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Immobilization of Antibodies on Ultraflat Polystyrene Surfaces

Weiping Qian,[1*] Danfeng Yao,[1] Fang Yu,[1] Bin Xu,[2] Rong Zhou,[1] Xiang Bao,[1] and Zuhong Lu[1]

Background: **Functional antibody surfaces were prepared on ultraflat polystyrene surfaces by physical adsorption, and the uniform distribution of monoclonal antibodies against hepatitis B surface antigen (anti-HBs) on such surfaces and the presence of dense hepatitis B surface antigen (HBsAg) particles captured by immobilized antibodies were identified.**

Methods: **A model polystyrene film was spin-coated directly onto a silicon wafer surface. Atomic force microscopy was used to directly monitor the immobilization of anti-HBs antibodies and their specific molecular interaction with HBsAg. Enzyme immunoassay was also used to characterize functional antibody surfaces.**

Results: **A mean roughness of 2 Å for areas of 25 μm^2 was produced. We found a uniform distribution of anti-HBs antibodies on ultraflat polystyrene surfaces and the presence of dense HBsAg particles bound to such anti-HBs surfaces after incubation with HBsAg.**

Conclusions: **This study confirmed the potential of preparing dense, homogeneous, highly specific, and highly stable antibody surfaces by immobilizing antibodies on polystyrene surfaces with controlled roughness. It is expected that such biofunctional surfaces could be of interest for the development of new solid-phase immunoassay techniques and biosensor techniques.**

ELISAs have become routine in medical diagnostics and other areas of medical and biological science because of their high sensitivities *(1–3)*. In an ELISA, a biological substance (antibody or antigen) must first be immobilized onto the inner surfaces of polystyrene microtiter wells, usually by physical adsorption. However, the immobilization is usually too inefficient to give good yields and does not always proceed in a dose-dependent manner *(4–7)*. Butler and co-workers *(7, 8)* have reported that >90% of monoclonal and 75% of polyclonal anti-fluorescyl capture antibodies are denatured by physical adsorption. The adsorption of proteins on polystyrene surfaces has been studied extensively *(9–14)*. From these studies it can be concluded that surface characteristics such as hydrophobicity, surface charge, and co-adsorption of or exchange with surfactants, copolymers, or other proteins are key controlling factors for the adsorption of proteins, particularly for the stability and specificity of adsorbed antibodies in immunoassays. It is evident that the orientation and structural integrity of an adsorbed antibody and the related activity of the antigen-binding sites are essential factors in the preparation of a reliable immunoassay. In addition to the amount adsorbed, the orientation and conformation of adsorbed antibodies are very important to the performance of the assay.

The immobilization of biological substances onto polystyrene surfaces is governed by several factors, but currently considerable efforts have been expended primarily in immobilizing biological substances onto microtiter well surfaces with various reagents. Isosaki et al. *(15)* have reported the successful use of methyl vinyl ether-maleic anhydride copolymer for immobilizing protein ligands. Other methods using pretreated microtiter wells to efficiently immobilize target substances have been reported, e.g., treatment with Triton X-100, poly-L-lysine, or Alcian blue *(16–18)* as well as the methyl vinyl ether-maleic anhydride copolymer method. There is no doubt, however, that many facets of ELISA can be improved *(19)*. It is likely that better substrate materials remain to be discovered and that the nature of the interactions between antibody and antigen remains to be explored. We therefore compared polystyrene microtiter wells coated with physically adsorbed antibodies against hepatitis B surface

[1] National Laboratory of Molecular and Biomolecular Electronics, Southeast University, Nanjing 210096, Peoples Republic of China.
[2] Center for Clinical Laboratory Science, Jiangsu Province, Nanjing 210009, Peoples Republic of China.
*Author for correspondence. Fax 86-25-7712719; e-mail wqian@seu.edu.cn.
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antigen (anti-HBs)[3] with silicon wafer substrates coated with site-specifically immobilized anti-HBs antibodies *(20, 21)*. It was interesting to see that the sensitivity of the ELISA could be improved by site-specific immobilization. However, simpler methods for the direct and efficient immobilization of antibodies still remain to be found.

Roberts et al. *(22)* and Davies et al. *(23)* have reported that long parallel ridges exist on blank polystyrene microtiter well surfaces and that the typical height from valley to peak is 20–30 nm with a periodicity of 50–250 nm. We also observed such ridges in our experiments using atomic force microscopy (AFM). The ridges result from the molding process used to fabricate the microtiter wells. The use of such a polystyrene microtiter well to immobilize one reactant also means that the eventual antigen-antibody interaction takes place within a small interface of unknown dimension and unknown reaction volume. We found that a sandwich ELISA, in which monoclonal anti-HBs antibodies are immobilized onto polystyrene microtiter well surfaces, did not always proceed in a highly sensitive and dose-dependent manner in our experiments. The amount of hepatitis B surface antigen (HBsAg) bound to immobilized anti-HBs antibodies showed only a proportional increase with increasing concentrations of added HBsAg from 1.0 μg/L to 100 μg/L in the dose curve (0.1–2000 μg/L), and the detection limit was ~1.0 μg/L.

In all future studies, a vital ingredient for ELISA is to understand, control, and optimize the interactions occurring at the two interfaces: (*a*) between a solid material surface and antibodies, and (*b*) between immobilized antibodies and their specific antigen molecules. One important task is the preparation of dense, homogeneous, highly specific, highly stable surfaces by immobilizing biomolecules with controlled chemical and topographic properties. It is expected that such biofunctional surfaces would be of high interest for the development of new solid-phase immunoassay techniques and biosensor techniques *(24)*.

We describe here a newly developed method to immobilize antibodies onto ultraflat polystyrene surfaces by physical adsorption. For surfaces in both model studies and applications, the topographies of substrate surfaces are important for immobilized biological substances *(25, 26)*. This is true because biological recognition is characterized by both its (bio)chemical nature and its sophisticated three-dimensional topographic aspects. In our experiments, HBsAg and its monoclonal anti-HBs antibodies were used as a model system. The molecular resolution of AFM images provides an important insight into the exact nature of anti-HBs antibodies after their adsorption and the eventual antigen-antibody interactions. The uniform distribution of anti-HBs antibodies on ultraflat polystyrene surfaces and the presence of dense HBsAg particles bound to such anti-HBs surfaces after further incubation with HBsAg was identified. Enzyme immunoassays (EIAs) were also used to detect functional surfaces.

[3] Nonstandard abbreviations: anti-HBs, anti-hepatitis B surface antigen; AFM, atomic force microscopy; HBsAg, hepatitis B surface antigen; EIA, enzyme immunoassay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; and HRP, horseradish peroxidase.

Materials and Methods

CHEMICALS

The standard polystyrene microtiter wells and monoclonal anti-HBs antibodies used in this study were gifts from Xiamen Advanced Scientific, INC. (Xiamen, China). Yeast HBsAg standards at different concentrations were purchased from the National Center for Clinical Laboratory Science. Bovine serum albumin (BSA) was purchased from Sigma Chemicals. A standard EIA kit for the determination of HBsAg was purchased from Kuhua Biotech. All aqueous solutions and buffers were prepared with water purified with the MilliQ system (Millipore).

PREPARATION OF ULTRAFLAT POLYSTYRENE SURFACES

Single crystal silicon wafers (n-type) were from the microelectronics center of Southeast University (Nanjing, China) and were cleaned with "Piranha solution", a 30:70 mixture (by volume) of 30 g/L H_2O_2 and concentrated H_2SO_4. The cleaned wafers were thoroughly rinsed in MilliQ-grade deionized water, absolute ethanol, and deionized water with sonication, respectively, and then dried in a stream of nitrogen. Silicon wafer surfaces were spin-coated with polystyrene immediately after the cleaning process. A silicon wafer surface was first wetted with toluene and spun at 2000 rpm for 1 s until the surface looked dry. As soon as the toluene disappeared from the surface, spinning was stopped. Polystyrene from microtiter wells was dissolved in toluene, and 300 μL (10 g/L) was used to coat a silicon wafer surface for 15 s. Each silicon wafer was then spun to complete dryness at 2000 rpm (~60 s).

IMMOBILIZATION OF MONOCLONAL ANTI-HBs ANTIBODIES ON POLYSTYRENE SURFACES

The silicon wafers spin-coated with polystyrene were mounted on Teflon cuvettes with a diameter of 7.0 mm. Monoclonal anti-HBs antibodies (200 μL) at a concentration of 5 mg/L in 50 mmol/L carbonate buffer, pH 9.6 (coating buffer), were added to each cuvette. After overnight incubation at 4 °C, the antibody solutions were removed. The cuvettes were washed twice with deionized water and then dried under a nitrogen stream for AFM imaging. As a comparison, antibodies were immobilized on a polystyrene microtiter well surface using the above experimental procedures.

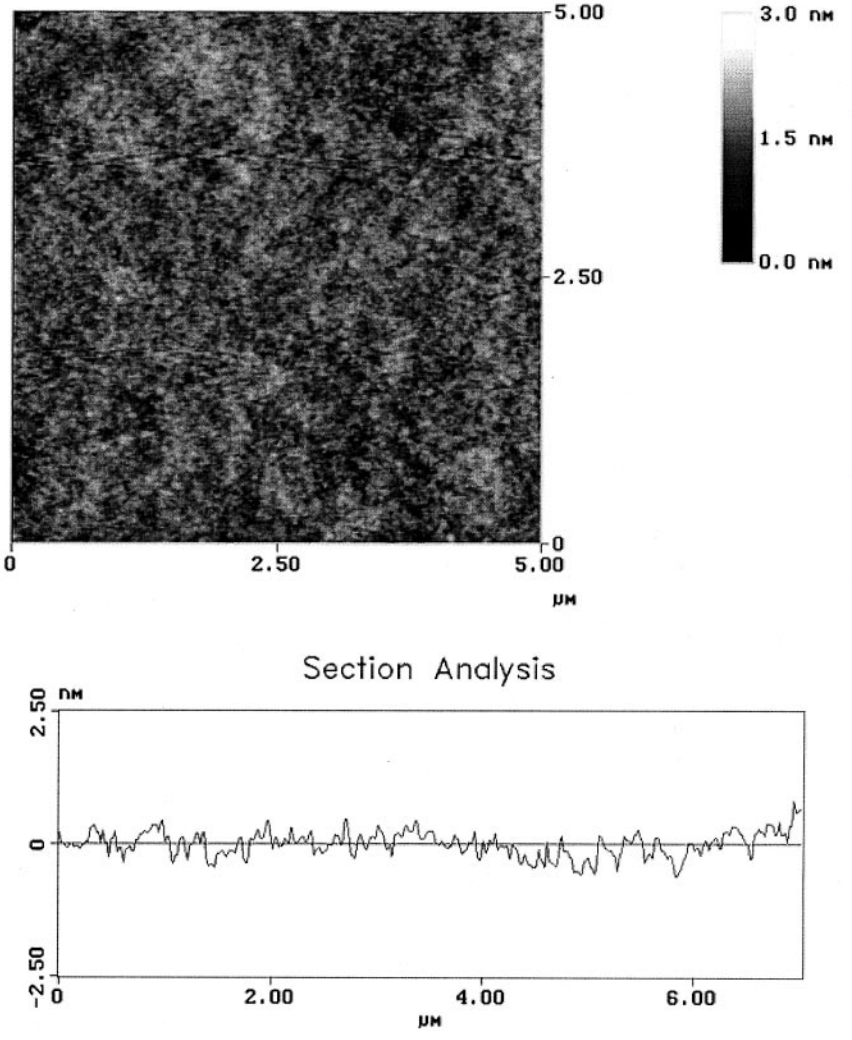

Fig. 1. AFM image (*top*) and cross-sectional analysis (*bottom*) of a spin-coated polystyrene film prepared on a silicon wafer substrate at a polystyrene concentration of 10 g/L.

The cross-sectional analysis (*bottom*) was carried out along a line extending from the *top right corner* to the *bottom left corner* of the AFM image (*top*). Note that all images reported were obtained with a steep contrast gradient (see the *z-range bars* in this figure and Figs. 2–5). The *vertical bar* shows the mapping of the sample height (in nm) to gray scale to aid in visualizing the depth of the features.

Interactions between yeast HBsAg and immobilized anti-HBs antibodies

The HBsAg standard at a concentration of 10 μg/L in 50 mmol/L phosphate-buffered saline (PBS), pH 7.4, was added to each cuvette, and the cuvettes were incubated for 60 min at 37 °C on a shaker-incubator. After incubation, the cuvettes were rinsed five times with 250 μL of Tween 20 (200 mg/L) in PBS and five times with 250 μL of deionized water, and dried under nitrogen for AFM imaging.

AFM imaging

Imaging of the surfaces was carried out using a commercial AFM (Nanoscope IIIa; Digital Instruments). The instrument was operated in Tapping Mode using silicon cantilevers oscillating with an average amplitude of 100 nm and a resonance frequency between 200 and 450 kHz. The scanning rate selected was <2 Hz. All of the images presented here were obtained repeatedly and were stable under the experimental conditions.

EIA detection

A commercial EIA kit for the determination of HBsAg was used in a standard EIA detection procedure. Silicon wafers (4 × 4 × 1 mm) coated with immobilized monoclonal anti-HBs antibodies were placed in individual wells of a polystyrene microtiter plate. The blank wells were preincubated overnight at 4 °C with 10 g/L BSA in PBS to block nonspecific adsorption of HBsAg or antibodies in the next steps. Each well was filled with 200 μL of PBS containing 10 g/L BSA for 2 h at 37 °C and shaken constantly to prevent artifacts produced by the adsorption of HBsAg or antibodies in the next steps. The BSA solution was removed, and the wells were rinsed with 250 μL of Tween 20 (200 mg/L) in PBS and 250 μL of distilled water. HBsAg standards (100 μL) at different concentrations in PBS were added to each well for 60 min at room temperature. The HBsAg solution was removed, and wells were rinsed with 250 μL of Tween 20 (200 mg/L) in PBS and 250 μL of distilled water. Polyclonal goat IgG labeled with horseradish peroxidase (HRP) was used as

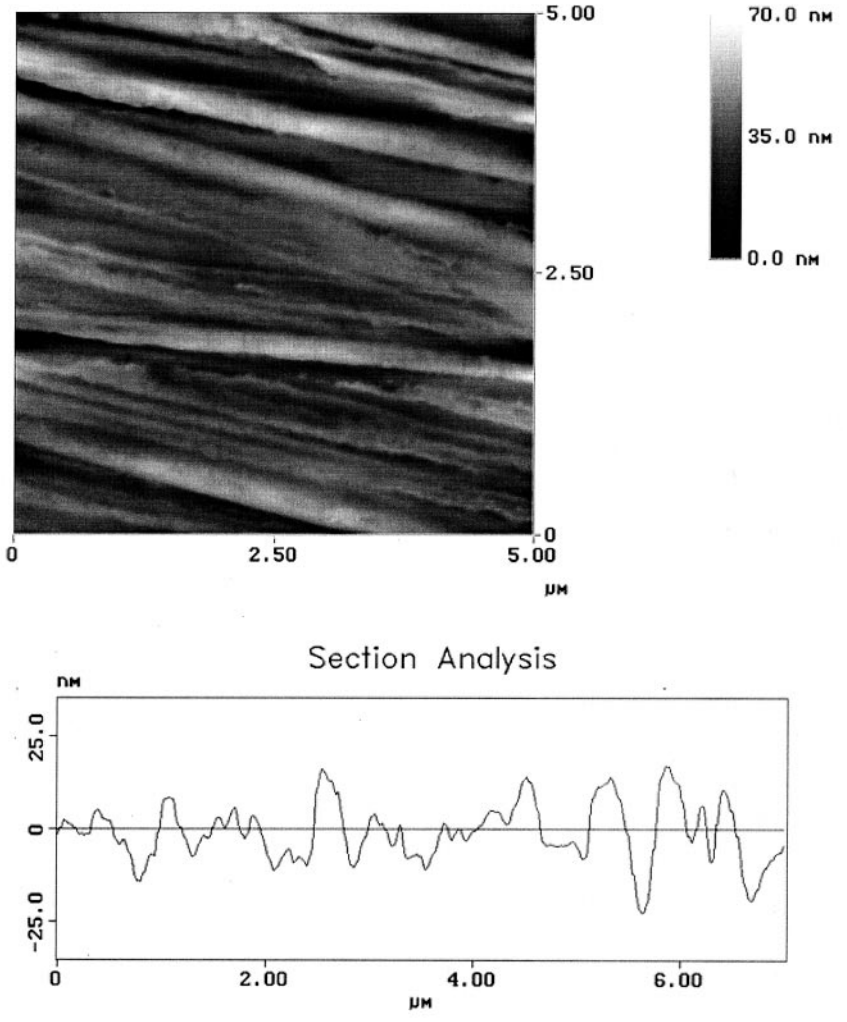

Fig. 2. AFM image (*top*) and cross-sectional analysis (*bottom*) of a standard polystyrene microtiter well surface.

The cross-sectional analysis (*bottom*) was carried out along a line extending from the *top right corner* to the *bottom left corner* of the AFM image (*top*).

the labeled antibody; 100 μL of HRP-IgG solution was added to each well. After a 60-min incubation at 37 °C, the excess HRP-IgG was removed, and each well was washed with 250 μL of Tween 20 (200 mg/L) in PBS and 250 μL of distilled water. A drop of solution A (tetramethyl benzidine; 50 μL) and a drop of solution B (H_2O_2; 50 μL) from a standard EIA kit were added to each well. The wells were incubated for 1 min at 37 °C. Finally, a drop of ending solution (50 μL) was added to each well to stop the reaction. The absorbance at 450 nm (A_{450}) of 150-μL aliquots from each well was measured by a CliniBIO 128. As a comparison, the EIA detection procedure for antibodies adsorbed on polystyrene microtiter well surfaces was the same as above.

Results

Spin-coated polystyrene films were first prepared as described previously *(27, 28)*. To determine the optimum film flatness, surfaces prepared from five solution concentrations (1.0, 2.5, 5.0, 10, and 20 g/L) were examined. The topography and continuity of each film were studied using AFM. Fig. 1 (top panel) shows a representative AFM image of a spin-coated polystyrene film at a concentration of 10 g/L; the corresponding roughness data along the cross-section are also indicated (Fig. 1, bottom panel). The mean roughness (R_a) of the whole image for areas of 25 μm^2 was 2 Å, and the method used to calculate R_a was the same as described in the literature *(29)*. This concentration (10 g/L) of polystyrene solution and the present procedure are appropriate for preparing ultraflat polystyrene films.

As a comparison, the AFM image of a standard blank polystyrene microtiter well surface is shown in Fig. 2 (top panel), and the corresponding roughness data along the cross-section are also indicated (Fig. 2, bottom panel). The most prominent types of structures observed were the long parallel ridges. The typical height from peak to valley was 20–30 nm with a periodicity between 50 and 250 nm. The R_a of the whole image for areas of 25 μm^2 was 78 Å, which showed that the surface of a standard microtiter well is not smooth.

The topographic images of anti-HBs antibodies physically adsorbed on an ultraflat polystyrene surface are shown in Fig. 3. Monoclonal anti-HBs antibodies formed

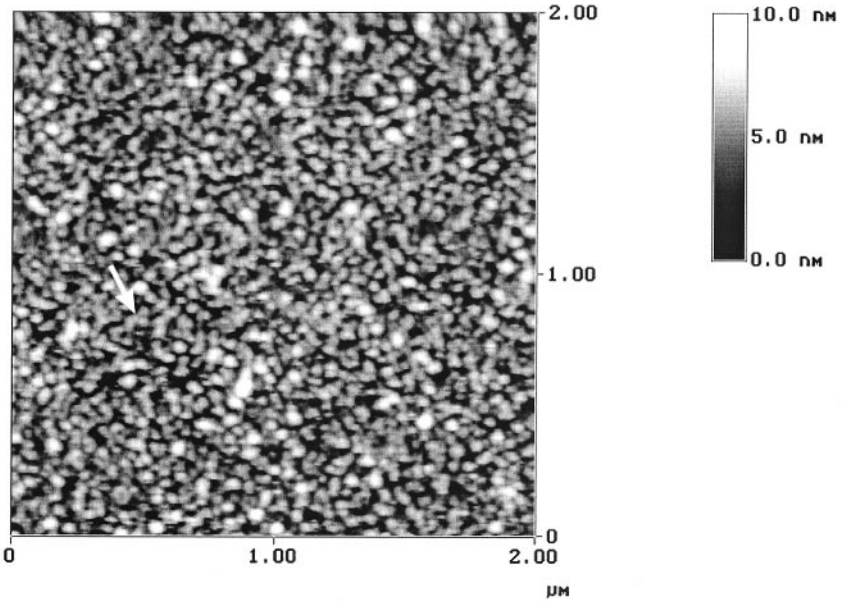

Fig. 3. AFM image of anti-HBs physically adsorbed on an ultraflat polystyrene surface.

The image consists of isolated molecules and packed molecular arrays and shows many examples of the characteristic Y shape (*arrow*).

a densely packed two-dimensional antibody film under our adsorption conditions. The sample consisted of isolated molecules and packed molecular arrays. The images of anti-HBs molecules also showed many examples of the characteristic Y shape (Fig. 3, arrow).

The topographic image obtained from anti-HBs antibodies adsorbed on a standard microtiter well surface is shown Fig. 4. Anti-HBs antibodies appear to adsorb to the polystyrene surface in clusters, and although they cover a major part of the well surface, there are distinct regions where no antibody adsorption has occurred (Fig. 4, arrow).

The AFM topography in Fig. 5 is typical of an adsorbed anti-HBs sample on the ultraflat surface after incubation with yeast HBsAg solution at a concentration of 10 μg/L. HBsAg particles captured by adsorbed anti-HBs antibodies are also indicated (Fig. 5, arrow). The average diameter of the HBsAg particles captured by the anti-HBs based on visual inspection was ~30 nm.

In Fig. 6, the dose curve of an immunoassay on ultraflat polystyrene surfaces for areas of 16 mm^2 is compared with the immunoassay on standard microtiter wells using the same antibody and antigen for the same areas. According to the dose curve of the immunoassay on ultraflat polystyrene surfaces (0.1–2000 μg/L), the amount of HBsAg bound to the immobilized anti-HBs antibodies increased proportionally with increasing concentrations of added HBsAg from 1.0 μg/L to 500 μg/L, and then

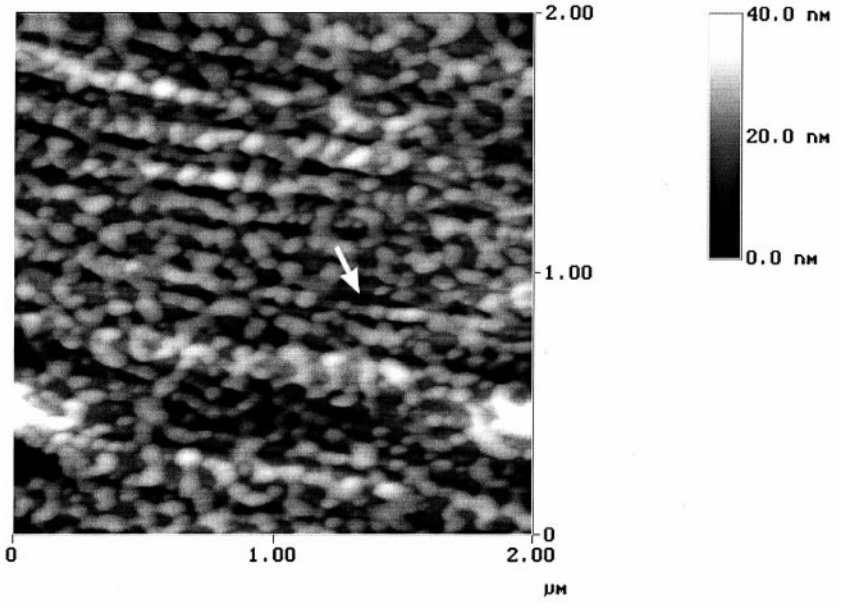

Fig. 4. Topographic image obtained from anti-HBs antibodies adsorbed on a standard microtiter well surface.

Anti-HBs antibodies cover a major part of the well surface, but there are distinct regions where no antibody adsorption has occurred (*arrow*).

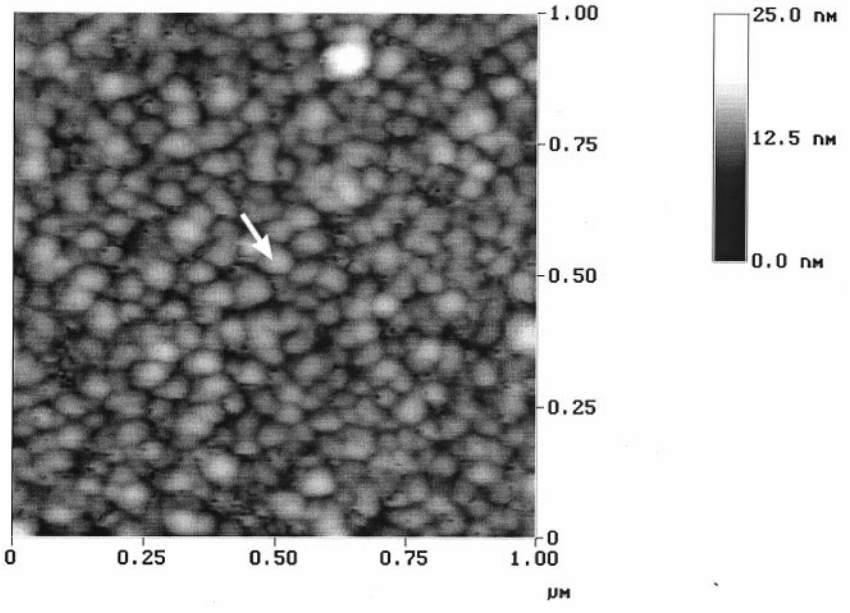

Fig. 5. AFM image displaying HBsAg particles (*arrow*) captured by adsorbed anti-HBs antibodies at a HBsAg concentration of 10 μg/L.

The average diameter of the HBsAg particles captured by the anti-HBs antibodies based on visual inspection is ~30 nm.

reached a plateau. In the dose curve of the immunoassay on standard microtiter wells (0.1–2000 μg/L; Fig. 6), two stages of binding were seen. In the first stage, the amount of HBsAg bound with coated anti-HBs initially increased with increasing concentrations of added HBsAg (1–100 μg/L), and then reached a plateau (100–500 μg/L). In the second stage, a hook effect was observed when a high concentration of HBsAg was present (500–2000 μg/L). Interestingly, the absorbance values at 450 nm were higher in the immunoassay on ultraflat polystyrene surfaces. The difference between immunoassays on two substrates with different topographies seems to occur at higher concentrations of HBsAg.

Discussion

To achieve optimal thickness and flatness of polystyrene films, we prepared a series of polystyrene films at various polystyrene concentrations. Considerable care needs to be taken in the production of polystyrene-coated silicon wafer surfaces because films are required to be of sufficient thickness to coat the entire substrate, leaving no silicon wafers exposed, but thin enough to ensure the uniformity of the polystyrene film. Studies using the spin-coating conditions described above indicated that ultraflat polystyrene surfaces are easy to obtain at rotation speed of 2000 rpm at a polystyrene concentration of ~10 g/L. The ridges observed on standard blank microtiter

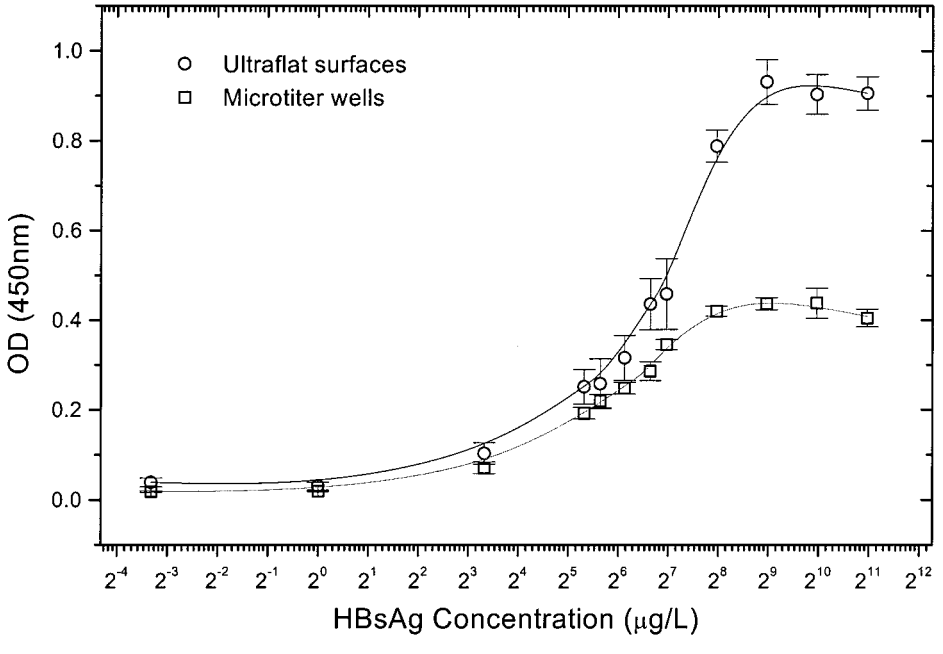

Fig. 6. Comparison of the performance of EIA on ultraflat polystyrene surfaces for areas of 16 mm^2 and EIA on standard microtiter wells using the same antibody and antigen for the same areas.

Bars, SD. *OD*, absorbance.

well surfaces result from the molding process used to fabricate the microtiter wells.

From crystallographic data it is known that an IgG antibody (M_r 146 000) is arranged in three discrete domains: two Fab fragments and one Fc *(30–32)*. It is also known that the hinge region between the two Fab domains is extremely flexible, and as a consequence, it is usually difficult to predict the exact conformation, and hence size, of an IgG antibody adsorbed onto a surface. An estimate of the dimensions that would be observed by AFM can be made from x-ray crystallographic analysis of isolated Fab fragments *(33)* and transmission electron microscopy images *(34, 35)*, giving an expected upper limit to the molecular dimensions on the surface of ~16–19 nm. AFM has become an important technique in biology and chemistry because of its unique ability to image and characterize the structure and/or function of a biological specimen at the solid-liquid interface. We have used AFM to directly monitor the adsorption of anti-HBs antibodies on the ultraflat polystyrene surface. AFM results provided a detailed understanding of the nature of the interactions between antibodies and surface. Isolated molecules and packed molecular arrays were observed in our experiments, and the images of the anti-HBs antibodies also showed many examples of the characteristic Y shape (Fig. 3, arrow), although the adsorption of antibodies on polystyrene can lead to the loss of protein structure and function. Because of the intrinsic conformational flexibility of IgG and hydrophobic interactions between antibodies and surfaces, we do not expect all molecules adsorbed from solution to exhibit the Y shape. In addition, the image (Fig. 3) clearly displayed a densely packed two-dimensional protein film covering an ultraflat polystyrene surface. The distribution of anti-HBs antibodies adsorbed on a standard microtiter well surface (Fig. 4) is different from that of antibodies immobilized on the above substrate, and only sparse anti-HBs molecules can be found on a microtiter well surface. From the above comparison, we know that the topographies of surfaces are as important for antibody adsorption as other surface characteristics such as hydrophobicity, surface charge, and co-adsorption of or exchange with surfactants, copolymers, or proteins. Perturbation by the surface is known to affect the protein behavior through both surface chemistry and topography *(25, 26, 36–39)*, but the details remain unknown.

A yeast recombinant, which was constructed by Miyanohara et al. *(40)*, can produce a HBsAg with properties similar to human HBsAg. Yeast HBsAg is also immunologically similar to its human counterpart and is now widely used in vaccines against hepatitis B. The AFM topography in Fig. 5 is a typical image of anti-HBs antibodies adsorbed on an ultraflat polystyrene surface after incubation with yeast HBsAg, and dense HBsAg particles captured by capture anti-HBs antibodies are also shown (Fig. 5, arrow). The average diameter of the HBsAg particles based on visual inspection is ~30 nm. The ultrastructure of yeast HBsAg particles produced by recombinant yeast cells was examined using high-resolution negative, and ice embedding, electron microscopy *(41)*. With negative staining, the HBsAg particles were spherical to slightly ovoid with a mean diameter of 27.5 nm. The size of the HBsAg particles bound by the capture antibodies on the polystyrene surface measured by AFM was the same as the individual particle size.

Compared with the immunoassay on standard wells (Fig. 6), the working range of the immunoassay on ultraflat surfaces is wider. The difference between the two immunoassays seems to occur at higher HBsAg concentrations, which also indicates that increasing the binding capacity of the capture surface does not improve detection at lower HBsAg concentrations.

Adsorption of antibodies on surfaces of standard wells is usually too inefficient to give good yields and does not always proceed in a dose-dependent manner. Quantitative data have shown that the method most commonly used to immobilize antibodies in microtiter immunoassays functionally inactivates most of the antibodies *(7, 8)*. Immobilization of antibodies onto ultraflat polystyrene surfaces can improve the performance of immunoassays. We have shown that it is possible to increase the number of active antibody-binding sites by controlling surface roughness.

The demand for increased sensitivity and dose responses in immunoassays has led to the development of new solid-phase materials and new immobilization methods on polystyrene. On the other hand, more work is needed to define the exact nature of the antibodies or antigens that cover the polystyrene surface after their immobilization and the eventual antigen-antibody interactions. It seems appropriate to conclude that in the area of immunoassays now, "the technological cart is ahead of the scientific horse". This study confirmed the potential for preparing dense, homogeneous, highly specific, and highly stable antibody surfaces by immobilizing antibodies on a polystyrene surface with controlled roughness. It is expected that such biofunctional surfaces could be of interest in the development of new solid-phase immunoassay and biosensor techniques.

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