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Chemical modification of polystyrene's surface and its effect on immobilized antibodies

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Abstract

The quantity and immunoreactivity of antibodies coupled to treated polystyrene beads were studied as a function of the chemical treatments used to prepare the polystyrene surface. Injection-molded polystyrene beads were nitrated with sulfuric and nitric acids followed by reduction to form amine groups. The nitration conditions used affect the quantity of nitro and amine groups introduced, and ultimately the quantity of antibody coupled to the surface.

Aminated beads were also grafted with monomethoxypolyethylene glycol (mPEG) prior to antibody coupling to determine if this modification could lower non-specific protein binding to the beads. At high surface levels of mPEG, antibody coupling was completely blocked. At lower surface levels of mPEG, antibody coupling was accomplished, but with no reduction of the non-specific protein binding to the beads. © 1998 Elsevier Science B.V.

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1. Introduction

The adsorption of antibodies onto polystyrene surfaces is a common practice in the development and production of immunometric assays [1,2]. Covalent immobilization results in better assay reproducibility and a more stable surface due to the minimization of antibody desorption [3,4]. The use of an appropriately sized cross-linker can preserve the immunoreactivity of the immobilized antibody by limiting its interaction with the surface and the subsequent conformational changes that ensue [5]. Polystyrene must be chemically modified to make its surface amenable to covalent cross-linking with proteins. The types of chemical group introduced, and the physical effects to the surface from the modification, play important roles in the immunoreactivity of the immobilized antibodies.

Primary amines are convenient groups for cross-linking proteins and they can be readily introduced onto polystyrene's surface. One method of aminating the surface is by nitrating the polystyrene with sulfuric and nitric acids [6], followed by reduction of the nitro groups. The number of nitro and amine groups introduced defines the electronic nature of the surface, and ultimately plays a role in determining the amount of antibody immobilized and its antigen binding capacity. The harsh acid conditions used in the nitration can cause degradation and changes to the surface roughness of the polystyrene which also affects the quantity and reactivity of the immobilized antibodies. The amine groups can be cross-linked with polyethylene glycol to introduce additional surface characteristics with respect to antibody immobilization.

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In this paper, we discuss how different nitration conditions with nitric and sulfuric acids modify the surface of polystyrene beads with respect to the quantity of nitro and amine groups introduced. How these surface groups affect the quantity and immunoreactivity of covalently immobilized antibodies is presented. The role of surface roughness, polyethylene glycol grafting, and the water contact angle are all discussed with respect to the quantity and reactivity of the immobilized antibody.

2. Experimental

2.1. Acid treatment of polystyrene beads

Polystyrene beads of 7.94 mm diameter produced from thermal injection molding of polystyrene pellets were obtained from Hoover Precision Products. The beads were nitrated with varying mixtures of concentrated sulfuric and nitric acids (Olin). The nitro beads were then reduced with stannous chloride in a hydrochloric acid solution. Beads with varying nitro levels were made by diluting the nitric and sulfuric acids, or by varying the nitration reaction time. Beads with varying amine levels were made by varying the reduction reaction times.

2.2. Quantification of amine and nitro groups per bead

The number of amine groups on the bead surface were quantified spectrophotometrically with trinitrobenzene sulfonic acid (TNBS) [7–9]. Acid-treated and reduced beads were reacted with a 5 to 6 molar excess of TNBS in a borate-buffered solution for 24 h with shaking at room temperature. Untreated polystyrene beads were also reacted with the TNBS solution as the negative control. During the incubation, the amine groups on the bead surface displace the sulfonate group bonding trinitrobenzene to the bead. At the end of 24 h, the unreacted TNBS was quenched with a 1.0 mg ml^{-1} glycine solution in borate buffer, pH 10.3, for 10 min. The glycine amine displaces the sulfonate group forming a soluble, bright orange, TNBS–glycine adduct. The concentration of the TNBS solution was determined from the absorbance of the TNBS–glycine adduct in acidic media, $\epsilon = 1.25 \times 10^{-4}$ M^{-1} cm^{-1}. The concentration of amine groups per bead was determined from the absorbance difference between the negative control and the aminated bead.

The number of moles of nitro groups remaining on the surface of the bead after the reduction step was determined in a second reduction step. Total reduction was accomplished by reacting the beads with a reducing metal solution for at least 24 h. The additional amine groups were then quantified using the TNBS reagent.

2.3. Surface measurements of treated and untreated polystyrene beads

The surface of several 7.94 mm virgin polystyrene beads was collected by filing the beads. The polystyrene powder was mixed to about 10% with KBr and pressed into a window with a KBr press. The IR spectrum was collected on a Nicolet 205 FTIR from 64 scans.

The surface of several beads that had been aminated was also collected by filing, and pressed into a KBr window. Its FTIR spectrum was acquired similarly and compared to the non-treated polystyrene beads. This method of looking at the chemical groups on the surface of the bead provides a rapid confirmation of the identity of the groups added on the bead's surface. Additionally, the surface layer of several aminated beads was taken up in deuterated chloroform and its ^{1}H NMR spectrum determined.

Bead roughness measurements were measured by Hoover Precision Products Inc. (Saulte Ste. Marie, MI) using a Stylus 400. Roughness values are reported in R_a units. The larger the R_a value, the rougher the bead surface. Water contact angle measurements were determined to assess the effect of the various treatments on the wettability of the bead surface. The contact angle for a droplet of water on a surface is measured as the angle, inclusive of the water, formed between the surface and the plane of the liquid where it meets the surface. The wettability of a surface for water is inversely proportional to the water contact angle. Contact angles were measured by Advanced Surface Technology, Inc. (Billerica, MA) using a

high resolution video contact angle system, VCA-2000®. Measurements were done in triplicate using deionized water delivered from the VCA-2000® precision liquid dispensing system.

2.4. Coupling of antibodies to modified polystyrene beads

The antibodies used in these studies were all IgG murine monoclonals and were specific for the prostate-specific antigen (anti-PSA), the carcinoembryonic antigen (anti-CEA), and alpha-fetal-protein (anti-AFP). All the antibodies investigated were coupled to the various modified beads according to the following abbreviated protocol. The beads are rinsed with deionized water, followed by immersion in pH 9.3 triethanolamine buffer. A bifunctional cross-linker is dissolved into the buffer. After the beads have incubated with the cross-linking reagent, the solution is drained, and the beads rinsed with sodium phosphate, pH 8. The desired antibody is dissolved in sodium phosphate, pH 8, and incubated with the activated beads. After the incubation, the beads are rinsed with a phosphate-buffered Tween 20 solution. The beads are blocked in the final step to cover any exposed polystyrene sites.

To demonstrate that antibodies coupled to beads by this procedure are covalently attached, a radiolabeled antibody was coupled to the treated beads and tested for passive vs. covalent attachment. Anti-AFP antibody was radiolabeled with $Na^{125}I$, using glucose oxidase-lactoperoxidase beads (Bio-Rad), and adjusted to a specific activity of 3 nCi μg^{-1}. Two replicate lots of H_2SO_4/HNO_3-treated beads were prepared for antibody coupling. The radiolabeled antibody was both covalently and passively attached to the two bead lots in separate experiments. The average amount of antibody attached to each bead type after buffer rinsing was measured from 20 replicate beads. Each bead lot was subjected to a 15–20 min 0.2% Tween 20 wash. The amount of antibody remaining after the Tween 20 wash was determined for each bead type.

2.5. Grafting of aminated polystyrene with methoxypolyethylene glycol

Methoxypolyethylene glycol (mPEG) with an average molecular weight of 5000, and activated with cyanuric chloride was obtained from Sigma. It was dissolved in a minimal volume of 0.1 M borate buffer at 4°C and pH 9.2, at molar ratios ranging from 4 to 0.0001 relative to the amine content on the aminated polystyrene beads [10,11]. The beads were reacted with shaking (170 rev min^{-1}) at 4°C for 30 min, followed by an additional 1.5 h reaction at room temperature with shaking. The beads were rinsed with copious amounts of water and stored in water at 4°C. The amount of mPEG coupled to the bead was determined from the difference in amine groups on the bead before and after mPEG coupling. The uncoupled amine groups were quantified colorimetrically using the TNBS reagent [7,8].

2.6. Measurement of antibody bound to the bead

Antibodies bound to the treated polystyrene surfaces were quantified using a bead ELISA method [12]. This method is based on the binding of a goat anti-mouse kappa alkaline phosphatase conjugate, GAMKAP, which binds to the mouse antibodies coupled at the bead's surface. The amount of antibody coupled to a derivatized polystyrene bead is proportional to the 405 nm absorbance signal generated by the alkaline phosphatase. The method is executed as follows: triplicate beads are placed in plastic tubes and incubated with 0.25 ml of a (20/80) ratio of GAMKAP/GAMK (goat anti-mouse kappa) at 5 μg ml^{-1} in PBST (0.5% Tween-20/phosphate-buffered saline) for 1.5 h in a 37°C water bath shaker. Beads were washed three times with (0.1% Tween-20/0.1 M PBS, pH 7.4), followed by a 5 min incubation with 0.2 ml per bead of *p*-nitrophenyl phosphate substrate. After 5 min the reaction was stopped with 1.5 ml of an EDTA quench solution, and absorbance read at 405 nm.

3. Results

3.1. Effect of varying nitration conditions on the amine quantities produced at the bead surface

Polystyrene beads of two roughness levels were nitrated with varying sulfuric:nitric acid ratios.

The nitro beads produced from these ratios were reduced with stannous chloride in a hydrochloric acid solution. The amine groups generated from these experiments were quantified with the TNBS reagent. As the proportion of nitric acid in the sulfuric:nitric acid ratio was increased, a corresponding increase in the amine per bead level resulted, Table 1.

3.2. Spectroscopic analysis of the nitrated and reduced beads

IR spectroscopy was used to compare the chemical groups at the surface of the bead. FTIR spectra of both untreated polystyrene beads and nitrated/reduced beads were compared. The untreated and treated bead spectra are shown in Fig. 1. The treated bead spectrum differs from the raw bead spectrum by the presence of two peaks in the 1525 and 1350 cm^{-1} regions. These peaks are consistent with the asymmetrical and symmetrical stretches of aromatic NO bonds of nitro groups. The peak at 850 cm^{-1} is characteristic for para-substituted aromatics. This finding agrees with the aromatic splitting pattern in the NMR spectra that suggests a para nitration. The amine NH stretch, which should appear in the 3500 cm^{-1} region, has a weaker intensity than the NO stretches, and is at too low a surface concentration to be detected by the FTIR.

Table 1
Amine per bead values from varying sulfuric/nitric acid ratios after reduction[a]

Acid ratio	Amines per bead[b] (nmol)	Surface roughness R_a
2.00:1	36±1	86–108
1.65:1	48±1	86–108
1.50:1	83±2	86–108
2.45:1	50±1	200–210
2.30:1	44±1	200–210
2.18:1	54±1	200–210
2.00:1	64±1	200–210
1.85:1	53±1	200–210
1.70:1	68±2	200–210
1.50:1	70±1	200–210

[a] The acid ratios are reported as sulfuric:nitric (v/v). Surface roughness values are reported as R_a ranges of the polystyrene beads prior to acid treatment.
[b] Amine per bead values and standard deviations were determined from five replicate beads.

3.3. Effects of antibody cross-linking through the surface amines

Fig. 2 shows that antibodies that were attached to the bead surface using a bifunctional cross-linker retained more antibody when subjected to surfactant washing studies vs. passively coated beads. This result suggests the antibody is covalently attached through the amine group in the coupling procedure. Beads that were passively attached lost more than 50% of the adsorbed antibody after the Tween 20 wash, whereas the chemically cross-linked beads lost less than 10%.

3.4. Effects of the acid treatment ratio on wettability and antibody bead activity

The wettability of the polystyrene surface can be increased by introducing polar groups at the surface. These groups create surface sites for interaction with water molecules resulting in increased wettability. As the wettability increases there is a corresponding decrease in the water contact angle [13]. When the acid treatment ratio was varied from (H_2SO_4:HNO_3) 1.70:1 to 2.45:1, the water contact angle remained constant at 127±4°. This result indicates that the change in surface polar groups over the treatment range was not large enough to alter the surface wettability.

Results presented in Table 1 demonstrate that varying the sulfuric acid to nitric acid ratio affects the quantity of amine groups when the reduction conditions are held constant. The acid ratio affects the antigen binding capacity of the antibody coupled to the bead's surface. The bead antibody's antigen binding capacity is proportional to the "hook ratio". The hook ratio is a value generated from two separate binding experiments, both of which form the [bead antibody–antigen–antibody conjugate] sandwich. The ratio is the signal generated using an excess of antigen, resulting in a hook effect [14], divided by the signal generated using a limiting amount of antigen.

Hook ratio values were determined for a series of anti-PSA antibody beads that were coupled to

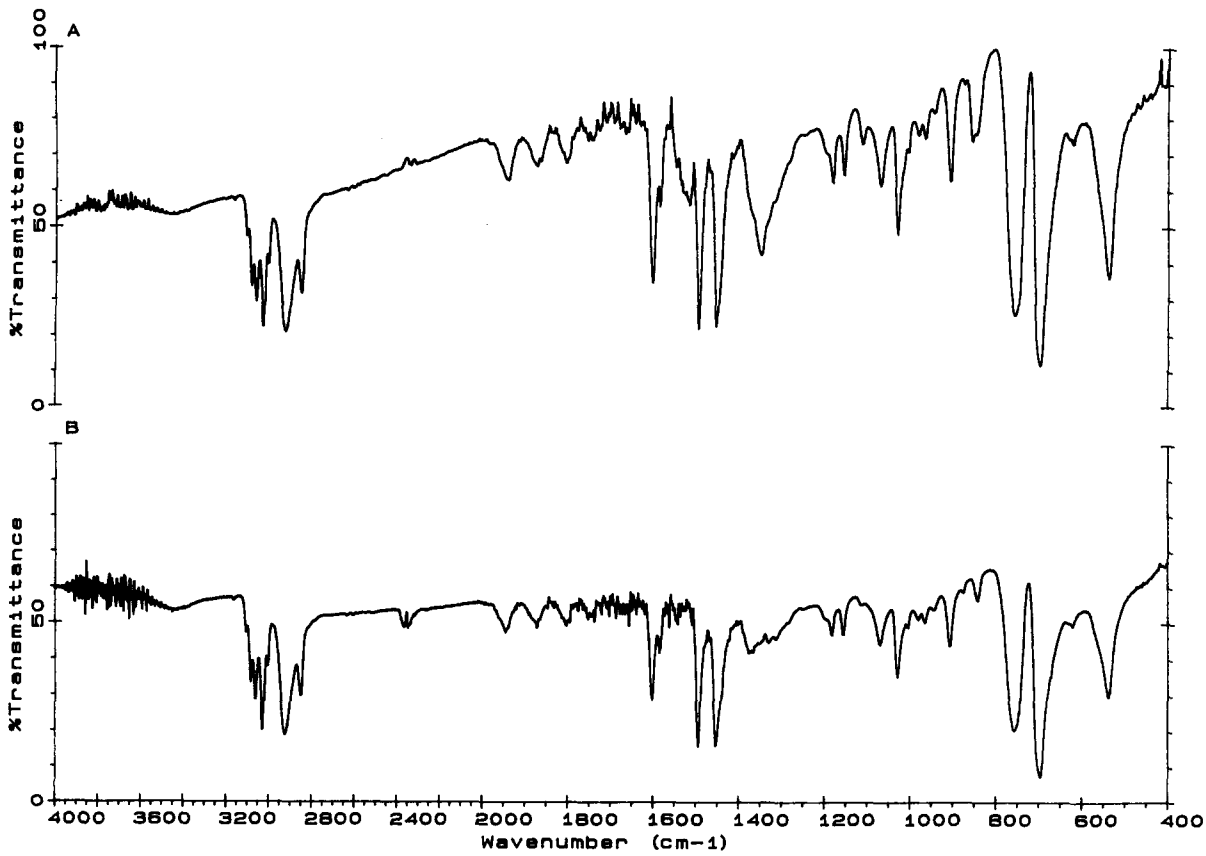

Fig. 1. FTIR spectra of the treated (A), and untreated (B) polystyrene bead surface. Surface filings of each bead were collected and the powder mixed to 5–10% with KBr.

beads with varying surface amine concentrations (Fig. 3). These figures show that a minimal level of 4–8 nmol of amine are necessary to produce active bead-bound anti-PSA antibody. Aside from this lower limit, the antibody functioned well over a wide range of amine levels. Other assay parameters, such as the minimal level of antigen detection, $<0.17\ \mu g\ l^{-1}$, and within assay sample precision, $<5\%$, were unaffected by the varying amine levels, and were comparable to values reported in the literature [15,16]. The relative amount of antibody immobilized to the bead in this series was also measured and correlated with the hook ratio, Fig. 4.

Hook ratio values were measured on a series of anti-AFP antibody-coupled beads. The same lot of anti-AFP antibody was coupled to beads that had been treated with the acid ratios (H_2SO_4:HNO_3) 2.45:1, 2.30:1, 2.18:1, 2.00:1, 1.85:1, 1.70:1, and reduced under equivalent conditions. The beads were assayed for their hook ratio values, Fig. 5. The results indicate that the different acid ratios used to generate the nitro groups modify the surface of the polystyrene bead and modulate the antigen binding capacity of the immobilized antibody. At higher nitric acid levels the polystyrene may be degraded by oxidation of the methine group on the aliphatic chain. These changes may control the amount of antibody that is conjugated to the surface of the bead.

The antigen binding capacity of the bead was also affected by the surface roughness of the bead. To investigate this effect, treated beads with a wide range of surface roughness values were generated. Aminated beads were made from different acid treatments of several raw bead lots of varying

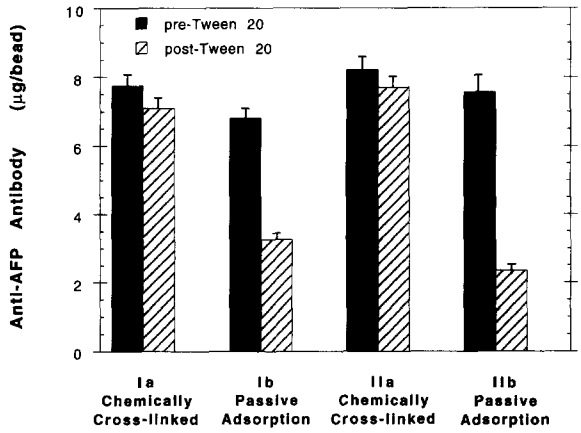

Fig. 2. Covalent vs. passive antibody attachment was tested by chemically cross-linking radiolabeled anti-AFP antibody to two acid-treated bead lots, Ia and IIa. The same radiolabeled antibody was also passively adsorbed on the same treated bead lots, Ib and IIb. The amount of antibody attached to the bead from the cross-linking or adsorption procedures was measured. The beads were washed with a 0.2% Tween 20 solution, and re-measured for the amount of remaining antibody.

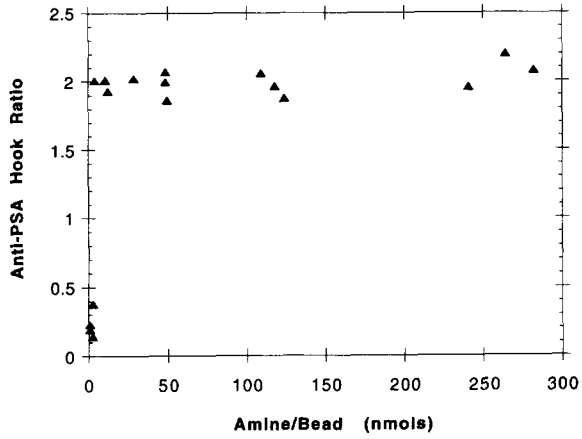

Fig. 3. Effect of the bead amine content on the anti-PSA antibody antigen binding capacity as measured by the hook ratio.

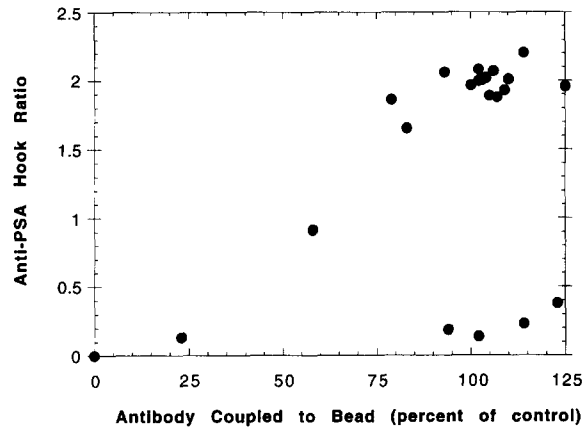

Fig. 4. Effect of the quantity of antibody coupled to the bead on the hook ratio of beads coupled with anti-PSA antibody. Values were measured using GAMKAP ELISA, described in Section 2. The anti-PSA antibody was coupled to beads with varying amine levels, as shown in Fig. 3. The four points in the lower right corner of the plot have sufficient antibody coupled to the surface, but lack activity due to low amine levels.

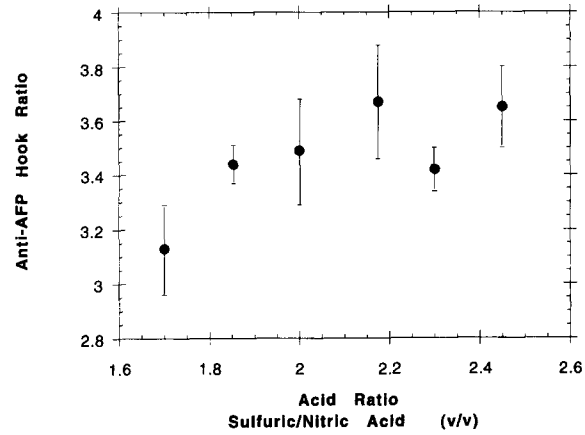

Fig. 5. Effect of the sulfuric/nitric acid ratio on the resultant anti-AFP bead hook ratio and the percentage of anti-AFP antibody per bead. Couplings were performed with the same anti-AFP antibody lot for all beads. Bead ELISA and hook assays were run in triplicate using the same lot of AFP antigen.

roughness. The resulting aminated beads were measured for their surface roughness. Samples of each bead type were coupled with an anti-AFP antibody and the hook ratio of each was measured. The correlation between the anti-AFP antibody hook ratio and the treated bead roughness is shown in Fig. 6.

3.5. *Grafting of mPEG to aminated beads*

Aminated beads were coupled using mPEG activated with cyanuric chloride at five levels with molar ratios (mPEG:bead amine) ranging from of 4:1 to 0.25:1. It was possible to quantify the amount of mPEG coupled to the beads by measuring the

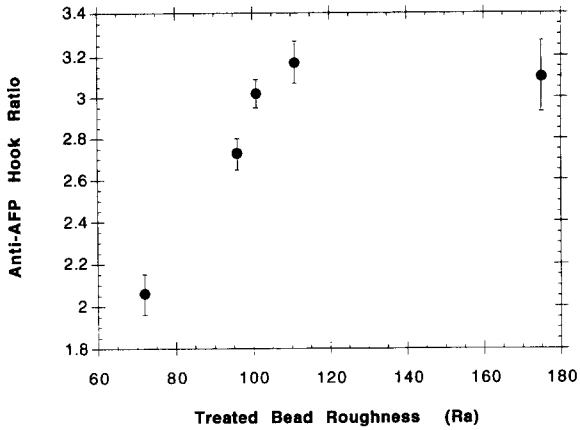

Fig. 6. Effect of the treated bead surface roughness on the anti-AFP antibody bead hook ratio. Acid-treated beads were measured for surface roughness prior to coupling with anti-AFP antibody. All beads were coupled with the same lot of anti-AFP. The coupled beads were assayed in triplicate at the same time using the same AFP antigen lot.

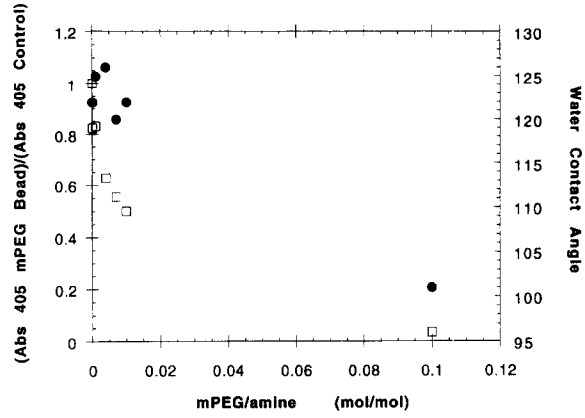

Fig. 7. Effect of mPEG grafting on the water contact angle (●). When mPEG was reacted with the bead at an mPEG/amine molar ratio range from 0 to 0.01 there was no change in the water contact angle. A significant drop in the water contact angle occurred when the mPEG/amine reaction ratio was raised to 0.1. The average standard deviation for these water contact angle measurements was ±2°. Effect of mPEG grafting on the amount of antibody coupled to the bead surface (□). The amount of anti-PSA antibody coupled to the mPEG grafted beads was measured using a goat anti-mouse alkaline phosphatase conjugate. The measurements were normalized against a control anti-PSA antibody bead that was not grafted with mPEG.

loss of free amine groups at the bead surface with the TNBS reagent. The final free amine level dropped exponentially with the amount of activated mPEG used. An initial value of 61 nmol amine per bead resulted in a final value 39 nmol per bead when reacted with a fourfold molar excess of activated mPEG. At lower ratios of mPEG, 0.1 and lower, the TNBS assay was not sensitive enough to differentiate the surface amine levels between pre- and post-mPEG-coupled beads. Beads coupled with mPEG at molar ratios (mPEG:bead amine) ranging from 0 to 0.1 were analyzed for their water surface contact angles (Fig. 7). As more mPEG groups were coupled to the bead surface a decrease in the surface hydrophobicity was expected to occur, resulting in decreased water contact angles. The surface mPEG groups did not affect the water contact angle until the highest mPEG ratio, 0.1, and the contact angle fell from 124° to about 100°. At this level of mPEG, it was impossible to couple a measurable amount of antibody to the bead.

The amount of antibody immobilized on the mPEG amino bead was dependent upon the amount of mPEG coupled to the bead. When mPEG coated the bead at 5 to 20 nmol per bead, there was essentially no antibody coupling to the bead. At this level of mPEG the antibody was effectively blocked from covalently or passively coating the bead surface. At lower mPEG molar reaction ratios, 0.01 mPEG per amine and lower, the quantity of antibody coupled to the surface begins to rebound, as shown in Fig. 7. It is apparent from this figure that grafted mPEG reduces the amount of antibody that can be coupled to the bead surface. The use of PEG-grafted surfaces is reported throughout the literature for its ability to reduce protein adsorption on the surface [17–20].

Anti-PSA and anti-CEA antibodies were each cross-linked to a series of mPEG-grafted beads as described in Section 2. The resultant antibody beads were assayed with control beads to determine if non-specific binding could be reduced by the mPEG, Table 2.

4. Discussion

The role of the surface amine groups was studied by quantifying these groups and comparing the results with the bead assay performance. Our results

Table 2
Non-specific ^{125}I-tracer binding to mPEG grafted antibody beads

mPEG ratio[a]	Anti-PSA (cpm)	Anti-CEA (cpm)
0.0000	110	383
0.0001	117	NA
0.0010	100	350
0.0040	111	346
0.0070	101	NA
0.0080	NA	335
0.0100	114	NA
0.0120	NA	329
0.0160	NA	344

[a] The molar ratio of mPEG to bead amine used in the grafting of the bead. Values listed are the average of 10 to 21 replicate measurements of ^{125}I tracer non-specific binding to the antibody bead in a matrix free of antigen.

with bead amine levels ranging from 0 to 282 nmol per bead, show that the anti-PSA monoclonal antibody requires a minimum of 4–8 nmol of amine per bead for an optimal antigen binding capacity when immobilized on the bead. While it was found that polystyrene beads with amine groups below this threshold level could adsorb comparable amounts of the anti-PSA antibody, these beads showed a lower antigen binding capacity than beads modified with amine groups. Bale et al. have shown a higher retention of immunoglobulin biological activity on polystyrene copolymers, which include hydrophilic or hydrogen bonding monomers, than on the polystyrene homopolymer [21]. Our results indicate that the activity of the anti-PSA antibody is enhanced by the electronic surface environment created by the chemical modification.

For the anti-PSA antibody, our results indicate that as the amount of antibody coupled to a bead increases, so does the total antigen capacity of the bead, Fig. 4. An upper limit of bead antigen capacity seems to occur after which further antibody immobilization does not increase the antigen capacity of the bead. Antigen–antigen and antibody–antibody steric hinderance may limit the antigen immobilization potential of the bead.

Our studies demonstrate that the antigen binding capacity of the bead is reduced when the nitration reaction is carried out at higher nitric acid concentrations, Fig. 5. While the higher proportion of nitric acid results in more nitro and, subsequently, amine groups, it also results in a greater smoothing of the bead surface, as shown by our surface roughness measurements. The surface smoothing is probably caused by oxidative degradation of the polystyrene. Degradative sulfonation of polystyrene has been reported in the literature [22,23]. Smoothing results in a loss of active surface and contributes to the reduction in antigen binding capacity of beads treated with higher proportions of nitric acid, Fig. 6.

Our results show that mPEG can easily be coupled to the amine groups at the bead surface through the cyanuric acid ring. The amount of mPEG bound can be determined using the TNBS reagent when more than 5 nmol of the bead amines have been coupled. Water contact angle analysis shows that the contact angle is not affected until the beads are coupled with at least a 0.1 molar ratio of mPEG to bead amine groups. At this ratio, 4 nmol or less of mPEG may be coupled to the bead's surface. This is an interesting result, since the 0.1 molar ratio of mPEG to bead amines also corresponds to the mPEG level on the bead that completely prevents coupling of the antibody to its surface. Hence, both the surface water contact angle measurement and the bead ELISA of the mPEG beads suggest that the 0.01 molar ratio (mPEG to amines), which corresponds to 0.4 nmol of mPEG or less, is the highest amount of mPEG that can be grafted to the bead before the mPEG groups begin effectively blocking the surface from coupling antibody. At a molar ratio of 0.1 the surface is completely blocked from coupling.

Amine beads that were grafted at 0.01 and lower molar ratios of mPEG, relative to the amines groups, were cross-linked with either anti-PSA or anti-CEA antibodies. These beads were tested for performance against identically cross-linked beads without mPEG; there was essentially no difference in the performance of the beads. Additionally, grafting of mPEG to the amine bead surface did not lower the non-specific binding for either the anti-PSA beads or the anti-CEA beads.

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