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Simultaneous detection of free and total prostate specific antigen on a screen-printed electrochemical dual sensor

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ABSTRACT

Voltammetric enzyme dual sensors for simultaneous determination of free and total prostate specific antigen (fPSA and tPSA) are described. Alkaline Phosphatase (AP) and a mixture solution of 3-indoxyl phosphate and silver ions were used as the enzymatic label and substrate, respectively. 8A6 or 5G6 antibodies specific for free and total PSA, respectively, were immobilized on different screen-printed electrodes (SPEs) – screen-printed carbon electrodes, screen-printed gold electrodes and screen-printed carbon electrodes modified with nanogold – in order to be able to select one of the surfaces as the most adequate one to develop the dual sensor. Screen-printed carbon electrodes modified with nanogold were the SPEs with the best analytical characteristics and lead to the most repeatable bioelectrodes, so they were selected for the development of the dual sensor. On *Dualsensor-nAu* electrodes, 8A6 antibody was immobilized on one working electrode and 5G6 antibody was immobilized on the other one by deposition of a drop of solution of each antibody and left overnight at 4 °C. Biotinylated anti-PSA antibody and streptavidin-AP conjugate were used as detection reagents, giving rise, to our knowledge, to the first simultaneous electrochemical biosensor for free and total PSA. The PSA dual sensor was used to monitor PSA production from three different cultures of human androgen-sensitive prostate tumor cells.

1. Introduction

Prostate specific antigen (PSA) is a glycoprotein (93% peptide, 7% sugar) produced by prostate to liquefy the seminal fluid, and is the most reliable tool for diagnosing prostate cancer. PSA in serum circulates non-complexed (free PSA, fPSA, MW 33 KDa) and complexed in several forms, being the predominant one the complex with α1-antichymotripsin (PSA-ACT, MW 90 KDa) (Lilja et al., 1991); total PSA (tPSA) is defined as the combination of both fPSA and PSA-ACT. In prostate cancer diagnosis, a tPSA measurement above 10.0 ng/mL is regarded as positive and indicates high probability of prostate cancer; below 4.0 ng/mL the result is considered negative and indicates low probability of prostate cancer; between 4.0 ng/mL and 10.0 ng/mL the result is in the so-called "grey zone", and fPSA/tPSA ratio indicates cancer specificity; ratios above 0.25 indicate low risk of prostate cancer.

Although there are some approaches that use nucleic acids for PSA detection (Hoshi et al., 1999), most of the methods developed to detect PSA are immunoassays based on different detection protocols. Kuriyama et al. (1980) described the first immunoassay for PSA, which was a sandwich-type enzyme-linked immunosorbent assay (ELISA). Some other ELISA immunoassays for PSA have been reported (Chen et al., 1997; Zhang et al., 2007a,b), as well as other immunoassays based on fluorescence microscopy, surface plasmon resonance (SPR), immunochromatography or surface enhanced Raman scattering (SERS) (Kerman et al., 2007; Cao et al., 2006; Yuhi et al., 2006; Grubisha et al., 2003).

There are several commercial assays (immunoassays) for the total PSA detection for clinical use, i.e. Roche's Elecsys assay, Abbott's IMx assay or Diagnostic Products Corporation's Immulite assay, which are large automatic high-throughput systems used for centralized laboratory testing (Healy et al., 2007). However, the recent emphasis placed on the need for point-of-care (POC) patient management has led to the development of novel biosensor detection strategies that are suitable for the miniaturization of assays for PSA. The development of biosensors for the measurement of PSA has almost exclusively used the immunosensor format, binding PSA by high-affinity antibodies to form the recognition element of the devices.

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Immunosensor technology makes use of immunochemicals as molecular recognition elements to construct self-contained devices that also comprise a physicochemical transducer. Due to the high affinity constants of the antigen–antibody interactions, most of them are single-use systems. In spite of the high selectivity and sensitivity that immunosensors normally exhibit, the lack of stability, limited reproducibility and the necessity for the addition of external reagents of most of the approaches developed so far are probably the major reasons for their limited commercial success.

A number of immunosensor designs for the detection of PSA that rely on optical (fluorescence, laser-based) (O'Neill et al., 1995; Wu et al., 2001), electrochemical (Sarkar et al., 2002, 2008; Zhang et al., 2007b; Panini et al., 2008; Okunoa et al., 2007; Liu et al., 2008; Liu, 2008; Jie-Hua et al., 2008) and piezoelectric (Lee et al., 2005; Zhang et al., 2007a; Ding et al., 2008) transduction modes have been reported.

In an effort to provide a POC alternative to centralized PSA testing, immunochromatographic membrane tests, also known as immunostrip tests, based on capacitance, colorimetric or electrochemical measurements have been developed (Fernández-Sánchez et al., 2004a,b, 2005; Liu et al., 2007). The major advantage of immunostrip devices is their low cost, robust nature and ease of use. Although there are some commercial immunostrip PSA tests (Biosign, Chembio, Medpro), their main drawback is a general lack of sensitivity and, at best, semi-quantitative nature.

An alternative for POC analysis is screen-printed electrodes-based biosensors. Screen-printed electrodes are versatile tools for biosensor development. The screen-printing microfabrication technology is nowadays well established for the production of thick-film electrochemical transducers. This technology allows the mass production of reproducible yet inexpensive and mechanically robust strip solid electrodes. Other important features that these electrodes exhibit are related to the miniaturization of the corresponding device along with their ease of handling and manipulation in a disposable manner, as well as the possibility of developing sensitive and quantitative methodologies. Sarkar et al. (2002, 2008) developed the first immunosensor for PSA (total PSA) on screen-printed electrodes, using a carbon working electrode and amperometric detection, and then developed another approach for free PSA.

In this work we report the development of an immunosensor for the determination of free PSA and an immunosensor for the determination of total PSA using and comparing different kinds of screen-printed electrodes. Furthermore, we report the development of a novel dual sensor for the simultaneous determination of free and total PSA. Indirect detection of PSA based in the use of Alkaline Phosphatase (AP) as an enzyme label is accomplished using a substrate solution that combines an indoxyl compound, called 3-indoxyl phosphate (3-IP), and silver ions. Although the methodology developed in this work does not strictly adheres to the POC area, it is a first step toward the first POC PSA detection on screen-printed electrodes.

2. Experimental

2.1. Materials

Gold tetrachloroaurate ($AuCl_4^-$) was obtained from Merck. Tris(hydroxymethyl)aminomethane (Tris), magnesium nitrate, silver nitrate and streptavidin labeled with alkaline phosphatase (Strp-AP) were purchased from Sigma. 5% Alkali soluble casein (casein) was obtained from Novagen and 3-indoxyl phosphate disodium salt (3-IP) was purchased from Biosynth. $AuCl_4^-$ dilutions were prepared in 0.1 M HCl. Casein was diluted in 0.1 M Tris–HNO_3 pH 7.2. Working solutions of Strp-AP were prepared in 0.1 M Tris–HNO_3 pH 7.2 containing 2 mM $Mg(NO_3)_2$. A mixture solution of 5.6 mM 3-IP and 0.4 mM silver nitrate (Substrate) was prepared daily in 0.1 M Tris–HNO_3 pH 9.8 containing 20 mM $Mg(NO_3)_2$, and stored in opaque tubes at 4 °C.

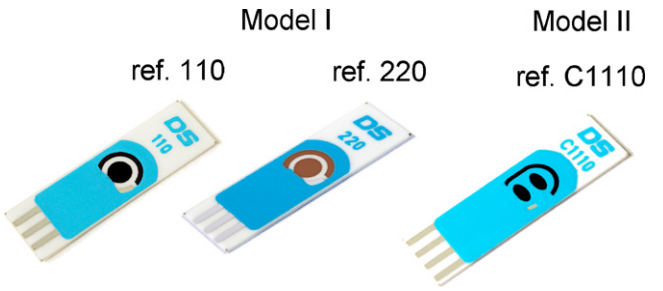

Fig. 1. Models of screen-printed electrodes used: Model I (single-working electrode): ref. 110- Screen-Printed Carbon Electrode (SPCE), ref. 220- Screen-Printed Gold Electrode (SPGE); Model II (two working electrodes): ref. C1110- Screen-Printed Carbon Dual sensor (dual sensor).

Mouse monoclonal anti-PSA antibody 8A6 (capture antibody specific for free PSA) and mouse monoclonal anti-PSA antibody 5G6 (capture antibody used to detect total PSA) were purchased from Abcam; mouse monoclonal biotinylated anti-PSA antibody PSA10 (detecting antibody for both free and total PSA) was obtained from Fujirebio Diagnostics. PSA standard solutions, calibrated against the International Standard Stamey 9010, were obtained from Fujirebio Diagnostics. Working solutions of anti-PSA antibodies and PSA were made in 0.1 M Tris–HNO_3 pH 7.2.

2.2. Apparatus and electrodes

SEM images were taken with a JEOL JSM-6100 scanning electron microscope.

Voltammetric experiments were performed with an Autolab PGSTAT 10 (bi)potentiostat controlled by the Autolab GPES software version 4.8 for Windows 98; Autolab GPES Multichannel software was used in bipotentiostat mode. All measurements were carried out at room temperature.

SPEs were obtained from DropSens. Two different electrode designs were used, one (Model I) with a single-working electrode and other one (Model II) with two working electrodes (dual sensor). Fig. 1 shows the models.

The single-working electrode model comprises a carbon (screen-printed carbon electrode, SPCE) or gold (screen-printed gold electrode, SPGE) disk-shaped working electrode (12.6 mm^2), a carbon or gold, respectively, counter electrode and a silver pseudo-reference electrode, all of them screen-printed on a ceramic substrate (3.4 cm × 1.0 cm); a dielectric paste is screen-printed over the electrode system, leaving uncovered the three-electrodes area of 7 mm × 6 mm (electrochemical cell with volume of 50 μL) and the electric contacts.

The dual sensor comprises two ellipse-shaped carbon working electrodes (6.3 mm^2 each one), a carbon counter electrode and a silver pseudo-reference electrode, all of them screen-printed on a ceramic substrate (3.4 cm × 1.0 cm); a dielectric paste is screen-printed over the electrode system and covers all the surface excepting the electrodes, in order to facilitate independent coating of each working electrode (the electrochemical cell is delimited by counter and reference electrodes).

Two different edge connectors from DropSens were used: a three-electric contacts connector for single-working electrode models and a four-electric contacts connector for the dual sensor.

2.3. Cell culture preparation

Three cell cultures of human androgen-sensitive LNCaP prostate tumor cells (used in prostate cancer research) were kindly provided

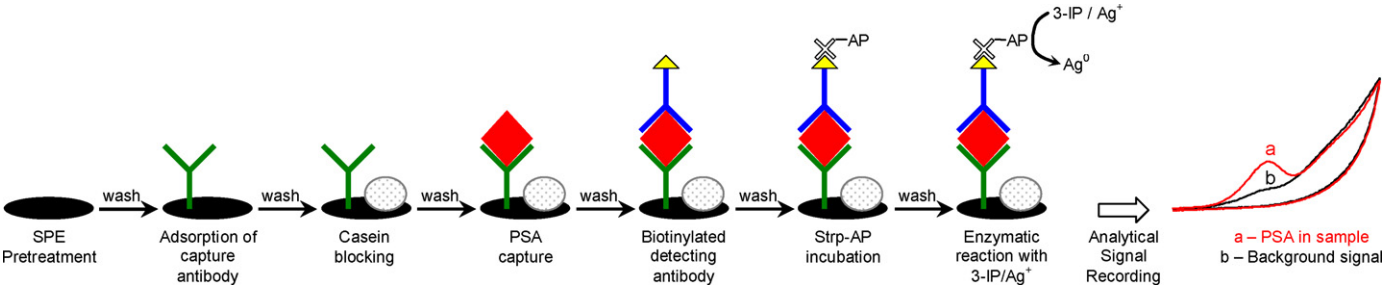

Fig. 2. Schematic representation of the analytical procedure followed for the detection of PSA.

by IUOPA (Instituto Universitario Oncológico del Principado de Asturias–Oncologic University Institute of Principado de Asturias).

The first culture (denoted as *CON*) were LNCaP cells maintained in RPMI 1640 culture medium supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 15 mM HEPES and an antibiotic–antimycotic cocktail containing 100 U/mL penicillin, 10 μg/mL streptomycin and 0.25 μg/mL amphotericin B. This cell line was grown at 37 °C in a humified 5% CO_2 atmosphere and the medium was changed every 2 days.

In a second culture (denoted as $FBS_{CH\text{-}ST}$), charcoal-stripped FBS was used instead of normal complete FBS. Serum was stripped from small molecular weight molecules such as androgens; for this purpose, FBS was incubated with a mixture of 2.5% charcoal activated and 0.025% Dextran T 70 overnight at 4 °C and then centrifuged twice at 5000 × *g* to eliminate charcoal/dextran from $FBS_{CH\text{-}ST}$, which was then added to the culture medium and filtered through a 0.22 μm sterile filter.

In the third culture (denoted as *DHT*), dihidrotestosterone, the active form of testosterone, was used at 10 nM (added to the culture medium from a 10 μM stock solution in 100% DMSO).

2.4. Methods

The general scheme for PSA detection is shown in Fig. 2.

Different SPEs were tested and their analytical characteristics toward PSA detection were compared; these SPEs were: *SPCEs*, *SPGEs* and SPCEs modified with nanogold (*SPC-nAu-Es*). Also, PSA sensors were developed on dual sensors modified with nanogold (*Dualsensor-nAu*).

Gold nanostructuration of carbon surfaces was achieved placing a 50 μL drop of 0.1 mM $AuCl_4^-$ on the SPCE or on the dual sensor, and applying a cathodic current of −100 μA for 4 min; then a potential of −0.1 V for 2 min was applied. Fig. 3 shows an electron micrograph of the gold-nanostructured carbon surface (average diameter of gold nanoparticles is 70 nm) compared to one of the unmodified surface.

The different steps of the analytical procedure showed in Fig. 2 were as follows:

2.4.1. Electrode pretreatment

An electrode pretreatment was carried out with the aim of improving the repeatibility of the results. The pretreatment applied for each type of SPE was different:

SPCEs: A 50 μL drop of 0.1 M H_2SO_4 was placed on the SPCEs, and an anodic current of +5.0 μA was applied for 2 min. Then the electrodes were washed using 0.1 M Tris–HNO_3 buffer pH 7.2.

SPGEs: A 50 μL drop of 0.1 M H_2SO_4 was placed on the SPGEs and ten cyclic voltammograms from 0 to +1.25 V were recorded, using a scan rate of 100 mV/s. Then the electrodes were washed using 0.1 M Tris–HNO_3 buffer pH 7.2.

Gold nanostructured carbon electrodes (*SPC-nAu-Es* and *Dualsensor-nAu*) did not need any additional pretreatment.

2.4.2. Immobilization of capture antibody onto the electrode surface

On single-working electrode models, immobilization of 8A6 or 5G6 antibodies was performed placing an aliquot of 40 μL of a 6 μg/mL antibody solution (on *SPCEs*) or of a 10 μg/mL antibody solution (on *SPGEs* and *SPC-nAu-Es*), and leaving it overnight at 4 °C.

On *Dualsensor-nAu* electrodes, 8A6 antibody was immobilized on one working electrode and 5G6 antibody was immobilized on the other one, in order to perform the simultaneous detection of free PSA and total PSA; aliquots of 4 μL of 50 μg/mL of each antibody solution were dropped on their corresponding working electrodes and left overnight at 4 °C.

In all cases, after capture antibody immobilization, the electrode was washed with 0.1 M Tris–HNO_3 buffer pH 7.2 to remove the excess of antibody, and finally free surface sites were blocked

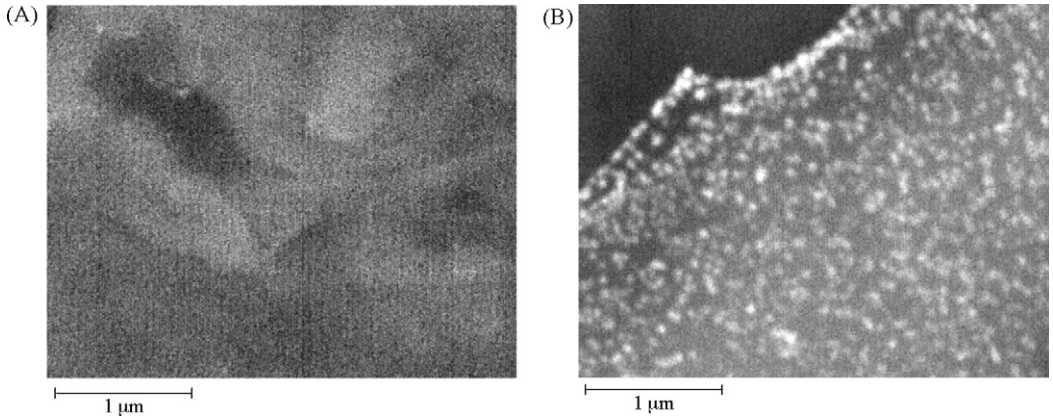

Fig. 3. Electron micrographs (recorded in retrodispersed mode) of a section (3.35 μm × 2.70 μm) of the carbon electrode surface before gold nanostructuration (A) and after gold nanostructuration (B).

placing a drop of 40 μL of a 2% (w/v) solution of casein for 20 min followed by a washing step with 0.1 M Tris–HNO_3 pH 7.2.

2.4.3. Immunoassay procedure

Once the sensing phase was formed, an aliquot of 40 μL of PSA solution was placed on the electrode surface for 60 min and then the electrodes were washed with 0.1 M Tris–HNO_3 pH 7.2. Afterwards, a drop of 40 μL of 300 ng/mL (on *SPCEs*, *SPC-nAu-Es* and *Dualsensor-nAu*) or of 100 ng/mL (on *SPGEs*) biotinylated anti-PSA detecting antibody PSA10 was placed onto the electrode surface for 60 min and then the electrodes were rinsed with Tris–HNO_3 pH 7.2 containing 2 mM $Mg(NO_3)_2$. Finally, an aliquot of 40 μL of 5×10^{-10} M Strp-AP (on *SPCEs*, *SPC-nAu-Es* and *Dualsensor-nAu*) or of 1×10^{-9} M Strp-AP (on *SPGEs*) was dropped on the electrodes for 60 min, followed by a washing step with Tris–HNO_3 pH 9.8 containing 20 mM $Mg(NO_3)_2$ to remove the excess of protein.

2.4.4. Enzymatic reaction and metallic silver deposition

The enzymatic reaction was carried out by dropping an aliquot of 35 μL of a mixture of 5.6 mM 3-IP and 0.4 mM silver nitrate solution for 20 min, protected from light.

2.4.5. Analytical signal recording

The analytical signal was then recorded using Linear Sweep Voltammetry (LSV); the SPEs were held at a start potential (−0.12 V for *SPCEs*; 0.00 V for *SPGEs*, *SPC-nAu-Es* and *Dualsensor-nAu*) for 3 s, and then a voltammogram was recorded from the start potential to +0.4 V at a scan rate of 50 mV/s.

3. Results and discussion

In this work detection of free PSA and total PSA was studied and optimized for the three types of single-working electrode SPEs: *SPCEs*, *SPGEs* and *SPC-nAu-Es*. The same optimization studies were performed for each SPE, in order to be able to select one of the surfaces and develop a dual sensor able to detect simultaneously fPSA and tPSA.

3.1. Free PSA and total PSA detection approach

Epitopes in PSA molecule can be divided in two groups, depending on their position in the glycoprotein: one group are the so-called "hidden epitopes", those covered by α1-antichymotripsin (ACT) in PSA–ACT complex and, therefore, not accesible to react with the antibodies when PSA is complexed to ACT; other group are the so-called "exposed epitopes", those able to be detected by the antibodies in PSA–ACT complex because they are not covered by ACT. For this reason, antibodies against hidden epitopes are used to detect fPSA, while antibodies against exposed epitopes are used to detect tPSA. The approach followed in this work was the formation of a different sensing phase for fPSA and tPSA (using 8A6 antibody for fPSA – detects a hidden epitope – and 5G6 antibody for tPSA – detects an exposed epitope –), and then the same immunoassay procedure using PSA10 antibody, a biotinylated antibody against an exposed epitope, for both detections.

3.2. Electrochemical detection scheme

The electrochemical detection scheme proposed in this work relies on an enzyme-catalyzed metal precipitation, a metal enhancement technique based on an enzymatic process that enables metal deposition at the enzyme, thus allowing the development of different detection strategies in biosensor designs (Pavlov et al., 2005; Moller et al., 2005).

In this work we use a mixture solution of 3-indoxyl phosphate and silver ions as substrate of alkaline phosphatase (Fanjul-Bolado et al., 2007). In the presence of AP, 3-IP is hydrolyzed giving an indoxyl intermediate, which is oxidized by atmospheric oxygen to give indigo blue, the final enzymatic product. However, the indoxyl intermediate can act as a reducing agent, and is able to reduce silver ions to metallic silver, which is deposited on the electrode surface. Then, silver stripping allows the indirect detection of AP and, hence, of PSA.

Detection of enzymatically deposited silver is achieved recording an anodic voltammetric scan, where silver stripping peak appears. For this reason, it is important to select an adequate start potential where silver is not electrochemically deposited, which would cause background signals due to non-enzymatically generated metallic silver. Start potential was studied for each type of SPE recording cyclic voltammograms starting at different potentials (from −0.20 V to 0.00 V) and reversing at +0.40 V, at a scan rate of 50 mV/s. On *SPCEs*, a start potential of −0.12 V was enough to avoid silver electrodeposition, but on *SPGEs* and *SPC-nAu-Es* it was necessary to start the anodic scan at 0.00 V because more negative potentials provoked silver stripping peaks.

3.3. Optimization of the experimental variables

Each experimental variable that affects the immunosensor response was optimized for the three SPEs. Studies on *SPC-nAu-Es* are shown below, but Table 1 summarizes the results for all the SPEs.

Preliminary studies showed that if free surface sites were not blocked with casein after capture antibody immobilization, nonspecific adsorptions of PSA10 biotinylated antibody and, mainly, of streptavidin labelled with alkaline phosphatase occurred. Different concentrations of casein were assayed, finding that a 2% casein solution dropped on the electrode surface for 20 min was necessary to avoid the nonspecific adsorptions (data not shown).

The immobilization of capture antibodies on the electrode surface occurred through direct physical adsorption of the molecules. Different concentrations between 3 μg/mL and 20 μg/mL of 8A6 antibody and 5G6 antibody were tested. These studies were performed using a PSA10 biotinylated antibody concentration of 300 ng/mL and a Strp-AP concentration of 5×10^{-10} M; incubation time of all steps was 60 min. The peak current increases with the 8A6 concentration up to 6 μg/mL where it shows a much lower increase, while background signals do not increase (data not shown). A concentration of 10 μg/mL was selected for further studies with this electrode. A similar result was obtained for 5G6 antibody.

The influence of the PSA10 biotinylated antibody concentration on the analytical signal was studied between 50 ng/mL and 600 ng/mL. In this study the concentration of Strp-AP was 5×10^{-10} M and incubation time of all steps was 60 min. The peak current slightly increases with the PSA10 biotinylated antibody concentration and background signals do not increase (data not shown). A good signal to background ratio was obtained at 300 ng/mL with a moderate reagent consumption, so a concentration of 300 ng/mL was used in further studies with this electrode.

The Strp-AP concentration was also studied. The peak current increases upon raising the Strp-AP concentration but for concentrations higher than 5×10^{-10} M an appreciable increase in the background current was observed due to the nonspecific adsorption of Strp-AP on the electrode surface (data not shown). Therefore a Strp-AP concentration of 5×10^{-10} M was chosen for further work.

Using the conditions shown in Table 1 for each kind of electrode, calibration plots for fPSA and tPSA were performed with PSA standard solutions. Linear plots were obtained for all cases. The results are shown in Table 1.

Table 1
Optimized experimental variables that affect the immunosensor response and analytical characteristics of the calibration plots for tPSA and fPSA for the three SPEs.

		[anti-PSA] μg/mL	[PSA10] ng/mL	[Strp-AP] nM	Linear range (ng/mL)	Slope μA/(ng/mL)	*r*
SPCE	tPSA	6	300	0.5	10–100	0.587	0.990
	fPSA	6	300	0.5	10–100	0.377	0.999
SPGE	tPSA	10	100	1	10–100	0.496	0.990
	fPSA	10	100	1	10–100	0.277	0.990
SPC-nAu-E	tPSA	10	300	0.5	1–10	12.300	0.994
	fPSA	10	300	0.5	1–10	6.081	0.992

As can be seen, only with *SPC-nAu-Es* is possible to detect PSA levels in the required 1–10 ng/mL range, giving rise to a limit of detection of 1 ng/mL (calculated as the PSA concentration yielding a signal three times the standard deviation of the blank response), and a relative standard deviation of 7.0% ($n = 6$, $i_p = 27.53$ μA, 5 ng/mL fPSA). Moreover, sensitivity of the biosensor improves considerably with these electrodes. Gold nanoparticles seem to be the responsible of the improved performance of the bioelectrodes. It is known that gold enhances protein adsorption if compared with carbon. On the other hand, gold nanoparticles formed *in situ* are probably most adequate for protein adsorption. The nanogold surface is quite different from bulk flat gold; nanogold particles have very high surface to volume ratio, high surface energy and are very active, so they can strongly act with protein molecules. Nowadays it is well established that the performance of biosensors depends greatly on the influence imposed on biomolecules by immobilization. The ability of gold nanoparticles to provide a suitable microenvironment for stable immobilization of increased amount of biomolecules retaining their bioactivity is a major advantage for the preparation of biosensors with enhanced analytical performance with respect to other biosensor designs (Pingarrón et al., 2008).

For this reason, nanogold modified screen-printed carbon electrodes were selected as the most suitable electrodic surface to develop further studies.

3.4. Simultaneous detection of fPSA and tPSA

The simultaneous detection of fPSA and tPSA levels was achieved using the gold modified screen-printed carbon dual sensor (*Dualsensor-nAu*). This was accomplished coating one of the working electrodes with 8A6 and the other one with 5G6 antibodies, and then performing the analytical procedure, so one of the working electrodes gives the analytical signal corresponding to free PSA and the other one the analytical signal corresponding to total PSA, respectively.

One of the main drawbacks when working with electrochemical multisensor platforms is the diffusion of the electroactive indicator to adjacent electrodes, giving rise to false results. However, the substrate used in this work overcomes completely this problem because the measurable product is silver precipitate, which deposits only on the surface of each electrode, avoiding product migration from one electrode to other. Fig. 4A shows the cyclic voltammograms recorded on a *Dualsensor-nAu* where working electrode 1 was coated with 5G6 antibody and working electrode 2 was not coated with capture antibody, demonstrating that no silver migration occurs from the coated electrode to the uncoated one; for comparison purposes, Fig. 4B shows the cyclic voltammograms obtained when both electrodes were coated, one of them with 5G6 antibody and the other one with 8A6 antibody.

The experimental conditions used with *Dualsensor-nAu* were the same as those optimized for *SPC-nAu-Es* excepting the capture antibody coating; the smaller and independent working electrode area allows using only 4 μL, so the antibody concentration was increased up to 50 μg/mL in order to use the same density of capture antibody than in *SPC-nAu-Es*, i.e. approximately 32 ng/mm^2.

The following calibration plots were obtained in the range 1–10 ng/mL:

tPSA: $i_p(\mu A) = 4.40\ [PSA](ng/mL) + 11.32$; $r = 0.999$
fPSA: $i_p(\mu A) = 2.53\ [PSA](ng/mL) + 0.27$; $r = 0.991$

showing the feasibility of simultaneous detection of fPSA and tPSA without loss of performance if compared with *SPC-nAu-Es*.

3.5. Test with real samples

The PSA dual sensor was used to monitor PSA production from three different cultures of human androgen-sensitive LNCaP prostate tumor cells (used in prostate cancer research), denoted as *CON*, *FBS$_{CH-ST}$* and *DHT*, and previously described in Section 2.

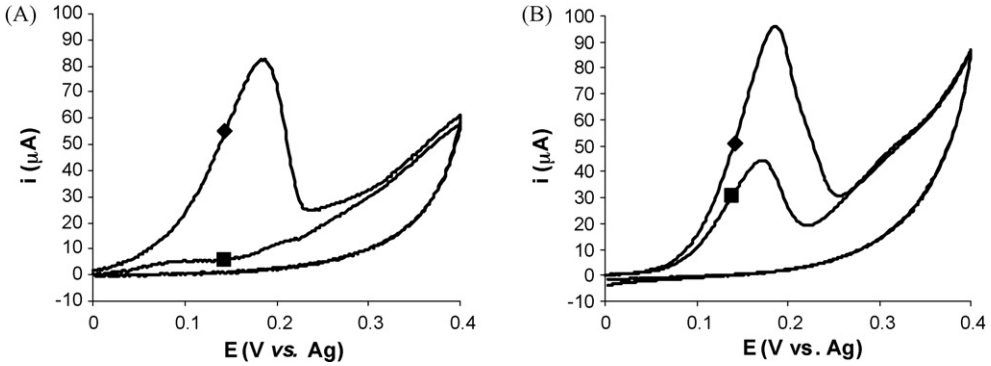

Fig. 4. Cyclic voltammograms recorded on a *Dualsensor-nAu* where one of the working electrodes (♦) was coated with 5G6 antibody (50 μg/mL) and the other working electrode (■) was not coated with capture antibody (A) or was coated with 8A6 antibody (50 μg/mL) (B). [PSA]: 20 ng/mL; [PSA10]: 300 ng/mL; [Strp-AP]: 5×10^{-10} M. Incubation time of all steps: 60 min.

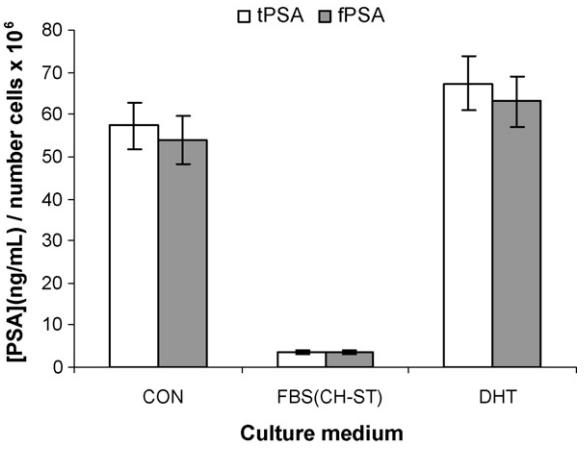

Fig. 5. tPSA (white bars) and fPSA (black bars) levels in one week *CON*, $FBS_{CH\text{-}ST}$ and *DHT* cultures. Total number of cells/mL, *CON:* 315555; $FBS_{CH\text{-}ST}$: 234889; *DHT*: 306222. Data given as average ± SD ($n = 3$).

One week after cultures preparation, cells were counted and serum samples were collected from the three different cultures, they were diluted 1/2 and then PSA levels were measured with the dual sensor. Before that, a calibration plot in the range 1–10 ng/mL was obtained using the FBS culture medium spiked with PSA standards; the results for tPSA: $i_p(\mu A) = 4.21$ [PSA](ng/mL) + 16.40 ($r = 0.990$) and for fPSA: $i_p(\mu A) = 1.61$ [PSA](ng/mL) + 2.84 ($r = 0.990$) showed that no matrix effect was observed if compared with calibration plots calculated in buffer solution.

Fig. 5 represents PSA levels (in terms of the ratio [PSA]/number of cells) for the three cultures, showing lower levels in $FBS_{CH\text{-}ST}$ and higher levels in *DHT* than those in *CON*. This is in agreement with the fact that $FBS_{CH\text{-}ST}$ lacks of androgens, molecules resposible of PSA production, so PSA levels decrease; on the other hand, dihitrotestosterone promotes PSA production, so PSA levels increase in *DHT* culture. Finally, as can be seen, PSA detected is free PSA, as in the culture medium there are no protein binding molecules to PSA. These results demonstrate that the PSA sensor can be used in these cancer research studies, as an alternative to the classical used Western Blot tests, which would improve and accelerate the research.

4. Conclusions

Simultaneous detection of free and total Prostate Specific Antigen can be performed with the screen-printed electrochemical dual sensors developed in this work, based on an immunosensor format with final enzymatic label. Gold nanoparticles, *in situ* generated by controlled electrochemical deposition of gold on screen-printed carbon electrodes, allow the detection of PSA within the required clinical range (1–10 ng/mL), thus providing a tool to perform the simultaneous analysis on a single device. So, one single test offers the data for fPSA, tPSA and the valuable ratio fPSA/tPSA, which, to our knwoledge, had not been described before in an electrochemical biosensor format.

The simultaneous detection of the enzymatic product has been achieved designing a specific dual sensor model on screen-printed carbon electrodes and using a combination of 3-indoxyl phosphate and silver ions as electrochemical substrate of alkaline phosphatase, which gives rise to metallic silver as product of the enzymatic reaction; silver particles deposit specifically on the electrode surface where are generated, avoiding diffusion that would lead to false results.

The PSA dual sensors have been tested to monitor PSA production from tumor cells, as an alternative to the classical Western Blot analysis. Now work is in progress testing the dual sensors in human samples and trying to shorten the analysis time, as well as testing different nanostructuration pathways; early very preliminary results not shown in this paper seem to indicate that nanoparticles with bigger mean diameter offer better responses, thus indicating that more sensitive devices could be developed. All this would allow in the future the development of easy-to-use and portable devices for the rapid monitoring of PSA.

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