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Research paper

Impact of surface defects and denaturation of capture surface proteins on nonspecific binding in immunoassays using antibody-coated polystyrene nanoparticle labels

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ABSTRACT

Microtiter wells are commonly used for bioassays. The sensitivity of such an assay depends on several instrumental and biochemical parameters such as the signal-to-background ratio and nonspecific binding of the label molecules. In this study, we have investigated the possible effects of well surface defects, well edges and denaturation of capture antibodies on the assay sensitivity. We used internally dyed Eu(III) chelate polystyrene nanoparticles as high specific activity labels in a thyroid stimulating hormone (TSH) sandwich-type model assay. The nanoparticle labels provide a high signal-to-background ratio in assays but the major limiting factor of the assay sensitivity is nonspecific binding of the labels. In our model assay the capture monoclonal antibodies were immobilized on microtiter wells passively or through streptavidin (SA)–biotin linkage. At first, commercially manufactured microtiter well surfaces were probed with an atomic force microscopy and significant structural inhomogeneities were found. The nonspecific binding of the nanoparticle conjugates did not appear to follow any of the microtiter well surface defect patterns in a number of experiments. In addition, the microtiter well edges did not increase the nonspecific binding. Denaturation of capture antibodies on solid surfaces has been proposed to expose amino acid sequences promoting nonspecific binding. This was studied by intentionally denaturing the surface capture antibodies by heat, detergent or acid treatment prior to the assay. Although specific signal was almost entirely lost no significant effect on nonspecific binding was observed. The passively adsorbed antibodies denatured at lower temperatures than those captured through streptavidin–biotin linkage. Evidently, the additional protein (SA) layer protected the capture antibody from denaturation whereas the solid surface appeared to act as a "catalyst" making the passively adsorbed antibody more susceptible to denaturation.

1. Introduction

Sensitivity of a sandwich immunoassay is dependent on the combination of imprecision, affinity of the antibody, specific activity of the label and nonspecific binding of labelled antibody (Ekins and Chu, 1991). Nanoparticle labels have proved to possess high specific activity and even single binding events can be observed due to the extremely intense luminescence of the particles as compared to molecular labels (Hall et al., 1999; Härmä et al., 2001; Schultz et al., 2000; Zijlmans et al., 1999). In addition, affinity of a nanoparticle label coated with multiple antibodies has been shown to exceed that of the soluble labeled antibody. More than ten-fold higher affinity constants have been measured for nanoparticle bioconjugates than their molecular counterparts (Soukka et al., 2001a). Due to the high specific activity of the nanoparticle labels, nonspecific binding has become an

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important sensitivity limiting factor of nanoparticle-based assays in a variety of applications in diagnostics and drug development.

Nonspecific binding of the labeled binding partner is a universal problem in immunoassays and bioaffinity assays in general. The origin of the phenomenon has been widely speculated but it has not been clearly characterized due to its diverse nature. There are at least three possible reasons for this nonspecific binding: 1) the surface itself adsorbs the label (labeled antibody) or 2) the capture protein attached to the capture surface binds the label or the antibody attached to it or 3) surface structure of the solid phase promotes non-specific binding. Generally, nonspecific binding is a sum of a wide variety of interferences (Selby, 1999). This interference can be caused by the sample matrix e.g. human anti-mouse antibodies, rheumatoid factors, complement reaction, heterophilic antibodies or autoantibodies in blood or serum (Selby, 1999; Eriksson et al., 2000). Adding an excess of related nonspecific bovine or murine immunoglobulin or other proteins to the assay buffer has been shown to reduce the problem (Soukka et al., 2001b) but not to eliminate it. Nonspecific signal has also been reported to increase in relation to the specific signal in immunoassays performed in a non-clinical buffer matrix (Soukka et al., 2001b).

Creating binding surfaces with high specific binding activity and low nonspecific binding is a prerequisite for a high sensitivity bioaffinity assay. The most common solid surface of choice has been polystyrene due to its ability to adsorb proteins, form relatively strong hydrophobic bonds upon adsorption and the ease of fabrication (Butler, 2000). Different methods, irradiation, plasma-treatment etc., are available to modify surface characteristics altering the profile of adsorbed proteins from hydrophobic to hydrophilic (Boudet et al., 1991). In most cases the capture protein layer is not complete and there is a need for blocking the surface with additional proteins or detergents (Butler, 2000; Reimhult et al., 2008). Blocking molecules are usually chosen to readily adsorb to polystyrene surface and have no affinity towards binding molecules in order to prevent their non-specific binding.

The process of passively adsorbing proteins on any surface will alter the conformation of the adsorbed proteins (Nyilas et al., 1974). In case of polystyrene and silicone there is evidence that proteins unfold (Butler, 2000) permitting internal hydrophobic side chains to form strong hydrophobic bonds with the surface. While the bond between surface and proteins is strong and relatively stable, the altered conformation has been proven to affect antigen specificity and function. The protein surface adsorption has also been reported to denature a large portion of the adsorbed antibodies and significantly reduce the amount of binding sites (Butler, 2000) and to change enzyme substrate specificity (Kennel, 1982; Hollander and Katchalski-Katzir, 1986). It has been shown that the method for attaching capture antibodies has an impact on the functionality of the antibody layer (Butler et al., 1992; Davies et al., 1994). In the case of the scFv-fragments lacking the stabilizing constant domains it has been suggested that partial unfolding of the antibody fragments creates sticky patches on the surface leading to increased nonspecific signal (Scheuermann et al., 2003). However, we have previously shown that whole monoclonal antibodies and their fragments are feasible for assay purposes when attached to microtiter well surfaces via capture proteins and no change in respect to nonspecific binding was found when fragmented antibodies were applied in the nanoparticle assays (Näreoja et al., 2009).

Nanoparticle labels are large in size when compared to molecular labels with size variation typically from ten to hundreds of nanometers. The large size of the labels may promote nonspecific binding to surfaces if uneven surfaces at nanoscale are utilized. Cavities in solid-phase surface could potentially retain nanoparticles due to larger surface area available for interactions. Also other features present on polystyrene surfaces have been suggested to induce patches with alternating binding properties which could lead to nonspecific binding of nanoparticle labels or uneven antibody coating of the polystyrene surface (Davies et al., 1994; Allen et al., 2001).

In this study we have imaged antibody-coated microtiter well surfaces in nanoscale in search for evidence of sites that are prone to nonspecific binding. Detailed topographic images from coated surfaces are presented. We have also studied the effect of the microtiter well edges on nonspecific binding of nanoparticle labels. The large surface area in the edges could potentially lead to higher degree of nonspecific binding compared to flat surface areas containing no edges. Moreover, the effects of denaturing treatments with heat, acid and detergents on nonspecific binding properties of capture antibodies on two differently-coated surfaces were studied. Finally, stabilities of capture antibodies attached via passive adsorption and streptavidin–biotin linkage were compared.

2. Experimental

2.1. Reagents

Europium(III)-chelate-doped Fluoro-Max™, monodisperse, carboxyl-modified, polystyrene nanoparticles of 92 nm in diameter were purchased from Seradyn (Indianapolis, IN). The fluorescent properties of the particles have been described previously (Härmä et al., 2001; Soukka et al., 2001a). Anti-TSH monoclonal antibody clones anti-TSH 5404 SP-1 and anti-TSH 5409 SPTNE-5 were purchased from Medix Biochemica (Kauniainen, Finland). Antibody fragments were produced at the Department of Biotechnology in the University of Turku (Eriksson et al., 2000; Ylikotila et al., 2005). Affinity constants for the antibodies were: anti-TSH 5409 9.3×10^{8} L mol^{-1} and anti-TSH 5404 2.2×10^{10} L mol^{-1} (Helenius and Tikanoja, 1986). The antibodies were adsorbed on NUNC clear MaxiSorp™ microtitration wells (Thermo Fisher Scientific, Rochester, USA). White, black and clear NUNC PolySorp™ and MaxiSorp™ and Greiner high- and medium-binding (Greiner Bio-One GmbH, Frickenhausen, Germany) surfaces were scanned in surface topography experiments. InnoSA96™ streptavidin coated microtitration low fluor plates, assay buffer [50 mmol L^{-1} Tris–HCl (pH 7.8), 150 mmol L^{-1} NaCl, 7.7 mmol L^{-1} NaN_3, 76 μmol L^{-1} bovine serum albumin, 80 μmol L^{-1} Tween 40, 3 μmol L^{-1} bovine γ-globulin, 20 μmol L^{-1} diethylenetriamine pentaacetic acid, 20 mg L^{-1} Cherry Red] and washing solution (5 mmol L^{-1} Tris–HCl, pH 7.8 containing, 150 mmol L^{-1} NaCl, 3.5 mmol L^{-1} Germall II and 40 μmol L^{-1} Tween 20) were from InnoTrac

Diagnostics (Turku, Finland). *N*-hydroxysulfosuccinimide (NHS) was obtained from Fluka (Buchs, Switzerland), bovine serum albumin fraction V (BSA), biotin and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) were from Sigma (Steinheim, Germany).

2.2. Conjugations and coatings

The solid-phase antibody mAb anti-TSH 5409 was immobilized on clear MaxiSorp™ microtitration wells by physical adsorption. The wells were coated for over night at 37 °C with 100 ng of antibody in 50 µL of 10 mmol L^{-1} phosphate buffer, pH 7.0. The coated wells were washed twice with wash solution and saturated over night at 23 °C with 200 µL of 10 mmol L^{-1} phosphate buffer (pH 7.0) containing 76 µmol L^{-1} bovine serum albumin and 27 mmol L^{-1} d-sorbitol. After the saturation, the wells were aspirated and dried in a laminar hood for 1 h. The wells were stored at 4 °C in a sealed package with desiccant. MAb anti-TSH 5404 was covalently coated to nanoparticles according to previously described procedure using 6 µmol L^{-1} mAb (Härmä et al., 2001). The monoclonal antibodies were biotinylated randomly through lysines according to protocol described earlier (Ylikotila et al., 2005). The Fab-fragments were produced, site-specifically biotinylated and coated according to previously described procedure (Näreoja et al., 2009).

2.3. Instrumentation

Time-resolved fluorescence imaging was performed with Signifer—a modified Nikon fluorescence microscope (excitation 340 nm, emission 615 nm) with 10× objective and 10 s exposure time using LSR-Ultra software (Wallac, PerkinElmer Life Sciences, Turku, Finland). Time-resolved fluorescence of plate assays was measured with Victor^2 1420 Multilabel counter (Wallac). AFM measurements were performed using NTEGRA Prima scanning probe microscope (NT-MDT, Russia). Surface topography was measured with intermittent-contact mode under ambient conditions ($T = 24$–25 °C, RH = 17–34%) using uncoated rectangular silicon cantilevers (MikroMasch, model NSC14/NoAl or NSC15/NoAl). Images were recorded in the repulsive regime using a damping ratio of 0.7 and a scan speed of 0.25–0.50 Hz. The images were processed with scanning probe image processor (Image Metrology) software.

2.4. Immunoassays

Streptavidin-coated microtitration wells were first washed twice with the washing solution to remove preservatives and loosely bound proteins. In the second step 3×10^{-13} mol of biotinylated Fab-fragment or 1.6×10^{-13} mol of monoclonal antibody (mAb) as capture antibody was incubated in 30 µL of assay buffer for 20 min. Microtitre wells with passively adsorbed mAbs were prewashed four times to remove preservatives. This was followed by denaturing treatments in a volume of 50 µL in case of acids and detergents and 100 µL when heating was applied. A washing step was performed to remove denaturing solution before continuation of the assay. Thereafter, analyte, thyroid stimulating hormone (TSH, 1 mIU L^{-1}), was added in 35 µL of assay buffer and incubated for 20 min. After the reaction, the wells were washed twice to remove unbound analyte. The nanoparticle labels, 1×10^8 nanoparticle bioconjugates in 40 µL of assay buffer, were sonicated with a bath sonicator, vortexed vigorously, added to wells and incubated for 120 min. In AFM studies, the well edges were removed to make the sample accessible to AFM. In addition, in all experiments measured with AFM, we used 1×10^9 nanoparticle bioconjugates in 15 µL of assay buffer in order to promote nonspecific binding. Finally, the wells were washed six times, and the time-resolved fluorescence from the nanoparticle–antibody bioconjugates was measured at 615 nm using the plate fluorometer.

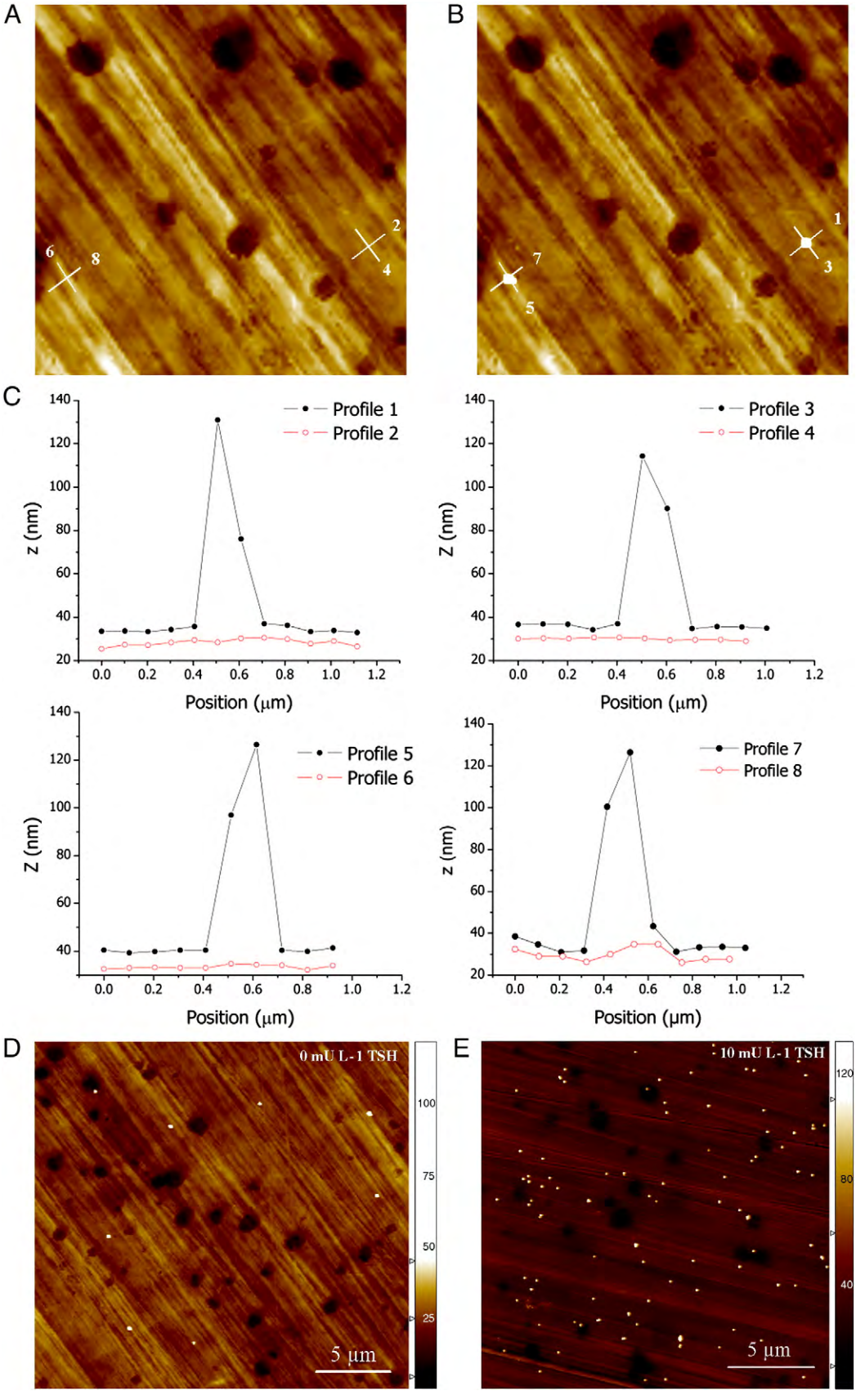
3. Results and discussion

Our first aim was to find out if surface defects can be attributed as an origin of nonspecific binding of the bioconjugated nanoparticle label on capture surface. Therefore, microtiter well surfaces and their patterns were studied with scanning probe microscopy (AFM) and fluorescence microscopy. Our second line of approach was to determine to which extent denaturing of surface-bound and soluble antibodies caused unwanted nonspecific binding and to study if the nonspecific binding of bioconjugated nanoparticles having large surface area compared to soluble label antibodies was connected to the specific binding activity of an antibody. The model assay used monoclonal antibodies, biotinylated antibodies and biotinylated antibody fragments for assaying of clinical analyte standard TSH under optimized buffer conditions and antibody-conjugated nanoparticle labels. Standardized buffer conditions were applied to avoid adverse nonspecific phenomena induced by clinical samples and to visualize clearly the core effects without disturbance from the sample matrix such as serum. To maintain the relevance to the analytic field the conditions were chosen in a way that they can also be applied for other analytes.

3.1. Effect of microtiter plate surface defects on nonspecific binding

All commercial microtiter well bottoms were investigated with AFM and their surfaces were found to be relatively uneven at nanoscale (Fig. 1). This was in agreement with previous studies (Davies et al., 1994; Allen et al., 2001). We studied more carefully NUNC MaxiSorp™ plate for nonspecific binding of nanoparticle labels. The observed cavities of this plate were up to 5 µm in diameter and from 10 to 40 nm in depth. The depth of the observed grooves was up to 10 nm.

Fig. 1. Topographic AFM-images measured in intermittent-contact mode from a passively coated, prewashed NUNC MaxiSorp™ microtiter well bottom before (A) and after (B) the zero calibrator assay. The images present typical results from the studied microtiter well plate bottoms. The scans revealed the typical topography of a polystyrene surface. C) AFM height profiles from marked lines in images A (even numbers) and B (odd numbers). The nonspecific binding did not seem to be dependent on the surface patterns. AFM-images from a passively coated microtiter well in an assay containing (D) 0 and (E) 10 mU L^{-1} TSH concentration. The scans revealed that the specific or nonspecific binding of nanoparticle labels was independent of the defects or inhomogeneous surface sites of the solid surfaces.


A
B
C
D
E
Profile 1
Profile 2
Profile 3
Profile 4
Profile 5
Profile 6
Profile 7
Profile 8
z (nm)
Z (nm)
Position (μm)
0 mU L-1 TSH
10 mU L-1 TSH
5 μm

Both features may arise from the manufacturing process; the cavities from air bubbles trapped between the mould and polystyrene and grooves from polishing marks on the mould. Nonspecific binding of bioconjugated nanoparticles onto the well surface of the MaxiSorp™ plate was monitored when no TSH analyte was added. In Fig. 1C, height profiles made from scans of the capture antibody surface (even numbers) provide a closer investigation of the sites where nonspecifically bound nanoparticle labels were attached (odd numbers). The data showed height differences of 8 nm in maximum indicating that the surface under the nonspecifically attached nanoparticles was relatively even. Topographical features were much smaller than the size of the nanoparticles studied suggesting that the nonspecific binding sites are not dependent on the surface roughness. In a number of experiments, we found that the particles were randomly distributed over the entire area of microtiter wells rather than followed any surface patterning (Fig. 1D). In addition, the same observation was evident for the assay where specific binding was studied after applying TSH: no pattern-based specific binding was observed (Fig. 1E). Topography images revealed no indication of antibody aggregates as a result of either of the coating procedures. We also speculated that the microtiter well bottom-wall edges could be potential sites for high nonspecific binding. This was investigated with time-resolved fluorescence microscopy (data not shown). No indication of increased nonspecific binding was observed. This suggests that the surface structure had insignificant impact on either specific or nonspecific binding through the analyte or nanoparticle labels, respectively.

3.2. Effect of capture antibody denaturation on nonspecific binding

Nonspecific binding is speculated to originate partially from denatured antibodies (Scheuermann et al., 2003; Wörn and Plückthun, 2001; Ewert et al., 2004). Antibody conformational changes can reveal amino acids that are attracted to surrounding biomolecules or solid surfaces and thus lead to nonspecific binding. This hypothesis was examined by subjecting the capture antibodies to denaturing conditions using heat, acid and detergent. Experiments were performed on two differently-coated surfaces: monoclonal antibodies passively adsorbed on microtiter wells and biotinylated monoclonal antibodies attached onto microtiter wells coated with SA. Instead of biotinylated monoclonal antibody a site-specifically biotinylated Fab-fragment of the anti-TSH clone 5409 antibody was studied under the same assay conditions in experiments with heat, acid and detergent treatments. The biotinylated Fab resulted in similar results compared to the biotinylated monoclonal antibodies and, therefore, solely data for monoclonal antibody is presented. The chaotropic steps were applied before the TSH analyte incubation.

First hydrochloric acid at 0.6 M concentration was incubated on the capture surface and binding activity of the capture surface was observed by determining the specific binding in an immunoassay. While most of the specific signal was lost the nonspecific signal was not affected in comparison to non-treated surface. The layer of passively adsorbed antibodies was more sensitive to denaturation treatment and all specific binding properties were lost whereas

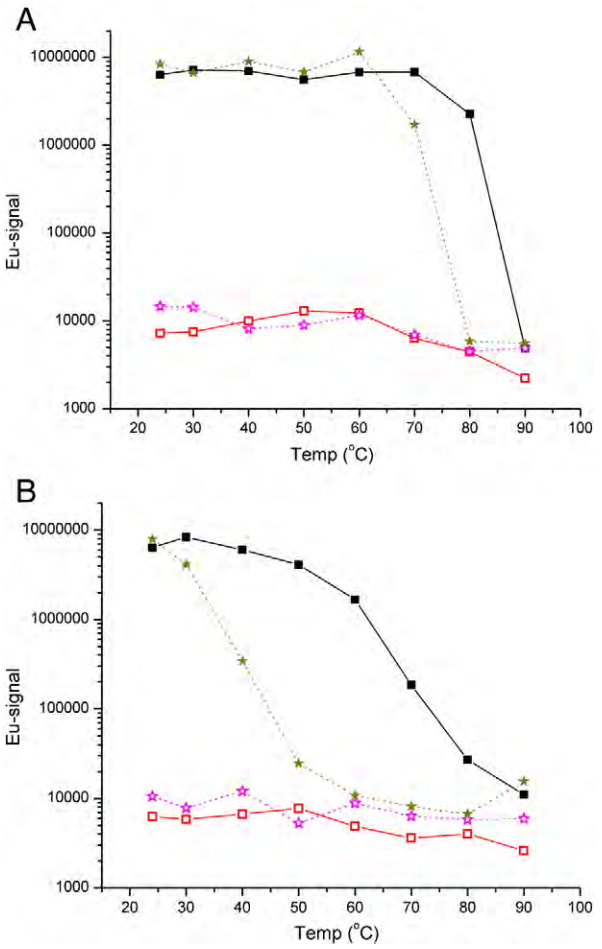


Fig. 2. Heat denaturation experiment of passively adsorbed mAb (stars) and bio-mAb captured through SA (squares) on polystyrene surface. Specific signals in closed and background signals are shown in open symbols. The heat treatment denatured passively adsorbed mAb at lower temperature compared to bio-mAb capture on the surface through SA. Heat denaturation was less pronounced in water (A) than in 20 mM SDS (B).

biotinylated antibodies attached to SA retained approximately 15% of the binding activity. There was a general trend that was consistently observed throughout this study—antibody coating using an SA protein layer provided more stability against all denaturation methods used. Similar effects have been reported when capture antibodies were immobilized on preadsorbed layer of anti-mouse IgG or SA (Butler et al., 1992; Butler et al., 1997).

In the next experiment the effect of temperature to binding activity of the capture antibodies was examined. This was carried out before the TSH analyte incubation by adding water to the microtiter plate and heating the plate for 15 min. Temperatures higher than 60 °C were required to have an impact on binding activity of passively adsorbed capture antibodies whereas the biotinylated antibody attached through SA required higher than 80 °C before a decrease in binding activity was observed (Fig. 2A). To assess the stability of the antibody at high temperature we incubated the soluble antibody in water in a test tube before the assay. The binding activity after heat treatment at 90 °C was retained indicating

that the surface has an impact on denaturation even when the SA–biotin linkage was separating the antibody directly from the surface. In addition, the heating experiment was performed in microtiter plates containing 20 mM SDS. The denaturation of the capture antibodies on the surfaces was more pronounced and it was observed in temperatures 20–40 °C lower than with water only (Fig. 2B). However, the denaturation of the antibody did not affect the nonspecific binding of the nanoparticle label. In every experiment conducted the nonspecific binding was shown not to be increased at conditions where the specific binding activity was lost. This indicates that nonspecific binding is not due to denaturation of the capture antibody. The data rather suggests that the nonspecific binding was slightly decreased when the capture antibody was denatured.

4. Conclusions

The microtitre well bottoms were found to contain structures at nanometer and micrometer scales where nanoparticles could potentially be accommodated nonspecifically. However, at nanoscale the surface was found to be smooth under the nonspecifically bound particles and no surface pattern specific binding was observed suggesting that surface structures do not lead to increased nonspecific binding of nanoparticle labels. The specific binding activity was evenly distributed over the antibody-coated polystyrene surface with both coating protocols used.

Our data suggests that relatively harsh denaturing conditions are required in order to deteriorate the capture antibody surface. A striking difference in the stability of capture antibody layer passively adsorbed on polystyrene or attached to streptavidin coated surface via chemically coupled biotin was observed. The antibodies attached through SA layer were significantly more resistant to denaturing treatments than those passively adsorbed to the surface. This suggests that the solid surface may act as a "catalyst" and make the antibodies more susceptible to denaturation. Apparently antibody on capture surface was relatively stable at ambient assay conditions and, therefore, did not create sites on the surface that would promote nonspecific binding of the nanoparticle conjugate. In fact, our data shows that nonspecific binding seems not to originate from denatured capture antibodies on surfaces because intentional denaturation of the capture surface did not increase nonspecific binding of the nanoparticle labels. The structural characteristics on the capture surface or denaturation of capture antibody did not promote nonspecific binding. This led us to believe that addition of sufficient blocking proteins to capture surface and in assay buffer is sufficient to reduce surface sites susceptible to nonspecific binding.

We have previously studied the effect of bioconjugate nanoparticle size and fragmented antibodies on nonspecific binding. Neither of the assay components showed significant effect on increasing nonspecific binding. The same conclusion can be drawn from this study: microtiter plate surface defects and denaturation of capture protein did not increase the nonspecific signal in a sandwich immunoassay utilizing bioconjugated nanoparticle labels. Therefore, we are inclined to think that improvements in assay performance and nonspecific binding should be made through manipulation of nanoparticles and such studies are currently ongoing in our laboratory.

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