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7 Integrated amperometric affinity biosensors using
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11 Co^{2+} -tetradentate nitrilotriacetic acid modified
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16 disposable carbon electrodes. Application to the
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20 determination of β -lactam antibiotics
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Abstract

A novel strategy for the construction of disposable amperometric affinity biosensors is described in this work. The approach uses a recombinant bacterial Penicillin Binding Protein (PBP) tagged by an N-terminal hexahistidine tail which was immobilized onto Co^{2+} -tetradentate nitrilotriacetic acid (NTA) modified screen-printed carbon electrodes (SPCEs). The biosensor was employed for the specific detection and quantification of β -lactam antibiotics residues in milk, which was accomplished by means of a direct competitive assay using a tracer with horseradish peroxidase (HRP) for the enzymatic labeling. The amperometric response measured at -0.20 V vs the Ag pseudo-reference electrode of the SPCE upon the addition of H_2O_2 in the presence of hydroquinone (HQ) as redox mediator was used as the transduction signal. The developed affinity sensor allowed limits of detection to be obtained in the low ppb level for the antibiotics tested in untreated milk samples. Moreover, the biosensor exhibited a good selectivity against other antibiotics residues frequently detected in milk and dairy products. The analysis time was of approximately 30 min.

Keywords: modified electrodes, penicillin binding protein (PBP), Co^{2+} -NTA, screen-printed carbon electrodes, β -lactam antibiotics, milk.

1. Introduction

Many efforts have been done in the last years to achieve the immobilization of biological macromolecules on electrode surfaces with an entire retention of their activity. Among the large variety of immobilization strategies described (adsorption, covalent attachment, polymer encapsulation, electrostatic or hydrophobic interactions, etc.), methods based on affinity ligands or biospecific recognitions are particularly attractive because (i) the activity of the immobilized biomolecule is generally preserved, (ii) the spatial distribution can be well-controlled, (iii) the efficiency of the specific immobilization can be high, making it possible to work with low biomolecule loadings, (iv) the stability of the biomolecule is usually improved.¹ One of the most versatile and powerful affinity ligand methods is based on the use of metal ion chelating agents and their specific affinity for proteins containing histidine residues, which constitutes a standard tool in affinity chromatography (IMAC). The coordination of appropriate chelating agents such as the tridentate iminodiacetic acid (IDA) or the tetradentate nitrilotriacetic acid (NTA) to bivalent metal cations (e.g. Cu^{2+} , Ni^{2+} , Zn^{2+} , Co^{2+}) allows further ligation of the free coordination sites of the chelator-metal complex to the imidazole moieties of the histidine tags thus enabling a localized, oriented and reversible immobilization of proteins.² The histidine-tag possesses a small size and its position at the protein surface can be controlled, thus leading to an oriented immobilization without interfering with structure and function of the protein³. This latter point is of significant importance in analytical approaches involving heterogeneous biomolecular recognitions because the random attachment of an affinity protein can alter its binding capacity resulting from steric hindrance of adjacent immobilized proteins or with the surface itself.¹ This histidine-tagged protein immobilization chemistry has been widely reported on gold supports using different approaches.⁴⁻⁸ However, carbon electrodes have been scarcely used with this purpose due to the laborious and time-consuming multiple chemical steps of functionalization required for their modification.¹

On the other hand, the development of receptor-ligand based biosensing strategies can result in rapid and sensitive analytical methodologies for a wide range of analytes. In this work we used penicillin-binding proteins (PBPs) as molecular receptors. PBPs are enzymes found in the cytoplasmic membrane of bacteria involved in the growth of the microorganisms by maintaining and determining cellular morphology.⁹ They catalyse transpeptidation reactions resulting in glycan chains crosslinking by short peptides, as well as the elongation of these

glycan chains by transglycosilation.¹⁰ β -lactams bind irreversibly to the active site of PBPs due to their analogy with the 2-peptide D-alanyl-D-alanine, leading to the PBPs inactivation by acylation of the active site serine residue involved in the transpeptidase reaction.¹¹

β -lactams are frequently prescribed in veterinary medicine for the treatment of mastitis in lactating dairy cows¹² and constitutes one of the most commonly identified antimicrobial group found in milk. Misuse of antibiotic drugs rely in public health risks causing allergic reactions in sensitised individuals or affecting the intestinal flora of consumers and financial losses due to the inhibition of growth of starter cultures used for food technological processes.¹³ Moreover, it is also important the fact that low level doses of antibiotics in foodstuffs during long periods may led to the spread of drug-resistant microorganisms.¹⁴ Maximum residue limits (MRLs) have been established by the European Union for common β -lactams antibiotics based on their potency. For example, the MRL for amoxicillin, ampicillin, and benzylpenicillin is 4 $\mu\text{g kg}^{-1}$ in milk, while for cefapirin is 60 $\mu\text{g kg}^{-1}$.^{15,16} Although the analysis of veterinary drugs residues is generally made by liquid chromatography, there is a high demand for rapid and inexpensive analytical methods for the screening of β -lactam antibiotics in certain samples. Actually, a variety of commercial test kits and strips are available.^{17,18} In this context, immunoanalytical methods show some limitations since many are unspecific or not sufficiently sensitive because the chemical reactivity of the β -lactam ring avoid the raise of specific and sensitive antibodies against β -lactam antibiotics.¹⁹ Moreover, microbial inhibition tests, which are the only tests recognized by the European Union as screening methods for penicillins, require a few hours period of incubation and, since they are not selective among antibiotics, sometimes may give false positives.¹⁸ All these considerations support the development of receptor ligand based strategies leading to rapid, specific and sufficiently sensitive analytical methodologies available to be used by untrained personnel.

In this paper, we describe a novel, extremely simple, versatile and fast approach for the functionalization of carbon electrodes in a single-step with dense monolayers of chelating agents without need for applying any previous harsh pre-treatment to the electrode surface. We report herein the first successful adsorption of long alkyl chains-NTA derivatives on disposable carbon electrodes and the use of the resulting film for the subsequent coordination of cobalt ions and the histidinyl residues in the tagged PBP, allowing the immobilization of the receptor protein in a reproducible and reversible manner, with a controlled molecular orientation and maintaining its

entire biological activity. The resulting integrated amperometric affinity sensor allows the quantification of β -lactam antibiotics residues in milk at the low ppb concentration level through competitive binding between sample β -lactam and a HRP-labelled specific tracer for the binding sites of the immobilized PBP. The electrochemical detection of the enzyme product was carried out using hydroquinone (HQ) as electron transfer mediator and H_2O_2 as the enzyme substrate.

2. Materials and Methods

2.1 Apparatus and electrodes

Amperometric measurements were performed with a CHI812B potentiostat (CH Instruments, Inc., USA) controlled by software CHI812B. All measurements were carried out at RT. Impedance and voltammetric measurements were accomplished using a μ Autolab Type III FRA 2 instrument. A Bunsen AGT-9 Vortex was used for homogenization of the samples.

The transducers employed for the development of the integrated amperometric affinity sensors were screen-printed carbon electrodes (SPCEs) (DRP-C1110, DropSens) consisting of two elliptic carbon working electrodes ($\phi \sim 2$ mm), a carbon counter electrode and a Ag pseudoreference electrode. A specific cable connector (DRP-BICAC also from DropSens) acted as the interface between the SPCE and the potentiostat.

2.2 Reagents and solutions

All the reagents used were of the highest available grade. *N*-[N_α, N_α -Bis(carboxymethyl)-L-lysine]-12-mercaptododecanamide (HS-NTA), N_α, N_α -bis(carboxymethyl)-L-lysine hydrate (ANTA), Tween[®]20, $\text{K}_3\text{Fe}(\text{CN})_6$, $\text{K}_4\text{Fe}(\text{CN})_6$, sodium acetate, hydroquinone (HQ), hydrogen peroxide (30 %, w/v), lauroyl chloride, 4-(*N,N*-dimethylamine)pyridine (DMAP), ampicillin sodium salt (AMP) and tetracycline hydrochloride (TC) were purchased from Sigma-Aldrich. The enzyme tracer, HRP-conjugated β -lactam (PENG-HRP) was acquired from United States Biological. Sulfapyridine (SPY), cefapirin sodium salt (CEF) and enrofloxacin (ENRO) were from Fluka. Sodium dihydrogen phosphate, di-sodium hydrogen phosphate, potassium chloride, sodium hydrogen carbonate and sodium chloride were purchased from Scharlau. Sodium carbonate anhydrous, dimethyl sulfoxide (DMSO) and cobalt (II) chloride were acquired from Panreac. Copper (II) chloride was from Merck, A.G. Nickel (II) nitrate and zinc nitrate were acquired from Probus S.A. Imidazol was purchased from Acros. All buffer solutions were

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3 prepared with Milli-Q water (18 M Ω cm at 25 °C). Acetate buffer, pH 4.5, was prepared from 0.1
4 M sodium acetate and carbonate buffer, pH 9.6 from 0.05 M carbonate buffer solution. Binding
5 and washing buffer (B&W) was 50 mM phosphate buffer solution, pH 8.0, containing 300 mM
6 NaCl and 0.01% Tween[®]20. Pull and down buffer (P&D) was 3.25 mM phosphate buffer
7 solution pH 7.4 containing 70 mM NaCl and 0.01% Tween[®]20. PBST is phosphate-buffered
8 saline (0.01 M phosphate buffer solution containing 137 mM NaCl and 2.7 mM KCl, pH 7.5)
9 with 0.05% Tween[®]20.
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16 10 mM stock solutions of the different antibiotics were prepared in DMSO (SPY and
17 ENRO) or in P&D buffer (AMP, CEF, and TC) and stored at 4 °C. Antibiotic standard solutions
18 were prepared daily upon dilution of the corresponding stock solutions in P&D buffer or in milk.
19 Hydrolyzed β -lactams solutions were prepared by dissolving 10 mg of the antibiotic (AMP or
20 CEF) in 9.8 mL methanol (Scharlab) and 0.2 mL of 1.0 M potassium hydroxide (Scharlab) and
21 left to react overnight at 4 °C.¹³
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27 Whole UHT milk samples were purchased in a local supermarket, spiked, and used for
28 the determination of β -lactams after 1:1 dilution with P&D buffer.
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31 32 *2.3 Synthesis of Lauroyl-NTA*

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34 Lauroyl-NTA (N²,N²-bis(carboxymethyl)-N⁶-dodecanoyllysine, LAU-NTA) was
35 synthesized as follows: 93 mg (0.76 mmol) of DMAP and 243 μ L (3.05 mmol) of pyridine were
36 successively added to a suspension of 200 mg (0.76 mmol) of ANTA in 5 mL of DMF at room
37 temperature while vigorously stirring. After 10 min, 220 μ L (0.92 mmol) of lauroyl chloride
38 were added and the mixture was stirred for two days at room temperature. Then the solvent was
39 evaporated in vacuum, and the crude dissolved in 2 mL of water. After carefully acidification
40 with 1.0 M hydrochloric acid, the resulting precipitate was filtered and successively washed with
41 water and diethyl ether to give the title compound (56% yield). Experimental data agree with
42 those previously described by Richard, et al.²⁰
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50 51 *2.4 Preparation of Co²⁺-NTA modified SPCEs.*

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55 In order to functionalize with the NTA layer, 5 μ L of a 1.0 mM HS-NTA or a 3.0 mM
56 LAU-NTA solution (prepared in carbonate buffer) were cast on the working electrode surface
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3 and incubated 2.5 h at room temperature in a humidified chamber. After washing with deionized
4 water and drying with nitrogen, the resulting NTA-modified SPCE was activated by
5 complexation of Co^{2+} by treatment with 5 μL of a 7.5 mM CoCl_2 solution (prepared in acetate
6 buffer) for 30 min, followed by thorough washing with deionized water and drying under a
7 nitrogen stream. The Co^{2+} -loaded electrodes showed no damage when they were left on the
8 benchtop for three days before use (no longer times were checked).
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14 15 16 *2.5 Penicillin binding protein production, overexpression and purification*

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18 Protocols used for recombinant His-tagged PBP protein production, overexpression,
19 purification and storage were the same than those employed in an earlier paper.²¹
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23 24 *2.6 Affinity biosensor preparation*

25 Recombinant PBP was immobilized on the Co^{2+} -NTA modified electrodes through the
26 protein His-tag. In order to do that, a 5- μL aliquot of the PBP solution (880 $\mu\text{g mL}^{-1}$) was
27 dropped on the modified electrode surface and incubated at room temperature for 20 min.
28 Thereafter, the electrode was washed with PBST to remove any unbound protein and dried under
29 a nitrogen stream.
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35 36 *2.7 Electrochemical measurements*

37 Impedimetric and cyclic voltammetric measurements were carried out upon deposition of
38 50 μL of a $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$, 5.0 mM in each component, solution in 0.1 M phosphate
39 buffer pH 7.0 on the modified electrode surface. Electrochemical impedance measurements were
40 performed at the equilibrium potential of the $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ pair, with a 10 mV (rms)
41 sinusoidal excitation amplitude and an automatic analyzer integration time (0.001% S.D. of
42 $I(j\omega)$ correlator output) with a 100 s cut-off time. Measurements were made at 20 steps per
43 decade in the appropriate frequency range five times at each frequency and averaged during each
44 run. Cyclic voltammograms were recorded from -0.40 to 0.60 V at a scan rate of 50 mV s^{-1} .
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51 Amperometric measurements in stirred solutions were performed by applying the desired
52 potential and allowing the steady-state current to be reached. The sensors were transferred to an
53 electrochemical cell containing 10 mL of a 0.1 M phosphate buffer pH 6.0 and 1.0 mM HQ
54 (prepared daily just before the electrochemical measurement) and the amperometric responses at
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$E_{app} = -0.20$ V vs Ag pseudoreference electrode were recorded after the addition of 50 μ L of 0.1 M H_2O_2 . The time required to reach the steady-state current was approximately 1.5 min.

2.8 Affinity assay procedure

A calibration curve for AMP was constructed in the assay buffer (1.0 M NaCl-enriched P&D buffer) containing the optimized enzymatic tracer concentration and different concentrations of the β -lactam antibiotic. The solutions were placed in a vortex to ensure adequate mixing of the components. The competitive assay was performed by casting of a 50- μ L aliquot of the corresponding mixture solution on the biosensor surface and incubation at RT for 30 min. Thereafter, the electrode was thoroughly washed with PBST and the electrochemical measurement was carried out.

The recorded amperometric signals were the difference between the steady-state and the background currents. They were plotted as a function of the analyte concentration, and the experimental data were fitted to a four-parameter logistic equation (sigmoidal) according to the equation:

$$y = i_1 + \frac{i_2 - i_1}{1 + 10^{(\log IC_{50} - x) \cdot p}} \quad (1)$$

where i_2 and i_1 are the maximum and minimum current values in the plotted calibration graph, IC_{50} is the analyte concentration producing 50 % of the maximal amperometric signal and p is the Hill slope at the inflection point of the sigmoid curve. The limit of detection (LOD) was calculated as the analyte concentration for which the enzymatic tracer binding to the PBP was inhibited by 10 %. The dynamic range (DR) was defined as the analyte concentration interval for which the inhibition of the enzymatic tracer binding was between 20 and 80 %. Unless otherwise indicated, the presented data corresponded to the average of at least three replicates.

2.9 Analysis of AMP in milk

Commercial milk samples purchased in a local supermarket were appropriately diluted with P&D buffer solution and spiked with the β -lactam antibiotic and the enzymatic tracer. Aliquots (50 μ L) of these milk solutions were analyzed following the same methodology described above for standard solutions. Non-spiked samples were also checked to exclude the presence of naturally contaminating antibiotics.

3. Results and discussion

The strong adsorption of long alkylic chains leading to the formation of ordered physisorbed monolayers onto carbon electrode surfaces²² was profited to perform the modification of SPCEs with long alkyl-NTA derivatives.²³⁻²⁵ Thermodynamic studies demonstrated that the hydrocarbon molecules adsorb with high affinity to graphite surfaces and that the heat of adsorption increases with chain length suggesting that the molecules are adsorbed with their long axis parallel to the graphite surface as it was confirmed by STM, X-ray and neutron diffraction studies.²⁶ In order to verify this, we evaluated by electrochemical impedance spectroscopy (EIS) the adsorption of 2 long alkyl chain NTA derivatives, a thiolated HS-NTA and the non-thiolated analogue (LAU-NTA) on carbon electrodes (see Figure S1, Supporting Information). Although a slightly larger coverage, calculated from the charge-transfer resistance values at the modified and unmodified electrodes,²⁷ was obtained with the HS-NTA derivative (74.7 vs. 67.2 %), the obtained results demonstrated the successful adsorption of both compounds on SPCEs generating dense layers in only 2.5 h. This fact confirmed that the thiol moiety did not play a major role in the adsorption of long alkyl chain NTA derivatives on a carbon surface.

As commented above, recombinant PBP immobilization was accomplished through the interactions between the protein His-tag and the Co^{2+} -NTA functionality existing at the modified electrode surfaces. The employed scheme involved the competition of the unlabeled analyte and a fixed concentration of the enzymatic tracer (PENG-HRP) for the limited number of binding sites of the recombinant binding protein immobilized on the SPCE surface using the Co^{2+} /NTA chemistry (see Figure 1).

Firstly, the successful modification of the electrode surface with the Co^{2+} -NTA moiety and the subsequent immobilization of the PBP were verified by monitoring the stepwise assembly using cyclic voltammetry (CV) and EIS. Fig. 2 shows the CVs recorded for the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox pair at a bare SPCE, and after the successive modifications with HS-NTA, Co^{2+} , and PBP, whereas the inset of Figure 2 displays the corresponding EIS Nyquist plots. As expected, the quasi-reversible voltammetric response observed at the unmodified SPCE was strongly attenuated at the HS-NTA-modified SPCE, reflecting the electrostatic repulsion between the redox pair and the $-\text{COO}^-$ groups at neutral pH. A slight increase in the peak current

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3 was observed after Co^{2+} complexation which can be attributed to the partial shielding of the
4 negative surface charges by complexed Co^{2+} ions.²⁸ The PBP immobilization on the Co^{2+} -NTA-
5 SPCE caused an additional barrier for the access of the redox probe to the electrode thus leading
6 to a further decrease in the voltammetric signal showing the ability of the Co^{2+} -NTA-SPCEs to
7 bind recombinant PBP via N-terminal hexahistidine tail. These results are also consistent with
8 the changes observed in the electron transfer resistance (Figure 2 inset).
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14 On the other hand, similar amperometric responses were obtained for solutions containing
15 no β -lactam antibiotic with biosensors constructed using both HS-NTA and LAU-NTA-modified
16 SPCEs (Figure S2, Supporting Information). Therefore, taking into account that the adsorption of
17 both compounds takes place by the long alkyl chain and not by the terminal functional group, we
18 decided to continue working only with the commercial HS-NTA derivative.
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24 *3.1 Optimization of experimental variables*

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26 The optimum experimental conditions for achieving the best affinity assay performance
27 were sought out. All the experimental variables involved in the affinity sensor preparation and
28 functioning were, therefore, optimized by taking as the selection criterion the maximum current
29 measured at -0.20 V in the absence of β -lactam antibiotics.
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34 Firstly, in order to evaluate the extent of enzymatic tracer non-specific binding to the
35 Co^{2+} -NTA-SPCE surface, we compared the responses measured with and without the PBP
36 immobilized on the modified SPCE in a solution of the enzymatic tracer in P&D buffer. Non-
37 specific adsorption of PENG-HRP occurred at the PBP-free Co^{2+} -NTA-SPCE surface, leading to
38 an amperometric signal only 1.2 times lower than at the PBP- Co^{2+} -NTA-SPCE. Therefore, we
39 performed the competitive reaction in different working media with the aim of minimizing the
40 non-specific signal. The ratios between the specific and the unspecific responses were calculated
41 for P&D buffer solution supplemented with NaCl (1.0 M) and for P&D buffer 1:1 diluted UHT
42 whole milk. As it can be seen in Figure 3, that ratio increased notably in both media as a
43 consequence of a remarkable decrease in the non-specific binding of the enzymatic tracer due to
44 the presence of the high ionic salt or milk matrix components.¹⁵ Accordingly, a P&D buffer
45 solution containing 1.0 M NaCl was chosen as the working medium to evaluate the analytical
46 performance of the affinity biosensor.
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Thereafter, the variables involved in the affinity biosensor preparation and functioning were optimized according to the selection criterion mentioned above. The ranges tested for all these variables as well as the selected values are collected in Table 1. It is worth to remark that the functionalization of the SPCE surface with the NTA derivative provided a stable monolayer with the thiol tether not attached to the electrode thus implying a larger accessible potential window when compared with thiolated chains-modified gold electrodes. Moreover, this modification took only 2.5 h which is a significant advantage with respect to the modification of gold electrodes with NTA-terminated thiolated SAMs which requires 12-24 hours of incubation in the thiol solutions.^{8,29}

Cu^{2+} , Ni^{2+} , Co^{2+} and Zn^{2+} were checked as metal ions to be coordinated with the NTA derivative attached to the electrode surface (Figure S3, Supporting Information). Similar specific-to-unspecific responses ratios were obtained using Cu^{2+} , Ni^{2+} and Co^{2+} . However, the use of Co^{2+} allowed the regeneration of the affinity sensor as it will be commented below. This was attributed to the lower stability constant of the corresponding complex ($\text{pK}_a = 10.4$).³⁰ Furthermore, the influence of the Co^{2+} concentration used for the coordination with the immobilized NTA derivative on the amperometric response obtained in the absence of free β -lactam antibiotic was evaluated (Figure S4, Supporting Information). It can be observed as the measured current increased with the Co^{2+} concentration which confirmed that the metal ion played an essential role in the PBP binding on the modified SPCE. As expected, the amperometric response obtained when no metal ion was present was only slightly higher than the non-specific signal resulting in the absence of immobilized His-PBP.

3.2 Analytical characteristics of the affinity sensor

The performance of the integrated affinity biosensor was tested using AMP as a model target penicillin. The calibration graph constructed for AMP in P&D buffer solution containing 1.0 M NaCl is displayed in Figure 4. The DR extended between 1.3 and 9.9 ng mL⁻¹ with an IC₅₀ value of 3.6 ng mL⁻¹ and a LOD of 0.7 ng mL⁻¹, which is approximately 6 times lower than the limit regulated by the EU (4 $\mu\text{g kg}^{-1}$ AMP content in milk samples). Therefore, the obtained results are promising and encouraging for the further application of the disposable amperometric affinity sensor in the analysis of food samples such as milk.

3.3 Analytical characteristics of the affinity sensor in milk samples

The usefulness of the affinity biosensor for the determination of β -lactam antibiotics in milk was evaluated selecting a penicillin (AMP) and a cephalosporin (CEF) as target analytes. Calibration curves were obtained for both compounds in whole UHT milk with only a 1:1 dilution with P&D buffer as sample treatment. It is important to remark that the addition of 1.0 M NaCl to the buffer solution was not needed now because, as commented before, the milk matrix components acted as an effective *in situ* blocking agent able to minimize the non-specific signals.¹⁵ The obtained calibration graphs are shown in Figure 5 and the corresponding analytical characteristics are summarized in Table 2. As it can be observed in both cases the LODs are well below the MRLs established by current legislation.

The reproducibility of the responses obtained with different sensors prepared in the same manner was evaluated both within and between days by performing measurements in 1:1 P&D buffer-diluted UHT whole milk spiked with the enzymatic tracer and in the absence of β -lactam antibiotics. Six different sensors prepared the same day yielded a RSD value for the steady-state current of 4.6 %. Moreover, the RSD value was 5.7 % for five sensors prepared in different days. These results demonstrated a good reproducibility of the affinity sensor fabrication procedure. The storage stability of the integrated affinity sensors was evaluated by preparing on the same day different PBP-Co²⁺-HS-NTA pre-modified SPCEs which were stored at 4 °C under dry conditions. The sensors were tested periodically by evaluating their response in a fresh solution of PENG-HRP. A control chart was constructed by taking the mean value of 5 measurements obtained the first working day as the central value and setting the upper and lower control limits at $\pm 3 \times SD$ of this initial value. The responses obtained remained within the control limits for 8 days without a noticeable decrease in their original response and kept the 91.2% of it after 11 days.

Furthermore, the reversibility of the oriented immobilization of the histidine-tagged protein onto the Co²⁺-NTA-modified electrode and hence the reusability of the developed affinity sensor was also verified. In order to do that, we tested the amperometric responses obtained with four different electrodes after incubation of the PENG-HRP-PBP-Co²⁺-NTA-SPCEs during 3 h in a 50 mM phosphate solution pH 8.0 containing 300 mM NaCl, 300 mM imidazol and 0.01 % Tween[®]20 under continuous stirring and after 40 min in deionized water. This buffer is the one recommended for reuse of His-tag-MBs. The measured currents were in all

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3 cases similar to that obtained with no enzymatic tracer which was attributed to the replacement
4 of the histidine ligand by imidazole and, consequently, to the release of the anchored PENG-
5 HRP. When the Co^{2+} coordination, PBP immobilization and PENG-HRP recognition protocols
6 were repeated with these regenerated sensors, the initial amperometric response was restored
7 thus evidencing the specificity and reversibility of this binding approach, in contrast with the
8 commonly used biotin-avidin immobilization which leads to irreversible anchoring of
9 biotinylated biomolecules. Therefore, the reusability of the affinity sensors was demonstrated.
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17 *3.4 Affinity sensor selectivity*

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19 The selectivity of the developed affinity sensor was tested against 3 non-target antibiotics
20 (TC, SPY and ENRO) which may be present in milk and dairy products. The amperometric
21 signals obtained with the affinity sensor in the absence and in the presence of these potentially
22 interfering antibiotics at two different concentration levels (500 and 10,000 nM) in 1:1 P&D-
23 diluted UHT whole milk were compared. The obtained results (data not shown) demonstrated
24 that no significant cross-reactivity occurred for the 3 non-target antibiotics tested even in the
25 presence of a high excess (10,000 nM). In fact, the percentages of cross-reactivity calculated
26 from the corresponding IC_{50} values³¹ were <1% in all cases indicating that no significant
27 competition was produced and the remarkable group specificity of the PBP used to prepare the
28 affinity sensor.
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37 An important practical aspect is that MRLs are defined for β -lactams with a non-
38 hydrolyzed β -lactam ring. Therefore, the specificity of PBP for active β -lactams should be also
39 tested. In order to do that, we spiked milk samples with hydrolyzed AMP or CEF (according to
40 the procedure described in *Section 2.2.*) at two concentration levels, 10^2 and 10^3 ng mL^{-1} . The
41 amperometric responses obtained with the affinity sensor showed that no detectable active
42 antibiotic residues were found in these samples demonstrating that no cross-reactivity occurred
43 with hydrolyzed β -lactams (open-ring metabolites or degradation products).
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51 *3.5 Application to the analysis of spiked milk samples*

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53 The amperometric affinity sensor was applied for the analysis of UHT whole milk
54 samples (antibiotic-free) spiked with AMP at a 30.0 nM (10.5 ng mL^{-1}) concentration level. The
55 quantification of AMP was accomplished by interpolation of the measured current values into
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3 the calibration plot constructed for the antibiotic in the same matrix sample (Figure 5). The
4 analysis of 10 milk samples yielded an AMP mean concentration of (10.8 ± 0.8) ng mL⁻¹ with a
5 mean recovery of (103 ± 8) % (RSD = 9.9 %), the confidence interval being calculated for $\alpha =$
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7 0.05. Taking into account the type of analysis carried out and the straightforwardness of the
8 experimental protocol, these good results demonstrated the usefulness of the affinity sensor for
9 the determination of β -lactam antibiotics, in a total time for the assay of approximately 30 min
10 (once the sensor is prepared).
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17 *3.6 Comparison with other reported sensors and methodologies*

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19 The performance of the developed amperometric affinity sensor was compared with that
20 provided by other approaches reported in the literature (Table 3). As it can be seen, most of the
21 previously developed methodologies used SPR detection whereas only two involved
22 electrochemical transduction.^{15,21} While offering similar analytical performance some important
23 advantages can be claimed for the approach described here against non-electrochemical
24 methodologies. These include the inherent simplicity and much easier potential automation and
25 miniaturization thus making the developed methodology ideal as a field-based screening tool for
26 β -lactam detection in milk. When compared with the other available electrochemical sensors, it
27 is important to remark that, conversely to that occurred with the only integrated affinity sensor
28 reported until now, the non-specific binding of the PENG-HRP conjugate was independent of the
29 free β -lactam concentration in solution.¹⁵ Moreover, the LOD achieved is 15 times lower than
30 that reported for an amperometric affinity PBP magnetosensor.²¹ It is worth to remark also that
31 this is the first time that an integrated amperometric sensor is developed by immobilization of a
32 recombinant PBP using the His-tag/M²⁺/NTA chemistry.
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46 **4. Conclusions**

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48 This work showed for the first time the successful, simple and one step modification of
49 SPCEs with a dense monolayer of long alkyl chain NTA ligands offering an attractive platform
50 for the development of affinity biosensors by reproducible, stable, reversible and oriented
51 immobilization of His-tagged recombinant proteins. Using this novel platform, an integrated
52 amperometric affinity sensor for the specific and sensitive detection of β -lactam antibiotics in
53 milk was developed by immobilization of a recombinant PBP on the surface of the modified
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3 SPCEs using His-tag/Co²⁺/NTA chemistry. The obtained results demonstrated the ability of the
4 developed sensor to detect and quantify low levels of β -lactam antibiotics residues, either
5 penicillins or cephalosporins, in milk samples in a reliable, specific and rapid manner (30 min)
6 with no need of complex sample treatment steps. On the other hand, it is anticipated that the
7 simple, versatile, efficient and fast immobilization approach developed, based exclusively on
8 commercially available chemicals, should be useful in the reversible immobilization, with a
9 controlled molecular orientation, of a wide variety of histidine-tagged proteins on carbon
10 electrode surfaces without significant loss of their biological activity finding application in the
11 development of other bioanalytical strategies of interest.
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21 Acknowledgements

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32 References

- 33
34 (1) Blankespoor, R.; Limoges, B.; Schöllhorn, B.; Syssa-Magalé, J.-L.; Yazidi, D. *Langmuir*
35 **2005**, *21*, 3362–3375.
36
37 (2) Haddour, N.; Cosnier, S.; Gondran, C. *J. Am. Chem. Soc.* **2005**, *127*, 5752–5753.
38
39 (3) Ley, C.; Holtmann, D.; Mangold, K.-M.; Schrader, J. *Colloid. Surface. B* **2011**, *88*, 539–551.
40
41 (4) Sigal, G.B.; Bamdad, C.; Barberis, A.; Strominger, J.; Whitesides, G.M. *Anal. Chem.* **1996**,
42 *68*, 490–497.
43
44 (5) Madoz-Gurpide, J.; Abad, J.M.; Fernández-Recio, J.; Velez, M., Vázquez, L.; Gómez-
45 Moreno, C.; Fernández, V.M. *J. Am. Chem. Soc.* **2000**, *122*, 9808–9817.
46
47 (6) Benson, D.E.; Conrad, D.W.; Lorimier, R.M.; Trammel, S.A.; Hellinga, H.W. *Science* **2001**,
48 *293*, 1641–1644.
49
50 (7) Gizeli, E.; Glad, J. *Anal. Chem.* **2004**, *76*, 3995–4001.
51
52 (8) Valiokas, R.; Klenkar, G.; Tinazli, A.; Reichel, A.; Tampé, R.; Piehler, J.; Liedberg, B.
53 *Langmuir* **2008**, *24*, 4959–4967.
54
55 (9) Harris, A.G.; Hazell, S.L.; Netting, A.G. *J. Antimicrob. Chemoth.* **2000**, *45*, 591–598.
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59
60
- (10) Cacciatore, G.; Petz, M.; Rachid, S.; Hakenbeck, R.; Bergwerff, A.A. *Anal. Chim. Acta* **2004**, *520*, 105–115.
- (11) Jamin, M.; Hakenbeck, R.; Frere, J.-M. *FEBS Lett.* **1993**, *331*, 101–104.
- (12) Fernández, F.; Hegnerová, K.; Piliarik, M.; Sanchez-Baeza, F.; Homola, J.; Marco, M.-P. *Biosens. Bioelectron.* **2010**, *26*, 1231–1238.
- (13) Lamar, J.; Petz, M. *Anal. Chim. Acta* **2007**, *586*, 296–303.
- (14) Liu, W.; Zhang, Z.; Liu, Z. *Anal. Chim. Acta* **2007**, *592*, 187–192.
- (15) Setford, S.J.; Van Es, R.M.; Blankwater, Y.J.; Kröger, S. *Anal. Chim. Acta* **1999**, *398*, 13–22.
- (16) Commission Regulation (EU) No 37/2010, Official Journal of the European Union L 15/1 of 22 December 2009.
- (17) Kantiani, L.; Farré, M.; Barceló, D. *Trends Anal. Chem.* **2009**, *28*, 729–743.
- (18) Babington, R.; Matas, S.; Marco, M.-P.; Galve, R. *Anal. Bioanal. Chem.* **2012**, *403*, 1549–1566.
- (19) Grubelnik, A.; Padeste, C.; Tiefenauer, L. *Food Agr. Immunol.* **2001**, *13*, 161–169.
- (20) Richard, C.; Balavoine, F.; Schultz, P.; Ebbesen, T.E.; Mioskowski, C. *Science* **2003**, *300*, 775–778.
- (21) Gamella, M.; Campuzano, S.; Conzuelo, F.; Esteban-Torres, M.; de las Rivas, B.; Reviejo, A.J.; Muñoz, R.; Pingarrón, J.M. *Analyst* (submitted, 2012).
- (22) Bickerstaffe, A.K.; Cheah, N.P.; Clarke, S.M.; Parker, J.E.; Perdigon, A.; Messe, L.; Inaba, A. *J. Phys. Chem. B* **2006**, *110*, 5570–5575.
- (23) Martin, D.S.; Weightman, P.; Gauntlett, J.T. *Surf. Sci.* **1999**, *424*, 187–198.
- (24) Enevoldsen, A.D.; Hansen, F.Y.; Diama, A.; Criswell, L.; Taub, H. *J. Chem. Phys.* **2007**, *126*, 104703, 1–10.
- (25) Zhao, M.; Jiang, P.; Deng, K.; Yu, A.-F.; Hao, Y.-Z.; Xie, S.-S.; Sun, J.-L. *Appl. Surf. Sci.* **2011**, *257*, 3243–3247.
- (26) Bhinde, T.; Clarke, S.M.; Phillips, T.K.; Arnold, T.; Parker, J.E. *Langmuir* **2010**, *26*, 8201–8206.
- (27) Janek, R.P.; Fawcett, W.R.; Ulman, A. *Langmuir* **1998**, *14*, 3011–3018.

- 1
2
3 (28) Giess, F.; Friedrich, M. G.; Heberle, J.; Naumann, R. L.; Knoll, W. *Biophys. J.* **2004**, *87*,
4 3213–3320.
5
6 (29) Lee, J.K.; Kim, Y.-G.; Chi, Y.S.; Yun, W.S.; Choi, I.S. *J. Phys. Chem. B* **2004**, *108*,
7 7665–7673.
8
9 (30) Matheus, P.R.; Abad, J.M.; Fernández, V.M. *Rev. Téc. Ing. Univ. Zulia.* **2007**, *30*, 225–235.
10
11 (31) Conzuelo, F.; Gamella, G.; Campuzano, S.; Reviejo, A.J.; Pingarrón, J.M. *Anal. Chim. Acta.*
12 **2012**, *737*, 29–36.
13
14 (32) Gustavsson, E.; Bjurling, P.; Sternesjö, Å. *Anal. Chim. Acta* **2002**, *468*, 153–159.
15
16 (33) Gustavsson, E.; Degelaen, J.; Bjurling, P.; Sternesjö, Å. *J. Agric. Food Chem.* **2004**, *52*,
17 2791–2796.
18
19 (34) Sternesjö, Å.; Gustavsson, E. *J. AOAC Int.* **2006**, *89*, 832–837.
20
21
22
23
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Table 1. Optimization of experimental variables involved in the preparation and functioning of the PBP-Co²⁺-NTA-SPCE affinity biosensor

Experimental variable	Checked range	Selected value
[NTA], mM	0.0 – 25	1.0 (HS-NTA) / 3.0 (LAU-NTA)
t_{NTA}, h	0.5 – o/n	2.5
t_{Co(II)}, min	0 – 60	30
[Co(II)], mM	0.0 – 10.0	7.5
t_{PBP}, min	0 – 60	20
[PBP], μg mL⁻¹	440 – 1330	880
PENG-HRP, dilution	1/25 – 1/200	1/50

o/n: overnight

Table 2. Analytical characteristics for the determination of AMP and CEF in 1:1 P&D buffer-diluted UHT whole milk with the PBP-Co²⁺-NTA-SPCE affinity biosensor

	AMP	CEF
r²	0.996	0.997
IC₅₀ / nM	15.6	7.1
IC₅₀ / ng mL⁻¹	5.5	3.0
LOD / nM	2.5	0.9
LOD / ng mL⁻¹	0.9	0.4
MRL / ng mL⁻¹	4.0	60.0
DR / nM	5.0 – 49.2	1.9 – 26.8
DR / ng mL⁻¹	1.7 – 17.2	0.8 – 11.3

Table 3. Comparison of the analytical characteristics of the developed affinity sensor with those reported for other assays for β -lactam antibiotics determination

Assay protocol	Detection type	DR / ng mL ⁻¹	LOD / ng mL ⁻¹	Sample	Assay time	Reference
The sample was mixed and incubated with PBP 2X*. After, DIG-AMPI was added and incubated to the mixture. It was injected over a sensor chip modified surface modified with anti-DIG.	SPR	—	a) 5 (PENG) 7 (AMP) 8 (AMOX) b) 2 (PENG)	Milk (previously defatted) and raw milk (previously heated)	40 min	10
The PBP2X is immobilized by adsorption into a microplate. After adding the sample, a bifunctional reagent is added, (DIG-AMPI). The detection step uses anti-digoxigenin HRP labeled.	Spectrophotometry	—	—	Milk, meat, honey, egg	125 min	13
Immobilization of PBP by adsorption over SPE-RC surface. The assay consists of a competitive binding assay format using PENG and 7-ACA-GOD	Amperometry (+350 mV vs Ag/AgCl)	—	5 (PENG)	Milk (50% diluted and undiluted)	6 min	15
Competitive affinity assay using PENG-HRP on PBP-modified MBs	Amperometry (E _{app} = -0.20 V vs. Ag pseudo ref. electrode)	62.0-238.1 (AMP)	14.0 (AMP)	Milk (1:1 P&D diluted)	30 min	21
A 2-peptide was covalently bounded to the sensor surface. The sample was mixed and incubated with 3-peptide and R39. After, 2-peptide antibody was added to the sample and the mixture was injected over a sensor surface.	SPR	—	2.6 (buffer) 5.2 (Defatted milk)	Milk (previously defatted)	9 min	32
The sample was mixed and incubated with 3-peptide and R39. a) 2-peptide antibody was added to the sample and the mixture was injected over a 2-peptide-modified sensor surface b) 3-peptide antibody was added to the sample and the mixture was injected over a 3-peptide modified sensor surface	SPR	a)1.5-5.0	a) 1.2 (PENG) b) 1.5 (PENG)	Milk	7 min	33
The sample was mixed and incubated with 3-peptide and R39. a) 2-peptide antibody was added to the sample and the mixture was injected over a 2-peptide-modified sensor surface b) 3-peptide antibody was added to the sample and the mixture was injected over a 3-peptide modified sensor surface	SPR	—	a) 1.2 (PENG) b) 1.5 (PENG)	Milk	7 min	34
Competitive affinity assay using PENG-HRP on PBP-Co ²⁺ -HS-NTA-modified SPCEs	Amperometry (E _{app} = -0.20 V vs. Ag pseudo ref. electrode)	1.7 - 17.2 (AMP) 0.8 - 11.3 (CEF)	0.9 (AMP) 0.4 (CEF)	Milk (1:1 P&D diluted)	30 min	This work

Abbreviations: 7-ACA-GOD: 7-aminocephalosporanic acid modified with glucose oxidase, AMOX: amoxicillin; AMP: ampicillin; DIG-AMPI: digoxigenin-labelled ampicillin; HRP: peroxidase, PBP2X*: Penicillin binding protein from *Streptococcus pneumoniae*, PENG: Penicillin G; 3-peptide: (Ac-L-Lys-D-Ala-D-Ala); 2-peptide: (Ac-L-Lys-D-Ala); R39: D,D-carboxypeptidase from *Actinomadura* R39; R39*: D,D-carboxypeptidase from *Streptomyces* R39, SPE-RC: screen printed electrode with a rhodinated-carbon working electrode, SPR: surface plasmon resonance.

Legends to Figures

Figure 1. Schematic display of steps involved in the development of the affinity biosensor. Bottom inset: details of the surface chemistry involved on the immobilization of the recombinant histidine-tagged PBP by using Co^{2+} -NTA modified SPCEs.

Figure 2. Cyclic voltammograms and Nyquist plots ($-Z''$ vs Z') obtained at a bare SPCE, NTA-SPCE, Co^{2+} -NTA-SPCE and PBP- Co^{2+} -NTA-SPCE for 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (1:1) in a 0.1 M phosphate buffer pH 7.0 solution. EIS parameters: 0.01 – 10,000 Hz frequency range with a 0.01 V rms signal at +0.25 V (vs Ag pseudoreference electrode); CV, $v = 50 \text{ mV s}^{-1}$.

Figure 3. Amperometric responses measured at -0.20 V for solutions containing PENG-HRP in the absence of β -lactam antibiotics with the Co^{2+} -NTA-SPCE (non-specific signals) and with the PBP- Co^{2+} -NTA-SPCE (specific signal) as well as their corresponding ratios in: P&D buffer (1), 1.0 M NaCl-enriched P&D buffer (2) and 1:1 P&D buffer-diluted UHT whole milk (3). Error bars were estimated as a triple of the standard deviation ($n=3$).

Figure 4. Calibration curve constructed with the PBP- Co^{2+} -NTA-SPCE affinity sensor for AMP in P&D buffer containing 1.0 M NaCl. The experimental points were fitted to equation 1. Error bars were estimated as a triple of the standard deviation ($n=3$).

Figure 5. Calibration graphs obtained with the PBP- Co^{2+} -NTA-SPCE affinity sensor for AMP and CEF in 1:1 P&D-diluted UHT whole milk. The experimental points were fitted to equation 1. Error bars were estimated as a triple of the standard deviation ($n=3$).

Figure 1

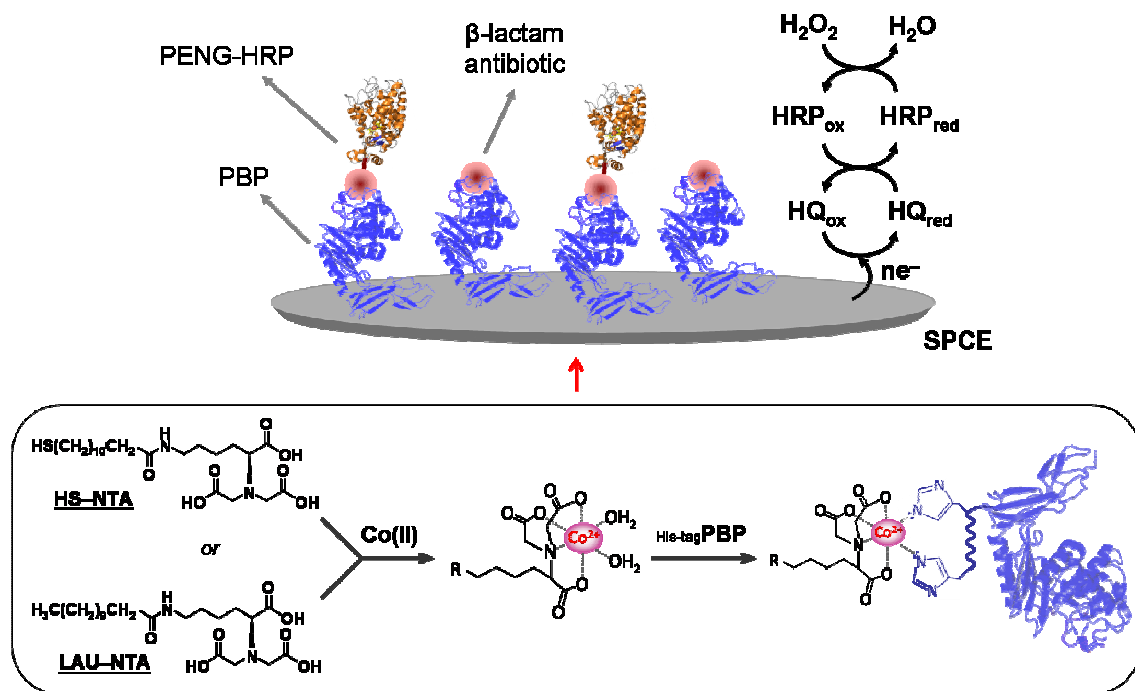


Figure 2

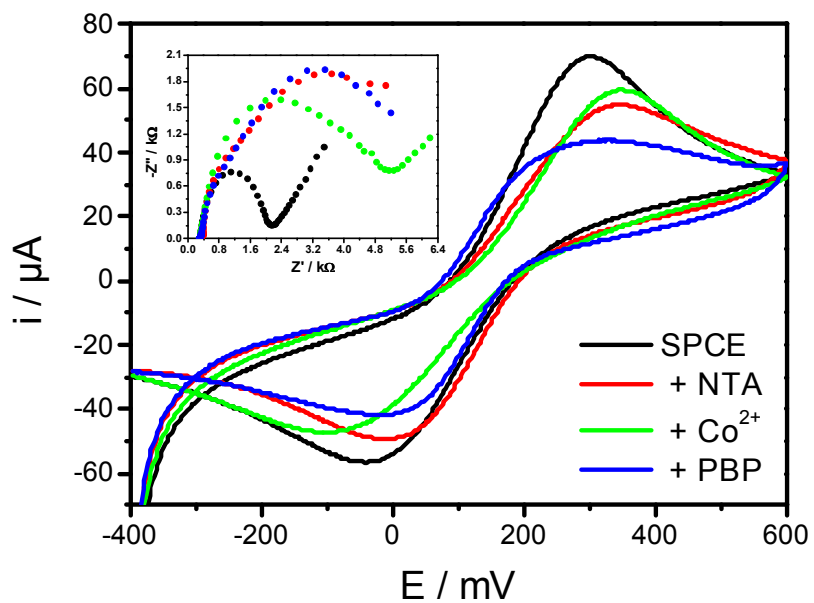


Figure 3

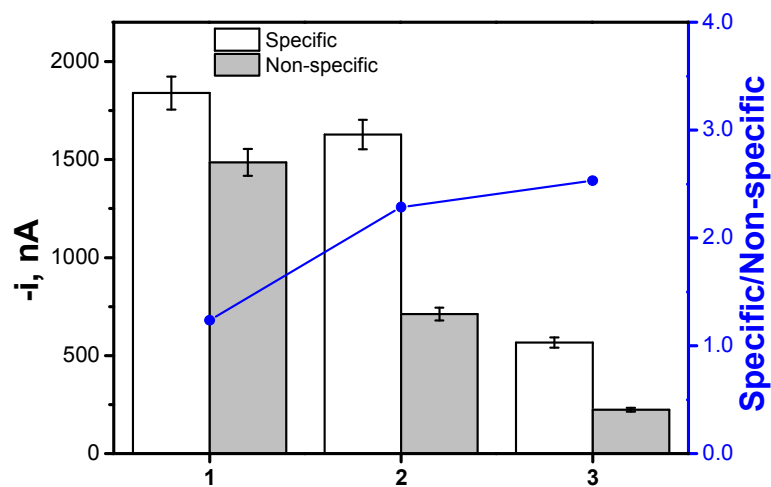


Figure 4

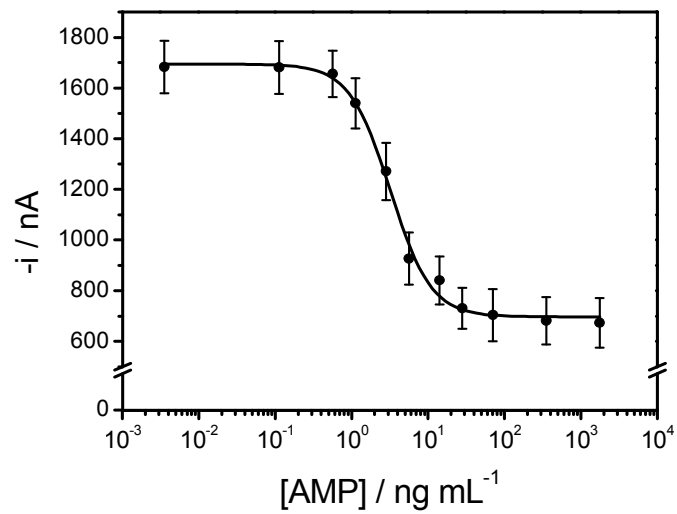
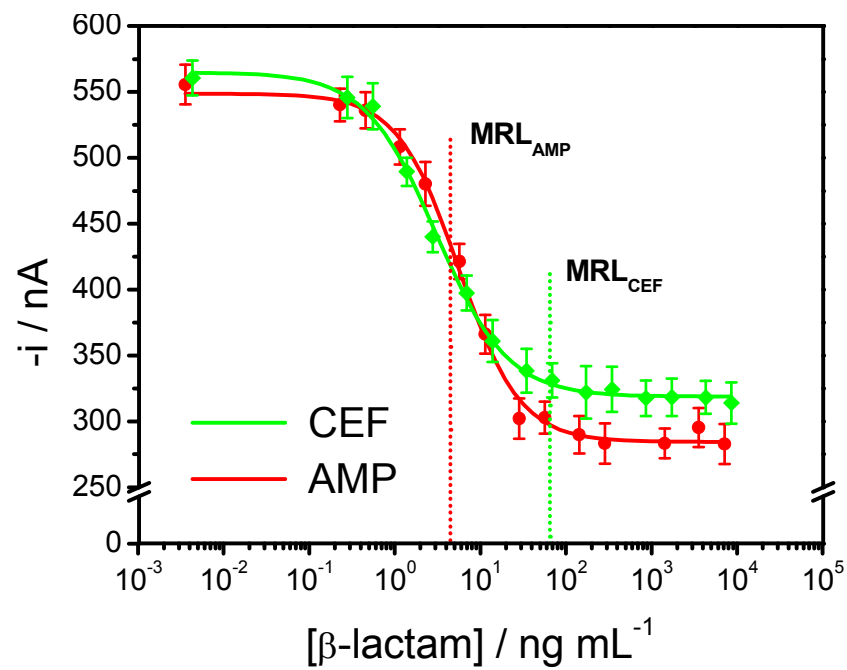
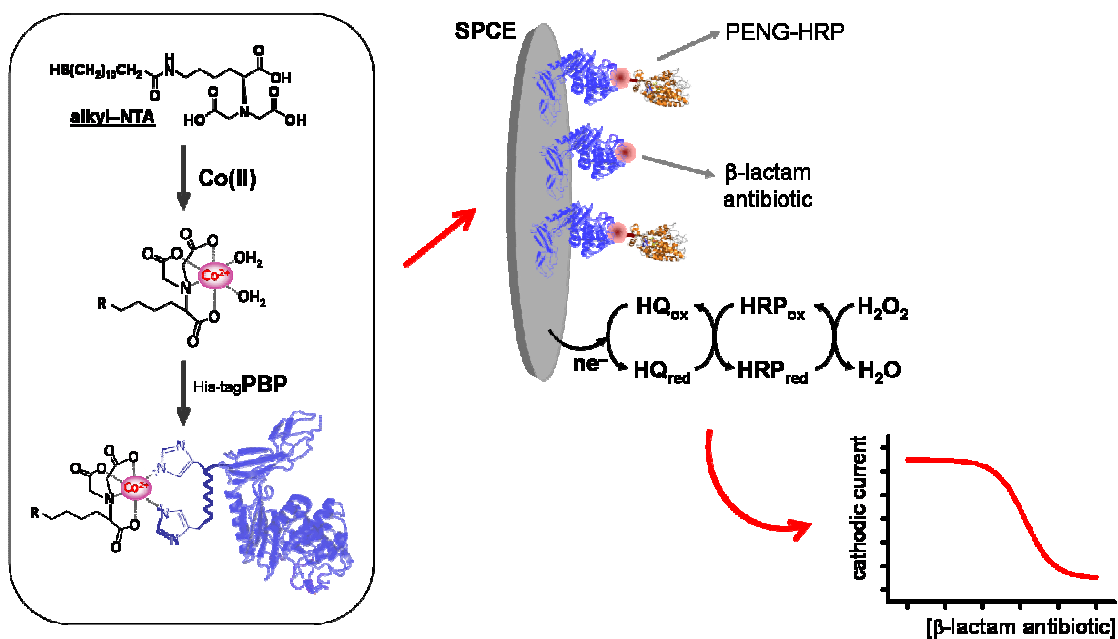


Figure 5



For TOC only:



Novel attractive platform for the development of affinity biosensors based on the successful, simple and one step modification of carbon electrodes with a dense monolayer of a long alkyl chain NTA derivative allowing the reproducible, stable, reversible and oriented immobilization of His-tagged recombinant proteins.