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Magneto-SPR Immunosensor

# Attomolar Detection of a Cancer Biomarker Protein in Serum by Surface Plasmon Resonance Using Superparamagnetic Particle Labels**

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Methods to measure protein biomarkers with ultralow detection limit (DL) and high sensitivity promise to provide valuable tools for early diagnosis of diseases such as cancer, and for monitoring therapy and post-surgical recurrence.[1,2] Surface plasmon resonance (SPR) utilizing nanoparticle–antibody labels for signal amplification in immunoassays is an emerging approach for detecting proteins in biomedical samples.[3–10] Herein, we show for the first time that clustering of superparamagnetic labels on SPR sensor surfaces leads to unprecedented sensitivity and ultralow DL for protein biomarkers in serum. Specifically, antibody bioconjugates on 1 μm diameter superparamagnetic particles (MP) used for off-line antigen capture enabled SPR detection of cancer biomarker prostate specific antigen (PSA) in serum at an ultralow DL of 10 fg mL$^{-1}$ (ca. 300 aM). This approach opens doors for accurate diagnostics based on new protein biomarkers with inherently low concentrations.

SPR immunoassays involve attaching capture antibodies ($Ab_1$) to an SPR chip and measuring signals after capture of the protein analyte from the sample. Since SPR lacks ultrahigh sensitivity, gold and magnetic nanoparticles have been used as labels on secondary antibodies ($Ab_2$) in sandwich assays to amplify SPR signals for biomarker detection by increasing average film thickness.[3–10] For example, Au nanoparticle–antibody conjugates were used to detect human IgG (DL 1 ng mL$^{-1}$),[3] and PSA with DLs 0.15,[4] 1.0,[6] and 0.01 ng mL$^{-1}$,[7] in buffer. Magnetic nanoparticles have been used for conjugate preparation, analyte capture, and magnetic-assisted separation.[8,11] Magnetic nanoparticle–$Ab_2$ conjugates were used to detect brain natriuretic peptide in plasma (DL 1 ng mL$^{-1}$),[8] heat shock protein-70 in buffer (DL 0.3 μg mL$^{-1}$),[9] and staphylococcal enterotoxin B in feces (DL 100 pg mL$^{-1}$).[10]

Sensitivity and DLs for proteins in buffer can degrade significantly in corresponding determinations in patient samples such as serum or saliva.[12] This is largely because non-specific binding (NSB) of any of the hundreds of non-analyte proteins in these media can seriously compromise DLs.[8] Here, we report a simple method using superparamagnetic particles (Dynabeads, Invitrogen), a commercial SPR flow sensor, and off-line analyte capture to attain ultrahigh sensitivity and ultralow DL for a cancer biomarker protein in serum. High sensitivity is related to off-line reduction of NSB combined with clustering of the supermagnetic particles on the SPR chip. No previous reports using magnetic particle labels in SPR have elucidated such large amplification afforded by magnetic particle clustering.[8–10]

We chose PSA as a model protein because of its established use as a clinical biomarker for prostate cancer.[13] For off-line PSA capture, we synthesized magnetic particle–$Ab_2$ bioconjugates (MP–$Ab_2$) using tosylated MPs (see the Supporting Information). Protein assays estimated the number of $Ab_2$ molecules per MP as $(1.0 \pm 0.1) \times 10^5$ and the number of $Ab_2$ molecules per control 1 μm silica particle as approximately $5 \times 10^4$. From the surface area of the 1 μm particles and the dimensions of one Ab molecule (14.5 nm × 8.5 nm × 4 nm) and considering the largest and smallest footprint orientations on the particle, we estimated a possible range of $3.2 \times 10^4$ to $2.5 \times 10^5$ antibodies per particle (see the Supporting Information). The experimental value of $(1.0 \pm 0.1) \times 10^5$ antibodies per MP is within the range of estimated coverage. In addition to advantages of lowering NSB by off-line PSA capture, this very large number of antibodies on the MP–$Ab_2$ serves to drive the $Ab_2 + PSA \rightleftarrows [Ab_2 \cdot PSA]$ equilibrium towards binding and facilitate efficient capture of PSA.

PSA in 40 μL calf serum was captured off-line by MP–$Ab_2$. The resulting MP–$Ab_2$–PSA particles were washed with blocking buffers, then injected into an SPR flow system and captured by antibodies on the gold SPR chip (Figure 1). Unless otherwise specified, all SPR measurements were done in pH 7.0 phosphate buffer saline (PBS, 0.1M in phosphate, 0.14M NaCl, 2.7 mM KCl) containing 0.05% Tween-20 (PBS-T).

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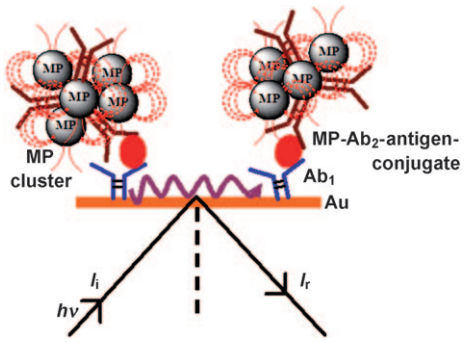


***Figure 1.*** SPR immunosensor utilizing a clustered magnetic microparticle–$Ab_2$–antigen bioconjugate for signal amplification (not drawn to scale).

Monoclonal antibodies ($Ab_1$) to PSA were covalently immobilized onto carboxylate-functionalized Au-SPR chips (Reichert Inc.) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). Unreacted carboxy groups were blocked by ethanolamine, and then treated with 2% bovine serum albumin (BSA) in PBS-T to minimize NSB (Supporting Information, Figure S1). SPR was monitored continuously after injection of the MP–$Ab_2$–PSA bioconjugates. As controls, 1 μm diameter non-magnetic silica particle–$Ab_2$–PSA (SP–$Ab_2$–PSA) conjugates were prepared similarly and used in SPR (Supporting Information).

SPR curves for binding of MP–$Ab_2$–PSA to $Ab_1$ are shown in Figure 2A in the fg mL$^{-1}$ PSA range in calf serum, which provides a good human serum surrogate for immunoassay standardization.[14] Corresponding SPR curves for silica controls, SP–$Ab_2$–PSA, were not observed in the fg mL$^{-1}$ range, but required pg mL$^{-1}$ concentrations of PSA before signals were observed (Figure S2). The SPR signal increase above zero PSA using MP–$Ab_2$ or SP–$Ab_2$ labels is plotted in Figure 2B. A single assay takes approximately 100 min (Supporting Information).

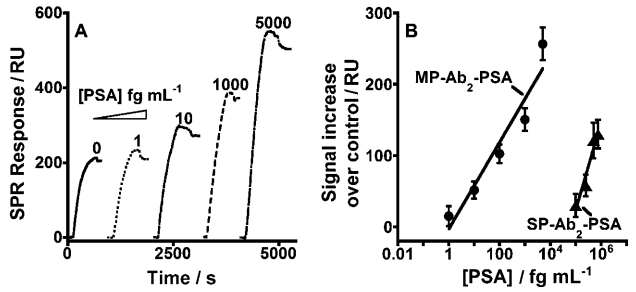


***Figure 2.*** Flow SPR for the binding of A) MP–$Ab_2$–PSA to covalently immobilized $Ab_1$ on the SPR sensor surface. B) Influence of PSA concentration on maximum SPR signals (less zero PSA control) for the binding of MP–$Ab_2$–PSA (●) and SP–$Ab_2$–PSA (▲) conjugates made from different PSA concentrations in calf serum (as denoted). The zero PSA signal results mainly from NSB. RU = response unit.

Using MP–$Ab_2$ for off-line PSA capture, we obtained a DL of 10 fg mL$^{-1}$ PSA (ca. 300 aM) as 3 standard deviation (SD) units larger than the zero PSA signal. This ultralow DL was 10000-fold better than 100 pg mL$^{-1}$ obtained with the non-magnetic SP–$Ab_2$–PSA control. Further, sensitivity as slope of the calibration curve in resonance units (RU) per fg mL$^{-1}$ PSA for MP–$Ab_2$–PSA was 220-fold larger than for SP–$Ab_2$–PSA (Figure 2B).

The DL for MP–$Ab_2$ with the SPR sensor was 1.5 times better than the previous most sensitive serum PSA determinations utilizing digital fluorescent enzyme-linked immunosorbent assay (ELISA, 14 fg mL$^{-1}$ PSA)[2a] and 33-fold better than by a DNA-based bio-barcode assay (330 fg mL$^{-1}$).[2b] However, a bio-barcode assay detected 30 aM PSA (1 fg mL$^{-1}$) in goat serum.[15] We report here the lowest DL achieved to our knowledge thus far for PSA in serum by an SPR immunosensor. A recent non-magnetic gold nanorod labeled SPR assay achieved a lower DL for IgE proteins,[16] but was not tested in serum.

We used magnetic and silica particles of the same size (1 μm) to make MP–$Ab_2$–PSA and SP–$Ab_2$–PSA conjugates, but MPs showed four orders of magnitude lower DL representing a 10000-fold signal enhancement compared to silica particles. This large amplification was attributed to MP–$Ab_2$–PSA aggregates that form on the SPR chip while no noticeable SP–$Ab_2$–PSA aggregates form in the low pg mL$^{-1}$ PSA range (Figure 3). Scanning electron microscope (SEM) images reveal these aggregates for MP–$Ab_2$–PSA at 5 pg mL$^{-1}$ PSA, but not with SP–$Ab_2$–PSA (5 pg mL$^{-1}$ PSA) (Figure 3, Figure S5). The linear increase in SPR signal with increasing [PSA] using the MP–$Ab_2$–PSA conjugate (Figure 2) suggests that SPR signal depends on PSA because the amount of PSA captured on the SPR chip depends on its concentration in solution, while the extent of aggregation is independent of PSA concentration. This latter observation was confirmed by size distribution analysis of particle

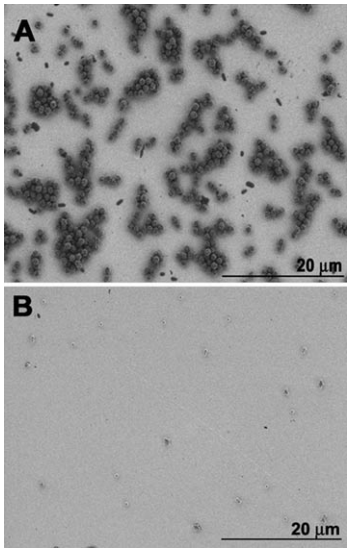


***Figure 3.*** Scanning electron microscopy images of A) magnetic MP–$Ab_2$–PSA (5 pg mL$^{-1}$ PSA) and B) nonmagnetic control SP–$Ab_2$–PSA (5 pg mL$^{-1}$ PSA) bound to $Ab_1$ on gold SPR surfaces.

  

dispersions using dynamic light scattering (DLS) which showed significant PSA-independent aggregation of MP–$Ab_2$–PSA, but no aggregation of SP–$Ab_2$–PSA (Figures S3 and S4).

SPR response (in RU) can be related by theory to the amount of bound antibody per unit area by the expression[17] $1\ RU \approx 1\ pg\,mm^{-2}$. Thus, relating the SPR signal response (in RU) to the number of magnetic particles on the SPR chip using the known density ($1.7\ g\,cm^{-3}$) and dimensions of our MPs, we estimated that $7\times10^3\ pg\,mm^{-2}$ MPs (i.e. $7.9\times10^3$ particles $mm^{-2}$) are required to give the observed $10^4$-fold SPR enhancement. This calculation assumes that the observed SPR amplification is a result of mass from the MP clusters bound within the 300 nm evanescent wave distance from the Au chip surface. Experimentally, from the analysis of MP–$Ab_2$–PSA particles $mm^{-2}$ area on the SPR chip from SEM images (Figure 3A), we obtained about $6.3\times10^3$ MP–$Ab_2$–PSA particles $mm^{-2}$ area over SP–$Ab_2$–PSA. This accounts for ca. 8000-fold signal enhancement for MP–$Ab_2$–PSA over SP–$Ab_2$–PSA. The remaining 20% signal enhancement may arise from the higher refractive index of MP (ca. 1.6)[18a] over SP (1.43). For comparison, a 0.04 unit change in refractive index of DNA amplified SPR by approximately 10%.[18b] Based on this, the higher refractive index of MP over SP can contribute up to 40% amplification. Also, we cannot rule out a minor contribution from the interaction of inherent magnetic field of the supermagnetic particle aggregates with the surface plasmons.[19]

The SPR immunosensor with off-line MP–$Ab_2$ sample capture was used to determine PSA in four human serum samples. Samples were diluted in buffer 20000-fold to correspond to the linear range of the PSA calibration. Results showed excellent correlation to determinations of PSA in undiluted samples by ELISA (Figure 4) over a clinically

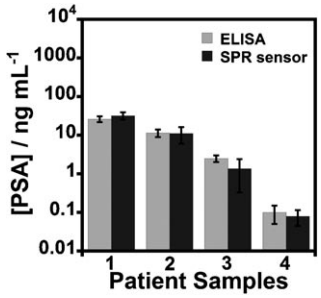


***Figure 4.*** SPR immunosensor results for patient serum samples assayed using magnetic particle labels and off-line capture compared to standard ELISA. Samples 1–3 were from males diagnosed with prostate cancer and 4 was from a cancer-free female. Error bars show standard deviations ($n=3$).

relevant range of PSA. There was no significant difference in PSA found between the two methods at the 95% confidence level (*t*-test). These results confirm good accuracy, as well as high selectivity for PSA in the presence of thousands of other proteins in serum.

In summary, we have demonstrated an unprecedented low DL for a cancer biomarker protein at aM levels in serum using an SPR immunosensor with MP amplification. The approach gave excellent correlation with ELISA for PSA in cancer patient serum. The added value of the low DL is in detection of recurrent prostate cancer, and in applications to future protein biomarkers with extremely low serum levels. The ultrahigh sensitivity is attributed mainly to mass and refractive index enhancement from clustered magnetic particle conjugates on the SPR chip. Given the good linear dynamic range of SPR response (Figure 2B), and the lack of dependence of MP aggregation on concentration of PSA (Figures S3 and S4), we speculate that an antibody bound analyte–MP particle or aggregate on the SPR surface may act as a nucleation site for the binding of further MP particles or aggregates, as predicted for single magnetic domain induced superparamagnetic particle aggregation.[20] The approach should also be applicable to other proteins and small molecules. Further studies are underway to uncover the full details of reproducible surface aggregation and signal amplification.

## *Experimental Section*

Materials and methods. Prostate-specific antigen (PSA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) were from Sigma. Monoclonal primary antihuman PSA antibody ($Ab_1$, clone no. CHYH1), and secondary anti-PSA antibody ($Ab_2$, clone no. CHYH2) were from Anogen/Yes Biotech Laboratory, Ltd. Tosyl-activated superparamagnetic microparticles (MP, Dynabeads, 1 μm diameter) were obtained from Invitrogen. Carboxy-functionalized silica microparticles (SP, 1 μm diameter) were from Bangs Laboratories Inc. (IN, USA). PSA standards were prepared in calf serum.[14] Human serum samples were from Capital Biosciences (Rockville, MD).

Surface plasmon resonance (SPR) was done using an SR7000DC dual channel flow SPR spectrometer from Reichert Analytical Instruments (NY, USA). SPR gold chips with a mixed self-assembled monolayer of 90% monothiol alkane $PEG_3$-OH and 10% monothiol alkane $PEG_6$-COOH were from Reichert (SR7000). SPR was done in pH 7.0 phosphate-buffered saline (PBS, 0.1M in phosphate, 0.14M NaCl, 2.7 mM KCl) containing 0.05% Tween-20 (PBS-T). Scanning electron microscopy (SEM) of MP–$Ab_2$–PSA and SP–$Ab_2$–PSA bound to capture antibodies on SPR chips was done using Zeiss field-emission scanning electron microscope (DSM 982 Gemini).

$Ab_2$ was covalently conjugated to tosyl-activated MP following the protocol provided by Invitrogen (see the Supporting Information for details of bioconjugate preparations). For standard curve generation, PSA in 40 μL calf serum was stirred with the MP–$Ab_2$ or SP–$Ab_2$ for 90 min at 37°C to capture PSA off-line.[15,21]

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[1] a) S. F. Kingsmore, *Nat. Rev. Drug Discovery* **2006**, *5*, 310–321; b) V. Kulasingam, E. P. Diamandis, *Nat. Clin. Pract. Oncol.* **2008**, *5*, 588–599; c) D. A. Giljohann, C. A. Mirkin, *Nature* **2009**, *462*, 461–464.

[2] a) D. M. Rissin, C. W. Kan, T. G. Campbell, S. C. Howes, D. R. Fournier, L. Song, T. Piech, P. P. Patel, L. Chang, A. J. Rivnak,

  

E. P. Ferrell, J. D. Randall, G. K. Provuncher, D. R. Walt, D. C. Duffy, *Nat. Biotechnol.* **2010**, *28*, 595 – 599; b) C. S. Thaxton, R. Elghanian, A. D. Thomas, S. I. Stoeva, J.-S. Lee, N. D. Smith, A. J. Schaeffer, H. Klocker, W. Horninger, G. Bartsch, C. A. Mirkin, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 18437 – 18442.

[3] L. A. Lyon, M. D. Musick, M. J. Natan, *Anal. Chem.* **1998**, *70*, 5177 – 5183.

[4] G. A. J. Besselink, R. P. H. Kooyman, P. J. H. J. van Os, G. H. M. Engbers, R. B. M. Schasfoort, *Anal. Biochem.* **2004**, *333*, 165 – 173.

[5] H. J. Lee, A. W. Wark, R. M. Corn, *Analyst* **2008**, *133*, 975 – 983.

[6] L. Huang, G. Reekmans, D. Saerens, J.-M. Friedt, F. Frederix, L. Francis, S. Muyldermans, A. Campitelli, C. V. Hoof, *Biosens. Bioelectron.* **2005**, *21*, 483 – 490.

[7] J.-W. Choi, D.-Y. Kang, Y.-H. Jang, H.-H. Kim, J. Min, B.-K. Oh, *Colloids Surf. A* **2008**, *313–314*, 655 – 659.

[8] Y. Teramura, Y. Arima, H. Iwata, *Anal. Biochem.* **2006**, *357*, 208 – 215.

[9] Y. Sun, Y. Bai, D. Song, X. Li, L. Wang, H. Zhang, *Biosens. Bioelectron.* **2007**, *23*, 473 – 478.

[10] S. D. Soelberg, R. C. Stevens, A. P. Limaye, C. E. Furlong, *Anal. Chem.* **2009**, *81*, 2357 – 2363.

[11] L. Gao, J. Zhuang, L. Nie, J. Zhang, Y. Zhang, N. Gu, T. Wang, J. Feng, D. Yang, S. Perrett, X. Yan, *Nat. Nanotechnol.* **2007**, *2*, 577 – 583.

[12] J. F. Rusling, C. V. Kumar, J. S. Gutkind, V. Patel, *Analyst* **2010**, *135*, 2496 – 2511.

[13] H. Lilja, D. Ulmert, A. J. Vickers, *Nat. Rev. Cancer* **2008**, *8*, 268 – 278.

[14] X. Yu, B. Munge, V. Patel, G. Jensen, A. Bhirde, J. D. Gong, S. N. Kim, J. Gillespie, J. S. Gutkind, F. Papadimitrakopoulos, J. F. Rusling, *J. Am. Chem. Soc.* **2006**, *128*, 11199 – 11205.

[15] J. M. Nam, C. S. Thaxton, C. A. Mirkin, *Science* **2003**, *301*, 1884 – 1886.

[16] H. R. Sim, A. W. Wark, H. J. Lee, *Analyst* **2010**, *135*, 2528 – 2532.

[17] E. Stenberg, B. Persson, H. Roos, C. Urbaniczky, *J. Colloid Interface Sci.* **1991**, *143*, 513 – 526.

[18] a) L. E. Helseth, H. Z. Wen, P. Heinig, T. M. Fischer, *Langmuir* **2004**, *20*, 6556 – 6559; b) B. P. Nelson, T. E. Grimsrud, M. R. Liles, R. M. Goodman, R. M. Corn, *Anal. Chem.* **2001**, *73*, 1 – 7.

[19] a) Y. Souche, E. Wagner, T. Fournier, A. D. Santos, *J. Magn. Magn. Mater.* **2004**, *272*, 2288 – 2290; b) B. Sepúlveda, A. Calle, L. M. Lechuga, G. Armelles, *Opt. Lett.* **2006**, *31*, 1085 – 1087; c) P. K. Jain, Y. Xiao, R. Walsworth, A. E. Cohen, *Nano Lett.* **2009**, *9*, 1644 – 1650.

[20] a) G. Fonnum, C. Johansson, A. Molteberg, S. Mørup, E. Aksnes, *J. Magn. Magn. Mater.* **2005**, *293*, 41 – 47; b) H. Kachkachi, D. A. Garanin in *Surface Effects in Magnetic Nanoparticles* (Ed.: D. Fiorani), Springer, New York, **2005**, pp. 75 – 104.

[21] S. I. Stoeva, J.-S. Lee, J. E. Smith, S. T. Rosen, C. A. Mirkin, *J. Am. Chem. Soc.* **2006**, *128*, 8378 – 8379.

  