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Testing the variability of PSA expression by different human prostate cancer cell lines by means of a new potentiometric device employing molecularly antibody assembled on graphene surface

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ABSTRACT

Prostate Specific Antigen (PSA) is widely used as a biomarker for prostate cancer. Recently, an electrochemical biosensor for PSA detection by means of molecularly imprinted polymers (MIPs) was developed. This work evaluated the performance and the effectiveness of that PSA biosensor in screening the biomarker PSA in biological media with complex composition, collected from different human prostate cell line cultures. For that, the prostate cancer LNCaP and PC3 cells, and the non-cancerous prostate cell line PNT2 were cultured for 2, 7 and 14 days in either α-MEM or RPMI in the presence of 10% or 30% fetal bovine serum. Human gingival fibroblasts were used as a non-cancerous non-prostatic control. The different culture conditions modulated cellular proliferation and the expression of several prostate markers, including PSA. The electrochemical biosensor was able to specifically detect PSA in the culture media and values obtained were similar to those achieved by a commercial Enzyme-Linked Immunosorbent Assay (ELISA) kit, the most commonly used method for PSA quantification in prostate cancer diagnosis. Thus, the tested biosensor may represent a useful alternative as a diagnostic tool for PSA determination in biological samples.

1. Introduction

Prostate cancer (PCa) is the third most common cancer diagnosed in men in Europe, with an incidence of 416.7 cases per 100,000 men and 92.2 deaths [1]. Its early detection is fundamental for the successful treatment of the disease, currently established by monitoring Prostate Specific Antigen (PSA) in serum.

PSA is a glycoprotein that belongs to the kallikrein family of proteases, with an androgen-regulated serine protease function. It has several isoforms, with isoelectric points ranging from 6.8 to 7.2 [2]. It is mainly produced by prostate secretory epithelia and its gene is located in chromosome 19, at locus 19q13.4 [3]. Although PSA is secreted in the seminal plasma of healthy man, low levels of PSA may be found in the blood. Nowadays, the PSA quantification test measures the total amount of PSA in the blood, and the generally accepted upper cut-off level of PSA to be considered normal is 4.0 ng/mL [2].

Despite its utilization as, at the present, the most used PCa biomarker, it is well known that PSA production and secretion are affected by a high number of factors, like several physiological/pathological conditions, as well as a consequence of different therapeutic approaches [4]. Moreover, several types of non-prostatic neoplastic cells can express PSA [5]. Also, among cancer cells, expression of PSA is very variable and, further, it appears to be significantly affected by the surrounding environment [4,6]. Due to this significant variability, it is important to have accurate and reliable methods for PSA detection within a broad range of protein concentrations, in biological fluids with complex composition.

Several detection methods based on biosensors for PSA have been reported, although they use different transduction modes, ranging from electrochemical [7], optical [8], frequency [9] to mass [10] detection methods. Currently, the method for PSA screening used in routine analysis in medical practice is the immunoassays like Enzyme-Linked Immunoabsorbent Assay (ELISA), which rely on the presence of antibodies against PSA, associated to enzymes that produce a response in the presence of the antigen [11].

Abbreviations: GUSB, beta-glucuronidase; KLK2, kallikrein-2; KLK4, kallikrein-4; PCTA1, prostate carcinoma tumor antigen-1; PrLZ, prostate leucine zipper; PSA, Prostate Specific Antigen; PSCA, Prostate Stem Cell Antigen; PSMA, Prostate-Specific Membrane Antigen; PSMB6, proteasome subunit beta type-6; TGM4, protein-glutamine gamma-glutamyltransferase 4 (TGM4).

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0928-4931/

The advantages of immunoassay technology relative to other analytical techniques include low detection limit, high selectivity, high throughput of samples, versatility and reduced sample preparation, which makes this method most commonly used in clinical routine. The use of specific natural antibodies and the special handling/storage conditions make this method expensive. In addition, the antigen binding to the antibody is very strong, turning this method irreversible and of single use.

Recently, an electrochemical biosensor for screening of PSA has been developed, having as main advantage the use of artificial antibodies instead of natural ones [12]. More precisely, the PSA recognition is made by means of molecularly imprinted polymers (MIPs) that are a kind of plastic antibodies, designed over the surface of graphene layers extracted from graphite [12]. Globally, MIPs show high sensitivity/selectivity and present much longer stability and much lower price than natural antibodies. This electrochemical biosensor has the advantages of high robustness, easy miniaturization, excellent detection limits with small analytic volumes, and ability to be used in turbid biofluids with optically absorbing and fluorescent compounds. The biosensor was already tested to determine PSA levels in artificial serum, with recoveries ≥96.9% and relative errors of ~6.8%, with the results suggesting that the sensor may have successful results under real applications [12].

The aim of this study was to test the effectiveness of the electrochemical biosensor in screening PSA in different in vitro conditions. In vitro models present as main advantage the fact that monitoring all variables is easier because some interfering conditions, present in in vivo models, can be avoided. In order to simulate different biological contexts, with different complex compositions, detection of PSA was conducted in the culture medium collected from several prostate cell lines, cultured in a variety of experimental conditions (different culture periods and media composition), thus with an expected wide range of PSA levels, and also different concentrations of many other metabolites. Cell behavior in the different culture conditions was assessed by cell proliferation analysis and qPCR expression of several prostate-related genes. The tested prostate cell lines included the cancer cell lines LNCaP (positive for androgen receptors) and PC3 (negative for androgen receptors) and the non-cancerous prostate cell line PNT2. In parallel, human skin fibroblasts were used as a non-cancerous control. Validation of the results was performed by assessing PSA levels in the same media samples by the conventional ELISA assay. MALDI-TOF mass spectrometry was also used to identify the protein.

2. Materials and methods

2.1. Setup of the electrochemical biosensor

The construction of the solid-contact PSA selective electrode was made similarly to that described by Kamel et al. in [13] and is shown in Fig. 1. The electrode body was replaced by a 10 mL syringe, using its smaller end to pack the conductive material – a mixture of graphite (Sigma-Aldrich) and epoxy resin (Sigma-Aldrich) – and bind the cupper electrical wire. The outer graphite layer on top of the syringe was removed to create a small cavity (~1 mm deep), where the selective membrane would be deposited, drop-by-drop.

The selective membrane was prepared by mixing 1 mg of plastic antibody material, 33 mg of *o*-Nitrophenyloctyl ether (oNPOE, Fluka), and 16 mg of poly(vinylchloride) (PVC, Fluka). The mixture was stirred until the PVC was well humidified, and dispersed in 2.0 mL tetrahydrofuran (THF, Riedel-deHäen). The dispersion was kept uniform by continuous agitation on a magnetic stirrer. The membrane was casted on the graphite support drop-by-drop, allowed to dry for 24 h, and was conditioned in PSA (Sigma-Aldrich) solution, 20 ng/mL, in Hepes buffer (Sigma-Aldrich), before use. Due to the instability of PSA, this conditioning was made at 4 °C.

The plastic antibody was prepared by obtaining a 1 mg/mL of graphene oxide (GO) solution from graphite powder. This procedure followed the method of Hummers and Offeman [14], and its subsequent modifications described by Shenguang et al. [15]. GO solution was mixed with 50 mg/mL *N*-hydroxysuccinimide (NHS, Fluka) aqueous solution and 10 mg/mL *N*-ethyl-*N*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, Sigma-Aldrich) aqueous solution for activation of the –COOH groups. The obtained colloidal GO started to flocculate and was removed from the solution by filtration, washed with a 1×10^{-4} mol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, Sigma-Aldrich) buffer, and allowed to dry in a desiccator under nitrogen atmosphere. After, about 1.5 mg of the previous solid was immersed in 2.5×10^{4} ng/mL PSA (Sigma-Aldrich) solution in Hepes buffer for protein binding. The carboxylic groups that remained active after the reaction with PSA were blocked by adding 0.1 g/mL 2-aminoethyl methacrylate hydrochloride 90% (AMH, Sigma-Aldrich) to the solid. The next stage consisted on incubating the solid in 2.9×10^{-6} g/mL (vinylbenzyl)trimethylammonium chloride 97% (VTA, Acros Organics) solution, 6.8×10^{-7} g/mL vinyl benzoate (VB, Sigma-Aldrich) solution. The resulting solid material was again isolated from the solution and moved to polymerization. The polymeric film was obtained by adding to the solid, 3.56×10^{-4} g/mL acrylamide (AA, Sigma-Aldrich), acting as functional monomer; 7.72×10^{-3} g/mL *N*,*N*-methylenebis(acrylamide) (NMAA, Sigma-Aldrich), acting as cross-linker; and 1.2×10^{-3} g/mL benzoyl peroxide (BOP, Riedel-deHäen), acting as radical initiator. Finally, 0.5 g/L trypsin (Sigma-Aldrich) solution was added to the solid for removing the protein. The resulting solid was isolated, washed with Hepes buffer and dried in a desiccator under nitrogen atmosphere. Non-imprinted materials were prepared in parallel by replacing the PSA solution by Hepes buffer.

The pH of solutions was measured by a Crison GLP 21 combined glass electrode connected to the above pH meter. An SBS vortex, MVOR 03, was used to grant a good mixing of the reacting solutions. Insoluble materials were suspended in a Sonorex digitec sonicator.

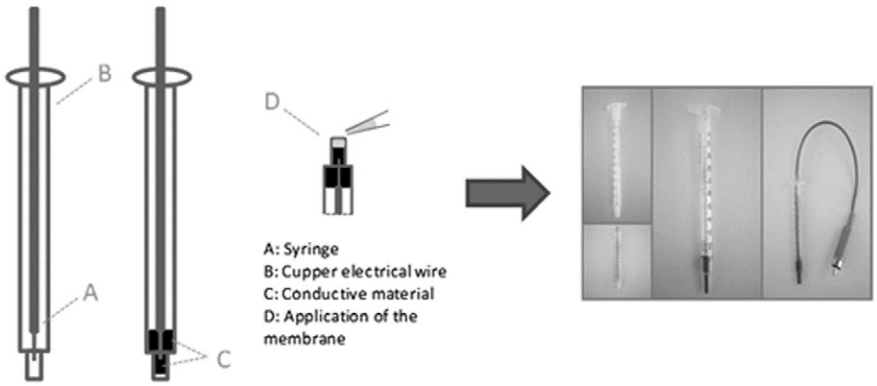


Fig. 1. Schematic representation of the assembly of the conductive support (left) and the picture of the several integrant parts of final device (right).

2.2. Cell cultures. Characterization of the cell behavior

The prostate cell lines LNCaP, PC3 and PNT2 were purchased from ATCC. Human gingival fibroblasts (FB) were obtained from explants collected from healthy donors with 25–35 years old, after informed consent. Cells were cultured in 100 mm culture plates and were maintained in standard culture conditions, i.e., α-minimal essential medium (α-MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 2.5 μg/mL fungizone (Gibco) and penicillin–streptomycin (Gibco) (100 IU/mL and 10 g/mL, respectively). Cells were incubated in a humidified atmosphere with 5% CO_2 in air, at 37 °C, and culture medium was changed twice a week. At 70–80% confluence, adherent cells were enzymatically released with a solution of 0.05% trypsin (Sigma-Aldrich) in 0.25% EDTA (Sigma-Aldrich).

The cells were seeded (10^4 cells/cm^2) in culture plates, and were incubated for 2, 7 and 14 days, without any further medium change. Cell cultures were performed in four culture media with different compositions: (*i*) α-MEM with 10% FBS, (*ii*) α-MEM with 30% FBS, (*iii*) RPMI 1640 (Gibco) with 10% FBS, and (*iv*) RPMI 1640 with 30% FBS. All culture media were supplemented with 2.5 μg/mL fungizone and penicillin–streptomycin (100 IU/mL and 10 μg/mL, respectively). In parallel, the four tested culture media were incubated under the experimental conditions described above, but in the absence of any cell type, and were used as a negative control.

Cultures were characterized for DNA content throughout the culture time, as a measure of cell proliferation. Further, at day 14, the cell layer was analyzed for several prostate markers by qPCR. At the end of each culture period, the medium was collected, centrifuged at 400 *g* for 10 min, aliquoted and frozen for subsequent analysis of PSA levels.

2.2.1. Total RNA extraction and qPCR analysis

RNA isolation was performed with RNeasy® Mini Kit (QIAGEN) according to manufacturer's instructions. Quantification of RNA was conducted at 260 nm on an Elisa plate reader (Synergy HT, Biotek). cDNA synthesis was performed using the DyNAmo cDNA synthesis kit (Finnzymthes, Finland) and random hexamers according to the manufacturer's instructions. Each cDNA template (~1.5 ng) was amplified with the DyNAmo Flash SYBR green qPCR kit (Finnzymes) on a Rotor-Gene thermocycler (Qiagen), according to the manufacturer's instructions. Validation of the reactions was performed by the presence of a single peak in the melt curve analysis. Cells were assessed for the expression of the housekeeping genes beta-glucuronidase (GUSB) and proteasome subunit beta type-6 (PSMB6), and the prostate associated markers PSA, Kallikrein-2 (KLK2), Kallikrein-4 (KLK4), Prostate Carcinoma Tumor Antigen-1 (PCTA1), Prostate Stem Cell Antigen (PSCA), Prostein, Prostate-Specific Membrane Antigen (PSMA), Protein-glutamine gamma-glutamyltransferase 4 (TGM4) and Prostate Leucine Zipper (PrLZ) [16,17]. In addition, LNCaP cells were also characterized regarding the expression of p53, androgen receptor (AR) and FKBP52 [18–20]. Primers used are listed in Table 1. qPCR results were analyzed using the standard curve analysis method. Briefly, the number of cycles required for the fluorescent signal to cross the threshold and exceed the background level, defined as cycle threshold (CT), was converted in relative expression levels, with the slope and the Y intersect extracted from the standard curve and applying the equation 10(Y intersect − CT / slope) [21]. The values obtained were normalized with the values obtained for both housekeeping genes.

2.2.2. DNA content

DNA content was quantified as a measure of cell proliferation, with the PicoGreen DNA quantification assay (*Quant-iT™ PicoGreen®* dsDNA Assay Kit, Molecular Probes Inc.), according to manufacturer's instructions. At each culture time, cultures were treated with Triton X-100 (0.1%) (Sigma-Aldrich) and fluorescence was measured on an Elisa plate reader (Synergy HT, Biotek) at wavelengths of 480 and 520 nm, excitation and emission respectively, and corrected for fluorescence of reagent blanks. The amount of DNA was calculated by extrapolating a standard curve obtained by running the assay with the given DNA standards, and is expressed as ng/mL.

Table 1
Primers used in RT-PCR analysis of cell cultures.

Gene	5′ Primer	3′ Primer
GUSB	TGCAGCGGTCTGTACTTCTG	CCTTGACAGAGATCTGGTAAT
PSMB6	GCCGGCTACCTTACTACCTG	AAACTGCACGGCCATGATA
PSA	ACCAGAGGAGTTCTTGACCCCAA	CCCCAGAATCACCCGAGCAG
KLK2	GGTGGCTGTGTACAGTCATGG	TGTCTTCAGGCTCAAACAGGTT
KLK4	GGCACTGGTCATGGAAAACGA	TCAAGACTGTGCAGGCCCAGCC
PCTA1	CGTAGTGTTCTTTGGACACG	CTACCAGCTCCTTACTTCCAG
PSCA	TGCTTGCCCTGTTGATGGCA	CCAGAGCAGCAGGCCGAGTGC
Prostein	CCTTCACGCTGTTTTACACG	CTACGCTGAGTATTTGGCCAAG
PSMA	CCAGGTTCGAGGAGGGATGGT	GCTACTTCACTCAAAGTATCTG
TGM4	CATCATTGCGGAAATTGTGG	CTACTTGGTTGATGAGAACAA
PrLZ	GTAGAGAGATGGACTTATATG	TCACAGGCTCTCCTGTGTCTT

2.3. PSA levels in the culture media

PSA levels were quantified in the culture medium from the cell cultures maintained in all tested conditions, and collected after 2, 7 and 14 days of culture. Quantification was performed with the Biosensor and with a commercial ELISA kit (CanAg PSA EIA). Results were normalized to the DNA content of the cell layer.

2.3.1. Electrochemical biosensor

Calibration plots were used for determination of the PSA concentrations in the culture media. Decreasing concentration levels of PSA were obtained by transferring 5 μL of 2.5×10^4 ng/mL PSA standard solution to a 75 mL beaker containing 375 μL of each tested medium and 620 μL Hepes buffer 1.0×10^{-4} mol/L. Potential readings were recorded after stabilization to ±0.2 mV and emf was plotted as a function of the logarithm of the PSA concentration. After calibration, the diluted samples (375 μL of sample and 625 μL buffer) were analyzed. All potentiometric measurements were carried out at room temperature and in stirred solutions of pH 7.3.

The measurements were made in a Crison pH-meter GLP 21 (±0.1 mV sensitivity). The simultaneous reading of multiple potentiometric devices was enabled by a home-made commutation unit with six ways out. The assembly of the potentiometric cell using the solid-contact support was as follows: conductive graphite | PSA selective membrane | buffered solution (Hepes buffer 1 × 10–4 mol/L, pH 5.2, or artificial serum, pH 7.3) || electrolyte solution, KCl |AgCl(s) | Ag. The reference electrode was an Ag/AgCl electrode of double-junction from Crison 5240.

2.3.2. ELISA assay

The same samples used to quantify PSA levels with the biosensor were assessed by ELISA, with a commercial ELISA kit (CanAg PSA EIA), according to the manufacturer's instructions. The minimum dose of PSA detectable by the kit was 0.1 ng/mL. The concentration of PSA in each sample was determined at 405 nm in an ELISA plate reader (Synergy HT, Biotek).

2.3.3. Statistical analysis

Results are expressed as the mean ± standard deviation. Groups of data were evaluated using a two-way analysis of variance (ANOVA). Statistical differences between controls and experimental conditions were assessed by Bonferroni's method. Values of $p \leq 0.05$ were considered significant.

2.3.4. PSA identification by MALDI-TOF mass spectrometry

2.3.4.1. In solution digestion of proteins. MALDI-TOF mass spectrometry analysis was performed on all tested culture medium samples. However, the presence of PSA was only detected on the samples from 14-day LNCaP cell cultures. Sample treatment followed essentially López-Ferrer [22] and Santos [23] with minor modifications. Briefly, protein samples were resuspended in 12.5 mM ammonium bicarbonate solution (NH_4HCO_3, Sigma-Aldrich) and mixed using a vortex for 1 min followed by adding 2 μL of 110 mM dithiothreitol (DTT, Sigma-Aldrich) in 12.5 mM NH_4HCO_3 (Sigma-Aldrich) as reduction step of protein disulfide bonds. Then, samples were sonicated in a sonoreactor UTR200, from Dr. Hielsher (Teltow, Switzerland) for 1 min at 50% amplitude and continuous mode. After cooling to room temperature, it was added 600 mM iodoacetamide (IAA, Sigma-Aldrich) in 12.5 mM NH_4HCO_3, for alkylation, and again sonicated in the sonoreactor (1 min; 50% amplitude; continuous mode). The sample solutions were diluted in 72 μL of NH_4HCO_3. Then, 4 μL of trypsin sequencing grade (Sigma-Aldrich) (0.025 mg/mL in 25 μL of 12.5 mM NH_4HCO_3) was added to each sample and incubated overnight (37 °C) with trypsin for digestion. Afterwards, 2 μL of formic acid (50% v/v) (Fluka) was added to each sample to stop enzyme activity and mixed using the vortex.

2.3.4.2. Intact protein by MALDI-MS

(i) Sample clean-up

To improve data quality, prior to MALDI-TOF-MS intact protein analysis, the sample was purified and concentrated using $ZipTip_{C4}$ pipette tips (Merck-Millipore). The protocol for $ZipTip_{C4}$ sample preparation was adapted from manufacturer's guidelines. The micropipette was set to 10 μL and $ZipTip_{C4}$ equilibration step was performed, first by aspirating and dispense a solution 50% methanol (Sigma-Aldrich) and 0.1% TFA in MilliQ water (3 cycles), and then by aspirating and dispense the washing solution, 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich) in MilliQ water (3 cycles). After ZipTip equilibration the protein binding step was carried out by aspiration and dispense of the sample (10 cycles in 50 μL of sample), followed by a washing step with 0.1% TFA in MilliQ water (5 cycles). Sample elution was accomplished by aspirating and dispensing 10 μL of a solution 75% methanol 0.1% TFA in MilliQ water that was previously added to a clean vial (5 cycles).

(ii) MALDI-TOF-MS analysis

Prior to MALDI-TOF-MS analysis the sample was mixed with an equal volume of the MALDI matrix solution 10 mg/mL α-cyano-4-hydroxycinnamic acid (α-CHCA, Sigma-Aldrich) in TFA 0.1% (v/v) and acetonitrile (Panreac AppliChem) 50% (v/v). An aliquot of the sample/matrix solution (0.5 μL) was hand-spotted onto the MALDI sample plate and the sample was allowed to dry at room temperature. Intact protein data was obtained using a ABI 4700 Proteomics Analyzer with time-of-flight (TOF)/TOF optics (Applied Biosystems, Foster City, USA) equipped with a 355-nm Nd:YAG laser and the laser intensity was set just above the threshold for ion production. Laser shots of 600 per spectrum were used to acquire spectra within a mass range of 10 to 50 kDa. Spectra were acquired in the linear positive ion mode with a 20 kV acceleration voltage, 16 kV grid voltages and a delay time of 240 ns. All the mass spectra were processed using Data Explorer™ software, version 4.5 (Applied Biosystems, USA). MS acquisition data was calibrated externally using the ProteoMass Protein MALDI-MS Calibration Kit (MSCAL2) from Sigma-Aldrich as mass calibration standard for MALDI-TOF-MS.

(iii) Data analysis and database searching

All data were processed using DataExplorer 4.5 software from Applied Biosystems. Peptide Mass Fingerprint (PMF) data were used to search for candidate proteins using the MASCOT database search (http://www.matrixscience.com) engine. SwissProt database was selected by default for all Mascot searches. NCBInr database was used each time, and no significant identification was obtained with SwissProt. Database searches were, by default, performed with no taxonomy restriction and allowing up to a maximum peptide mass tolerance of 100 ppm. The number of allowed missed cleavages for trypsin was set to one. Carbamidomethylation of cysteine and methionine oxidation were selected as fixed and variable modifications, respectively. In order to provide accurate results, protein identification was considered positive for MASCOT protein scores higher than 56 ($p < 0.05$), that presents a minimum of 4 peptides matching.

3. Results

3.1. Characterization of the cell cultures

3.1.1. Cell proliferation

The different cell types were stained for cytoplasm and nucleus with hematoxylin and eosin, respectively, and were visualized under a light microscope (Fig. 2A). It was observed that the cells displayed a uniform distribution in the wells and revealed the expected morphology and pattern of cell growth for each tested cell type.

Fig. 2B shows the DNA content of the cell cultures in the different experimental conditions. Cells proliferated throughout the culture time in the tested culture conditions. Fibroblastic cells presented a higher proliferation in α-MEM (with slightly higher values in the medium with 30% FBS). The prostate cell lines showed a higher growth rate during the first week. LNCaP cells presented higher DNA values in α-MEM and RPMI containing 10% FBS. PC3 cells showed a slight preference for RPMI media and, α-MEM and RPMI containing 30% FBS yielded slightly increased DNA values. Regarding PNT2 cells, values were only somewhat higher in RPMI, and the percentage of FBS did not affect the cell behavior.

3.1.2. Expression of prostate genes

Cell cultures were assessed for the expression of several genes reported to be specifically or preferentially expressed by normal and malignant prostate cells, namely, PSA, KLK2, KLK4, PCTA1, PSCA, Prostein, PSMA, TGM4 and PrLZ. Results are presented in Fig. 3A.

Fibroblasts did not reveal the expression of any of the analyzed genes. The prostate cancer cell line LNCaP expressed all the tested genes, with responses globally higher or similar, when grown in α-MEM, compared to those achieved with RPMI. PSA gene presented the highest expression values, particularly in α-MEM supplemented with 10% FBS. A similar behavior was observed for KLK2 gene. In the case of PC3 cell line, no expression was observed for PSA, KLK2 and PSMA. Furthermore, PSCA expression was not detected when cells were grown in α-MEM, and when cells were maintained in RPMI, they did not reveal the expression of Prostein. The non-cancerous cell line PNT2 did not express PSA, KLK2 and KLK4, but the expression of the remaining genes was observed.

Following, LNCaP cells were analyzed for the expression of several genes known to be involved in the regulation of PSA expression, namely, p53, AR and FKBP52 (Fig. 3B).

It was observed that p53 expression was higher in cultures performed in RPMI, and the lowest value was achieved in α-MEM supplemented with 10% FBS. AR and FKBP52 expression was similar in all the tested conditions, though the expression values were lower for the latter gene.

3.2. PSA levels in the culture medium

3.2.1. Biosensor

Calibration curves were performed in a range of PSA concentrations 2.0 to 89.0 ng/mL. The sensor showed a good potentiometric response, with a slope of −44.16 mV/decade and a limit of detection (LOD) of

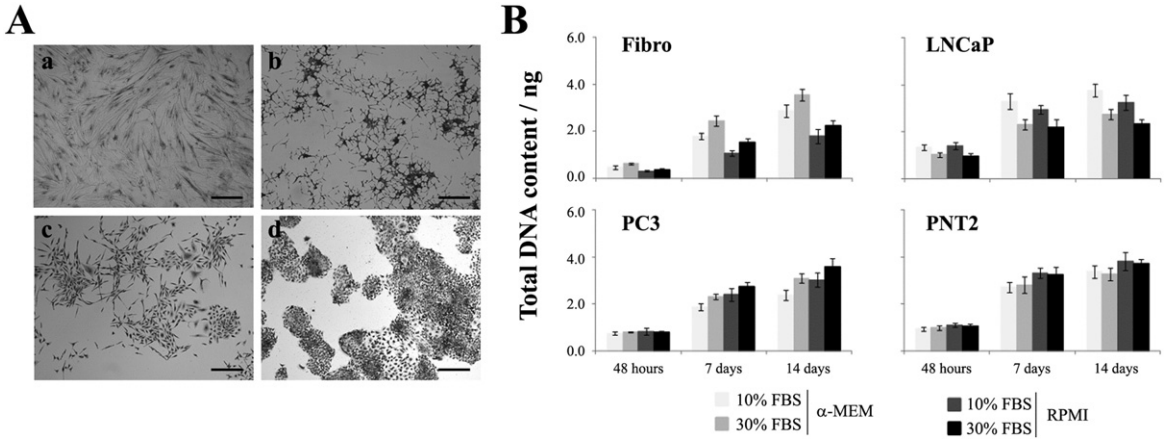


Fig. 2. Cellular characterization of cell cultures. A — cellular morphology at 7 days of culture, after hematoxylin/eosin staining method. Cell lines: a-human skin fibroblasts, b-LNCaP, c-PC3 and d-PNT2. Bar represents 300 μm. B — cell proliferation, assessed by total DNA quantification, of cell cultures maintained in different culture media for 14 days.

2.0 ng/mL (Fig. 4). A negative control of non-imprinted polymer (NIP) was prepared by following the same steps. In general, the time required for the electrodes to make a steady potential (±0.2 mV) was always less than 20 s, even for the highest concentrations tested.

The quantification of PSA levels by potentiometry was performed in the culture medium collected from the cell cultures. Only LNCaP cell line was able to produce detectable amounts of PSA. Levels were higher at day 14 in all conditions, and the highest values were found in α-MEM, especially with 10% FBS. In the RPMI medium, the presence of 30% FBS induced a greater production of PSA. The biosensor was unable to quantify PSA levels at day 2 for all culture media, nor at day 7 in RPMI medium. The results are shown in Table 2.

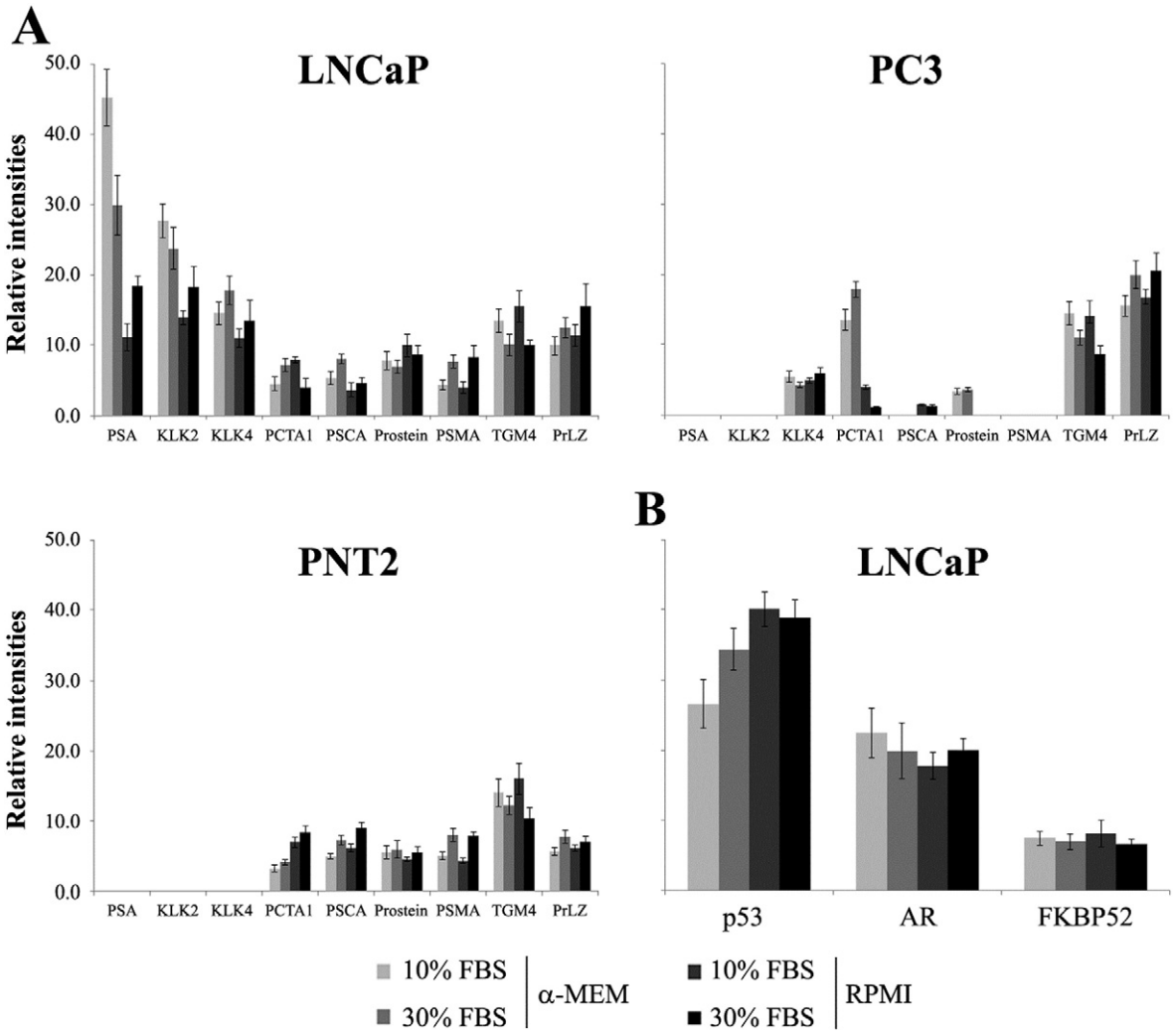


Fig. 3. qPCR analysis of cell cultures. A – PSA, KLK2, KLK4, PCTA, PSCA, Prostein, PSMA, TGM4 and PrLZ expressions by LNCaP, PC3 and PNT2 cell lines. B — p53, AR and FKBP52 expressions by LNCaP cell line.

3.2.2. ELISA assay

The quantification of PSA was carried out in the same samples used for the analysis with the biosensor. Also, PSA was only detected in the culture medium from the LNCaP cell line. The pattern of PSA production in the different culture media was similar to that described for the quantification with the biosensor. Results are shown also in Table 2.

3.2.3. Data correlation

Quantification of PSA levels by the biosensor and by the ELISA assay revealed similar concentrations, with recoveries ranging from 72.07 to 110.36%. The obtained results are summarized in Table 3.

3.3. PSA identification by MALDI-TOF mass spectrometry

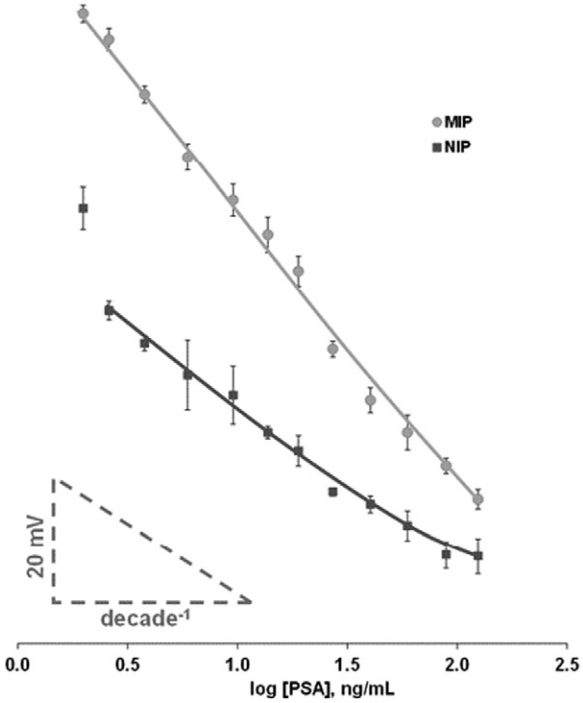
Samples were also analyzed by MALDI-TOF and compared with a PSA protein standard solution, in order to have information about potential changes in the size/composition of PSA in the different experimental conditions, Fig. 5. PSA was only detected in the samples collected from LNCaP cell line, at day 14. The protein appeared in a sharp peak corresponding to $[M + H]^+$ at 33,440 Da. $[M + H]^{2+}$ was also observed at 16,690 Da. In order to confirm the identity of PSA, the identification by PMF was performed. Positive identification was achieved with a score of 70 and a sequence coverage of 26% corresponding to 8 peptide matches.

4. Discussion

PCa is a public health problem, which can reduce the quality of life and even lead to the death of the patients [24]. However, if detected early it can be treated and even cured. PSA is a protein that has been used for the screening of PCa and monitoring patients after therapy, being considered an important biomarker for this pathology [25]. Recently, a PSA electrochemical biosensor was developed and reported to present a good response in the determination of PSA levels in non-biological fluids with a simple composition [12]. In addition to the observed high sensitivity/selectivity, this methodology presents a significantly lower price of analysis than the currently used ELISA technique, which makes it a potentially useful routine tool for PCa diagnosis.

Despite its recognized potential as a PCa biomarker, PSA expression is not only confined to prostate cancer cells. In fact, normal prostate cells have the ability to produce low levels of this protein, and also it is reported that some non-prostatic cancer cells may express PSA [6]. Moreover, the synthesis and secretion of PSA is under a complex regulation, which responds to many different exogenous stimuli, being significantly affected by the cellular metabolic context [4,6]. Taking this in account, in this study, the effectiveness of PSA quantification by the biosensor was assessed in culture media from human prostate cell lines, as representative of biological complex environments. For that, two human prostate cancer cell lines and one non-cancerous prostate cell lines were cultured in different conditions, which are expected to modulate the PSA expression and, thus, to create a wide range of PSA concentrations in environments with different complex compositions. Cells were maintained in two different but widely used culture media (α-MEM and RPMI), that present significantly different composition, especially in the proportions of the standard amino acids. In order to create more pronounced differences in the culture media composition, the concentration of fetal bovine serum (FBS), was also changed, being tested at a final concentration of 10% and 30% of FBS. In parallel, human gingival fibroblasts were used as a non-cancerous, non-prostatic control.

Regarding cell viability/proliferation in the different culture condition, it was observed that cell cultures presented different behaviors during the 14-day culture period. For fibroblasts, the culture medium that elicited a higher cellular growth was α-MEM supplemented with 30% FBS. It is important to note that α-MEM is one of the most widely used culture medium for this cell type [26,27], although there also studies conducted in DMEM [28,29] or RPMI [30,31]. In the case of prostate cell lines, studies are usually performed in RPMI [32,33]. In line with this, PC3 and PNT2 revealed a somehow higher growth in this medium. PNT2 behavior was not significantly affected by the different concentrations of FBS, while PC3 viability/proliferation was increased in the presence of 30% FBS. LNCaP cell line exhibited a slightly higher cellular response in α-MEM. In addition, in both culture media, the presence of 10% FBS seemed to promote a higher cell viability/proliferation of LNCaP cells. This differential behavior observed in the presence of culture media with different compositions is in line with the known specific needs of each cell line, when maintained in culture [34].



Biosensor	MIP	NIP
Slope (mV/decade)	-44.16 ± 2.89	-24.80 ± 1.91
R^2 (n=3)	0.991	0.994
LOD (ng/mL)	2.0	2.6
Linear range (ng/mL)	2.0 – 89.0	2.6 – 89.0

MIP - Molecularly Imprinted Polymer; NIP - Non-Imprinted Polymer and LOD - Limit of detection

Fig. 4. Potentiometric response of PSA selective electrodes prepared with imprinted and non-imprinted materials (ranging from 2.0 to 89.0 ng/mL, in 1×10^{-4} mol/L Hepes buffer).

Table 2
Quantification of PSA in culture media.

Culture		Biosensor		ELISA		*F*-test		*t*-test	
Media	Time	PSA (ng/mL)[a]	RSD	PSA (ng/mL)[a]	RSD	Calculated	Critical	Calculated	Critical
α-MEM 10% FBS	7 days	17.86 ± 2.17	12.15	16.19 ± 1.65	10.19	1.73	19.30	1.16	2.36
	14 days	206.93 ± 7.97	3.85	196.91 ± 15.56	7.90	3.81	5.79	1.32	2.36
α-MEM 30% FBS	7 days	12.40 ± 0.90	7.26	14.01 ± 1.17	8.35	1.69	5.79	2.32	2.36
	14 days	74.75 ± 2.54	3.40	94.86 ± 8.58	9.04	11.41	5.79	3.97	4.30
RPMI 10% FBS	14 days	9.35 ± 2.0	21.40	11.54 ± 1.45	12.56	1.90	5.79	1.67	2.36
RPMI 30% FBS	14 days	29.83 ± 1.73	5.80	33.83 ± 4.84	14.31	7.83	5.79	1.39	4.30

RSD — relative standard deviation.
[a] Results were normalized to the corresponding DNA content of the cell layer.

Also, cells were assessed by qPCR for the expression of different prostate marker genes, namely, PSA, KLK2, KLK4, PCTA1 PSCA, Prostein, PSMA, TGM4 and PrLZ [16,17]. KLK2 and KLK4 are two members of the kallikrein protein family that are thought to be important for the activation of several prostate zymogens, including pro-PSA [35,36]. PCTA1, also known as galectin-8, is over expressed by prostate cancer cells. Its main function is thought to be related to cell adhesion and growth regulation [37]. PSCA, Prostein, TGM4 and PrLZ are proteins that are related with prostate cancer progression and metastatic behavior [38–41]. PSMA seems to be important for angiogenesis regulation during prostate cancer development [42]. It was observed that all the markers were expressed by at least LNCaP cells, although with evident differences between them. Furthermore, the medium composition had a significant effect on the express of the genes by the different cell types. None of the markers were expressed by the fibroblasts. PSA was only expressed by LNCaP cells, which is in agreement with previously published reports [16,43] and its expression was significantly modulated by the culture media composition. Interestingly, KLK2 and, in a lesser extent, KLK4 (which was also expressed by PC3 cell line, at low levels) revealed a similar culture media-dependent expression pattern, which is in line with their proposed role in pro-PSA activation [35,36]. PCTA1 was expressed differentially by the three prostate cell lines. Generally, LNCaP and, particularly, PC3 cells revealed high expression values when maintained in α-MEM supplemented with 30% FBS. PNT2 revealed a higher cell response in RPMI in the presence of 30% FBS. Up to our knowledge, this is the first time that PCTA1 expression is observed in PNT2 cell line [16]. Regarding PSCA expression, a similar pattern was observed for LNCaP and PNT2 cells. However, in the case of PC3, only a residual expression was found in cell cultures conducted in RPMI supplemented with 30% FBS. Although there are studies that point to the expression of PSCA in the three tested cell lines [16], others did not observe its expression by PC3 cell line. The gene coding for Prostein was expressed by LNCaP and PNT2 cells, as reported previously [16] and also at low levels by PC3 cell line, though in this case it was only observed when cells were cultured in α-MEM. A residual Prostein expression by PC3 was observed by others [44], although there are also reports that point for an inability of this cell line to express that gene [16]. PSMA was only expressed by LNCaP and PNT2 cells, while TGM4 and PrLZ were expressed by all the tested cell lines. Taken together, since the different culture conditions elicited significant differences in the expression levels of several genes in all tested cell lines, the effect that the metabolic environment has in gene expression may account for some apparent contradictions with previously published data and even among the literature. Furthermore, it reinforces the importance that culture media composition may have in the cell response observed in this kind of analysis.

Table 3
Data correlation between the biosensor and the ELISA analysis.

Culture media	Culture time	% error	% recovery
α-MEM 10% FBS	7 days	10.36	110.36
	14 days	5.09	105.09
α-MEM 30% FBS	7 days	−11.5	88.50
	14 days	−21.2	78.80
RPMI 10% FBS	14 days	−27.93	72.07
RPMI 30% FBS	14 days	−11.83	88.17

PSA production is known to be under a complex network of regulatory mechanisms [3,45]. In this context, the expression by LNCaP of some important modulatory proteins, namely p53, AR and FKBP52 [18–20], in the different culture conditions was also investigated. p53 is a transcription factor encoded by a tumor suppressor gene that inhibits the expression of different prostate cancer biomarkers, including PSA [18]. AR is the intracellular receptor of androgen molecules, and its activation promotes the expression of PSA [19]. FKBP52 is a cochaperone that functions as a positive modulator of AR [20]. It was observed that p53 expression is inversely correlated with the expression profile observed for PSA, which is in line with the proposed negative role of p53 in the expression of PSA [18]. Regarding AR and FKBP52, no significant differences were observed in the different tested conditions. Although androgens appear as key players in the regulation of PSA expression [19,20,46], no hormonal treatment was performed in cell cultures, which might help to explain the observed results.

The production of PSA was analyzed by a recently developed electrochemical biosensor [12]. Electrochemical sensors are emerging as a promising alternative tool to the conventional methodologies in determination of biological and environmental analysis [47–51]. These sensors have boosted the development of new diagnostic tools [52] displaying high sensitivity, specificity, and fast/accurate analysis. They also offer experimental simplicity, low cost, portability allowing the possibility to carry out on-site analysis and adjust the technique to disposable devices. It was observed that PSA was only detected in culture media from LNCaP cell line, which increased with the culture period. The relative production of PSA was higher in α-MEM supplemented with 10% FBS. A similar behavior was observed for RPMI, although in this case the values were significantly lower. The ability that human prostate cancer cell lines, and, more precisely, LNCaP cell line have to express PSA is far from being elucidated. This appears to be strongly affected by the culture conditions and culture time, which reflects the high variability of PSA production values found in literature. Even when the media composition is similar, significant differences are reported. For instance, it was observed that PSA concentration on LNCaP cell line culture medium (RPMI supplemented with 10% FBS) ranges between 0.1–110 ng/mL [18,46,53,54]. This apparent heterogeneity of responses is in agreement with what happens in vivo, since both acute and chronic stimuli (ex: neuropeptides and androgens, respectively) may modulate PSA expression [55,56]. Moreover, PSA production and secretion appears to be regulated by complex mechanisms, which are affected not only by growth factors and hormones, but also by cellular interactions with the extracellular matrix [43,53]. Taken together, it is noteworthy to highlight that although LNCaP produced PSA in all tested conditions, the culture media composition and the concentration of FBS markedly affected this ability, which demonstrates that PSA

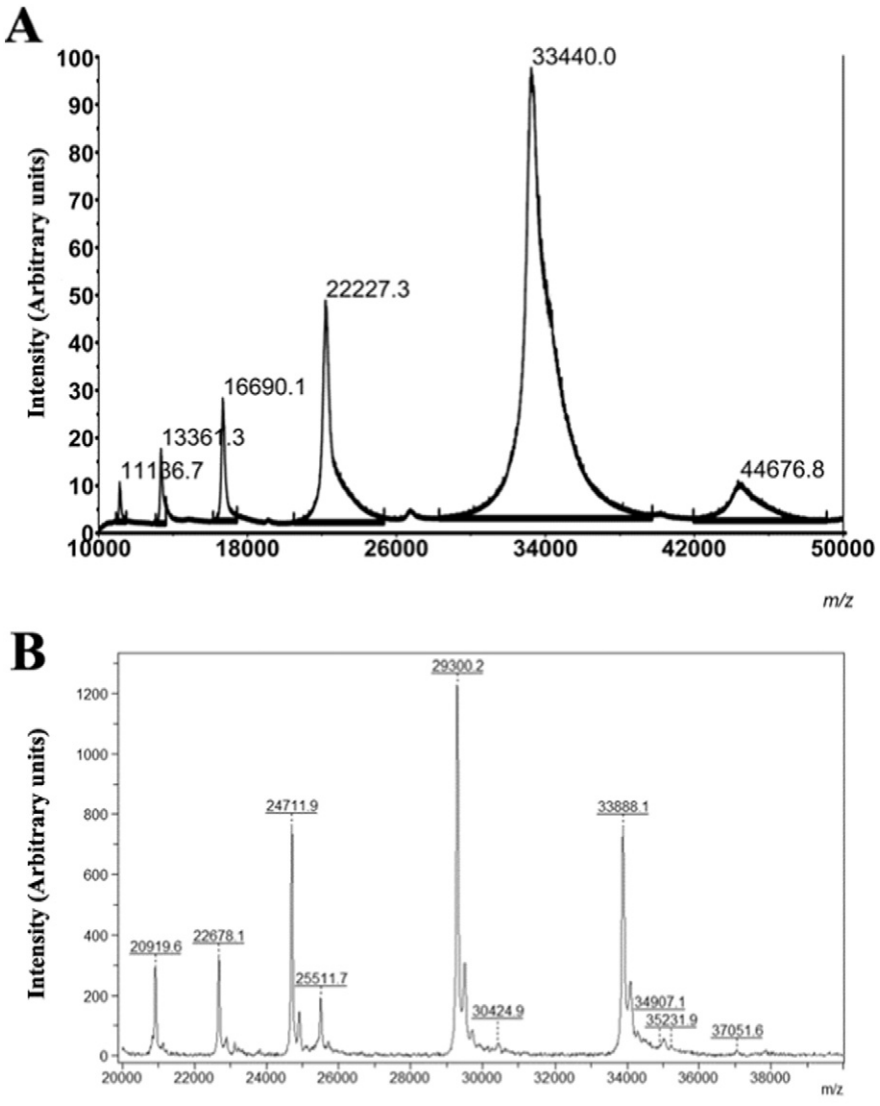


Fig. 5. MALDI-TOF MS analysis of the (A) PSA from LNCaP cell culture and (B) standard PSA in solution.

expression is strongly modulated by the cellular environment. Also, although the production of the protein increased with the culture period, this increase was particularly evident during the second week of culture, which coincided with a period of a lower proliferation rate. This suggests that cell density and, consequently, the establishment of proper cell-to-cell contacts may play an important role in the ability of prostate cancer cells to produce PSA. Regarding PC3 cell cultures, the absence of PSA expression is in line with previous studies [57].

Results showed that the PSA levels measured with the biosensor were in line with those obtained by the ELISA method. The accuracy and precision of the data was assessed by *t-Student* and Fisher tests, respectively. Considering as null hypothesis that the two methods agree, an unpaired single-tail test for 5% level of significance gave calculated *F-values* almost always below the tabulated one (Table 2), therefore accepting the null hypothesis for most samples observed. Samples outside this validation showed RSD values of the biosensor that were much lower than the ones obtained by the ELISA method, meaning that the biosensor proposed herein is displaying a better analytical performance than the ELISA itself. The calculated t value used the same assumptions, using homoscedastic or heteroscedastic populations (according to the F test) and confirmed for all cases the null hypothesis.

Despite the complexity of the samples, the biosensor was able to detect with selectivity and sensitivity the presence of PSA. The utilization of the biosensor presents several advantages when compared with the traditional immunoassays and, specifically, ELISA assays, such as its inexpensiveness, simplicity of construction, high robustness and easy miniaturization. This method appears to be particularly suitable for screening assays carried out in analytical laboratories. The detection limit and the linear response were significantly lower than the cut-off value for PSA levels (2.0 vs 4.0 ng/mL, respectively), which supports its potential application as a diagnosis tool for PCa. In order to confirm this potential, an assay with different biological samples from healthy individuals and from those with PCa is required. This study is now underway.

As can be seen in Table 4, various methodologies have been described before for the determination of PSA, including Surface-Plasmon Resonance (SPR), Quartz Crystal Microbalance (QCM), ELISA and electrochemical approaches, among others. These methods presented similar concentration linear ranges, with the exception of SPR and piezoresistive micro-cantilever, in which the linear ranges and LODs obtained are higher. On the other hand, sensors based on ELISA and amperometric methods allowed to obtain lower LODs than the

Table 4
Biosensors for PSA biomarker with different transducers and their detection range.

Recognition element	Transduction platform	Response range (ng/mL)	LOD (ng/mL)	Reference
MIP	Potentiometric	2.0–89.0	2.0	[12]
Antibody	Potentiometric	4.0–13	3.4	[58]
Antibody	Amperometric	4–10	0.4	[59]
Antibody	Amperometric (CV)	1–10	—	[7]
Antibody	SPR	—	100	[60]
Antibody	SPR	100–10,000	—	[61]
Antibody	UV–Vis spectroscopy	5–8	4	[62]
Antibody	Fluorimetric	4–12.6	4	[63]
Antibody	Piezoresistive micro-cantilever	10–1000	—	[9]
Antibody	Piezoelectric	1.5–40	—	[64]
Antibody	QCM	2.3–150	4.7	[10]
Antibody	ELISA	0.12–25	0.1	[11]
Antibody	ELISA	0.9–60	0.4	[65]

LOD — limit of detection; SPR — Surface-Plasmon Resonance; QCM — Quartz Crystal Microbalance (QCM); ELISA — Enzyme-Linked Immunosorbent Assay.

sensor tested in the present work, although they use antibodies as recognition element, with the associated disadvantages previously stated.

In order to evaluate if the PSA produced in the different experimental conditions presented some composition changes, like proteolytic cleavages or post-translational changes, the samples were analyzed by MALDI-TOF mass spectrometry. The digested PSA was analyzed by PMF search, using the MASCOT search engine, for putative identification of the protein, resulting in unambiguous PSA identification. In addition, the results from intact protein analysis revealed that PSA (from LNCaP cell line) appeared as a uniform population of molecules with about 33,400 Da, which corresponds to the full-length PSA molecular ion. The observed peak corresponding to a mass of 16,690 Da may correspond to the PSA molecular ion with double charge. However, the MALDI-TOF analysis of the standard PSA show a main peak corresponding to a mass of 29,300 Da followed by other prominent peaks that corresponds to 24 712 Da and 33 888 Da which are compatible with findings from Végvári et al. [66] In fact, these results may be explained by the fact that PSA can occur in three major isoforms and it was suggested that these isoforms may appear upon translation of alternative hKLK3 transcripts [67].

Taken together, PSA is not expressed equally by the different prostate cell lines. Besides the individual ability of the cell lines to produce the protein, the cellular environment is a key modulator of the PSA expression. The recently developed PSA electrochemical biosensor based/employing on molecularly imprinted polymers was able to specifically detect PSA in the samples, with values similar to those achieved by a commercial ELISA kit, and in levels well below the upper cut-off values for PCa. Thus, the tested biosensor may be regarded as a potentially useful diagnostic tool for PCa, due to the advantages that it offers when compared with the current assays employed in PSA quantification.

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