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Amperometric biosensors for detection of the prostate cancer marker (PSA)

Priyabrata Sarkar [a,*], Partha S. Pal [a], Dipankar Ghosh [a], Steve J. Setford [b], Ibtisam E. Tothill [b]

[a] *Department of Polymer Science and Technology, University of Calcutta, 92 A.P.C. Road, Calcutta 700009, India*
[b] *Cranfield Biotechnology Centre, Cranfield University, Silsoe, Bedfordshire, MK45 4DT, UK*

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Abstract

Prostate specific antigen (PSA) has been identified as the most reliable clinical tool for diagnosing and monitoring prostate cancer (CAP). Since, there is no curative therapy available for prostate cancer, detecting the disease at the early stage is the best hope of increasing mortality rate. There are some procedures available for the detection of prostate cancer e.g. Tandem-R PSA, Hybritech Inc. (USA), IMx-PSA Abbott Laboratories (USA). However, these are time consuming and costly. We have developed a very simple and cost effective technique for identification and monitoring of prostate cancer using amperometric immunosensor. PSA is a glycoprotein with 93% peptide and 7% sugar content and isoelectric pH of 6.9. It may exist in the human serum as free (f-PSA) and complex (PSA-ACT) forms. Normally if the total PSA (t-PSA) level is more than 10 ng/ml, CAP is suspected. This paper presents an amperometric detection procedure for t-PSA using three electrode system in which working electrode (WE) is made of hydroxyethyl cellulose (HEC) and rhodinised carbon. The method used is rapid, very easy to use and involves low cost compared with other procedures. The electrochemical response was directly observed due to enzymatic reaction via a sandwich immunoassay on the WE. Monoclonal capture antibody (Mab) to PSA was immobilised on the WE and the other Mab labelled by the enzyme marker, horseradish peroxidase (HRP), was used as a tracer antibody.

Keywords: Biosensor; Prostate Cancer; Prostate Specific Antigen; Electrode; Amperometric; Antibody

1. Introduction

Prostate Carcinoma (CAP) is a deadly malignancy and major cause of death in men population aged between 55 and 80 years. At present, there is no curative therapy available once the disease spreads the limits of the organ. The best way to control and improve mortality rate from CAP is to detect the disease at early stage, while it is localised and organ-confined. Urologists have believed that the digital rectum examination (DRE) was sufficient for the detection of prostate cancer. However, this lacks the desirable sensitiv-

* Corresponding author. Tel.: +91-33-4852-975; fax: +91-33-4852-976.
E-mail address: psarkar@cubmb.ernet.in (P. Sarkar).

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ity and specificity for early CAP detection. Experimental studies investigated that prostate specific antigen (PSA) is a potential marker for CAP and established itself as the most reliable clinical tool for diagnosing and monitoring the disease (Chu, 1994). Although, serum PSA elevations are associated with CAP, these are also due to benign prostatic hypertrophy (BPH). A major use of PSA testing has been to monitor patients diagnosed with CAP and it has been shown to be the most useful serologic test in staging and monitoring CAP especially in the early detection of recurrent disease (Blijenberg et al., 1996).

Although unique, PSA has limitations that prevent it from being the perfect tumour marker for early detection of CAP. Nevertheless, several studies have demonstrated that PSA alone detects a greater number of malignancies than DRE or any other isolated parameter. The majority of tumours detected by PSA have histopathologic features of clinically important, life-threatening cancer. Serum testing for PSA is widely used for early detection and monitoring the therapy of prostate cancer. Kuriyama and others (Kuriyama et al., 1980) described the first immunoassay for PSA, which was a sandwich-type, enzyme-linked immunosorbent assay (ELISA) sufficiently sensitive to detect PSA in the serum of normal men and patients with BPH and prostate cancer.

PSA is a serine protease composed of a single chain glycoprotein. It exhibits proteolytic activity similar to chymotrypsin. The molecular weight of PSA as estimated by sodium dodycyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is about 33–34 kDa. It is composed of 237 aminoacids, with a mol. wt. of 26 079 for the peptide moiety of the molecule (Lilja, 1995). Prostate cancer tissue generally contributes about ten times more to serum PSA levels than does an equal amount of normal prostate tissue. The major forms of PSA in serum are complex with α1-antichymotrypsin (bound) PSA-ACT (90% of all complexed forms) and as a free form (f-PSA). It is also bound to α2-macroglobulin (PSA-AMG) but this form is not immunoreactive. The minor forms include complexes with protein-C inhibitor (PSA-PCI), α1-antitrypsin (PSA-AT), and inter-α trypsin (PSA-IT). Total PSA refers to the sum of all immunodetectable species of PSA, primarily free-PSA and PSA-ACT. The free form is lower in patients with prostate cancer. CAP is present in half of the patients having t-PSA >4 μg/l and 70% for >10 μg/l in blood serum (Blijenberg et al., 1996).

ACT is a major protease inhibitor in the blood occurring at 4–8 ng/ml. Others are available at very low concentrations. Reaction between PSA and ACT results in inactivation of the enzyme. This is due to the formation of covalently stabilised 1:1 molar ratio complexes of about 90 kDa. A number of antigenic epitopes on the f-PSA are not accessible on the PSA-ACT (Chu, 1994).

Lilja (1995) found that the ratio of PSA-ACT to t-PSA was significantly higher in CAP than BPH and ratio of f-PSA to t-PSA was found to significantly lower in CAP. This measurement increases diagnostic specificity by 15–20% compared with usual method of measuring t-PSA. PSA exhibits a mild but unique protease activity, the substrate specificity being different from other known protease. It is a chymotrypsin like enzyme. PSA screening for CAP appears to have produced an acceptable morbidity.

Zhou et al. (1998) reported five major epitopes named A through E on PSA recognised by monoclonal antibodies (MAbs). Monoclonal antibodies, which bind to epitopes A, B and C, appear to react with both free and ACT-complexed PSA. Epitope D appears to be partially occluded by ACT. Epitope E appears to be a free PSA epitope and is completely occluded by PSA-complexation with ACT. Consequently, the Mabs in this epitope group are potentially useful in a sandwich immunoassay for quantification of f-PSA in a serum.

A combination of immobilised and acridinium ester-labelled monoclonal antibodies were used to develop a two step 90-min chemiluminometric assay (Klee et al., 1994). The assay was standardised to match the Abbott Imx PSA assay.

Total PSA values were measured with a microplate ELISA using monoclonal antibody PR1 as capture antibody and horseradish peroxidase (HRP) labelled monoclonal antibody PR12 as tracer antibody. These PR1 and PR12 are iden-

tified as total PSA specific antibodies. There is correlation between this assay and the Tandem-R assay. Free PSA assay was done with a microplate ELISA using the free PSA specific monoclonal antibody 365 as a capture antibody and HRP labelled PR12 as tracer antibody (Elgamal et al., 1996).

Matrix metalloproteinase-2 (MMP-2) zymogen is another marker for CAP. It was shown that by immunoblotting and ELISA the MMP-2 enzyme (MMP-2a) was expressed in CAP and it increased with progression. Monoclonal antibodies specific for MMP-2a were used to investigate the expression of MMP-2a in human (Stearns and Stearns, 1996).

An optical immunosensor was developed (Daniels et al., 1995) using a fluorescence capillary fill device. Three fluorophores had been tried. The clinical data showed that this whole-blood assay correlated well with the Hybritech Tandem-R assay.

A monoclonal antibody based PSA enzyme immunoassay was used in dipstick test by Madersbacher et al. (1996). The dipstick was able to detect 4–10 ng/ml PSA with 75.6% success.

PSA assay calibration was done by Hoffman et al. (1996) using whole blood spotted on filter paper with the help of chemiluminescent immunoassay analyser.

Allard et al. (1998) had developed a novel immunoassay for complexed PSA accurately, and the measured values of free + complexed PSA in artificial mixtures and in patient sera were compared. It was shown that the measurement of PSA-ACT gives more specificity towards the detection of CAP.

Corey et al. (1997) studied binding characteristics of anti-PSA antibodies for better understanding of the distribution of antigenic determinants on the PSA molecules, which can help to identify Mabs that are more likely to distinguish between f-PSA and PSA-ACT. They have characterised ten new monoclonal antibodies against PSA.

An assay specific for PSA-ACT without the problem of high background had been developed by Wu et al. (1998). The assay comprised of a two-site ELISA format using polyclonal antibodies. The assay had a sensitivity of 0.05 ng/ml. Stability of f-PSA and t-PSA in serum of patients with CAP has been studied by Paus et al. (1998). Chen et al. (1997) developed an immunoassay using amperometric method for the detection of serum PSA. However, they used conventional glassy carbon electrodes and flow injection analysis (FIA) requiring elaborate instrumentation.

In spite of the growing interest in measurement of PSA level in patients suspected for CAP, no work has been reported on development of a screen-printed electrode for measuring PSA. We have tried to utilise the ELISA procedure on a screen-printed amperometric electrode and achieved good results. Before we describe our methods, we discuss below the assay techniques presently available.

1.1. Present assay techniques

A series of very specific and sensitive assays have been developed exploiting antigenic epitopes with unique binding characteristics. Today, there are five assays for the total PSA that have the approval of the food and drug administration (FDA) for clinical use: Tandem-R assay (Hybritech), Tandem-E assay (Hybritech), Imx assay (Abbott), Tosoh assay (Tosoh), and Immunolite assay (Diagnostic Products Corporation). The first three are widely used. There is no standardisation of the assay procedure. Tandem-R PSA, Hybritech Inc., uses a solid phase two site immunoenzymetric assay: Samples containing PSA are reacted with a plastic bead coated monoclonal antibody directed toward the PSA molecule, and with an enzyme labelled monoclonal antibody directed against a distinctly different antigenic site on the same PSA molecule. After washing and incubation with enzyme substrate the amount of substrate turnover is measured colorimetrically. IMx-PSA Abbott Laboratories uses monoclonal antibody coated microparticles to capture the PSA analyte and these are then reacted with a polyclonal goat antibody-alkaline phosphates immunoconjugate directed against PSA. After conversion of the substrate the fluorescent product, 4-methylumbelliferone, is measured.

2. Materials and methods

Human PSA, monoclonal antibodies to total PSA (clone: A45510259P and A45080020P) and a test kit containing PSA in human serum were purchased from Biospacific, CA, USA. All chemicals for preparation of buffer, glucose oxidase (GOX) (EC 1.1.3.4 from Aspergillus niger, 18 500 IU/g), HRP (EC 1.11.1.7, RZ 3.1), 2,2′-Azinobis(3-Ethylbenz-thiazoline-6-sulfonic acid) (ABTS), sodium periodate were supplied by Sigma Chemical Co. Ltd., Poole, Dorset, UK and used as received. Hydroxyethyle cellulose (HEC) was supplied by Fluka, (Gillinham, UK) and Sigma-Aldrich (Gillinham, Dorset, UK) supplied Polyethylenimine (PEI) and nafion (5% aqueous solution). The antibodies, antigens, and conjugates were obtained from Biospacific Inc., USA. Protein-G column was obtained from Pharmacia Biotech, Sweden.

3. Immunosensor construction

Three-electrode devices were mass-manufactured by a multi-stage screen-printing process using a DEK 248 machine (DEK, Weymouth, UK) and screens with appropriate stencil designs (60 per screen) fabricated by DEK precision screen division. The stainless steel screen mesh was mounted at 45° to the print stroke with 77 wires per cm and emulsion thickness of 13 and 18 μm for the solvent and water resistant screens, respectively.

Devices were printed onto 250 μm thick polyester sheet (Cadillac Plastic Ltd., Swindon, UK). The circular electrocatalytic working electrode (WE) (planar area: 0.16 cm^2) was fabricated from MCA 4a, a commercially available carbon powder containing 5% rhodium (MCA Services Ltd., Cambs. UK), made into a screen-printable paste by mixing 1:4 and 1:3 in 2.5% w/v HEC in buffer-electrolyte. The reference electrode ink contained 15% silver chloride in silver paste (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 125 °C for 2 h to cure the epoxy resin and to stabilise the electrocatalytic pad to allow prolonged use of the device in aqueous solutions. The electrocatalyst/HEC complex forms a thick, porous pad that is a suitable base for immobilisation of biocomponents (Sarkar et al., 1999; Sarkar, 2000).

4. Polymer deposition

The use of polymer in the construction of biosensor and also as an immobilising agent is well established. A number of polymers had been deposited to immobilise enzymes on the WE either by electropolymerisation or solvent evaporation technique to modify the WE surface evenly which in turn reduced the interference in current response (Emr and Yacynych, 1995). We have tried three polymers namely PEI, nafion, and polypyrrole. The first two were deposited by solvent evaporation technique and polypyrrole was deposited by electropolymerisation. About 5 μl of a 2% solution of PEI in methanol was deposited on the WE and the solvent was evaporated. A thin film of nafion was made by evaporating 5 μl of 5% nafion (as received) on the WE. A thin film of polypyrrole was deposited along with GOX on the WE by electropolymerisation of the monomer from an electrolyte of the monomer and GOX. Pyrrole (0.4 M) was dissolved in 0.05 M phosphate buffer (PB), pH 7 with 0.1 M KCl containing GOX (250 U/ml). Cyclic voltamogrammes (six scans) were taken (0–1.0 V) with a scan rate of 50 mV/s using a platinum reference electrode.

5. Preparation of Ab-HRP conjugate

The conjugate was made following the procedure of Hudson and Hay (1980). Four milligram HRP was dissolved in 1 ml double distilled water to which 200 ml freshly prepared sodium periodate (0.1 M) was added and stirred for 20 min. This was dialysed against 0.001 M sodium acetate

buffer overnight at 4 °C. Twenty millilitres of 0.1 M sodium carbonate buffer was then added to raise pH to 9 to 9.5 and immediately 1 ml of the Ab sample was added and left at room temperature for 2 h. One hundred millilitres of freshly prepared sodium borohydride (4 mg/ml) was mixed and kept at 4 °C for 2 h. The solution was dialysed against borate buffer (0.1 M, pH 7.4) and mixed with an equal volume of 60% glycerol in borate buffer and kept at 4 °C. The separation and purification of the conjugate was carried out by passing through a protein-G column.

6. Test procedure

The test procedure was controlled using an Autolab Electrochemical Analyser with GPES3 software (Ecochemie, Utrecht, The Netherlands). Ten ml of the 0.1 M PB, 0.15 M NaCl, 6 mM KI, 20 mM glucose was kept in a stirred condition. The electrode was dipped and the WE was poised at an appropriate potential. The system was allowed to equilibrate in the presence of sample for 180 s and depending on the format, either reagent was added or left undisturbed for 7 min. The changes in the current were monitored continuously and recorded. Tests were performed at ambient temperature.

Gox : Glucose + O_2 ⟶ H_2O_2 + gluconic acid

Ab1-Ag-Ab2-HRP

Polymer film $H_2O_2 + 2I^- + 2H^+$ ⟶ $I_2 + 2H_2O$

Reactions at WE release electrons

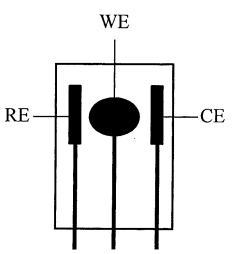

Fig. 1. Schematic diagram of the screen-printed sensor with circular WE on which antibody (Ab1) was immobilised. The WE is made of 5% rhodinised carbon, the reference electrode (RE) is made of 15% Ag/AgCl and the counter electrode (CE) carbon.

7. Experimental methods

7.1. Preparation of antibody electrodes

After polymer deposition as described above, monoclonal antibody (10 μl of 120 ng/ml) to total PSA (i.e. to both PSA-ACT and f-PSA) was immobilised on the WE with 1% glutaraldehyde and 200 mU GOX. These were then washed with 0.1 M PB, pH 7 and remaining active sites were blocked with 0.1 M glycine in 0.1 M PB, pH 7. The electrodes were again washed with buffer and dried and kept at 4 °C until further use.

This research was aimed to develop a rapid, simple and sensitive one-step amperometric detection of t-PSA. The schematic diagram of the sensor and the reactions occurring at the electrode surface are shown in Fig. 1. GOX hydrolyses glucose to produce hydrogen peroxide, whose concentration remains essentially the same around the WE. This hydrogen peroxide is dissociated into water in presence of HRP and KI and the number of electrons released on the WE will depend on the amount of HRP, which in turn depends on the PSA concentration of the analyte. The higher the PSA concentration, higher will be the response. The strategy involved signal amplification by regeneration of the enzymatic substrate within the membrane layer of the electrode and the accumulation of the redox mediators (I_2/I^-) at the membrane/electrode interface (Ivnitski and Rishpon, 1996). The iodine–iodide system, which is reversible with low redox potential and fast electrode kinetics, is stable with time and non-carcinogenic. When the substrate is present in amounts that limit the rate of the overall process, the enzymatic-electrochemical cycling can greatly increase the signal. We have followed two different formats for the immunoassay on the surface of the WE e.g. sandwich and enzyme channelling.

8. Sandwich immunoassay

The analyte containing t-PSA i.e. PSA-ACT and f-PSA (10 μl) was deposited on the WE of the previously described antibody electrode using a suitable 'O' ring and kept for 30 min at room

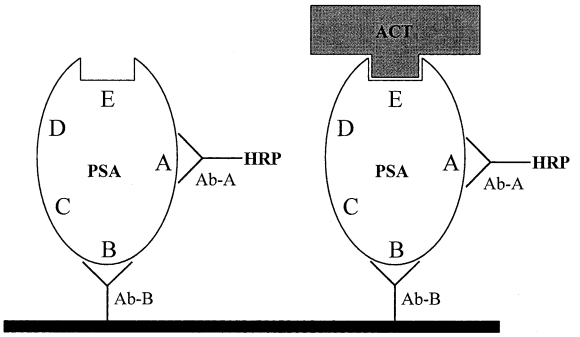

Fig. 2. Typical immunoassay format for total PSA on WE of screen-printed biosensor. Ab-A and Ab-B represent monoclonal antibodies with specific binding sites A and B, respectively.

temperature. The electrodes were then washed with buffer and dried. Aliquot (10 μl) of HRP-labelled monoclonal antibody (tracer) specific to t-PSA (ca. 120 ng/ml) was added in the 'O' ring and incubated for 30 min at room temperature. The electrodes were washed, dried and kept ready at 4 °C for analysis.

The response was measured at − 70 mV versus Ag/AgCl with a 20 mM glucose, 0.1 M PB, 0.1 5 M NaCl, 6 mM KI (Ivnitski and Rishpon, 1996). The response is directly proportional to the concentration of total PSA in the sample. The change in response with time was noted. The change in current after equilibration is proportional to the concentration of t-PSA.

9. Enzyme channelling immunoassay

In this case, antibody specific to t-PSA was immobilised on the WE same as before co-immobilising GOX. Whatman 114 filter were cut into circular discs (1.1 cm diameter) and was placed over the three-electrode assembly which, when wetted with sample, completed the electrochemical circuit. A 40 μl of the same PB containing 20 mM glucose was deposited onto the filter paper. An aliquot of the analyte containing PSA and Ab-HRP conjugate was added after equilibration was attained. Here the conjugate competes with unlabeled antigen to bind to antibodies on the surface of the electrode. The captured conjugate catalyses the reaction of iodide ions with the hydrogen peroxide that is generated by the immobilised GOX. The signal was amplified by enzyme channelling and the current was continuously monitored amperometrically by reduction at − 0.07 versus Ag/AgCl.

10. Results and discussion

A number of epitopes are present on PSA allowing the sandwich immunoassay using monoclonal antibodies having specificity for different epitopes. Fig. 2 shows the schematic illustration for the measurement of t-PSA by sandwich immunoassay on the electrode surface. ACT forms a complex with the PSA by capturing one specific epitope (E) on the free PSA. Ab-A and Ab-B are monoclonal antibodies having specificity for two different epitopes.

Initially experiments were carried out using Ab1-Ag-Ab1-HRP format to observe if any difference in response could be obtained for concentrations of t-PSA. As expected, no difference in the response was observed for different concentrations of t-PSA (Fig. 3).

However, when the capture antibody was changed to Ab2, the response is shown in Fig. 4 for two different types of pei coated electrodes namely 1:4 and 1:3 of MCA4a: HEC. It was observed that MCA4a: HEC of 1:3 gave better results in terms of response than the 1:4 electrodes. Thus, all experiments were conducted with this 1:3 type electrodes. Some experiments were conducted with pei and nafion coated electrodes and it was observed that nafion gave lower response than that given by pei. The results were shown for only pei coated electrodes except for the enzyme channelling immunosensor where the electrode was coated with polypyrrole by electropolymerisation. Fig. 5 shows the response for enzyme channelling sensor using a polypyrrole coated electrode. The results show that there is a distinct difference in response with various concentrations of t-PSA. The application of ELISA is therefore quite satisfactory on a screen-printed electrode.

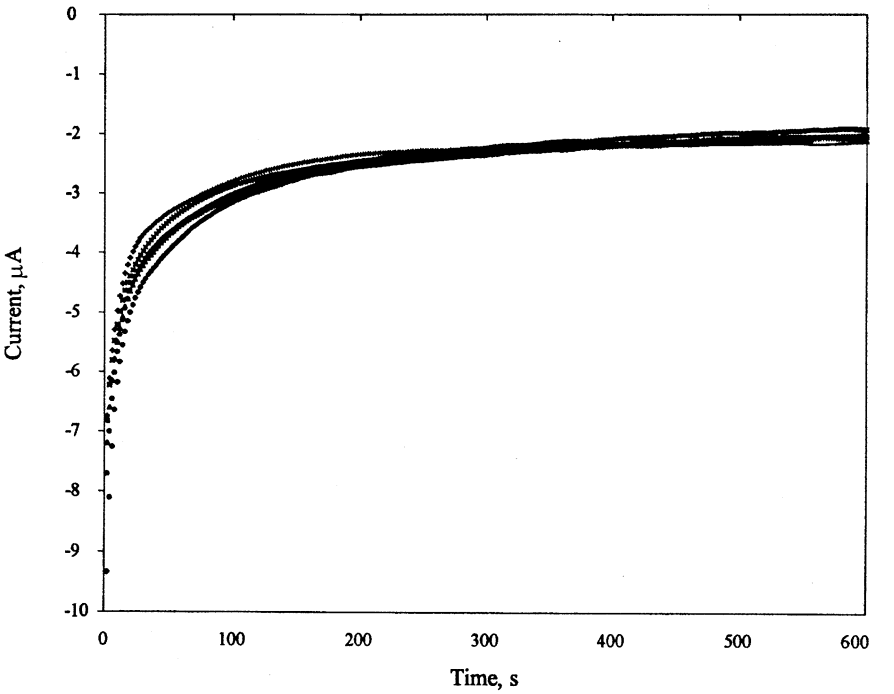

Fig. 3. Transient response of t-PSA electrodes with various concentrations of t-PSA using sandwich (Ab1-Ag-Ab1-HRP) format (− 70 mV across WE in 0.1 M PB, 0.15 M NaCl, 6 mM KI, 20 mM glucose).

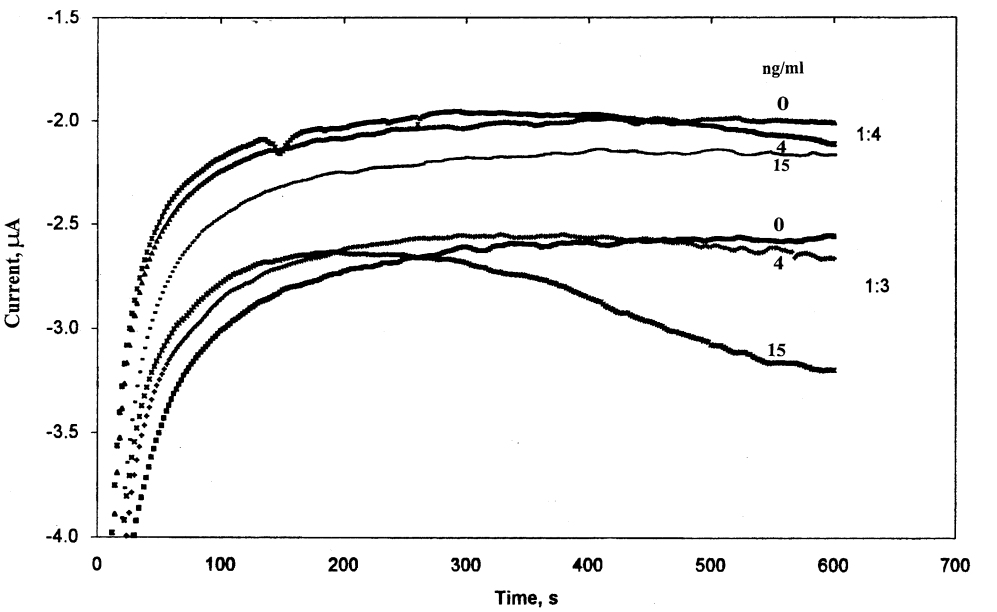

Fig. 4. Performance of the electrodes with different amount of rhodinised carbon i.e. MCA4a: HEC for t-PSA concentrations 0, 4, 15 ng/ml (− 70 mV across WE in 0.1 M PB, 0.15 M NaCl, 6 mM KI, 20 mM glucose).

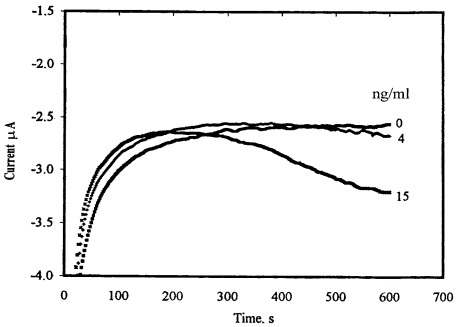

Fig. 5. Current response with enzyme channelling format for 0, 4, 15 ng/ml t-PSA (− 70 mV across WE in 0.1 M PB, 0.15 M NaCl, 6 mM KI, 20 mM glucose).

In another modification, ABTS was used as a substrate for HRP. The peroxidase-catalysed reduction of hydrogen peroxide to water is coupled to a one-electron oxidation of ABTS (Childs and Bardsley, 1975), forming a metastable radical cation. In this case, the electrode (without GOX) was dipped in 10 ml of PB (pH 7.0) having 0.8 mM hydrogen peroxide and was poised at + 350 mV. After equilibration, 10 µl of ABTS solution was added to make the concentration of ABTS 0.7 mM in the buffer. The change in response was noted for farther 2 min. Fig. 6 shows that the change in response with the concentration of PSA. In this case, the time required for measurement was only 2 min. Table 1 shows lower detection limits and coefficient of variation (CV) data for both the methods. All the above experiments have been performed with human serum in triplicate and the CV and limits of detection (IUPAC) are reported in Table 1.

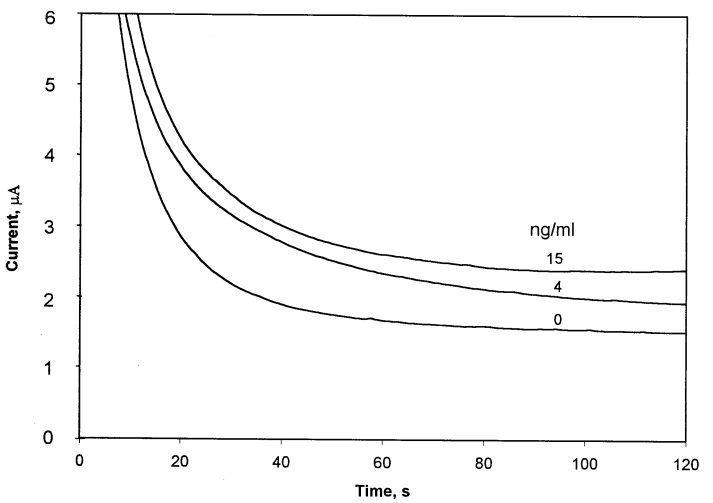

Fig. 6. Current response with ABTS as substrate using sandwich (Ab1-Ag-Ab2-HRP) format (0.35 V across WE, 0.8 mM H_2O_2 in PBS, 0.7 mM ABTS).

Table 1
Statistical data for two different immunoassays used for detection of PSA

Methods of immunoassay	PSA concentration (ng/ml)	CV (%)	Limit of detection (ng/ml)
Sandwich	0	4.46	0.25
	4	5.1	
	1.5	6.2	
Enzyme channelling	0	7.6	0.72
	4	9.1	
	15	5.2	

11. Conclusions

The method of immunoassay on amperometric screen-printed electrode for detection of cancer marker can be used for a new generation of assays for clinical laboratories and may be adapted to screening devices for physicians' offices and even home diagnostics. The most significant achievement of this work is that the sensor may be produced at a very low cost and detection of PSA is made within a short time. Future work will be directed towards measurement of PSA-ACT and f-PSA separately since the ratio of these two is very important to differentiate between BPH and CAP.

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