Supersensitive Time-resolved Immunofluorometric Assay of Free Prostate-specific Antigen with Nanoparticle Label Technology

Tero Soukka,* Janika Paukkunen, Harri Härmä, Stefan Lönnberg, Hanne Lindroos, and Timo Lövgren

Background: The extreme specific activity of the long-lifetime fluorescent europium(III) chelate nanoparticles and the enhanced monovalent binding affinity of multivalent nanoparticle-antibody bioconjugates are attractive for noncompetitive immunoassay.

Methods: We used a noncompetitive, two-step immunoassay design to measure free prostate-specific antigen (PSA). Europium(III) chelate nanoparticles (107 nm in diameter) were coated with a monoclonal anti-PSA antibody (intrinsic affinity, $6 \times 10^9$ L/mol). The nanoparticle-antibody bioconjugates had an average of 214 active binding sites per particle and a monovalent binding affinity of $7 \times 10^{10}$ L/mol. The assay was performed in a low-fluorescence microtitration well passively coated with another monoclonal anti-PSA antibody (affinity, $2 \times 10^{10}$ L/mol), and the europium(III) fluorescence was measured directly from the bottom of the well by a standard time-resolved microtitration plate fluorometer.

Results: The detection limit (mean ± 2 SD) was 0.040 ng/L ($7.3 \times 10^5$ molecules/mL), and the dynamic detection range covered four orders of magnitude in a 3-h total assay time. The imprecision (CV) over the whole assay range was 2–10%. The detection limit of the assay was limited by the fractional nonspecific binding of the bioconjugate to the solid phase (0.05%), which was higher than the nonspecific binding of the original antibody (<0.01%).

Conclusions: The sensitivity of the new assay is equal to that of the ambient-analyte, microspot immunoassay and will be improved by use of optimized, high binding-site density nanoparticle-antibody bioconjugates with reduced nonspecific binding and improved monovalent binding affinity.

Ultrasensitive immunoassay methods are developed and used in clinical diagnostics to measure extremely low concentrations of specific compounds in highly complex samples. Although the sensitivity, reliability, rapidity, simplicity, and cost of these methods have steadily improved, further improvements are still needed and possible (1–3). The current trend toward miniaturized, multi-analyte methods has introduced its own challenges and requirements for immunoassay technology (4, 5). The interest in new label technologies has especially increased because none of the commonly used direct or enzyme-amplified radioactive, colorimetric, luminescent, or fluorescent reporters fulfills all of the requirements for an ideal label, including specific activity, size, nontoxicity, cost, stability, localization, and detection. Directly detectable labels such as fluorophores suffer from limited sensitivity, and enzyme-amplified or dissociation-enhanced methods lose spatial information.

Recently, new detection methods based on high specific-activity particulate labels, such as quantum dots (6), luminescent inorganic crystals (7), up-converting phosphors (8), fluorescent nanoparticles (9–11), and plasmon resonant particles (12), have been introduced to respond to future demands for clinical diagnostics and biological, genomic, and pharmaceutical research. These submicrometer-sized labels are coupled to specific binding reagents such as nucleic acid probes, receptors, lectins, enzymes, and antibodies to detect specific molecules with sensitivities equal to or better than the best conventional labels available. In spite of the large molecular size and obvious steric problems, these particular labels have also been used successfully in solid-phase immunoassays.
(9, 12–16). It has been recognized, however, that the production, colloidal stability, and nonspecific binding of particle-protein bioconjugates may still require further improvements (7, 12, 17).

Time-resolved fluorometry and lanthanide labels were introduced for immunoassays 20 years ago (18, 19). Since then, the dissociation-enhanced lanthanide fluorimunoassay (DELFIA®) technology has been known as one of the most sensitive and reliable immunoassay platforms (20). The research on intrinsically fluorescent, inert, and stable lanthanide chelate and cryptate labels has led to the development of novel homogeneous (21, 22) and heterogeneous assays (23, 24) that are expected to be introduced into routine clinical diagnostics. Moreover, an advanced dissociation-enhanced technology, based on lanthanide cofluorescence, which amplifies the long-lifetime fluorescence of europium(III), terbium(III), samarium(III), and dysprosium(III), has been published (25). A unique feature of lanthanide chelate fluorescence, the absence of self-quenching effects from multiple labeling (26, 27), makes them ideal and suitable for high-density cluster labels such as dyed latex nanoparticles (28). The highly fluorescent chelates used in the DELFIA technology (29, 30) can also be used in fluorescent lanthanide(III) chelate nanoparticles (28) because the hydrophobic environment inside the latex protects the fluorescent chelates from environmental effects, such as solvent quenching, and stabilizes the kinetically weak complexes. The adaptation of appropriate chelates for all four lanthanides would enable a nanoparticle-based, quadruple-labeling technology with an extremely low detection limit and a time-resolved technique to demonstrate detection of individual, biospecifically bound nanoparticle-streptavidin bioconjugates on microtitration well surfaces in an immunoassay with a time-resolved fluorescence microimager and a standard time-resolved microtiter plate fluorometer with a sub-attomole detection limit (10, 13). This improved lower limit of detection in a standard microtitration platform was made possible by the extremely high specific activity of the nanoparticle label compared with the miniaturized assay setup (24) using intrinsically fluorescent lanthanide chelates and a time-resolved microfluorometer. Recently, we demonstrated the enhanced monovalent binding affinity of an multivalent nanoparticle-antibody bioconjugate (17). The enhanced affinity in combination with the high specific activity of the nanoparticle label allows next-generation immunoassays to be performed with superior sensitivity. The purpose of this study was to implement a supersensitive nanoparticle-based noncompetitive immunoassay of free prostate-specific antigen (PSA) in conventional microtitration wells and to demonstrate the methodologic potential still available for immunoassay research.

Materials and Methods

REAGENTS

Highly fluorescent europium(III) chelate-dyed, monodisperse, carboxylate-modified Fluoro-Max°F poly styrene nanoparticles (10 g/L, 107 nm in diameter, 1.8 carboxylic acids/nm²) were purchased from Seradyn. The fluorescence properties and standardization of the nanoparticles have been fully described in previous publications (10, 17). The europium(III) chelate of N²-(4-isothiocyanatobenzyl)diethylenetriamine-N¹,³,⁵,N⁷-tetraakis(acetic acid) [Eu(III)-N1-ITC], DELFIA enhancement solution, DELFIA enhancer, free-PSA calibrators, monoclonal antibody (Mab) H117-coated anti-PSA microtitration wells, streptavidin-coated wells, assay buffer [50 mmol/L Tris-HCl (pH 7.8), 9 g/L NaCl, 0.5 g/L NaN₃, 5 g/L bovine serum albumin, 0.1 g/L Tween 40, 0.5 g/L bovine γ-globulin, 20 μmol/L diethylenetriamine pentaacetic acid, 20 mg/L Cherry Red] and wash solution [5 mmol/L Tris-HCl (pH 7.8), 9 g/L NaCl, 0.05 g/L Tween 20] were from Perkin-Elmer Life Sciences (Wallac Oy). Additional free-PSA calibrators were prepared by dilution with the following buffer, used as the zero calibrator [50 mmol/L Tris-HCl (pH 7.8), 75 g/L bovine serum albumin, 9 g/L NaCl, and 0.5 g/L NaN₃]. Free-PSA-specific Mab 5A10

¹ Nonstandard abbreviations: PSA, prostate-specific antigen; Eu(III)-N1-ITC, europium(III) chelate of N²-(4-isothiocyanatobenzyl)diethylenetriamine-N¹,³,⁵,N⁷-tetraakis(acetic acid); and Mab, monoclonal antibody.
and total-PSA-recognizing Mabs H50 and H117 were produced in our laboratory. Eu(III)-N1-ITC-labeled Mab 5A10 was prepared as described previously (34). Noncoated, low-fluorescence MaxiSorp microtitration wells were purchased from Nunc. Bovine milk casein and cold-water fish skin gelatin were from Sigma Chemical, bovine serum albumin was obtained from Intergen, and Tween 85 was from E. Merck. The bacteriostatic agent Germall II was from Sutton Laboratories. Skim milk powder (Valio) was dissolved at a concentration of 50 g/L in 25 mmol/L Tris-HCl (pH 8.0), and noncovalent aggregates were removed before use by centrifugation at 2500 g for 5 min.

Nanoparticle-antibody bioconjugates

Mab 5A10 was covalently coupled to activated nanoparticles, and the bioconjugates were purified by ultrafiltration as described previously (17). The monodispersity and colloidal stability of the bioconjugates were evaluated by measuring the colloidal particle concentration after selective centrifugation steps and a prolonged storage at 4 °C. To investigate their effect on the nonspecific binding, the following proteins and compounds were attached to the nanoparticles, one at a time: Mab H50, bovine serum albumin, casein, milk powder, amino-caproic acid, glycine, and ethanolamine. Blocking with bovine serum albumin was performed only for antibodies. One lot of nanoparticle-Mab 5A10 bioconjugates was also purified by size-exclusion chromatography using Sephrose 6B (Amersham Pharmacia Biotech) matrix with 2 mmol/L Tris-HCl buffer (pH 8.0) containing 50 mmol/L salicylic acid and 0.1 g/L Tween 40 as the elution buffer. The number of active binding sites of covalently coupled Mab 5A10 or Mab H50 on a single nanoparticle-antibody bioconjugate was determined using terbium(III)-labeled PSA, measuring the ratio between terbium(III) fluorescence from particle-bound labeled PSA and europium(III) fluorescence from nanoparticles (17). Determination of the kinetic rates and affinity constants for the bioconjugates has been described in the same context.

Coating of microtitration wells

The solid-phase antibody Mab H117 was immobilized on low-fluorescence MaxiSorp microtitration wells by physical adsorption. The wells were coated for 2 h at 35 °C with 0.4 μg of antibody in 50 μL of 200 mmol/L phosphate buffer, pH 7.8. The coated wells were washed twice with wash solution in a Delfia plate washer (Perkin-Elmer Life Sciences) and saturated for 1 h at 23 °C with 100 μL of 50 mmol/L Tris-HCl buffer (pH 7.2) containing 1 g/L bovine serum albumin, 1 g/L Germall II, and 30 g/L trehalose. The microtitration wells were slowly shaken during the saturation on a Delfia plate shaker (Perkin-Elmer Life Sciences); after the saturation, the wells were aspirated and dried in a laminar hood for 1 h. The experimental wells were stored at 4 °C in a sealed package with desiccant.

Nonspecific binding

Both the experimental Mab H117-coated microtitration wells and commercial Mab H117 anti-PSA- and streptavidin-coated wells were tested for fractional nonspecific binding of different nanoparticle bioconjugates or unmodified blank nanoparticles. The assay buffer (100 μL/well) was first preincubated in the wells with shaking in a Thermomix shaker (Labsystems) at 900 rpm and 23 °C for 30 min to remove loosely bound protein and to ensure adequate blocking; the wells were then washed once with the wash solution. Subsequently, different nanoparticle bioconjugates (1 × 10⁹ particles/well) were added in 40 μL of the assay buffer. The wells were incubated on a shaker for 2 h at 1150 rpm and 23 °C and washed four times before immediate measurement of the surface-bound nanoparticle fraction. Time-resolved europium(III) fluorescence from the nonspecifically bound nanoparticle-antibody bioconjugates was detected at 615 nm from the bottom of the well by a Victor™ 1420 fluorometer (Perkin-Elmer Life Sciences) with a reduced focal distance.

Two-step PSA immunoassay

The experimental Mab H117-coated microtitration wells were used in two-step noncompetitive immunoassays of free PSA. The wells were preincubated as for the nonspecific binding experiments with the exception that modified assay buffer containing, additionally, 0.005 g/L milk powder and 0.05 g/L Tween 85 was used. In brief, 30 μL of calibrator or 5 μL of calibrator plus 25 μL of the modified assay buffer were added to each well, and the microtitration wells were incubated in the Thermomix shaker at 900 rpm and 23 °C for 45 min. The wells were then washed once with the wash solution. In the second step, nanoparticle-antibody bioconjugates (1 × 10⁹ particles/well) or labeled antibodies (75 ng/well) were added in 40 μL of the modified assay buffer. The wells were then incubated at 23 °C and 1150 rpm for 2 h, and then washed four times before measurement of the surface-bound fluorescence. A detailed description of the assay principle and the effect of different factors on assay performance are shown in Fig. 1. Time-resolved europium(III) fluorescence from the nanoparticle-antibody bioconjugates was detected as described above for the nonspecific binding experiments, using a damped emission aperture. The signal from the conventionally labeled antibody was measured at 615 nm with a standard europium(III) protocol after additional incubation with the Delfia enhancement solution (200 μL/well). The lower limit of detection of the assay was calculated from the signal of the zero calibrator plus 2 SD.

Fluorescence imaging of microtitration wells

Five separate immunoassays with two different nanoparticle-antibody bioconjugates were performed as described above for the two-step PSA immunoassay: (a) one immunoassay using commercial wells; (b) one performed without any modifications; (c) one not shaken during the
binding; 7). A small fraction of the labeled antibody is also bound nonspecifically simultaneously to more than one bound analyte molecule (i.e., multivalent surface or they have time to dissociate and rebind, the labeled antibody can bind hindrances. If analyte molecules are initially bound very near each other on the density on the measurement area.

Each microtitration well is passively coated with capture antibody in a 50-μL volume, so that the reactive binding area on the well (1) is larger than the surface area covered in the first incubation of a 30-μL sample volume (2a) with shaking. The second incubation, with detection antibody, is carried out in a 40-μL volume (2b) to ensure coverage of all bound analyte. The surface readout of bound labeled antibody (i.e., bioconjugate) is made from the bottom of the well, sampling the number of bound labeled detection antibodies (4) on the measurement area (3), but not detecting the possibly existing nonlabeled (i.e., nonconjugated) detection antibodies (5). Not all of the analyte molecules bound to the capture antibody (6) are detected, because of either equilibrium state or steric hindrances. If analyte molecules are initially bound very near each other on the surface or they have time to dissociate and rebind, the labeled antibody can bind simultaneously to more than one bound analyte molecule (i.e., multivalent binding; 7). A small fraction of the labeled antibody is also bound nonspecifically to the surface (8), and aggregated labeled antibodies (9) reduce the effective concentration and increase variation. The quality of the solid-phase coating (e.g., inactive antibodies (10) and occurrence of void, blocked surface (11)) contributes to the representativeness of the bound analyte and the labeled antibody density on the measurement area.

Fig. 1. Schematic drawing of the solid-phase, two-step noncompetitive immunoassay with surface readout detection.

Each microtitration well is passively coated with capture antibody in a 50-μL volume, so that the reactive binding area on the well (1) is larger than the surface area covered in the first incubation of a 30-μL sample volume (2a) with shaking. The second incubation, with detection antibody, is carried out in a 40-μL volume (2b) to ensure coverage of all bound analyte. The surface readout of bound labeled antibody (i.e., bioconjugate) is made from the bottom of the well, sampling the number of bound labeled detection antibodies (4) on the measurement area (3), but not detecting the possibly existing nonlabeled (i.e., nonconjugated) detection antibodies (5). Not all of the analyte molecules bound to the capture antibody (6) are detected, because of either equilibrium state or steric hindrances. If analyte molecules are initially bound very near each other on the surface or they have time to dissociate and rebind, the labeled antibody can bind simultaneously to more than one bound analyte molecule (i.e., multivalent binding; 7). A small fraction of the labeled antibody is also bound nonspecifically to the surface (8), and aggregated labeled antibodies (9) reduce the effective concentration and increase variation. The quality of the solid-phase coating (e.g., inactive antibodies (10) and occurrence of void, blocked surface (11)) contributes to the representativeness of the bound analyte and the labeled antibody density on the measurement area.

second incubation; (d) one not shaken during analyte or bioconjugate incubation; and (e) one that included an intense wash step instead of aspiration in the coating of microtitration wells. After the europium(III) fluorescence was measured, time-resolved fluorescence microscope images of the bottoms of the wells were shot at ×10 magnification using a Signifier time-resolved fluorescence microimager (Perkin-Elmer Life Sciences) as described previously (10). We evaluated the distribution of bound nanoparticle-antibody bioconjugates on the bottoms of the wells by combining several overlapping images taken from each well.

Results

PREPARATION OF IMMUNOASSAY COMPONENTS

Seven different nanoparticle-antibody Mab 5A10 bioconjugates with 8–130 binding sites for PSA on a single nanoparticle had been characterized in detail previously (17). The properties of the bioconjugates are summarized in Table 1. New bioconjugates had a higher active binding site density (>200 binding sites) and adequate colloidal stability. The fractional nonspecific binding was measured with a blank sample and as described above for the two-step PSA immunoassay. The typical storage concentration of all nanoparticle bioconjugates in suspension was 5×10¹² particles/mL (~3 g/L). The functionality of the experimental Mab H117 microtitration wells was tested using an immunoassay with a conventionally labeled antibody and commercial anti-PSA wells as reference. Fractional nonspecific binding of the labeled antibody to the commercial well was twofold, but the background-subtracted fluorescence signals were fully identical.

TABLE 1. Properties of the labeled compounds.

<table>
<thead>
<tr>
<th>Labeled compound</th>
<th>Monovalent affinity,a</th>
<th>Association rate,a</th>
<th>Nonspecific binding,b,c %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eu(III)-N1-ITC-labeled Mab 5A10</td>
<td>6.6 ± 0.3</td>
<td>12.6 ± 0.7</td>
<td>0.009 ± 0.002</td>
</tr>
<tr>
<td>Nanoparticle-antibody bioconjugates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.6 ± 0.5</td>
<td>2.8 ± 0.1</td>
<td>0.005 ± 0.0002</td>
</tr>
<tr>
<td>12</td>
<td>4.1 ± 0.9</td>
<td>4.1 ± 0.1</td>
<td>0.007 ± 0.0001</td>
</tr>
<tr>
<td>19</td>
<td>9.3 ± 0.7</td>
<td>6.1 ± 0.3</td>
<td>0.007 ± 0.0003</td>
</tr>
<tr>
<td>30</td>
<td>13.3 ± 1.7</td>
<td>8.3 ± 0.3</td>
<td>0.008 ± 0.0008</td>
</tr>
<tr>
<td>46</td>
<td>22.6 ± 3.0</td>
<td>11.8 ± 0.6</td>
<td>0.009 ± 0.0007</td>
</tr>
<tr>
<td>76</td>
<td>35.1 ± 5.7</td>
<td>15.3 ± 0.9</td>
<td>0.009 ± 0.0011</td>
</tr>
<tr>
<td>130</td>
<td>53.8 ± 7.9</td>
<td>24.8 ± 1.6</td>
<td>0.014 ± 0.0013</td>
</tr>
<tr>
<td>214d</td>
<td>74.6 ± 6.3</td>
<td>36.5 ± 3.2</td>
<td>0.042 ± 0.0021</td>
</tr>
</tbody>
</table>

a Mean ± SD.
b Fraction of total labeled compound nonspecifically bound to the experimental Mab H117-labeled microtitration wells at 75 ng/40 μL for Mab 5A10 and 1×10⁸ particles/40 μL for the nanoparticle-antibody bioconjugates.
c Shown according to number of active PSA binding sites/particle.
d Purified using size-exclusion chromatography (Sepharose 6B matrix).

Purification and colloidal stability of bioconjugates

The non-antibody (i.e., bovine serum albumin and ethanolaime) bioconjugates showed better monodispersibility than the antibody bioconjugates when the yields were compared after centrifugation, the last step in the purification procedure. The Mab H50 bioconjugate evidently had better colloidal stability than the corresponding Mab 5A10 bioconjugate, although the colloidal stability of nanoparticle-Mab 5A10 bioconjugates improved with lower antibody loading (data not shown). The performance of the nanoparticle-Mab 5A10 bioconjugates from the same coupling reaction, purified by either size-exclusion chromatography or ultrafiltration, was further compared in an immunoassay. The bioconjugates purified by size-exclusion chromatography gave higher specific and lower fractional nonspecific binding and thus improved detection limits (see Fig. 3).

Nonspecific binding

The fractional nonspecific binding of various nanoparticle bioconjugates to three different, protein-coated microtitra-
Table 2. Nonspecific binding of the bioconjugates.

<table>
<thead>
<tr>
<th>Nanoparticle bioconjugate</th>
<th>NSB&lt;sup&gt;b&lt;/sup&gt; to the coated microtitration wells, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank; carboxylic acid</td>
<td>0.0003</td>
</tr>
<tr>
<td>Aminocaproic acid</td>
<td>0.0051</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.0019</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>0.0005</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.0018</td>
</tr>
<tr>
<td>Bovine milk casein</td>
<td>0.0022</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>0.0003</td>
</tr>
<tr>
<td>Mab H50 (220)</td>
<td>0.12</td>
</tr>
<tr>
<td>Mab 5A10 (14)</td>
<td>0.031</td>
</tr>
<tr>
<td>Mab 5A10 (43)</td>
<td>0.16</td>
</tr>
<tr>
<td>Mab 5A10 (208)</td>
<td>1.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values in parentheses indicate number of active binding sites.
<sup>b</sup> NSB, fractional nonspecific binding; ND, not determined.

Two-step PSA immunoassay

The calibration curves for seven different bioconjugates with a variable number of binding sites are shown in Fig. 2. Both specific and nonspecific binding increased with a higher number of binding sites. When the number of binding sites increased from 8 to 130, the detection limit of the assay was improved because the number of specifically bound nanoparticles increased more than the nonspecific binding (five- vs threefold). The bioconjugates with a high number of binding sites, i.e., a higher monoclonal binding affinity, confirmed the potential for lower detection limits if the increase in nonspecific binding could be decreased. The specific binding was further increased when the bioconjugate with 214 active binding sites, purified with size-exclusion chromatography, was used to the two-step immunoassay (Fig. 3). The bioconjugate with 214 active binding sites had a higher association rate constant; thus, the binding reaction almost reached equilibrium (∼80% of equilibrium value) in the 2-h incubation. Unfortunately, the lower limit of detection of the immunoassay was no longer improved because the nonspecific binding was increased slightly more than was predicted from the inset of Fig. 2. The specific activity of the nanoparticle label was definitely not a limiting factor of assay sensitivity. The improved monodispersibility of the bioconjugate with 214 binding sites produced more reactive bioconjugate but increased the nonspecific binding. Apparently the improved sensitivity available with high binding-site density nanoparticle-antibody bioconjugates is partly a consequence of different kinetic behaviors of specific and nonspecific binding. The saturation point of the specific binding is limited by the concentration of the analyte, whereas nonspecific binding can increase slowly over time without obvious saturation.

In the two-step nanoparticle-based immunoassay (Fig. 3), a 70-fold improvement in the detection limit was achieved compared with the corresponding immunoassay using a highly labeled antibody detected by the DELFIA...
technology, one of the most sensitive conventional label technologies available (1). The detection limits achieved, 0.040 ng/L for free PSA using 30-μL sample (36 zmol) and 0.39 ng/L using 5-μL sample (59 zmol) are 25- and 2.5-fold lower, respectively, than the reported detection limits of the most sensitive immunoassays for free PSA (analytical limits of detection, 1 ng/L for 50-μL sample and 0.3 ng/L for 100-μL sample) (35, 36). In the nanoparticle-based assay, excellent reproducibility was achieved: the relative imprecision was <10% within the whole assay range. The dynamic range of the nanoparticle-based assay was four orders of magnitude, and the nonlinearity at concentrations >1000 ng/L was caused by the limiting concentration of the bioconjugate and the lack of a linear range for measurement of high signals.

**FLUORESCENCE IMAGING OF MICROTI TitRATION WELLS**

The surface readout detection of fluorescence from the bottom of the well increases variation in an immunoassay compared with dissociation-enhanced or enzyme-based methods. Sampling the surface density is susceptible to local variations in binding and thus is more dependent on uniform coating than methods that measure the total number of bound molecules (see Fig. 1). The effects of different modifications in solid-phase antibody coating and in shaking on assay performance are shown in Table 3. Typical examples of the images of the bottoms of the wells are shown in Fig. 4. The data suggest that the commercial anti-PSA wells may have a higher antibody density on the bottom because the specific signals were higher (the higher background also supports this). These wells were designed for dissociation-enhanced measurement, but they performed very well in the view of the variations in the surface measurement of fluorescence. Many of the commercial wells had a dark, i.e., empty area, at a position identical to the one shown in Fig. 4a, but

![Calibration curves for bioconjugate (214 active binding sites; □) and labeled antibody [8 europium(III) ions per antibody; □] based on two-step, noncompetitive free-PSA immunoassays using 5 μL (solid lines) and 30 μL (dashed lines) of sample. The arrows and concentrations indicate 2 SD of the blank sample and the lower limits of detection of the assay, respectively. The dashed and solid lines indicate 30 and 5 μL of sample, respectively, with the upper lines representing bioconjugate and the lower lines representing labeled antibody. The absolute signals cannot be directly compared between the bioconjugate and the labeled antibody because the nanoparticle-associated fluorescence is measured from the surface with a damped emission aperture. The relative imprecision (CV) over the whole assay range was 2–10% and 4–12% for the 30- and 5-μL samples, respectively. cts, counts.

**Table 3. Effect of solid-phase coating and shaking on assay performance.**

<table>
<thead>
<tr>
<th>Microtitration well</th>
<th>Incubation procedure</th>
<th>Backgrounda</th>
<th>Free-PSA calibrator,a 10 ng/L</th>
<th>Detection limit, ng/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Analyte</td>
<td>Bioconjugate</td>
<td>ctsb CV, %</td>
<td>ctsb CV, %</td>
</tr>
<tr>
<td>122 active PSA binding sites on the nanoparticle-antibody bioconjugate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Commerciala</td>
<td>Shaking</td>
<td>Shaking</td>
<td>14 942 14</td>
<td>100 066 3.5</td>
</tr>
<tr>
<td>Experimentalb</td>
<td>Shaking</td>
<td>Shaking</td>
<td>3989 8.1</td>
<td>75 133 8.7</td>
</tr>
<tr>
<td>Experimentalb</td>
<td>Shaking</td>
<td>No shaking</td>
<td>4644 7.6</td>
<td>88 893 6.4</td>
</tr>
<tr>
<td>Experimentalb</td>
<td>No shaking</td>
<td>No shaking</td>
<td>5125 1.4</td>
<td>139 931 8.2</td>
</tr>
<tr>
<td>Experimentalb</td>
<td>Shaking</td>
<td>Shaking</td>
<td>2430 17</td>
<td>27 922 11</td>
</tr>
<tr>
<td>49 active PSA binding sites on the nanoparticle-antibody bioconjugate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Commerciala</td>
<td>Shaking</td>
<td>Shaking</td>
<td>5378 5.8</td>
<td>65 818 2.6</td>
</tr>
<tr>
<td>Experimentalb</td>
<td>Shaking</td>
<td>Shaking</td>
<td>2805 4.5</td>
<td>59 034 7.4</td>
</tr>
<tr>
<td>Experimentalb</td>
<td>Shaking</td>
<td>No shaking</td>
<td>2925 8.0</td>
<td>65 395 6.2</td>
</tr>
<tr>
<td>Experimentalb</td>
<td>No shaking</td>
<td>No shaking</td>
<td>3143 2.2</td>
<td>97 895 5.2</td>
</tr>
<tr>
<td>Experimentalb</td>
<td>Shaking</td>
<td>Shaking</td>
<td>1795 6.3</td>
<td>26 650 17</td>
</tr>
</tbody>
</table>

a Mean and CV calculated from three replicates.
b cts, counts.
c Background-subtracted signal from 5 μL of 10 ng/L free-PSA calibrator.
d Commercial Mab H117 anti-PSA-coated microtitration wells.
e Mab H117 microtitration wells coated as described in Materials and Methods.
f Experimental microtitration wells prepared with an extensive postcoating wash step.
apparently it was outside the view of the surface detection. The typical excitation area for a Victor microtitration plate fluorometer is $\approx 1 \times 3 \text{ mm}^2$, but the damped emission aperture reduces the view to some extent.

The specific signals on the surfaces of the experimental wells were markedly increased when the wells were not shaken during the analyte incubation. The shaking extended the surface area in contact with the liquid and, hence, diluted the surface density of the bound analyte, decreasing the signal in the surface measurement. The distribution of the antibody on the experimental wells (Figs. 4, b–d) was more uniform, although densely patterned, irregular dark areas were found on all of the wells. The pattern may be caused by differences in or impurities on the surfaces of the wells or by the drying process after coating. Apparently, these areas do not contain reactive antibodies, but are blocked from further adsorption. Fig. 4e indicates that washing can also produce local variations in the antibody surface density.

**Discussion**

Our previous study of the kinetic properties of multivalent nanoparticle-antibody bioconjugates (17) revealed that the association rate constant and the monovalent binding affinity to the analyte were linearly related to the number of active binding sites on the surface of the nanoparticle. The association rate constant of the bioconjugate was shown to exceed the rate constant of the original, conventionally labeled antibody, whereas the dissociation rate constant of the monovalent complex was only weakly dependent on the antibody density. The dissociation rate constants were smaller than the rate constant of the original antibody. Although the fractional nonspecific binding of the bioconjugates also increased with antibody loading, it was obvious that utilization of the enhanced monovalent affinity could be a key issue to improved assay sensitivity. As a model, we constructed a free-PSA immunoassay on a standard microtitration well platform to demonstrate the potential design of a supersensitive immunoassay based on nanoparticle label technology.

Purification of nanoparticle-antibody bioconjugates from free, unconjugated antibody was essential to avoid a drop in a specific assay response (see legend for Fig. 1 for details). Production of monodisperse bioconjugates that tolerate long-term storage at 4°C without settling or aggregating was an equally obvious goal. The ultrafiltration in the purification procedure was replaced by size-exclusion chromatography for one lot of Mab 5A10 bioconjugates to improve purification and particularly to avoid induced aggregation. Chromatographic separation provided higher purification and a better yield of monodisperse bioconjugates than the ultrafiltration procedure. The accumulation of bioconjugates on the membrane during ultrafiltration caused aggregation as well as partial denaturation of antibodies.

The colloidal stability of the nanoparticle-antibody bioconjugates is required to maintain the effective concentration of separate particles. In the nanoparticle-based, solid-phase immunoassay, colloidal more stable bioconjugates than those used in agglutination assays can be applied to further reduce the nonspecific binding because no specific particle-particle interactions are required (37). The repulsive, negative charge of bioconjugates was maintained with passive adsorption of blocking proteins to the nanoparticle surface. The covalent coupling consumed most of the carboxylate groups on the nanoparticle, and not all of them were regenerated even when aminocaproic acid and glycine were coupled to the surface. A common approach to stabilizing nanoparticles, which uses less activation reagent in the coupling reaction and thus leaves more negative charge on the nanoparticle, would, however, sacrifice a part of the binding site potential and thus is advantageous only up to a certain point (38). The colloidal stability of the high binding-site density nanoparticle-antibody bioconjugates could be op-
tinally improved, e.g., by use of nanoparticles with a high surface density of carboxyl groups or high-quality, recombinant antibody fragments with an increased negative charge.

The antibody-antibody interaction was in accordance with the fact that the nonspecific binding of bioconjugates increased with the higher number of active binding sites (17). Skim milk powder has previously been shown to decrease the nonspecific binding of nanoparticle-antibody bioconjugates, but this effect alone is not sufficient to allow an extremely low detection limit. The nanoparticle approach enhances the nonspecific binding attributable to multiple, weak nonspecific interactions as in the case of high binding-site density bioconjugates. The phenomenon is also partly dependent on the antibody or the antibody quality because a difference was found between Mab H50 and Mab 5A10 bioconjugates with an equal number of binding sites. Both antibodies, Mab 5A10 and Mab H50, were of the IgG subclass and should have been identical in structure except in the variable area. Apparently the nonspecific binding and the colloidal stability of the nanoparticle-antibody bioconjugates are strongly connected.

The observed differences in the colloidal stability and the nonspecific binding among different nanoparticle-antibody bioconjugates can be related to either a difference in the quality of the immunocomponents or the importance of the paratopic regions to nonspecific interactions. In principle, lower nonspecific binding, as has been achieved with other proteins (<0.005%), should also be possible with the nanoparticle-antibody bioconjugates. The nonspecific binding of the nanoparticle-streptavidin bioconjugates in the assay buffer has been shown to be <0.001% (13). The nonspecific binding of the high binding-site density nanoparticle-antibody bioconjugates to the same commercial antibody-coated well was >0.05% (17) even in an optimized assay buffer. The coating process of the commercial wells utilized an acid treatment step, which produced partly denatured, defective, or misfolded immunoglobulins and increased the nonspecific binding. This treatment was omitted from the coating process described in Materials and Methods. The modified procedure had only a small effect on the binding capacity when compared with low-capacity surfaces blocked predominantly with bovine serum albumin (9, 16). A low capacity or binding-site density on the solid-phase surface will reduce the nonspecific binding caused by antibody-antibody interactions, but it affects other performance characteristics. Low capacity, but a high binding-site density on a small binding area, however, improves the sensitivity and could be applied to the current nanoparticle-based assay concept (39, 40).

The noncompetitive free-PSA immunoassay was carried out in two steps to avoid the kinetic problems related to a one-step nanoparticle-based solid-phase immunoassay (17). Assuming that the nanoparticle-antibody bioconjugate reacts first with antigen in solution, the binding of this complex to the solid-phase capture antibody would be slow because the complex has essentially only one binding site. The experimental Mab H117 wells and an optimized assay buffer were selected to reduce the nonspecific binding and thus improve the detection limit. The nanoparticle-antibody bioconjugates of Mab 5A10 were preferred for detection because the epitopes of the sandwich pair Mab H117 and Mab 5A10 are located on opposite sides of the free PSA molecule (41). The first incubation with the calibrators was for 45 min to ensure maximal binding, although 5 min should have been enough (13). In the second step, the time needed to reach equilibrium decreased with increasing number of binding sites on the nanoparticle as described previously (17), but none of the present bioconjugates was able to reach equilibrium in 2 h at a concentration of $1 \times 10^9$ particles/40 µL. Any matrix effects that cause fluctuations in the bioconjugate binding rate constants between wells, and thus increase variation, are avoided in the two-step immunoassay (5, 15) because the sample is removed before the second incubation.

The detection limit of the nanoparticle-based assay corresponded to $7.3 \times 10^5$ molecules/mL, which is equal to the detection limit of the ambient analyte, microspot immunoassay for thyroid-stimulating hormone (15, 42), which has the lowest detection limit reported to date. The detection limit for the microspot immunoassay, 0.0002 mIU/L ($8 \times 10^5$ molecules/mL), has been beyond the reach of any conventional immunoassay methods (1). It is also one order of magnitude lower than the detection limits of the best homogeneous immunoassay (32) and the ultrasensitive heterogeneous assay using the DELFIA technology (Perkin-Elmer Life Sciences), which have lower limits of detection for thyroid-stimulating hormone of 0.0012 and <0.005 mIU/L, respectively. For the nanoparticle-based assay in equilibrium, a detection limit of 0.078 ng/L was calculated from $2 \sigma[Ab^*]_nab [Ab]_nab (1 + [Ab]^*_{tot} K)/(2[Ab]^*_{tot} K)$, where $\sigma[Ab]^*_{nab}$ is the standard deviation for the nonspecifically bound labeled antibody, $[Ab]^*_{nab}$ is the concentration of the nonspecifically bound antibody, $[Ab]^*_{tot}$ is the total labeled antibody concentration, and $K$ is the affinity constant (43). The twofold difference between the theoretical calculation and the actual detection limit could be caused, for example, by the existence of different subpopulations of bioconjugate and by overestimation of the nonspecific binding.

The lower limit of detection of the assay was limited not by the specific activity of the label, but rather by the nonspecific binding of the nanoparticle-antibody bioconjugates. The functional sensitivity of the nanoparticle-based assay can be expected to not differ substantially from the lower limit of detection, and the assay can be applied to analyze serum samples with otherwise undetectable free-PSA concentrations. An additional decrease in the nonspecific binding of bioconjugates and optimization of the assay setup could enable an improved immunoassay setup.
no assay sensitivity. The supersensitive assay could be of great benefit in postsurgical follow-up of PSA for the earliest possible detection of biochemical relapse of prostate cancer (44). Detection of very low concentrations of free PSA is also suggested to have clinical applicability for breast cancer diagnosis (45). Furthermore, an extremely sensitive assay could be utilized in detection of human glandular kallikrein 2 concentrations in serum of healthy males and females (46).

The nanoparticle approach enabled an immediate surface-readout measurement from the excitation area without any enhancement or amplification steps. Only a fraction of analyte molecules was actually measured because the edges of the well were also coated with solid-phase antibody and incubated with analyte. It was particularly important that the antibody coating on the bottom of the well was dense and uniform because both of the factors have a direct effect on assay response. Use of a smaller antibody coating area could improve the detection limit more than fivefold, as reported previously for a fluorescent terbium(III) chelate label (40). However, the time-resolved microscope images of the bottoms of the wells (Fig. 4) did not encourage the use of the “microspot” approach unless the amount of the reactive antibody on wells (Fig. 4) did not encourage the use of the “microspot” approach unless the amount of the reactive antibody on the measurement area can be accurately controlled (47). Shaking and incubation (48) could also be optimized to allow equal mixing over the entire surface area. Nevertheless, the increase of the reactive solid-phase area with shaking increased the limit of detection as the measured signals decreased. The washing procedures had to be gentle because damage to the antibody coating increased variation. The extremely low detection limit achieved still required an efficient reduction of the unwanted nonspecific binding.

We previously discussed the further development of nanoparticle-antibody bioconjugates, especially the use of smaller nanoparticle labels and recombinant antibody fragments (17). Because the specific activity of the nanoparticle 107 nm in diameter was >10-fold higher than required, the particle diameter could be halved without a decrease in the assay sensitivity. In principle, this should enable the same sensitivity in a one-step immunoassay using the same or a shorter incubation time than in the two-step assay. The nanoparticle-antibody bioconjugates could also be advantageous in the ambient analyte immunoassay because the individual analyte molecules could be measured directly from the solid-phase with single-molecule resolution using the time-resolved fluorescence microimager (10). The multivalent nanoparticle-antibody bioconjugates could also be used to detect multivalent antigens, such as viruses, because the multivalent binding (49) could enable even a 100-fold decrease in the assay detection limit. This would allow a lower limit of detection near 1000 molecules/mL, challenging the current nucleic acid amplification-based methods.

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