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Protein imprinted materials designed with charged binding sites on screen-printed electrode for microseminoprotein-beta determination in biological samples

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ABSTRACT

In the past few years a large effort is being made aiming at the development of fast and reliable tests for cancer biomarkers. Protein imprinted sensors can be a fast and reliable strategy to develop tailor made sensors for a large number of relevant molecules.

This work aims to produce, optimize and use in biological samples a biosensor for microseminoprotein-beta (MSMB).

Caffeic acid (CAF) electropolimerization was performed in the presence of microseminoprotein-beta (MSMB) creating target protein specific cavities on the surface of a screen-printed carbon. Dopamine was introduced as charged monomer labelling the binding site and was allowed to self-organize around the protein. The subsequent electropolimerization was made by applying a constant potential of +2.0 V, for 30 s, on a carbon screen-printed electrode, immersed in a solution of protein and CAF prepared in phosphate buffer.

The sensor with charged monomers showed a more sensitive response, with an average slope of −7.59 μA/decade, linear concentration range of 0.5–100 ng/mL and a detection limit of 0.12 ng/mL. The corresponding non-imprinted sensor displayed an inconsistent response over the range of the calibration curve. The biosensor was successfully applied to the analysis of MSMB in serum and urine samples.

1. Introduction

Prostate cancer (PCa) is now the most common cancer disease in men over European [1]. There are no obvious preventive strategies and, therefore, screening has been considered an important approach to reduce the number of deaths. Monitoring the levels of the prostate specific antigen (PSA) biomarker is currently recommended by medical community. However, some limitations are strongly associated with PSA detection due to false negative/positive readings. An elevated PSA level in the range 4.0–10.0 ng/mL is not always due to PCa and can be associated with other prostate conditions, including benign prostatic hyperplasia, inflammation or prostatitis, and many patients are sent to unnecessary biopsy procedures. On the other hand, PSA levels below 4.0 ng/mL can be obtained in men with PCa [2]. Therefore, there is a need to seek for appropriate biomarkers for more accurate medical diagnosis and improve therapeutic treatments to increase long term survival rates.

MSMB and PSA are the two most abundant proteins in the secretions produced by the human prostate. MSMB is a small, nonglycosylated peptide consisting of 94 amino acids (10.7 kDa) [3], having systemic functions including growth regulation and induction of apoptosis in prostate cancer cells *in vitro* and *in vivo* [4]. In healthy men, MSMB levels are shown to be fairly constant independently of race and ethnicity [5]. Furthermore, MSMB levels measured in serum, urine, and prostate tissue have been shown to be statistically significantly lower in men with prostate cancer and even lower in men with aggressive disease [6–8]. MSMB holds a high expectation as a biomarker of PCa development, progression and recurrence and potentially as a target for

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therapeutic intervention [9]. Furthermore, the combined detection of MSMB and PSA, can be a powerful tool in diagnosing PCa in a clinical setting, obviating the drawbacks of single PSA detection.

The methods available for MSMB screening are immunoassays, such as enzyme-linked immunosorbent assay (ELISA) [6,7,10,11], immunohistochemistry methods [12,13], immunoprecipitation [14] and AutoDelfia automatic immunoassay system [5,8] based methods. The use of an antibody as biological receptor confers a selective response for MSMB detection, although the drawbacks related to the binding irreversible nature and its subsequent non-reutilization are not yet solved. The analysis is also very expensive, since it requires specific natural antibodies, which needs special handling and storage conditions. Thus, novel approaches with a simple procedure and low cost are highly needed and would be greatly appreciated.

As an alternative, a new strategy based on the use of artificial antibodies instead of the natural ones could offer higher chemical/thermal stability [15] and promote a reversible analytical response, enabling a re-use of the biosensor devices. Protein plastic antibodies are a new class of materials where proteins are imprinted within a polymer shell, which is grown around the protein. For further use of the protein plastic antibodies the encapsulated protein is extracted afterwards. These materials are also referred to as polymer imprinted materials (PIM) and they can be obtained by simple surface imprinting procedures [16]. Aiming at improving the protein binding, charged monomers are added to the imprinting solution [17,18]. In this way charged monomers interact with the protein charged binding sites, further stabilizing the protein. Upon polymerization, specific binding sites are generated within the frame work of the polymer matrix improving the sensor affinity towards the imprinted protein [16]. These materials are referred in this work as charge stabilized polymer imprinted materials (C/PIM).

The PIMs based on electrochemical procedures have been widely used for the recognition and detection of template molecules [19–21] and are a promising tool for the construction of simple design, high stability, rapid response and enhanced selectivity sensors devices [22]. Electrochemical polymerization is typically conducted by mixing the template and the monomer in solution and by applying the necessary electrical conditions to form a thin film above the electrode surface containing the template entangled in a polymeric network [23]. In this process, the monomer selection is crucial, leading to more or less conductive polymer layers [22], with different physical features.

CAF is a well-known natural phenol that can be found in seeds, fruits, tubers and herbaceous parts of many vegetable species [24]. CAF has been employed in the fabrication of electrochemical sensors for recognition and detection of some biomolecules [25–29] offering good biocompatibility properties and simple procedures for the immobilization of biomolecules.

This work proposes a novel PIM for MSMB built on the surface of a screen-printed carbon electrode and displaying charged labels on the binding site. The construction of an electrochemical biosensor based on the electropolymerization of CAF in the presence of MSMB and dopamine was tested aiming at increasing is site specificity. Dopamine was introduced as a charged monomer able to self-organize around the protein creating, in this way, upon polymerization binding sites that will increase the specificity of the polymer imprinted material cavities towards microseminoprotein-beta. For this purpose, this work presented a systematic investigation of several analytical parameters, such as sensitivity, dynamic linear range, limit of detection and selectivity, in order to evaluate the performance of the MSMB electrochemical biosensor for the PCa screening.

2. Experimental procedure

2.1. Reagents and solutions

Ultra-pure water (resistivity >18 MΩ cm at 25 °C) was used throughout the work for cleaning and solution preparation. All chemicals were of analytical grade and used without further purification. Caffeic acid (CAF), sodium sulfate, potassium phosphate, ammonium chloride, urea, creatinine, bovine serum albumin (BSA) and 3-hydroxytyramine (dopamine) were purchased from Sigma–Aldrich; Microseminoprotein-beta (MSMB) from OriGene; sodium chloride from Panreac; sodium hydrogen carbonate, calcium chloride dehydrate, potassium chloride, potassium ferricyanide ($K_3[Fe(CN)_6]$) and potassium ferrocyanide trihydrate ($K_4[Fe(CN)_6]$) from Merck.

2.2. Solutions

Phosphate buffer solutions (PBS) of pH 7.2 (0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4) were used in this work. Stock solutions of MSMB (C = 0.2 mg/mL) were prepared in PBS (pH 7.2) and less concentrated standards were also prepared by suitable dilution in PBS buffer solution. Electrochemical assays were performed in the presence of 5.0×10^{-3} mol/L $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ in PBS. The synthetic urine solution used had the following composition: calcium chloride dihydrate (1.103 g/L), sodium chloride (2.295 g/L), sodium sulfate (2.25 g/L), potassium phosphate (1.40 g/L), potassium chloride (1.60 g/L), ammonium chloride (1.00 g/L), urea (25.0 g/L) and creatinine (1.10 g/L) [30]. Artificial serum solution was prepared with the following composition: sodium chloride (7.01 g/L), sodium hydrogen carbonate (1.68 g/L) and BSA (30 g/L) [31].

2.3. Apparatus

The electrochemical measurements were conducted in a PGSTAT302N potentiostat/galvanostat from Metrohm Autolab, containing an impedimetric module and controlled by computer with GPES 4.9 software. Carbon screen-printed electrodes (SPEs, 4 mm diameter, DRP-C110) were used as sensor platforms and were from DropSens (Spain). SPEs were connected to the Autolab by means of a suitable box, also from DropSens.

2.4. Procedures

2.4.1. Fabrication of the PIM on the carbon-SPE

Before modification the carbon-SPE electrodes were electrochemically cleaned by cycling the potential from −0.2 V to +1.0 V, at a 100 mV/s scan-rate, in a 0.5 mol/L sulfuric acid solution. Cycling procedures were repeated until the resulting voltammogram are reproducible (~30 cycles were necessary). The electrodes were then thoroughly rinsed with ultra-pure water and dried under N_2 atmosphere.

Next, the imprinted layer of poly(CAF) was assembled on the cleaned carbon surface, adapting the procedure described in reference [27].

About 30 μL for the preparation of PIM materials, of a solution containing 2.0×10^{-4} mol/L of CAF and 5.0×10^{-3} mg/mL MSMB in PBS buffer (pH 7.2) were casted over the three-electrode system of the SPE. Electropolymerization was conducted by applying a constant potential of +2.0 V for 30 s. The polymer modified electrode was then thoroughly washed with ultra-pure water, dried under N_2 and incubated overnight in a 1 mol/L H_2SO_4 solution at 45 °C in order to remove the protein [20]. The resulting PIM layer was washed with PBS buffer for several times, aiming to remove the

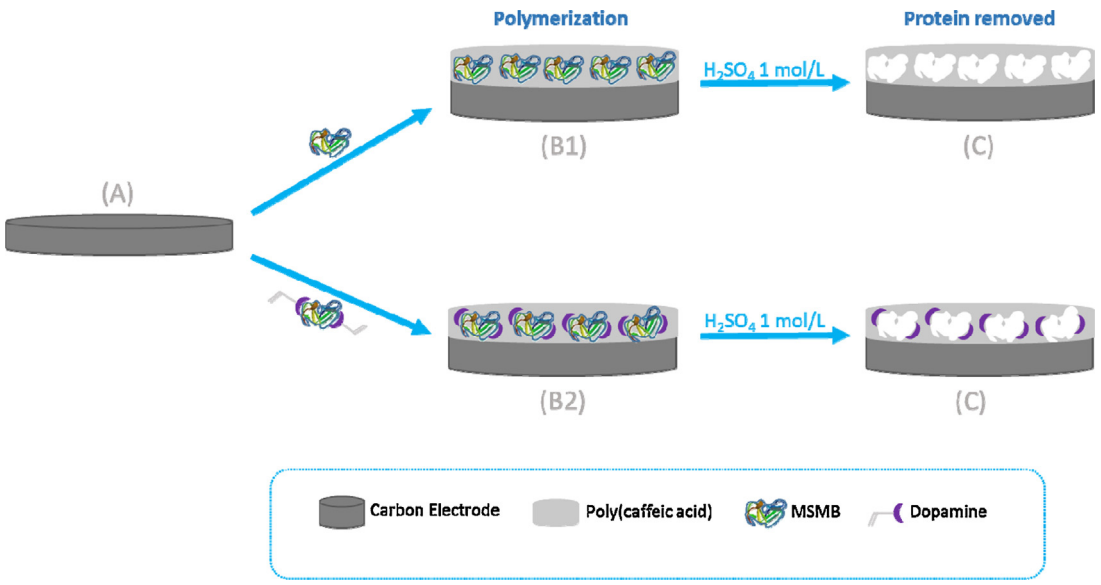

Fig. 1. Schematic representation of the synthetic process of PIM and C/PIM.
(A) Carbon-SPE; (B1) electropolymerization of caffeic acid with template; (B2) electropolymerization of caffeic acid after the addition of dopamine to template; (C) and binding site formation by template removal with H_2SO_4.

remaining protein fragments and H_2SO_4, and finally rinsed with ultra-pure water and dried under N_2.

The preparation of C/PIM was identical to PIM materials, the only difference being the addition of dopamine which was introduced as charged monomer labelling the binding site. About 30 μL of a solution containing 5.0×10^{-3} mg/mL of MSMB and 5.0×10^{-2} mg/mL dopamine in PBS buffer (pH 7.2) was incubated overnight in the fridge, at 4 °C. After that a solution with 2.0×10^{-4} mol/L of CAF was added.

The procedure adopted for the preparation of PIM and C/PIM are described in Fig. 1.

As a control, the non-imprinted materials, NIM and C/NIM modified carbon-SPE, were subject to the same procedure, except for the absence of MSMB in the electropolymerization process.

2.4.2. Electrochemical procedures

SWV and EIS measurements were conducted in triplicate and a redox probe solution containing 5.0×10^{-3} mol/L $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$, was prepared in PBS buffer of pH 7.2, was used. In SWV a potential window from −0.5 to 0.6 V was used at a frequency of 10 Hz, a step potential of 10 mV and amplitude of 50 mV. EIS was performed at open circuit potential (+0.12 V), using a sinusoidal potential perturbation with an amplitude of 10 mV and the number of frequencies equal to 50, logarithmically distributed over a frequency range of 0.1–100 kHz.

Calibration curves plotted peak current values obtained from SWV measurements as a function of MSMB concentration (between 0.1 and 200 ng/mL), in PBS buffer (pH 7.2).

All experiments were carried out using a 0.1 M PBS pH 7.2, an electrolyte solution close to the physiological conditions.

2.4.3. Determination of MSMB in synthetic urine and artificial serum

Synthetic urine and artificial serum solutions with different concentrations of MSMB were used for the evaluation of sensor response. They were prepared by adding a known amount of MSMB (from 0.2 to 20.0 ng/mL) to the synthetic urine or to the artificial serum solution.

3. Results and discussions

3.1. Imprinting stage

From previous studies (results not shown) the polymerization of CAF is favoured by applying a voltage of +2.0 V. Our previous experience demonstrated that a potential pulse of 30 s, with fixed

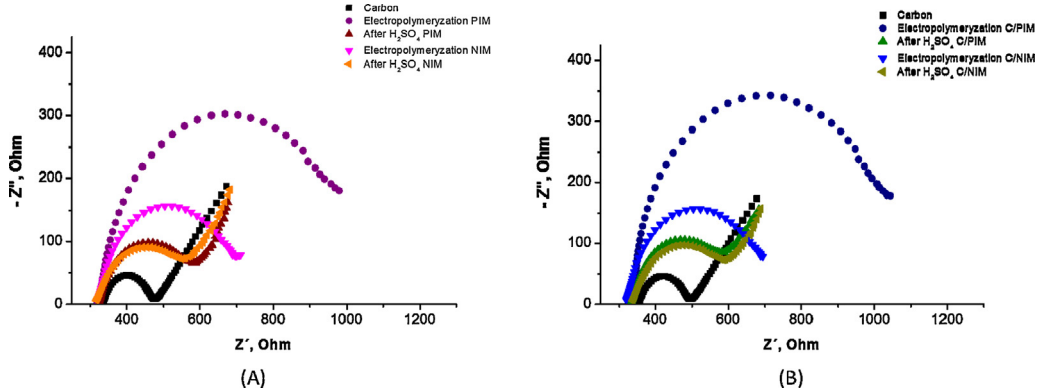

Fig. 2. EIS study over the subsequent modification steps of the carbon-SPE in 5.0 mM $[Fe(CN)_6]^{3-}$ and 5.0 mM $[Fe(CN)_6]^{4-}$ in PBS buffer.
(A) Protein imprinted material without oriented charges (PIM) and (B) protein imprinted material with charged binding sites (C/PIM).

Table 1
Fitting parameters extracted from electrochemical impedance data using the Randles type equivalent circuit.

(A)

	Carbon	Electropolymerization PIM	Electropolymerization NIM	After H_2SO_4 PIM	After H_2SO_4 NIM
R_s (Ω)	331.0	340.0	334.0	334.0	319.0
Q ($\Omega^{-1}s^{-n}$)	7×10^{-5}	2×10^{-5}	4×10^{-5}	1×10^{-5}	6×10^{-5}
n	0.86	0.92	0.81	0.71	0.80
R_{ct} (Ω)	150.0	582.0	481.0	202.4	274.4
W ($\Omega s^{-1/2}$)	6×10^{-3}	6×10^{-3}	6×10^{-3}	6×10^{-3}	6×10^{-3}

(B)

	Carbon	Electropolymerization C/PIM	Electropolymerization C/NIM	After H_2SO_4 C/PIM	After H_2SO_4 C/NIM
R (Ω)	331.0	343.0	336.0	327.0	345.0
Q ($\Omega^{-1}s^{-n}$)	7×10^{-5}	3×10^{-5}	2×10^{-5}	8×10^{-5}	9×10^{-5}
n	0.86	0.87	0.85	0.79	0.71
R_{ct} (Ω)	150.0	640.0	542.0	191.9	266.6
W ($\Omega s^{-1/2}$)	6×10^{-3}	6×10^{-3}	6×10^{-3}	6×10^{-3}	6×10^{-3}

Table 2
Selectivity study of the biosensors in the PBS, serum and urine artificial.

Characteristics	PIM			C/PIM		
	PBS	Serum	Urine	PBS	Serum	Urine
Slope (μA/decade)	−6.67	−5.50	−6.38	−7.29	−7.97	−13.52
LOD (ng/mL)	0.090	0.10	0.18	0.12	0.084	0.079
R^2	0.994	0.991	0.991	0.995	0.994	0.999
Linear concentration range (ng/mL)	0.1–200	0.5–200	0.5–200	0.5–100	0.5–100	0.5–100

concentration of 2.0×10^{-4} mol/L of CAF, and an MSMB concentration in the PIM construction equal to 5×10^{-3} ng/mL, were suitable conditions.

Charge/polar labels (*C*) were introduced to the binding site of the imprinted material (C/PIM), during the imprinting stage. This was done by adding to the solution protonated dopamine with a positive charge. Dopamine was present in a higher molar amount and was chosen due to the negative overall net charge of MSMB (isoelectric point of 5.6) under physiological conditions.

The imprinted sites were obtained by removing the protein template with 1 mol/L H_2SO_4 solution at 45 °C incubated overnight.

3.2. Control of the surface modification by impedance measurement

EIS studies were used to follow the carbon-SPE modification after each chemical change. These can be probed by monitoring the changes in the electron transfer properties of redox systems, such as $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$, as shown in Fig. 2. Data were fitted to the Randles equivalent circuit in order to extract the numerical values of the charge transfer resistance and the values obtained are displayed in Table 1. The diameter of the semicircle in the Nyquist plot corresponds to a pseudo charge-transfer resistance (*R*ct) of the redox probe at electrode/electrolyte interface.

Results obtained clearly show an increase in the resistance of charge transfer after polymerization due to the barrier effect of the polymer matrix decreasing the redox probe ability to access the electrode surface. This increase is visible both for PIM and C/PIM, due to the modifications made on the electrode surface which interfere (making the access to the surface more difficult) in the charge transfer process ($[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$) at the electrode surface. It is noteworthy that the increase in charge transfer resistance is greater in the case of C/PIM, where the polymerization was performed in presence of dopamine. This cannot, obviously, be related to an electrostatic effect but it is most probably due to the modification of the pathways within the polymer matrix affecting the redox probe ability to access the electrode surface.

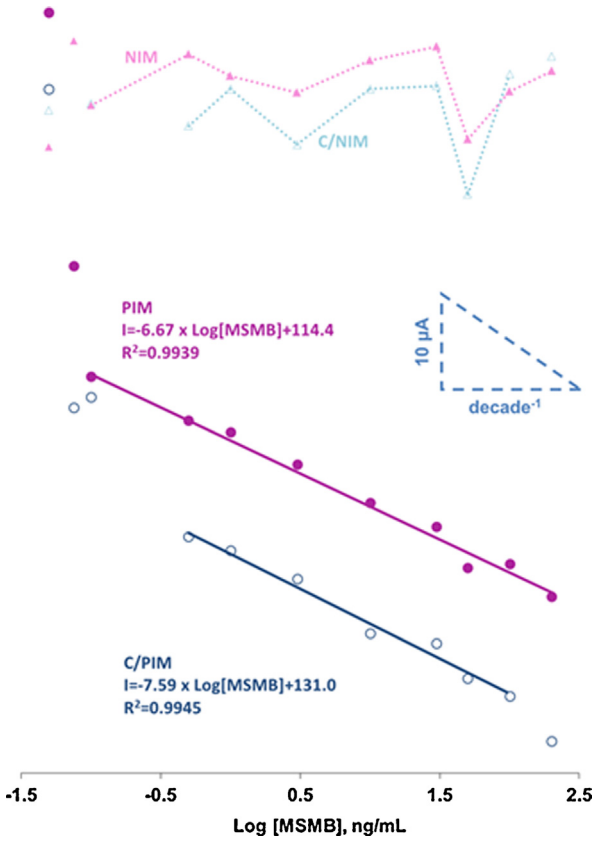

Fig. 3. Calibration curves of PIM, C/PIM, NIM and C/NIM based carbon-SPE biosensors obtained by SWV measurements in 5.0 mM $[Fe(CN)_6]^{3-}$ and 5.0 mM $[Fe(CN)_6]^{4-}$ PBS buffer.

In the final step of the PIM and C/PIM synthesis, after the protein removal with H_2SO_4, a decrease in the charge transfer resistance was observed, suggesting that MSMB was successfully extracted from the polymer.

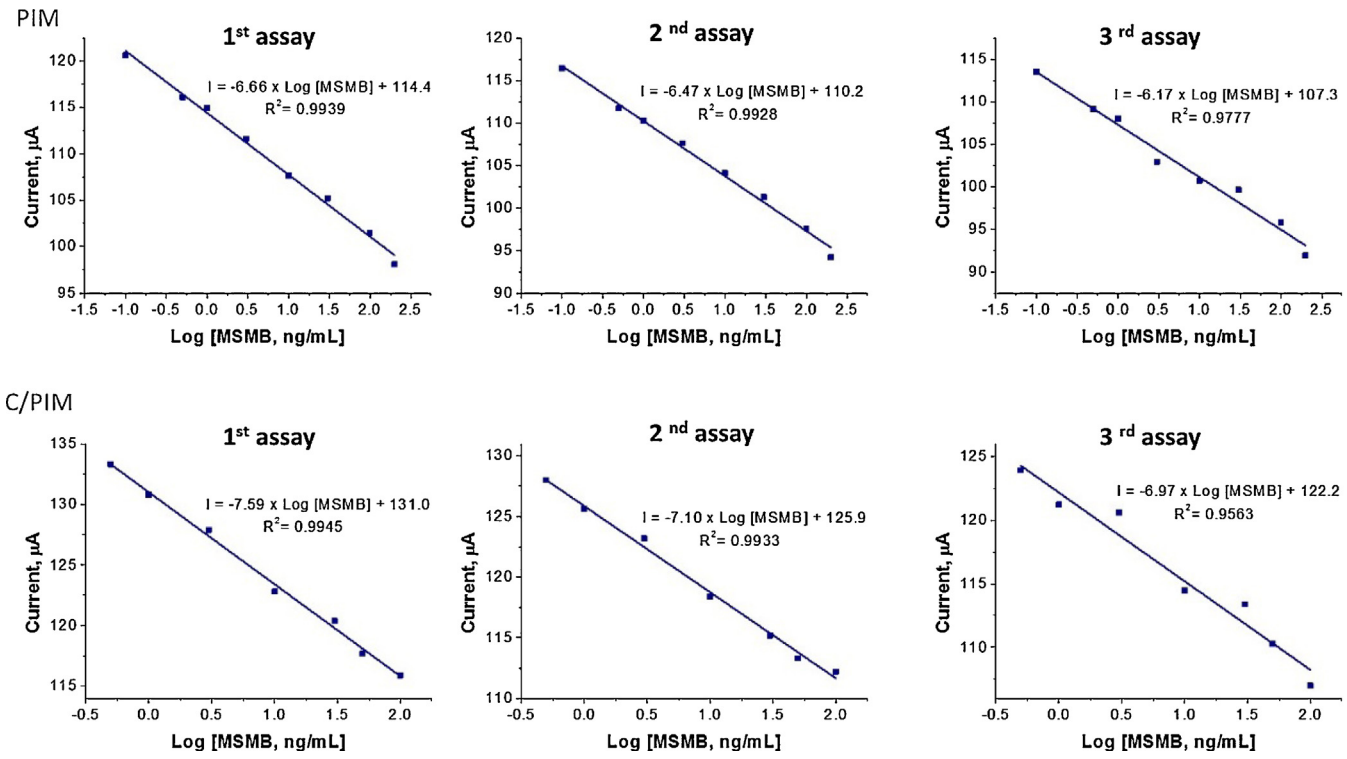

Fig. 4. Calibration curves displaying the effect of reused PIM and C/PIM carbon-SPE biosensors obtained by SWV measurements in 5.0 mM $[Fe(CN)_6]^{3-}$ and 5.0 mM $[Fe(CN)_6]^{4-}$ PBS buffer.

In the same way, an increase in the charge transfer resistance was observed for the NIM and C/NIM electrode after the electropolymerization of CAF in absence of MSMB. After the treatment of the electrode surface with H_2SO_4, the EIS profiles obtained were similar to the PIM and C/PIM electrodes, indicating the removal of a small fraction of polymer attached to the electrode surface.

3.3. Performance of the sensors

MSMB sensors were prepared without and with charged labels (PIM or C/PIM). The main analytical features of the devices were evaluated by SWV. This technique offers, as advantage, high sensitivity to surface-confined electrode reactions, along with suitable detection capabilities and rapidity.

The calibrations curves obtained are shown in Fig. 3 and plot peak current as function of MSMB logarithm concentration (between 0.050 and 200 ng/mL). MSMB binding was revealed by a decrease in the typical anodic peak current of the $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ redox probe. Higher MSMB concentrations yielded smaller current peaks. Observing Fig. 3 it is found that the calibration curves for PIM and C/PIM follow a linear pattern versus log[MSMB], respectively, in a concentration range from 0.1 to 200 ng/mL and 0.5–100 ng/mL and a correlation coefficient of 0.9939 and 0.9945. The limit of detection (LOD) was 0.090 ng/mL for PIM and 0.12 ng/mL for C/PIM which were estimated by the intersection of the two linear parts of the response function [32]. Analyzing the calibration curves we suggest that the best sensor would be PIM, because it has a larger working range and greater LOD, although it has lower sensitivity than the C/MIP sensor with slopes of −6.67 and −7.59 µA/decade [MSMB, ng/mL], respectively.

The NIM and C/NIM sensors feature an inconsistent response over the range of concentrations studied (Fig. 3). These results indicated that in this case the interaction between the protein and polymer was random and uncontrolled.

3.4. Interference study and electrode stability

The selectivity of the sensor is very important for a successful analytical application. The biosensors studied here have been exposed to many interferences that are present in biological fluids, when in contact with synthetic urine and serum, and showed a good analytical performance. Therefore, in the present study, instead of studying the individual effect of each interfering species, a global approach was adopted, studying the effect of using a biological fluid containing the most usual interfering species, accounting in this way possible synergetic effects.

The interference study was carried out by comparing the linear ranges, slopes and the LODs obtained through the concentration curves of PIM and C/PIM sensors in serum and artificial urine. The results were resumed in Table 2. The results obtained showed that PIM sensor in contact with biological fluids decreased its

Table 3
Determination of MSMB in serum and urine samples.

Sample	MSMB (ng/mL)	Serum			Urine		
		Found (ng/mL)	Recovery (%)	Relative error (%)	Found (ng/mL)	Recovery (%)	Relative error (%)
1	0.2	0.17 ± 0.05	114.6 ± 19.4	12.8	0.22 ± 0.05	90.0 ± 18.3	−11.1
2	1.0	1.1 ± 0.11	92.8 ± 9.2	−7.8	1.1 ± 0.13	91.5 ± 9.1	−9.2
3	3.0	2.9 ± 0.10	104.1 ± 3.7	4.0	2.9 ± 0.19	104.9 ± 7.9	4.7
4	20.0	19.4 ± 0.89	103.0 ± 3.8	3.0	20.6 ± 1.1	97.0 ± 7.0	−3.1

sensitivity (−5.50 μA/decade serum; −6.38 μA/decade urine), LODs (0.10 ng/mL serum; 0.18 ng/mL urine) and linear concentration range (0.5–200 ng/mL), while the C/PIM sensor increased sensitivity (−7.95 μA/decade serum; −13.52 μA/decade urine), LODs (0.084 ng/mL serum; 0.079 ng/mL urine) and kept the linear concentration range (0.5–100 ng/mL). Once average values of MSMB present in the serum are around 12 ng/mL and, in the worst case scenario, the present sensor is capable to measure concentrations of MSMB from 0.5 ng/mL it will be able to detect the decrease in MSMB concentrations due to prostate cancer related processes [5].

The biosensor offers a stable response within time and could be re-used a few times (≈3 times). Reusing was possible after cleaning with 1 mol/L H_2SO_4 for 12 h at 45 °C and subsequent washing with PBS and ultra-pure water. The graphs in Fig. 4 display the calibration plots for the reuse of different sensors. As observed, by the third time the sensor is used a loss of linearity of the sensor response is evident. This behaviour is however due to the physical degradation of the carbon SPE biosensor rather than a problem solely attributed to the degradation of the polymeric matrix. This is probably due to the repeated effect of the 1 mol/L H_2SO_4 solution at 45 °C causing the degradation of the carbon ink and of the polymer matrix.

In these conditions, the biosensor response has an average relative standard deviation of 5%.

As discussed previously in PBS, PIM showed better characteristics, however, when biosensors were studied in biological fluids C/PIM improved significantly its sensitivity (slope) and LOD.

Due to the mentioned above, C/PIM is selected to proceed with this study, although the decrease of linear concentration range in upper limits (which was not importance to our purpose, once 12 ng/mL is the upper limit in real cases).

3.5. Application

The C/PIM biosensor was used to determine MSMB in artificial urine and serum samples. Blank samples of synthetic urine and serum were spiked with MSMB in order to obtain concentrations ranging from 0.2 to 20.0 ng/mL. The results obtained for the four concentration values are summarized in Table 3.

For samples 2, 3 and 4 in serum, the recoveries ranged from 92.8 to 104.1% with an average relative error of 4.9%, in urine the recoveries ranged from 91.5 to 104.9% with an average relative error of 5.7%, these results suggesting that the proposed sensor may have success in real applications. Sample 1, in serum and urine, has weak recoveries and high relative errors, because the concentration level is out of the linear range of the biosensor, although it is higher than the LOD.

4. Conclusions

Molecular imprinting process over the surface of a carbon-SPE produced a simple and low cost electrochemical sensor for the determination of MSMB in biological fluids. The presence of charged labels in the imprinting phase enabled the synthesis of a more sensitive device. The selected biosensor was obtained by electropolymerization of CAF in the presence of MSMB and dopamine on carbon electrode.

In general, the C/PIM biosensor showed simplicity in design, short measuring time, reusability, low limit of detection and good selectivity. This biosensor was successfully applied to the analysis of MSMB in serum and urine artificial samples. For future developments, it is important to proceed with clinical trials and to establish a correlation between MSMB and PSA levels.

The performance of the electrochemical biosensor presented in this work enabled us to conclude that, in a near future, this can be a valuable alternative method for screening MSMB in point-of-care.

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