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Electrochemical Immunoassay for Free Prostate Specific Antigen (f-PSA) using Magnetic Beads

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Abstract

Prostate specific antigen (PSA) is a prominent marker for the prostate carcinoma. It is found in human blood in free (f-PSA) and complex forms. These two forms together are called total PSA (t-PSA). Estimation of both forms is essential to predict malignancy. In this study we report a unique and effective technique of electrochemical detection of f-PSA using magnetic beads on a three-electrode screen-printed sensor. A magnetic bead enzyme linked immunosorbent assay (ELISA) was performed in a cuvette. Following the immunoassay, magnetic beads were recovered by a magnetic concentrator and transferred on the working electrode of the 3-electrode assembly. The amperometric response, a measure of the amount of residual enzyme activity on the beads and hence the concentration of analyte in solution, was determined by addition of enzyme substrate. The device has a detection limit of < 0.1 ng mL^{-1} f-PSA and a linear range of 0 to 1 ng mL^{-1} f-PSA.

Keywords

Bioanalytical methods, Bioassays, Biosensors, Immunoassays, ELISA.

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INTRODUCTION

Prostate specific antigen (PSA) is recognized as the most effective marker for prostate cancer and the best tumor marker in oncology. With the exception of skin cancers, prostate cancer is the most common cancer in man. Prostate cancer is the second leading cause of cancer death in men in the United States, exceeded only by lung cancer. Prostate cancer accounts for about 10% of cancer-related deaths in men.

PSA is a serine protease composed of a single chain glycoprotein. The molecular weight of PSA, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), is approximately 33-34 kD. It is composed of 237 amino acids, with a mol. wt. of 26,079 for the peptide moiety of the molecule [1]. PSA in human body is mainly bound to alpha 1-antichymotrypsin (ACT) as a complex form and the remaining PSA is in the free form (f-PSA). The total amount of bound PSA and f-PSA is known as total PSA (t-PSA).

A number of epitopes are present on PSA allowing the development of sandwich assays using polyclonal and monoclonal antibodies that can measure both free PSA and PSA-ACT [2]. Prostate cancer is present in half of all patients having PSA-ACT at more than 4 ng mL^{-1} and 70% at more than 10 ng mL^{-1} [3]. Thus PSA-ACT levels of 4.0 ng mL^{-1} or higher are strong indicators of the possibility of prostate cancer (CAP). Elevated serum PSA levels have also been attributed to benign prostatic hyperplasia (BPH). PSA elevations may also occur with aging and with conditions such as prostatitis [4]. Thus high level of t-PSA may lead to a large percentage of false positive screening results.

A potential solution to this problem involves the determination of f-PSA levels. The ratio of f-PSA to t-PSA is very important in the screening of BPH from CAP [5]. The serum determination of the f-PSA / t-PSA ratio is an excellent index of prostate carcinoma.

Stancik et al. [6] showed that the ratio can differentiate BPH from CAP. Table 1 [7] will show the probability of cancer with % of f-PSA.

Besselink and others [8] have developed surface plasmon resonance (SPR)-based sensor for detection of prostate-specific antigen (PSA) using colloidal gold and latex microspheres (120 nm diameter) on planar- and gel-type sensor surfaces. Application of colloidal gold led to a sensitivity increase of approximately three orders of magnitude compared with no amplified detection. They achieved a limit of detection of 0.15 ng mL^{-1} of PSA, which is sufficient for measuring enhanced, clinically relevant PSA levels. They however measured t-PSA.

A two-step immunoassay was designed by Soukka and others [9] to measure free prostate-specific antigen (PSA) using europium(III) chelate nanoparticles coated with a monoclonal anti-PSA antibody. The assay was performed in a low-fluorescence microtitration well passively coated with an another monoclonal anti-PSA antibody and the europium(III) fluorescence was measured directly from the bottom of the well by a standard time-resolved microtitration plate fluorometer. Wu and his coworkers [10] have developed a PSA sensor by attaching PSA antibodies to a gold-coated silicon nitride microcantilever. They described the cantilever as "a microscopic diving-board-shaped mechanical structure." A sample containing PSA was flown over the microcantilever surface. The binding of PSA to the antibodies caused a deflection in the microcantilever that could be measured using a laser beam. They were able to detect PSA over a concentration range of 0.2 ng mL^{-1} to 60 µg mL^{-1}.

A particle-based renewable electrochemical magnetic immunosensor was developed by Lin Guodong and Lin Yuche [11] using magnetic beads and gold nanoparticle labels.

Anti-IgG antibody-modified magnetic beads were attached to a renewable carbon paste transducer surface by magnet that was fixed inside the sensor. Gold nanoparticle labels were capsulated to the surface of magnetic beads by sandwich immunoassay. The stripping signal of gold nanoparticles was related to the concentration of target IgG in the sample solution. The detection limit of 0.02 $\mu g\ mL^{-1}$ of IgG was obtained under optimum experimental conditions.

Fernandez-Sanchez et al. [12] developed a one-step lateral flow immunoassay on a strip format for detection of serum levels of free and total prostate specific antigen. The performance of the system described relied on non-competitive immunoassay protocols. Herein, f-PSA and t-PSA were sandwiched between anti-f-PSA and anti-t-PSA monoclonal antibodies immobilised on the strip and a colloidal gold anti-PSA antibody tracer. In the presence of PSA in the sample, the tracer accumulated on the strip results in the appearance of specific pink colour lines. A semi-quantitative estimation could be carried out visually. Quantitative analysis was also possible by densitometry. The detection limit was 1 $ng\ mL^{-1}$.

Immunoradiometric assay is based on the reversible and non-covalent binding of an antigen by a specific antibody labeled with a radioactive nuclide as a tracer. Kochańska-Dziurowicz and coworkers [13] used immunoradiometric assay using $Iodine^{125}$ labeled anti-psa. The diagnostic kit was manufactured by Euro-DPC Ltd. Llanberis, UK.

Jochen et.al. [Jochen Peter1, Carlo Unverzagt2, Thomas N. Krogh, Ole Vorm and Wolfgang Hoesel, Identification of Precursor Forms of Free Prostate-specific Antigen in Serum of Prostate Cancer Patients by Immunosorption and Mass Spectrometry, *Cancer*

Research 61, 957-962, February 1, 2001] isolated f-PSA from serum using streptavidin coated magnetic beads and estimated f-PSA by mass spectrometry.

In this presentation we describe a method of immunoassay for f-PSA using the magnetic bead. Particle-based electrochemical magnetic immunosensors can be readily used for disease diagnostics. The method is unique compared to other methods since it combines the efficiency of the separation by magnetic beads and sensitivity of electrochemical measurement. This method is also an improved one in many respects compared to our earlier work on t-PSA [14]. The method uses immunoassay product on the sensor surface rather than performing it on the electrode, avoiding interference. The separation of the immunoasasay product was made easy by use of magnet/magnetic beads. The detection limit is also much higher (<0.1 ng mL^{-1}). The method is very convenient and the detection may be carried out in non-laboratory locations with simple hand held instruments. The method was tested by hand held potentiostat (Whistonbrook Technologies Limited, Bedfordshire, UK). The antibody-coated magnetic beads have a shelf life of several months.

MATERIALS

Human prostate specific antigen (PSA), monoclonal antibodies to total PSA (clone: A45510259P and A45080020P), conjugates and a test kit containing PSA in human serum were purchased from Biospacific, CA, USA. All chemicals for preparation of buffer, horseradish peroxidase enzyme (HRP), biotinylated HRP, 3,3' 5,5'-,tetramethyl benzidine (TMB), 0.01M Phosphate buffer saline (PBS, pH- 7.4, 0.137M NaCl) were supplied by Sigma Chemical Co Ltd, Poole, Dorset, UK and used as received. Hydroxyethyl cellulose (HEC) was supplied by Fluka, (Gillingham, Dorset, UK).

Dynabeads ®M-280 Tosylactivated and streptavidin coated dynabeads were supplied by Dynal Biotech., USA.

EXPERIMENTAL

This paper presents amperometric detection procedure for PSA using a three-electrode screen-printed sensor. The benefits of using screen-printed electrodes is that they can be mass-manufactured at low cost and thus can be considered 'one-shot' single-use devices, thereby negating the problems of electrode regeneration required when using conventional solid state electrode materials. The immunoassays were based on a sandwich assay formats. Three different schemes were followed. They are shown in Figures 1, 2 and 3. In scheme I and III the magnetic beads were coated with anti-PSA antibody that could bind specifically to ACT-bound epitope or free epitope of PSA respectively. In scheme II the magnetic beads were coated with ACT that could bind with free epitope of PSA. In all the three schemes, a tracer material consisting of anti-PSA antibody (with specificity towards a second epitope on the target analyte) coupled to HRP enzyme was used to tag bead bound PSA. Following bead recovery by the use of a magnetic particle concentrator (Dynal Biotech ASA, Oslo, Norway), unbound material was washed from the system. The Dynal MPC-15 magnetic particle concentrator contains an acrylonitrile-butadiene-styrene (ABS) plastic housing incorporating high-energy neodymium magnets. The beads recovered were placed on the working electrode of the 3-electrode assembly. Enzyme (HRP) activity on the beads would be proportional to the amount of PSA present in the sample and this was determined by addition of enzyme substrate through the resultant production of electro active product. In this work, hydrogen peroxide was used as HRP substrate. HRP reduces the hydrogen peroxide with

concomitant oxidation of an electron donor (mediator, in this case TMB), the reducing equivalents being transferred to TMB via the enzyme active site. The mediator was re-reduced by accepting electrons from a suitably poised electrode. The resultant current is related to the amount of HRP, hence analyte present. The beads were held firmly on the surface of the working electrode by a Neodymium rare earth magnet (N42 disk magnet; 0.5"D x 0.25"T, Indigo Instruments, NY).

Washing of magnetic beads

The magnetic beads (Dynabeads M-280) are uniform supermagnetic polystyrene beads coated with a polyurethane layer. The polyurethane surface is activated by p-toluenesulphonyl chloride [15] that provides reactive groups for covalent binding of proteins (e.g. antibodies) or other ligands containing primary amino or sulphydryl groups. The standard protocol provided with the kit was followed to wash the magnetic beads before coating them with the anti-PSA antibody.

Three buffer solutions were prepared to follow the protocol.

Buffer A (0.1 M Na-phosphate buffer pH 7.4): 2.62 g Na H2 PO4 x H2 O (MW 137.99) 14.42 g Na2 HPO4 x 2H2 O (MW 177. 99) were dissolved in reverse osmosis (RO) water and the volume was adjusted to 1000 ml.

Buffer B (PBS pH 7.4 (phosphate buffered saline) with 0.1% (w/v) BSA): 0.88 g NaCl (MW 58.4) and 0.1% (w/v) bovine serum albumin (BSA) were added to 80 ml 0.01 M Na-phosphate pH 7.4. They were mixed thoroughly and the volume was adjusted to 100 ml with 0.01 M Na-phosphate pH 7.4.

Buffer C (0.2 M Tris pH 8.5 with 0.1% (w/v) BSA): 2.42 g Tris was dissolved in 80 ml RO water. The pH of the solution was adjusted to 8.5 using 1 M HCl. 0.1% BSA was added and the volume of the solution was adjusted to 100 ml.

Dynabeads were resuspended in it's container for approximately 1 min using a vortex mixer. Measured volume (10^7 beads per sample of 3μg) of suspension was pipetted out into a micro-centrifuge tube. The tube was placed on a magnetic concentrator (Dynal MPC) until the beads migrated to the side of the tube and the liquid was clear. The supernatant was discarded. The beads were washed carefully in ample volume of buffer A for 2 minutes. The beads were recovered by the magnetic concentrator and used for coating.

Coating of magnetic beads

Antibody or ACT solution in buffer A (3μg antibody or ACT per 10^7 Dynabeads) was added to the washed dynabeads and mixed for 1 minute. It was incubated for 24 h at 25°C with slow tilt rotation. After incubation, the tube was placed on the magnetic concentrator until the beads concentrated on the side of the tube, and the supernatant was removed. The coated beads were then washed four times, twice in buffer B for 5 minutes at +4°C, once in Buffer C for 24 h at +20°C and finally in buffer B for 5 minutes at +4°C. The coated Dynabeads were thus made ready for use.

Construction of screen-printed electrode

Devices were printed onto 250 μm thick polyester sheet (Cadillac Plastic Ltd., Swindon, UK). The circular electrocatalytic working electrode was fabricated from a commercially available carbon powder (MCA Services Ltd., Cambs. UK), made into a screen-printable paste by mixing 1:3 (w/w) with 2.5% w/v HEC in PBS. The reference electrode ink contained 15% w/w silver chloride in silver paste [16] (MCA). The counter electrode and basal tracks used to connect the electrodes to the measurement device were fabricated from I45R carbon ink (MCA). The basal tracks were insulated from the measurement solution using an epoxy-based protective coating ink 242-SB (Agmet ESL Ltd., Reading,

UK). The electrodes were then heat treated at 125ºC for 2 hours to cure the epoxy resin and to stabilize the electro-catalytic pad to allow prolonged use of the device in aqueous solutions [17]. Figure 4 shows the detailed schematic of the screen-printed electrode.

Detailed Procedure

Cyclic voltammetry was first performed to ascertain the appropriate potential for the HRP initiated redox reaction of hydrogen peroxide in presence of TMB. The optimum detection potential for reduction of hydrogen peroxide was selected as –100 mV (Figure 5). To find the optimum concentration of TMB substrate solution, different amounts of HRP were dissolved in the same buffer containing 1% v/v glutaraldehyde and 10µl volumes of this solution were deposited on the working electrodes of the sensors. The bifunctional activity of glutaraldehyde served as a means of cross-linking the HRP protein onto the carbon working electrode surface. Amperometry was performed at -100 mV. After electrochemical equilibration, 10 µl of 50% v/v TMB stock solution was added and response was noted. The effect of TMB solution concentration on amperometric response was studied across a range of HRP activities (0-30 mU, Figure 6). To confirm whether magnetic beads could be used for immunoassay on the screen-printed electrode, streptavidin-coated magnetic beads were used for conjugation with biotinylated HRP and used on the electrode for response of the HRP. As the basic objective of the method was to measure low concentration of HRP tagged bead-bound PSA using TMB solution, experiments were performed to find the detection limit for HRP. A stock solution of 1.2 mg of HRP-biotin was prepared in PBS and aliquots from this stock solution were diluted as appropriate. Initially 0.1 mg quantities of streptavidin-coated beads were transferred to 0.5 ml tubes and the volume made up to 100 µl with PBS. After thorough mixing, the beads were separated by a magnetic concentrator and

the supernatant was removed. This process was repeated once more in order to wash the beads. A proper aliquot of diluted HRP-biotin solution was added to provide the desired HRP activity. The magnetic beads were washed thrice by buffer after incubation for one hour at 20^0C. Volumes (10 μl) of the final bead mixture were prepared by adding 100μl of PBS containing 1% v/v gluteraldehyde and deposited on the WE and dried at 4^0 C. The dried electrodes were tested amperometrically with 100 μl 50% v/v TMB stock solution in PBS at -100mV as described earlier (Figure 7). The beads coated with t-PSA-Mab (scheme I)/ ACT (scheme II)/ f-PSA-Mab (scheme III) were divided into 5 equal portions to which were added 20 μL of f-PSA solutions of 5 different concentrations (0.1, 0.25, 0.5, 0.75 and 1.0 ng mL^{-1}). These preparations were incubated for 6 hours to allow specific binding of f-PSA to the Mab. After separation of beads and washing with buffer A, the conjugate f-PSA-Mab-HRP (scheme-I)/ t-PSA-Mab-HRP (scheme II and III) was added in excess (200μl) to each aliquot and incubated for 6 hours. The beads were again washed with buffer A and subjected to amperometric study.

Following the immunoassay, the magnetic beads were placed directly on the working electrode of a 3 electrode screen-printed sensor. A powerful magnet was placed underneath the working electrode to ensure proper retention of the magnetic beads on the working electrode. 90μL volume of 6 mM potassium iodide in PBS was applied to the electrodes (within the circular aperture defined by the insulation layer) and amperometry performed at -0.1 V (versus the Ag/AgCl reference electrode). After equilibration of the electrochemical response, a 50% dilution of the TMB stock solution was added and the electrochemical current response noted at 120 s.

RESULTS AND DISCUSSIONS

Figure 6 shows the improvement of response with increasing TMB mediator concentration. But the increase in response was not appreciable above 50% TMB solution. We have chosen 50% TMB solution for estimation of f-PSA as a tradeoff between cost of reagent and response. Thus optimum concentration was chosen as 50% v/v of the supplied TMB stock solution. Figure 7 indicates that there is a steady apparently first-order increase in device response up to 8 μU HRP, and a tendency towards a zero order saturated response as the enzyme loading is increased to 100 μU per electrode. The limit of detection with respect to enzyme loading, was calculated as 0.24 μU in accordance with the IUPAC convention. Hence it was considered this detection system had merit with respect to the estimation of f-PSA using HRP-labeled antibody. Further an experiment following Sarkar et al. [14] was conducted by performing the immunoassay for f-PSA right on the electrode to check the response and consider if there was a necessity for magnetic beads to improve performance. The response of f-PSA was studied in the absence of PSA-ACT complex. A 4 μl volume of f-PSA-Mab was immobilized on the working electrode of a 3 electrode screen-printed sensor using glutaraldehyde. Following Mab immobilisation, 4 μl volumes of f-PSA solution of different concentrations (0, 0.4, 0.8, 1.6 ng mL^{-1}) were applied to the biosensor, incubated for 24 h, and washed. 16 μl f-PSA-Mab-HRP solution was then added to the Mab-f-PSA complex, incubated for 24 h and washed. The response was measured with 50% TMB-H_2O_2 solution as substrate (Figure 8). The graph indicates only 20 nA response for addition of 1.6 ng mL^{-1} of f-PSA. As we needed detection limit of less than 0.25ngmL^{-1} of f-PSA (Lower limit of f-PSA being around 0.25 ngmL^{-1}which is 10 % of amount of t-PSA i.e., 2.6 ngmL^{-1}) to ascertain the ratio of f-PSA to t-PSA, hence prostate carcinoma,

this method did not serve the purpose and consequently we switched over to the magnetic bead route.

Different schemes were followed in order to determine an optimum approach towards f-PSA determination using the magnetic beads.

Figure 9 shows the response obtained in the presence of different concentrations of f-PSA according to scheme I. A linear response in the range of 0 ng mL^{-1} to 1.0 ng mL^{-1} f-PSA has been obtained.

Figure 10 shows the responses obtained due to the presence of different concentrations of f-PSA according to schemes II and III. A linear response in the range of 0 ng mL^{-1} to 1.0 ng mL^{-1} f-PSA is evident.

To test whether the system works in a mixture of f-PSA and PSA-ACT (as would be the case when dealing with clinical samples) measurements have been performed for determination of f-PSA in a mixture of f-PSA and PSA-ACT. Five different mixtures containing equal volumes of f-PSA and PSA-ACT complex at different concentrations were prepared as shown in Table 2. Scheme I might be used for both t-PSA and pure f-PSA. Thus to determine the concentration of f-PSA in a mixture of f-PSA and PSA-ACT this scheme is not suitable. On the other hand scheme II and III could be valid for only f-PSA and thus were chosen for determining f-PSA in t-PSA. The responses obtained for f-PSA in samples of t-PSA as per Table 2 using scheme II and III are shown in Figure 11. To ascertain that the methods used are also suitable for mixtures of f-PSA and PSA-ACT, a comparison of the device response to pure f-PSA and f-PSA/PSA-ACT mixtures is shown in Figure 12. It is seen that the slopes of the linear trend lines are almost unity. Thus there was practically no interference in determining f-PSA in presence of PSA-

ACT. Table 3 depicts an overall comparison of f-PSA sensors. This work presents improvement in major four aspects e.g. detection limit, cost, interference, usability.

CONCLUSIONS

A quantitative magnetic bead based ELISA sandwich immunoassay, with electrochemical determination of enzyme label activity at screen-printed electrodes has been demonstrated for the detection of f-PSA. The benefit of this retention procedure is that, no cross-linking agent or polymer membrane is required compared to standard electrochemical immunosensor devices. Also the electrode does not come into contact with the sample medium and hence is not prone to electrode fouling or interference problems. The detection method is simple and has potential to be used as a medical diagnostic tool. Three formats are reported here, each capable of detecting f-PSA to levels less than 0.1 ng/ml. A significant improvement in device performance is evident due to the use of magnetic beads as opposed to immobilizing immunoreagents to the screen-printed working electrode surface and performing the assay directly at the transducer surface. It is simple to perform using low cost reagents and detection methodologies coupled to sensitivities several-fold higher than the conventional colorimetric method.

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Table 1. Probability of cancer with concentration of f-PSA

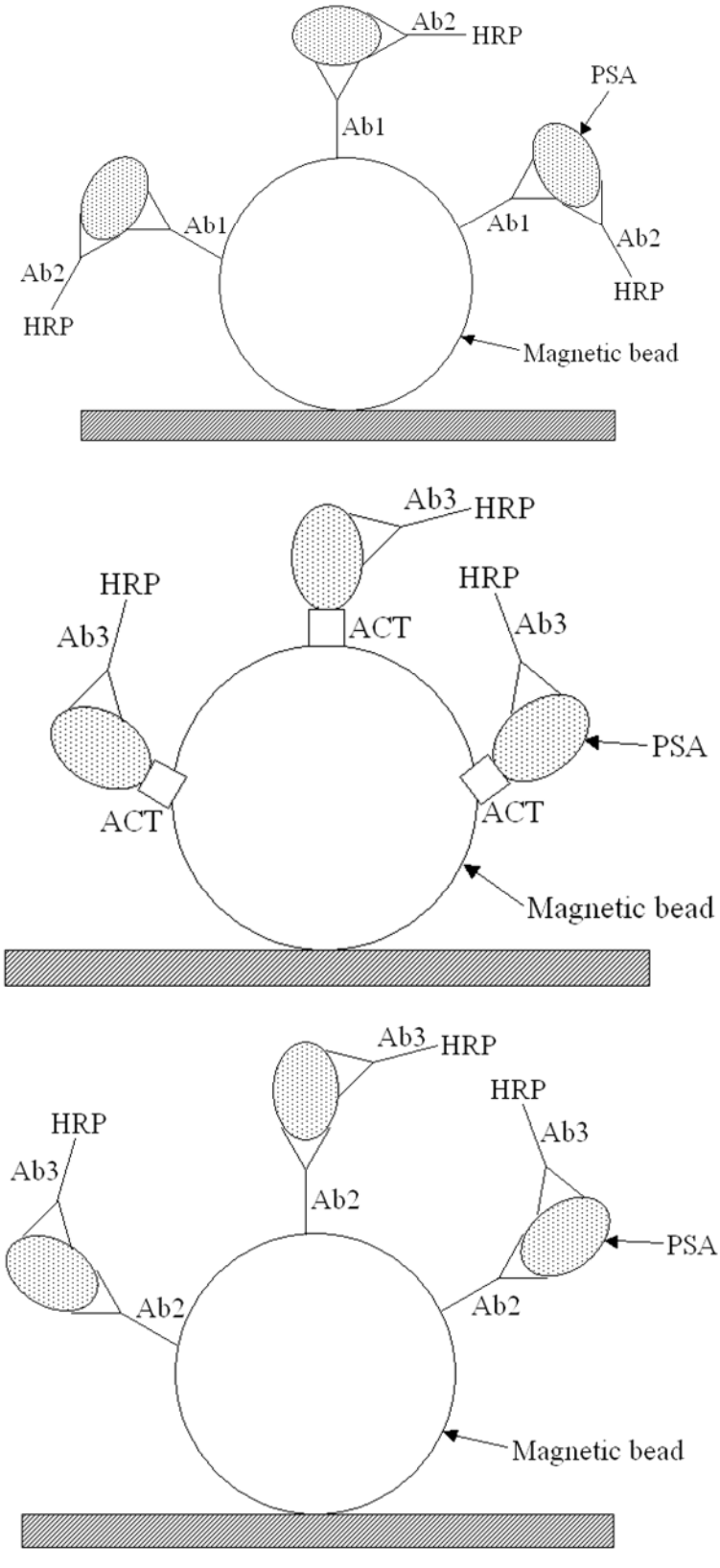
Standard PSA (ng mL^{-1})	**Probability of cancer (%)**	**Percent free PSA (%)**	**Probability of cancer (%)**
0-2	1	0-10	56
2-4	15	10-15	28
4-10	25	15-20	20
>10	>50	20-25	16
		>25	8

Table 2. Combinations of f-PSA and PSA-ACT complex used to determine assay performance for measurement of f-PSA

Sample No.	**Concentration of f-PSA (**ng mL^{-1})	**Concentration of PSA-ACT (**ng mL^{-1})
1	0	1.0
2	0.1	0.9
3	0.5	0.5
4	0.75	0.25
5	1.0	0

Table 3. Performances of different f-PSA sensors

Serial No.	Method	Remarks	Reference
1	Surface plasmon resonance	Gold was used, costly, limit of detection 0.15 ng mL^{-1}	[8]
2	Fluorescence	Costly europium(III) chelate nanoparticles were used, intensive sample preparation	[9]
3	Gold-coated silicon nitride microcantilever	Gold was used, costly, detection as low as 2 ng mL^{-1}	[10]
4	electrochemical magnetic immunosensor	gold nanoparticles were used, costly, detection limit of 2 ng mL^{-1}	[11]
5	Immunostrip	Costly, estimated semi-quantitatively by visual estimation of pink lines, detection limit of 1 ng mL^{-1}	[12]
6	Immuno-radiometric assay	Costly, radiation hazard, high sensitivity	[13]
7	Diposable electrochemical sensor using tosylactivated magnetic beads	Detection limit <0.1 ng mL^{-1}, less costly, no immoblilization on electrode, portable	Present work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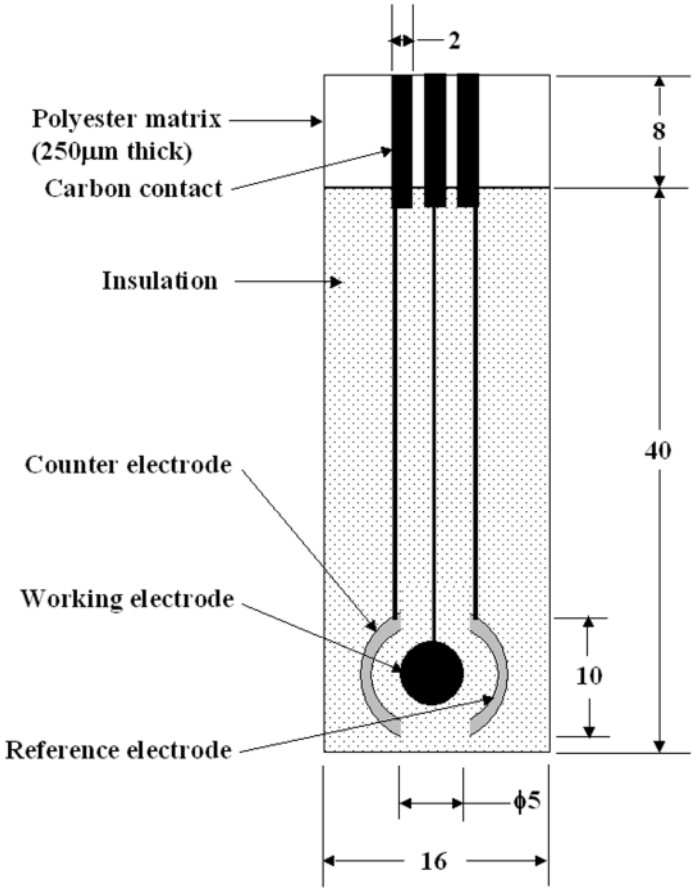
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Insulation
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Counter electrode
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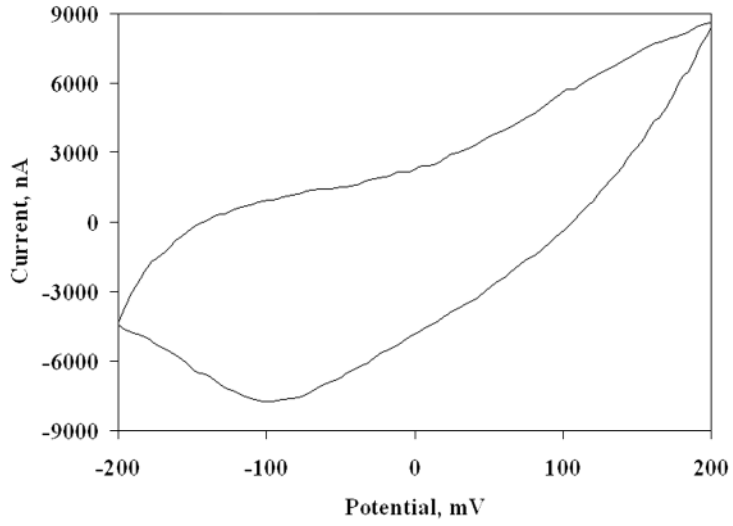
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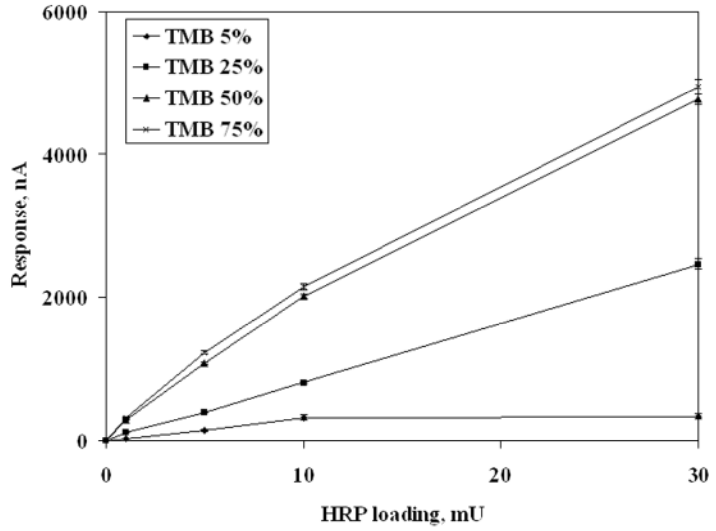
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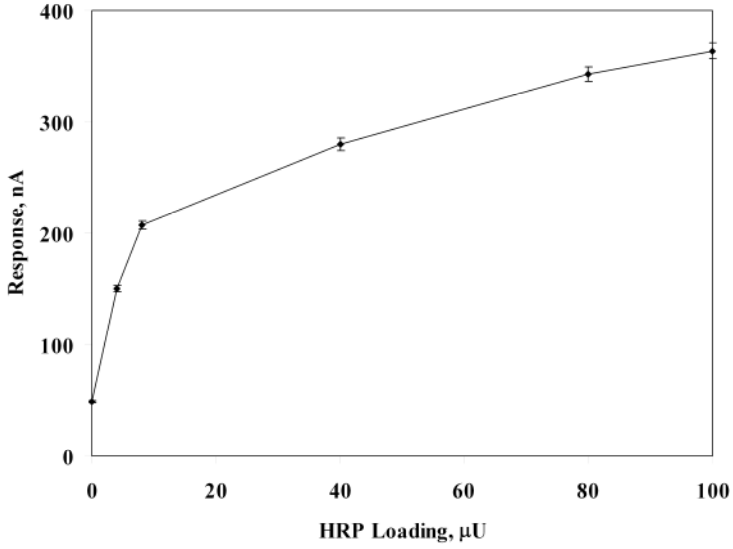
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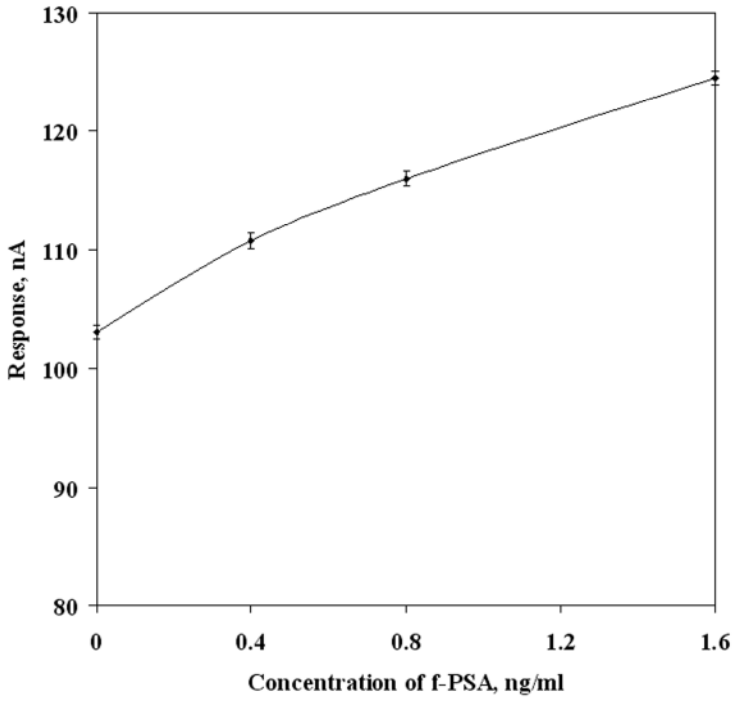
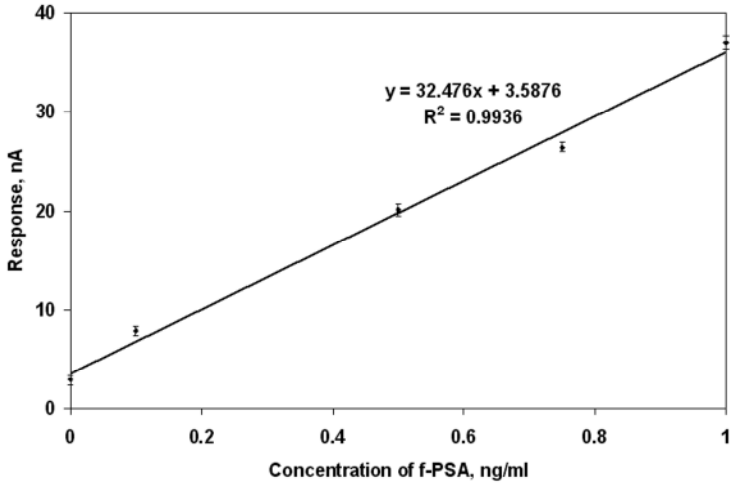
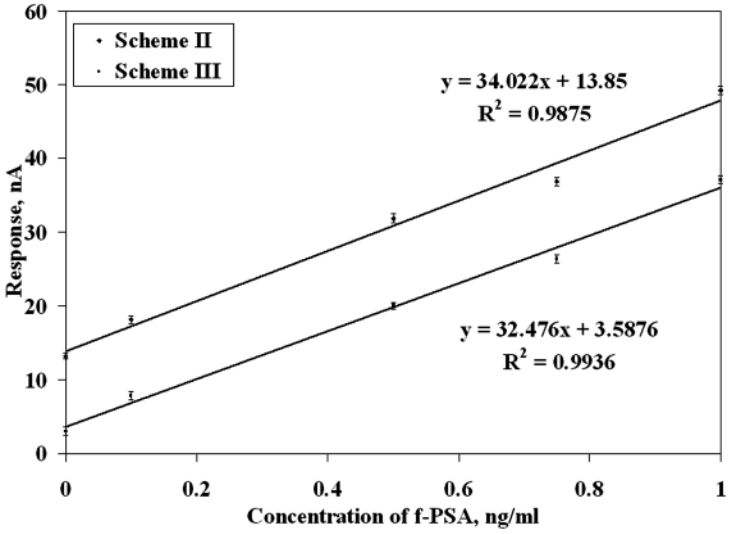
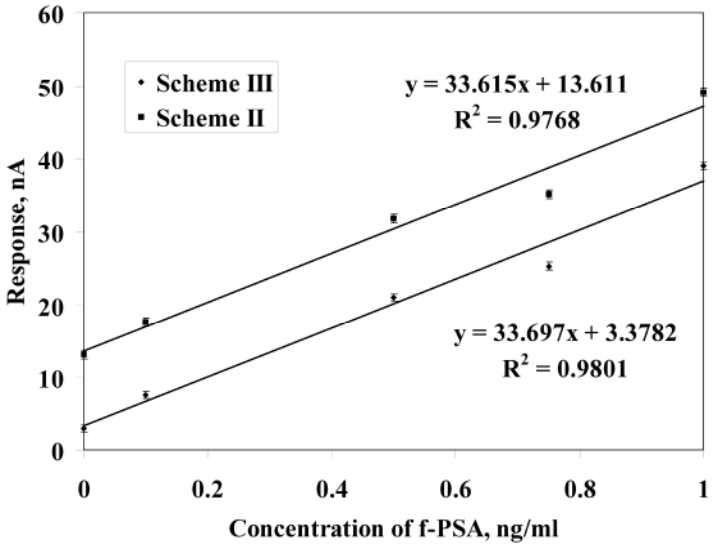
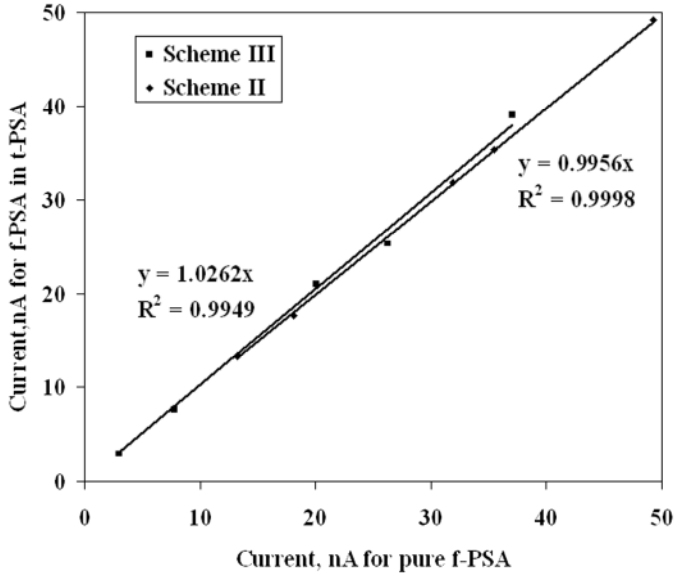

130
120
110
100
90
80
Response, nA
0
0.4
0.8
1.2
1.6
Concentration of f-PSA, ng/ml


40
30
20
10
0
Response, nA
y = 32.476x + 3.5876
R2 = 0.9936
0
0.2
0.4
0.6
0.8
1
Concentration of f-PSA, ng/ml


Scheme II
Scheme III
y = 34.022x + 13.85
R2 = 0.9875
y = 32.476x + 3.5876
R2 = 0.9936
Response, nA
Concentration of f-PSA, ng/ml


Scheme III
Scheme II
y = 33.615x + 13.611
R2 = 0.9768
y = 33.697x + 3.3782
R2 = 0.9801
Response, nA
Concentration of f-PSA, ng/ml


Scheme III
Scheme II
y = 0.9956x
R2 = 0.9998
y = 1.0262x
R2 = 0.9949
Current,nA for f-PSA in t-PSA
Current, nA for pure f-PSA

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Electrochemical Immunoassay for Free Prostate Specific Antigen (f-PSA) Using Magnetic Beads

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