protein immobilization was investigated by adsorbing different concentrations of ^{125}I-sIgG on gold coated polyurethane. A steady increase in the mass of immobilized protein was observed with increasing solution concentration for all the three types of electrodes (Fig. 2). Further, for a given protein concentration, there was no significant difference observed in the mass of protein immobilized on BG electrodes versus TA or ETA electrodes. Finally, for 2 mg/ml solution, multiple layers of protein were shown to have been immobilized on all the three types of electrodes. These data suggest that non-specific interactions are again playing a significant role in immobilization, and that the report of multiple layers of protein on nylon supported electrodes are not solely the result of porosity.

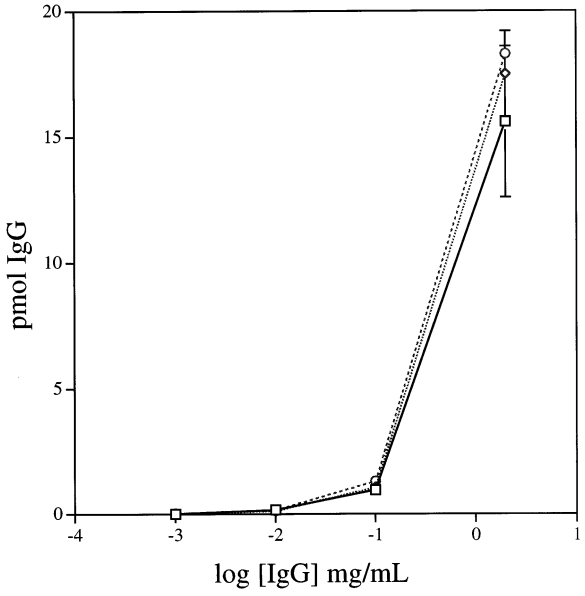

Fig. 2. Mass of protein immobilized on gold coated polyurethane chips (electroactive area, 0.221 cm^2) as a function of IgG concentration and gold treatment (BG, □) bare gold; (TA, ◇) gold treated with thioctic acid and (ETA, ○) gold treated with thioctic acid and EDAC ($n \geq 3$ for all points).

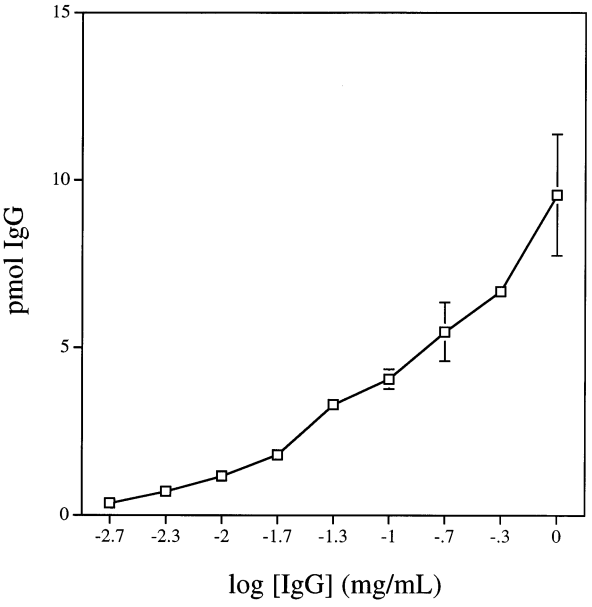

Fig. 3. Mass of protein immobilized on polystyrene tubes (geometrical area, 1.188 cm^2) as a function of sIgG concentration ($n = 3$ for all points).

The effect of thioctic acid concentration on the mass of protein immobilized was investigated by employing either a 0.01 or 2 mg/ml ^{125}I-sIgG solution for immobilization of protein on gold coated polyurethane. Polyurethane electrodes were treated with thioctic acid solutions ranging in concentration from 0 to 5% (w/v), which were subsequently activated with a 1.5% (w/v) EDAC solution in anhydrous acetonitrile. These thioctic acid concentration studies indicated that regardless of the concentration of thioctic acid employed, the mass of immobilized protein was statistically the same as the mass shown in Fig. 2. This result further confirms the assertion that non-specific interactions play a large role in the immobilization process. In fact, it is not statistically possible to determine if there is any covalent attachment occurring under these conditions. This lack of covalent attachment is probably the result of the protein being hindered sterically from interacting with the activated thioctic acid. This hypothesis was supported by grazing angle IR experiments which showed that butylamine, a small molecule, was attached to the thioctic acid (appearance of vibrational bands due to amide bond formation) when conditions similar to those used for protein immobilization in NEEIA were employed (data not shown).

3.6. Examination of adsorption of IgG on polystyrene

Protein immobilization to plain polystyrene was used to demonstrate that protein aggregation results in the formation of multiple protein layers even on a non-porous, non-gold surface. The titration curve shows a region of independence (region where surface coverage is relatively unaffected by concentration) at IgG concentrations between 0.1 and 0.2 mg/ml (Fig. 3). The use of higher concentrations, however, leads to continual increase in the mass of protein immobilized, which is reflective of IgG aggregation. This supports the earlier observations made with microporous and rough gold electrodes, where the mass of antibody immobilized was unaffected by both immobilization and washing conditions at low antibody concentrations.

3.7. The effect of gold treatment with thioctic acid on NEEIA performance

A comparison of the performance of ETA and BG style electrodes was made utilizing either 0 or 1 μM digoxin solutions in TBG to perform NEEIA experiments. The resulting currents measured (B_0 and B, respectively) were subsequently used to calculate a B/B_0 value for both the ETA and BG electrodes. The calculated B/B_0 value was determined to be statistically identical for both ETA and BG style electrodes (0.30 ± 0.032 and 0.289 ± 0.038, respectively; $n \geq 3$). This strongly suggests that there is no advantage gained in the NEEIA system when thioctic acid is employed in the Ab immobilization step, and that equivalent immunoassay performance can be achieved with non-specifically adsorbed Abs.

4. Conclusions

Immobilization of ^{125}I-labeled IgG on gold electrodes deposited on microporous nylon and on polyurethane, as well as, the immobilization of the protein on polystyrene was examined. Additionally, solution phase protein aggregation was studied using dynamic laser light scattering (LLS). Results demonstrate that immobilization of antibody to the gold surfaces is accomplished through non-specific surface–protein and protein–protein interactions, which are further enhanced by drying the microporous gold electrodes prior to washing. Results also demonstrate that immobilization occurs not only on the electrode surface, but within the pores of the nylon membrane as well. In short, the use of thioctic acid does not result in the covalent attachment of a monolayer of antibody to microporous gold electrodes as previously thought (Duan and Meyerhoff, 1995). Instead, immobilization is the result of the non-specific adsorption of aggregated protein. The lack of covalent attachment is believed to be the result of a steric effect since grazing angle IR experiments show that the small molecule, butylamine, can indeed be attached to the thioctic acid layer.

The results presented here, suggest a vastly different picture of the electrode surface than the one believed to exist previously (Duan and Meyerhoff, 1994). Instead of a gold electrode with a monolayer of protein attached covalently to the surface, these electrodes have multiple layers of aggregated protein, which extend into the pores of the nylon membrane, anchored only via non-specific adsorption. Nonetheless, such electrodes have proven useful for carrying out a number of separation-free enzyme immunoassays (NEEIA). Despite this fact, future studies will probably not use the ETA style electrodes but rather the BG style electrodes since both the electrodes perform the same but the ETA electrodes require more reagents and time to prepare. It is, however, still clear that a well-defined immobilization technique that would yield a controlled monolayer surface coverage of antibody, may further enhance the performance of this new assay format.

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