

AN AMPEROMETRIC AFFINITY PENICILLIN-BINDING PROTEIN
MAGNETOSENSOR FOR THE DETECTION OF β -LACTAM ANTIBIOTICS IN
MILK.

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Abstract

The preparation, characterization and performance evaluation of an amperometric affinity disposable magnetosensor, based on the use of a recombinant penicillin-binding protein (PBP) and screen-printed carbon electrodes (SPCEs), for the specific detection and quantification of β -lactams antibiotic residues in milk is reported. PBP was immobilized onto His-Tag-Isolation-modified magnetic beads (His-Tag-Isolation-MBs), and a direct competitive assay using a tracer with horseradish peroxidase (HRP) for the enzymatic labeling was performed. The amperometric response obtained at -0.20 V vs. the Ag pseudoreference electrode of the SPCE after the addition of H_2O_2 in the presence of hydroquinone (HQ) was used as transduction signal. The developed methodology showed very low detection limits (in the low ppb level) for the 6 antibiotics tested in untreated milk samples, and a good selectivity against other antibiotics residues frequently detected in milk and dairy products. Due to the bioreceptor employed, this methodology was able to detect only the active form of β -lactam antibiotics with high affinities for both penicillins and cephalosporins. Moreover, the analysis time took only 30 min.

Introduction

Penicillins and other β -lactam antibiotics (i.e. penicillins and cephalosporins) are the most frequently used antibiotics in veterinary medicine for treatment of various bacterial infections, e.g., mastitis in dairy cows. This may lead to the presence of antibiotics residues in milk and a small quantity of these compounds might be responsible for allergic reaction in humans and for the development of resistant strains of bacteria.¹ Consequently, detection of β -lactam antibiotics residues in food and environment is crucial for public health protection. In order to assure the safety and quality of dairy products, a number of regulatory authorities have decreed maximum residue levels (MRLs). For example, the European Union Regulation 508/1999 set the MRL for benzylpenicillin in milk at 4 ppb or 1.2×10^{-8} M.²

The most common methods for the analysis of veterinary drugs make use of liquid chromatography.^{3,4} These methods are reliable and possess adequate sensitivity for enforcement of the maximum residue limits or tolerance levels of antibiotic residues in different samples. However, HPLC methods require extensive sample pretreatment and are thus mostly used in centralized laboratories. As a consequence, the development of rapid, inexpensive, sensitive and on-site analytical strategies with high sample throughput suitable to be used in a wide variety of food matrices and by untrained personnel constitutes a challenge.⁵ A variety of commercial test kits and strips are available for rapid screening of β -lactam antibiotics in certain samples.^{6,7} These screening tests can be divided into microbial inhibitor assays,⁸ enzymatic assays,⁹ immunoassays^{10,11} and receptor assays.^{12,13} The first approach examines the growth of test microorganisms in the presence of the sample, and therefore it is time consuming and non-specific. Enzyme inhibition methods are based on the specific inhibitory effect of β -lactams on certain enzymes and are more rapid.¹⁴ In the case of immunoassays, the development of antibodies that are specific to the intact β -lactam structure has been achieved with limited success¹⁵ because the immunisation procedure leads to the

open-ring forms of the β -lactams and consequently to antibodies against hydrolysed penicillins.¹⁶ Moreover, these test systems can only detect penicillins and not the whole group of β -lactams including cephalosporins.

The receptor binding assays do not use antibodies to bind antibiotics although the principles of these tests are analogous to immunological methods.¹² The binding bioreceptors are bacterial proteins which are inhibited by β -lactams. The action mechanism is based on the inhibition of membrane-bound enzymes involved in the final stages of bacterial peptidoglycan synthesis.¹⁷ Due to their ability to covalently bind penicillins and other β -lactams antibiotics through the active-site serine, these enzymes are called penicillin binding proteins (PBPs).¹⁶ PBPs interact with the β -lactam structure forming a very stable complex and, as a result, the enzymatic activity is inhibited.¹⁸

PBPs have been used as binding reagents in β -lactam specific receptor binding assays and enzyme assays, the inhibition of the enzymatic DDCarboxypeptidase activity being measured in the latter case.¹⁶ One of the earliest receptor protein-based methods for detection of penicillins was reported by Frère et al.,¹⁹ and it relied on the enzymatic activity of an extracellular carboxypeptidase from *Streptomyces* R39 (R39). More recently, Surface Plasmon Resonance (SPR) biosensors based on the use of R39 were described.^{15,18,20} Concerning specific receptor binding assays, Cacciatore et al.¹² described an SPR biosensor based on a penicillin-binding protein derivate (PBP2X*) from *Streptococcus pneumoniae*, expressed in *E. coli*, while Lamar et al.¹⁶ reported a spectrophotometric assay based on the same PBP2X*.

This paper describes, for the first time, the design and development of an amperometric affinity magnetosensor with a broad specificity for the detection of active β -lactam antibiotics residues in milk at the low ppb concentration level, which can provide a rapid method suitable to be applied for on-site food quality control. The biosensor design involved the

immobilization of the recombinant PBP on the surface of modified magnetic beads. Quantification was achieved through competitive binding between the target β -lactam and a HRP-labelled specific tracer for the binding sites of the immobilised PBP. The electrochemical monitoring of the enzyme reaction using H_2O_2 as the enzyme substrate and hydroquinone (HQ) as electron transfer mediator was carried out at a disposable screen-printed carbon electrode (SPCE). The affinity sensor performance, including the analytical characteristics for the determination of different β -lactams as well as the selectivity against other antibiotics were evaluated, and the magnetosensor was successfully applied to the analysis of low concentration level spiked milk samples after just a simple dilution treatment.

Experimental

Materials

Amperometric measurements were performed with a BASi Epsilon-EC (Bioanalytical Systems, Inc) controlled by the BASi ComServer Ver. 3.09 software. All measurements were carried out at room temperature.

The transducers employed were screen-printed carbon electrodes (SPCEs, DRP-110, DropSens) consisting of a 4-mm carbon working electrode, a carbon counter electrode and an Ag pseudoreference electrode.

An Optic Ivymen[®] System constant temperature incubator shaker (Comecta S.A.) and a Bunsen AGT-9 Vortex were used for the incubation of the MBs and for homogenization of the spiked samples, respectively. Magnetic separations in the washing steps were performed using a Dynal MPC-S magnetic separator (product No. 120.20, Dynal Biotech ASA, Norway).

All the reagents used were of the highest available purity grade. Tween[®]20, hydroquinone (HQ), hydrogen peroxide (30%, v/v), penicillin G sodium salt (PENG), amoxicillin (AMOX),

ampicillin sodium salt (AMPI), cefapirin (CEF), enrofloxacin (ENRO), sulfapyridine (SPY), tetracycline hydrochloride (TET), oxacillin (OXA), cloxacillin (CLOX) were purchased from Sigma-Aldrich. Sodium dihydrogen phosphate, di-sodium hydrogen phosphate, potassium chloride, potassium hydroxide, methanol, sodium chloride and dimethyl sulfoxide (DMSO) were purchased from Scharlau. Stock solutions (10 mM) of the different antibiotics were prepared in DMSO (SPY and ENRO) or in Pull and down buffer (P&D) (PENG, AMOX, AMPI, CEF, TET, OXA and CLOX). Antibiotic standard solutions for affinity sensor calibration were prepared daily upon dilution of the stock solutions in P&D buffer or in the diluted milk matrix.

Tetradentate Metal Chelator-modified magnetic beads Dynalbeads His-Tag-Isolation and Pull down (His-Tag-Isolation MBs, 1.0 μm , 40 mg mL⁻¹, Dynabeads® His-Tag Isolation & Pull down), were purchased from Dnal Biotech ASA.

The enzyme tracer, HRP-conjugated Penicillin G (PENG-HRP) was acquired from US biological.

All buffer solutions were prepared with Milli-Q water (18 M Ω ·cm at 25°C). **Binding and** washing buffer (B&W) was 50 mM phosphate buffer solution pH 8.0 containing 300 mM NaCl and 0.01% Tween[®]20 and P&D buffer was 3.25 mM phosphate buffer solution pH 7.4 containing 70 mM NaCl and 0.01% Tween[®]20.

Hydrolysed β -lactams solutions were prepared by dissolving 10 mg of the specific antibiotic in 9.8 mL methanol (Scharlab) and 0.2 mL 1 M potassium hydroxide (Scharlab) and left to react overnight at 4 °C.¹⁶

Whole UHT milk samples (Ca²⁺ enriched) were purchased in a local supermarket and analyzed directly or after dilution with P&D buffer.

Penicillin binding protein production, overexpresion and purification

The *pbpX* gene (coding for the penicillin binding protein 2X) from *Streptococcus pneumoniae* R6 was PCR-amplified by HS Prime Start DNA polymerase (Takara) using the primers 699 (5'-GGTGAAAACCTGTATTTCCAGGGC**at**ggggacaggcactcgc) and 700 (5'-ATCGATAAGCTTAGTTAGCTATT**Ag**tctcctaaagttaatgtaatttt) (the nucleotides pairing the *pbpX* gene sequence are written in lowercase characters; the start and stop codons are indicated in bold). The *pbpX* gene was amplified lacking the 48 N-terminal amino acid residues of a short hydrophobic peptide involved in membrane anchor.²¹ The 2.1 kb purified PCR product was inserted into the pURI3-TEV-Km vector using a restriction enzyme-free and ligation-free cloning strategy.²² Previously, the vector pURI3-TEV-Km was constructed from pURI3-TEV vector following the same strategy used to clone any gene into pURI vectors.²³ The kanamycin resistance gene (*aph*), 813 bp, was amplified from pET28a vector (Novagen) by HS Prime Start DNA polymerase using the following oligonucleotides 755 (5'-CAATAATATTGAAAAAGGAAGAGT**at**gagccatattcaacgggaaacgt) and 756 (5'-ATGAGTAAACTTGGTCTGACAG**tt**agaaaaactcatcgagcatcaaatg) (the nucleotides pairing the *aph* gene sequence are written in lowercase characters; the start and stop codons are indicated in bold). After the amplification, the 813 bp fragment was used to substitute the ampicillin resistance gene present in pURI3-TEV vector.

The expression vector pURI3-TEV-Km contained the leader sequence MGGSHHHHHHGENLYFQG consisting of an N-terminal methionine followed by three spacer amino acids, a His₆ tag, a spacer glycine residue and the TEV protease recognition site.²³ Thus the final recombinant His₆-tagged PBP2X protein consisted of 720 amino acid residues with a molecular weight of 78 kDa. *Escherichia coli* DH10B cells were transformed and recombinant plasmids containing the correct insert (pURI3-TEV-Km-PBP2X) were isolated. Subsequently, pURI3-TEV-KM-PBP2X was transformed into *E. coli* BL21 (DE3) competent cells for PBP2X protein expression. Kanamycin was used as the selection marker.

Cells carrying pURI3-TEV-Km-PBP2X plasmid were grown at 37 °C in LB medium containing kanamycin (200 $\mu\text{g mL}^{-1}$) until they reached an optical density of 0.4 at 600 nm, and induced by adding IPTG (0.4 mM final concentration). After induction, the cells were grown at 22 °C during 20 h and collected by centrifugation. Cells were re-suspended in phosphate buffer (50 mM pH 7.0) containing 300 mM NaCl. Crude extracts were prepared by French press lysis of the cell suspension (three times at 1100 psi). The insoluble fraction of the lysate was removed by centrifugation at 47,000 g for 30 min at 4 °C and the supernatant was filtered through a 0.45 μm filter.

After filtration the supernatant was gently mixed for 20 min at room temperature with 1 ml TALON® Superflow resin (Clontech), equilibrated with the buffer described previously but containing 10 mM imidazole to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted using 150 mM imidazole in the same buffer. Protein purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at the various stages of the purification process (see Fig. 1). Protein concentration was determined by the Bradford' method.²⁴

The purified protein was stored in the elution buffer containing 10 % aqueous glycerol at -20 °C.

Affinity-magneto sensor preparation

3 μL of the His-Tag-Isolation-MBs suspension were transferred into an Eppendorf tube and re-suspended in 100 μL of a 0.07 mg mL^{-1} PBP solution prepared in B&W pH 8.0 buffer. After 10 min incubation at 25 °C under stirring at 600 rpm, the tube was placed on the magnet holding block for 2 min. Once MBs were migrated to the side of the tube, the supernatant was removed and the beads bearing the PBP were washed twice with 200 μL of B&W buffer solution (pH 8.0). Each washing step consisted of a beads re-suspension in the washing

solution and gentle stirring for 1 min (up to homogenization) followed by separation with the magnet for 2 min to remove the solution. The so prepared PBP-modified MBs were subsequently used for the determination of β -lactam antibiotics or re-suspended in 100 μ L of B&W buffer solution (pH 8.0) and stored at 4 °C until use.

Affinity assay procedure

The assay buffer solution (P&D buffer) containing the optimized concentration of the enzymatic tracer and the desired concentration of the β -lactam antibiotic was placed in a vortex for adequate mixing. In order to carry out the competitive assay, the PBP-modified MBs prepared as described in the previous Section were re-suspended in a 100 μ L-aliquot of the above mentioned mixed solution and incubated at 25 °C for 30 min with vigorous shaking (600 rpm). Thereafter, the tube with the beads was positioned on the magnetic separator and the supernatant was removed. The modified-MBs were then washed three times with 100- μ L aliquots of P&D buffer and re-suspended in 50 μ L of 0.05 M phosphate buffer pH 6.0.

Electrochemical Assay Procedure

To perform the electrochemical measurements the modified-MBs, re-suspended in 50 μ L of 0.1 M sodium phosphate buffer solution (pH 6.0), were captured on the SPCE surface. This was made by keeping the SPCE in horizontal position after positioning it on a homemade magnet holding block (see Fig. 2B2). In this way the MBs were localized in a reproducible way onto the working electrode surface, thus avoiding variations in bead layer thickness or spreading area on the electrode surface between different measurements.

Amperometric measurements in stirred solutions were performed by applying the selected detection potential and allowing the steady-state current to be reached. The magnet holding block-SPCE (with the modified MBs captured on its surface) assembly was transferred to an

electrochemical cell (see Fig. 2B3) containing 10 mL 0.05 M phosphate buffer pH 6.0 and 1.0 mM HQ (prepared daily just before the electrochemical measurement) and the amperometric responses at $E_{\text{opp}} = -0.20$ V vs. Ag pseudo-reference electrode were recorded upon addition of 50 μL of a 0.1 M H_2O_2 solution until the steady-state current was reached (approx. 100 s). Standard curves for different β -lactams were prepared in P&D buffer. The obtained responses were normalized according to:

$$S_n = \frac{i - i_\infty}{i_0 + i_\infty} \quad (1)$$

where i is the amperometric signal measured for a given analyte concentration, i_∞ is the current measured in the presence of an excess of β -lactam, and i_0 is the blank current in the absence of β -lactam. The plotted amperometric signals constituted the difference between the steady-state and the background current and experimental data were fitted to the following four-parameter logistic equation (sigmoidal):

$$S_n = i_1 + \frac{i_2 - i_1}{1 + 10^{(\log IC_{50} - x) \cdot p}} \quad (2)$$

where i_2 and i_1 are the maximum and minimum current values of the obtained 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 sigmoidal curve. The limit of detection (LOD) was calculated as the analyte concentration for which the amperometric signal was reduced by 10%. The dynamic range (DR) was taken as the analyte concentration interval for which the amperometric signal was between 20 and 80% of the maximum signal (obtained in the absence of β -lactam antibiotic).²⁵

Unless otherwise indicated, the reported data corresponded to the average of at least three replicates.

Selectivity studies

Cross-reactivity from other non-target antibiotics was evaluated by measuring the competitive signals (at 250 nM and 10,000 nM concentration levels) under the optimized conditions in 1:1 P&D-diluted UHT whole milk.

Milk Analysis

Samples of commercial Ca^{2+} -enriched UHT whole milk (undiluted or after dilution in P&D buffer solution) were spiked with the appropriate concentrations of the enzymatic tracer and the antibiotic according to the protocol described in Section Affinity assay procedure. Aliquots (100 μL) of these milk samples were analyzed following the same methodology described above for standard solutions. Non-spiked samples were also analyzed to exclude the presence of naturally contaminating antibiotics.

Results and discussion

Nitrilotriacetic acid (NTA) transition metal complexes are commonly used in metal-ion affinity chromatography (IMAC) for the selective retention of histidine-tagged proteins.²⁶ In this work, we followed this strategy to immobilize the histidine-tagged PBP onto the MBs functionalized with a Co^{2+} -based IMAC chemistry (see Fig. 2A), thus leading to its localized and oriented immobilization without interfering with the PBP structure and function.²⁷

Fig. 2A schematizes also the fundamentals of the competitive assay where the unlabeled analyte (the β -lactam antibiotic) competed with a fixed concentration of the enzymatic tracer (PENG-HRP) for the limited number of the binding sites of the recombinant binding protein immobilized on the His-Tag-Isolation-MBs. Since PENG is the most commonly used β -lactam antibiotic,¹⁵ we used this compound for the optimization of all the experimental variables involved in the analytical performance of the developed affinity magnetosensor.

The suitability of this assay design was verified by comparing the amperometric responses obtained at an applied potential of -0.20 V (vs. the Ag pseudo-reference electrode) after implementing the protocol with and without target antibiotic. The measured current was approximately ten times lower when the assay was performed in the presence of 5.0×10^{-7} M (178.2 ng mL $^{-1}$) PENG, thus showing that a smaller amount of PENG-HRP was attached in the presence of free antibiotic as a consequence of the competitive affinity assay and, therefore, demonstrating the suitability of the electrochemical detection of the antibiotic-PBP recognition process.

Optimization of the experimental variables

Firstly, in order to evaluate the existence of non-specific binding of the enzymatic tracer on the functionalized MBs surface, we compared the responses obtained with and without the PBP immobilized on the MB using a solution of the enzymatic tracer in P&D buffer.

The obtained results (not shown) showed low nonspecific adsorption of PENG-HRP at PBP-free MBs leading to amperometric signals 10 times smaller than those measured in the presence of PBP. In order to minimize even more this non-specific signal, we performed also the competitive reaction in working media consisted of P&D buffer solution supplemented with BSA (5% w/v) or NaCl (1M). Fig. 3 displays a comparison of the resulting amperometric measurements as well as the ratios between the specific and the unspecific signals. As it can be seen, such ratio increased around twice in the presence of BSA and NaCl. No further improvement was observed by increasing the blocking agent concentration or incubation time. Taking into account that a higher amperometric signal was obtained in the NaCl medium, we selected it for further work with PENG standard solutions. . It is important to remark that, conversely to that reported by other authors, the non-specific binding of the PENG-HRP conjugate was independent of the free PENG concentration in solution.^{14,28}

Once the non-specific adsorption was minimized, optimization of all other experimental variables involved in the affinity biosensor preparation and functioning was accomplished. The selection criterion taken was the magnitude of the current values measured with the biosensor **when no β -lactam antibiotic** was present. Table 1 summarizes the experimental conditions selected for variables such as the amount of functionalized MBs, the PBP concentration, the incubation time of the MBs with PBP, the dilution degree of the PENG–HRP enzymatic tracer as well as its incubation time and the detection potential applied to the affinity biosensor. The evaluated ranges for all these variables are also given in Table 1.

Analytical characteristics of the affinity magnetosensor

PENG was employed as target compound to quantify the analytical performance of the developed affinity magnetosensor. Fig. 4 displays the amperometric responses recorded under the optimized conditions for different PENG concentrations in P&D buffer (containing 1 M NaCl). The resulting calibration plot, shown in the inset of the Figure, displayed the expected shape for a competitive format. A linear dynamic concentration range ($r = 0.997$) was found between 6.6 and 160.9 nM (2.3 and 57.3 ng mL⁻¹). The EC₅₀ and LOD calculated values were 33.3 nM (11.9 ng mL⁻¹) and 2.6 nM (0.93 ng mL⁻¹), respectively. It is important to remark that this LOD value is approximately 4 times lower than the limit fixed by the EU legislation (4 ppb or 12 nM of PENG content in milk samples). Therefore, the obtained results were promising and encouraging for the further application of the electrochemical affinity magnetosensor in the rapid analysis of food samples such as milk.

Analytical characteristics of the affinity magnetosensor in milk

The applicability of the affinity magnetosensor to the analysis of milk samples was initially evaluated in PENG-spiked Ca-enriched whole UHT milk, either undiluted or after a 1:1

dilution with P&D buffer. PENG calibration curves were constructed for both samples. As it can be observed in Fig. 3 (bar no.4), the largest ratio between the specific and nonspecific current was observed in 1:1 P&D buffer-diluted UHT milk which can be attributed to a minimization of the non-specific binding of the enzymatic tracer due to the presence of some milk matrix components.¹⁴ An important consequence of this result is that no blocking agent was required in the analysis of the milk samples. Under these conditions, the maximum specific signal (**in the absence of free β -lactam antibiotic**) was 75 times higher than the one measured without PBP.

Table 2 summarizes the analytical characteristics obtained with the affinity magneto-sensor for the determination of PENG in the 1:1 diluted UHT whole milk. The DR ranged between 34.0 and 255.1 nM (12.1 and 90.0 ng mL⁻¹) and the IC₅₀ and LOD were 99.4 and 12.0 nM (35.4 and 4.3 ng mL⁻¹), respectively, thus demonstrating the usefulness of the affinity biosensor for detecting PENG at the limit set by the EU legislation. It is also important to mention that PENG could be also analyzed directly in undiluted UHT whole milk samples, although with a worsening of the LOD (67.1 nM or 23.9 ng mL⁻¹).

An important practical aspect to be tested was the broad specificity of the used PBP. Therefore, calibration curves were also constructed for other 5 commonly employed β -lactam antibiotics in 1:1 diluted UHT whole milk samples (Fig. 5). As can be observed, all the antibiotics tested (AMOX, AMPI, OXA, CLOX and CEF) were highly recognized by the affinity magnetosensor, with LOD values ranging between 12.0 and 44.8 nM, (see Table 2). The cross-reactivity data given in Table 2 allow deducing that the PBP exhibited similar recognition ability to that observed with PENG for AMOX, CEF and OXA, but it was remarkable lower for CLOX.

The reproducibility of the responses obtained with different affinity magnetosensors prepared in the same manner was evaluated both within the same day and between different days by

performing measurements in 1:1 P&D buffer-diluted UHT whole milk spiked with the enzymatic tracer and in the absence of penicillin antibiotic. Results for 5 different sensors prepared the same day yielded a RSD value of 5.1 %, whilst the RSD value was 6.61 % for six amperometric responses measured in different days. These results demonstrated that the affinity magnetosensor fabrication procedure was reliable and that reproducible amperometric responses could be obtained with different biosensors constructed in the same manner.

We also evaluated the reproducibility of the measurements obtained using 3 different batches of PBP resulting in a RSD value of 3.9 % (for the current values measured when no β -lactam antibiotic was present) thus demonstrating also the reliability of the enzyme overproduction and purification protocols.

The stability of the PBP-His-Tag-Isolation-MBs conjugates was evaluated by preparing on the same day different conjugates which were stored at 4 °C in plastic tubes containing P&D buffer. A couple of the prepared conjugates were incubated into a fresh solution of PENG-HRP or a mixture of PENG (500 nM, 178.2 ng mL⁻¹) and PENG-HRP, according to the protocol described in the Section Affinity assay procedure, each working day. A control chart was constructed by taking as the central value the mean of 5 measurements obtained the first day of the study. The upper and lower control limits were set at $\pm 3 \times SD$ of this initial value. The results obtained demonstrated that the responses both in the absence and in the presence of free antibiotic remained within the control limits for at least 9 days, indicating a high stability of the PBP-modified MBs conjugates. A similar experiment was also performed by storing the PENG-HRP/PBP-His-Tag-Isolation-MBs conjugate prepared in the absence of penicillin antibiotic. The affinity biosensors constructed with these conjugates kept 95.3, 80.9 and 52.5 % of the original amperometric signal after 24, 48 and 120 h of storage at 4 °C in P&D buffer. These results are in agreement with the reported stability of the β -lactam/PBP complexes (half-life 24-54 h).²⁹

Response of the affinity biosensor to active forms of antibiotics

The existing legislation concerning residue limits is only applicable to the active form (with closed ring structure) of β -lactams. Therefore, the specificity of PBP for the active (non-hydrolyzed) β -lactam forms was investigated. So, milk samples were spiked with PENG or CEF at a concentration level of 500 nM and hydrolyzed as described in Section Materials. These samples as well as non-hydrolyzed samples were analyzed using the affinity sensor (Fig. 6). As it can be seen, there were no detectable active residues in the hydrolyzed sample, whereas the concentration of the non-hydrolyzed samples was determined to be 500 nM. Therefore, it could be concluded that there was no cross-reactivity in the assay for the opening metabolites or degradation products. The fact that the developed methodology is able to detect only active forms of the antibiotics can be considered as an important practical advantage since marker residues for compliance with the MRL are the parent compounds.

Affinity magnetosensor selectivity

The selectivity of the developed affinity magnetosensor was tested against 3 non-target antibiotics which can be frequently present in milk and dairy products (SPY, ENRO and TC). The tests were performed by comparing the amperometric responses recorded with the affinity magnetosensor in the absence and in the presence of these potentially interfering antibiotics at two different concentration levels (250 and 10,000 nM) in 1:1 diluted UHT whole milk samples. The obtained results (not shown) demonstrated that no significant cross-reactivity occurred for the 3 non-target antibiotics tested even in the presence of a high excess (10,000 nM). The cross-reactivity percentage (CR%) for these non-target antibiotics was calculated according to the equation:

$$CR\% = \frac{IC_{50}(PENG)}{IC_{50}(\text{cross-reacting compound})} \times 100 \quad (3)$$

The obtained values were <1% in all cases thus demonstrating that no significant competition was produced and highlighting the remarkable group specificity of the PBP used to prepare the affinity magnetosensor.

Application to the analysis of spiked milk samples

The usefulness of the amperometric affinity magnetosensor for the analysis of real samples was evaluated by analyzing 1:1 diluted Ca-enriched UHT whole milk (antibiotic-free) spiked with PENG at a 1.0×10^{-7} M (35.6 ng mL^{-1}) concentration level which corresponded approximately to the IC_{50} value of the calibration curve in this medium. The determination of PENG was accomplished by interpolation of the measured current values into the calibration plot constructed for the antibiotic in the same matrix sample (Fig. 5). The analysis of 5 milk samples yielded a PENG mean concentration of $(9.8 \pm 0.8) \times 10^{-8}$ M with a mean recovery of $(98 \pm 8) \%$ (RSD = 6.2 %), the **confidence interval being calculated for $\alpha=0.05$** . Taking into account the type of analysis carried out and the straightforwardness of the experimental protocol, this can be considered as a very good result, demonstrating the usefulness of the disposable affinity sensor for the determination of β -lactam antibiotics in a total time for the assay of approximately 30 min (once the affinity magnetosensor is prepared).

In agreement with the great selectivity of the methodology towards β -lactam antibiotics, we also confirmed that it was possible to carry out the successful quantification of a β -lactam antibiotic in 1:1 P&D buffer-diluted UHT whole milk samples containing also three non-target antibiotics (SPY, TC and ENRO) at the same concentration or in a 40-fold excess with respect to the target antibiotic (results not shown).

In an attempt to decrease the LOD and achieve the MRL set by European Union in milk samples, a 2 steps assay design involving 30 min incubation of the PBP-modified MBs with

the milk samples spiked only with the antibiotic and a further 30 min incubation step in which the non-complexed PBP-MBs were then allowed forming complexes with PENG-HRP, was evaluated. The amperometric responses obtained for milk samples containing 4 ng mL^{-1} of PENG, AMOX or AMPI were 84.9, 84.4 and 74.3 % of the amperometric signal recorded in the absence of antibiotic which demonstrated the possibility to comply with the limits established by the legislation using the 2 steps format assay. Despite the higher sensitivity of this assay, we observed that the quantification of penicillin antibiotics using this protocol could not be achieved since the amperometric signals did not decrease when the antibiotic concentration increased above 4 ng mL^{-1} . However, taking into account that this modified methodology allows an easy differentiation between antibiotic free milk samples and samples containing only 4 ppb of PENG, AMOX and AMPI, the obtained results clearly demonstrate the usefulness of this approach as a quick tool for qualitative screening purposes able to provide reliable positive result for samples containing residues above a certain threshold.

Comparison with other sensors and methodologies

The performance of the amperometric affinity magnetosensor was compared with data provided by other approaches reported in the literature. As can be seen in Table 3, most of the currently developed methodologies are based on SPR detection and only one used electrochemical transduction.¹⁴ Offering similar analytical performance, some important advantages can be claimed for the approach reported in this article versus other described methodologies. These are related with the simplicity and much easier potential automation and miniaturization of the proposed affinity magnetosensor rendering the developed methodology in an ideal potential tool for field-based screening of β -lactam antibiotics presence in milk

Conclusions

This work describes for the first time a disposable amperometric affinity magnetosensor for the specific and sensitive detection of the whole group of β -lactam antibiotics in milk by immobilization of a recombinant PBP on the surface of commercial His-Tag-Isolation-MBs. The obtained results demonstrate that the use of a recombinant microbial protein as a receptor led to four major advantages. Firstly, the methodology is able to detect exclusively intact β -lactam and is fully non-reactive to the open ring forms, which is crucial to comply with MRL legislation. Secondly, both groups of β -lactam antibiotics, penicillins and cephalosporins, can be screened within one assay. Thirdly, due to its recombinant origin, the receptor protein can be produced in commercial and reproducible amounts. Fourthly, the developed methodology is able to detect and quantify low levels of these antibiotics residues reliably, specifically and rapidly in milk with no need for complex pre-treatment steps (just a 1:1 dilution with the working buffer).

The great exhibited analytical performance, together with the use of disposable mass-produced sensors, makes the developed affinity magnetosensing platform an interesting, useful and affordable alternative to classical assays for the detection of β -lactam antibiotics. Moreover, the simplicity and easy automation and miniaturization of the required instrumentation pave the way to integrate the quality control analysis in the milk intake process at the field or farm or just in the dairy tankers that arrived at the dairy industries.

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Figure Captions

Fig. 1 SDS-PAGE (8%) analysis of *E. coli*-PBP production, immobilization, and purification processes. Lanes: 1, soluble cell extract from *E. coli* BL21 (DE3) cells carrying pURI3-TEV-Km vector; 2, soluble cell extract from *E. coli* BL21 (DE3) carrying the recombinant pURI3-TEV-Km-PBP2X plasmid; 3, purified His-PBP after IMAC chromatography eluted with 150 mM imidazol. The molecular masses of the standard marker proteins (in kDa) are indicated at the left and right, respectively (200 kDa, 116 kDa, 97,4 kDa y 66 kDa; la proteína His-PBP, 78 kDa).

Fig. 2 A) Schematic display of the steps involved in the β -lactam antibiotics affinity magnetosensor developed. B) Picture showing the SPCE and the homemade magnet holding block (1), the deposition of the modified MBs on the SPCE assembled on the magnet holding block (2) and the assembled SPCE-magnet holding block immersed in the electrochemical cell used for the amperometric measurements (3).

Fig. 3 Comparison between the amperometric responses and the resulting specific/non-specific ratios after incubation of the PBP-immobilized or unmodified-MBs with an enzymatic tracer solution in pure P&D buffer solution (1), P&D buffer solution supplemented with BSA (5% w/v) (2), P&D buffer solution supplemented with NaCl (1M) (3) and 1:1 P&D buffer-diluted UHT whole milk samples (4).

Fig. 4 Amperometric recordings for different PENG concentrations: 0 a) , 6.0×10^{-9} b), 1.5×10^{-8} c), 1.0×10^{-7} d), 2.5×10^{-7} e) and 5.0×10^{-7} f) M. Inset: Calibration curve obtained with the developed affinity magnetosensor for PENG under the optimized conditions in P&D

buffer containing 1 M NaCl. Experimental points were fitted to equations (1) and (2). Error bars estimated as the triple of the standard deviation ($n=3$).

Fig. 5 Calibration graphs constructed with the affinity magnetosensor in 1:1 P&D-diluted Ca-enriched UHT whole milk samples for different β -lactam antibiotics (PENG, AMOX, AMPI, CEF, CLOX and OXA). Experimental points were fitted to equations (1) and (2). Error bars were estimated as the triple of the standard deviation ($n=3$).

Fig. 6 Amperometric signals recorded with the affinity magnetosensor for non-spiked and 5.0×10^{-7} M (178.2 ng mL^{-1}) PENG or hydrolyzed PENG spiked 1:1 P&D-diluted Ca-enriched UHT whole milk samples.

Table 1 Optimization of experimental variables

Experimental variable	Checked range	Selected value
Modified MBs amount, mg	0.04-0.28	0.12
[PBP], mg mL ⁻¹	0-0.25	0.07
Incubation time with PBP, min	5-20	10
PENG-HRP, dilution	1:25-1:100	1:50
Incubation time with PENG-HRP	10-45	30
Applied potential*	-0.25-0.05 V	-0.20 V

* vs. Ag pseudoreference electrode

Table 2 Analytical performance of the developed affinity magnetobiosensor for the determination of different β -Lactam antibiotics in 1:1 P&D buffer-diluted Ca-enriched whole UHT milk

	PENG	AMOX	AMPI	CEF	OXA	CLOX
r^2	0.997	0.996	0.995	0.995	0.997	0.996
IC ₅₀ / nM	99.4	83.2	124.2	102.8	110.7	199.8
IC ₅₀ / ng mL ⁻¹	35.4	30.4	46.1	43.5	44.4	87.1
LOD / nM	12.0	38.6	37.8	15.6	33.8	44.8
LOD / ng mL ⁻¹	4.3	14.1	14.0	6.6	13.6	19.5
MRL/ ng mL ⁻¹	4.0	4.0	4.0	60	30	30
DR / nM	34.0–255.1	52.9–129.5	62.0–238.1	33.5–290.8	49.3–270.2	75.3–531.2
DR / ng mL ⁻¹	12.1–90.9	19.3–47.3	23.0–88.4	14.2–123.1	19.8–108.5	32.8–231.5
CR/%	100	119.5	80.0	96.7	89.8	49.7

Table 3 Comparison of the analytical characteristics of the developed affinity magnetosensor with other reported immunoassays for β -lactam antibiotics

Assay protocol	Detection type	DR / ng mL ⁻¹	LOD / ng mL ⁻¹	Sample	Assay time	Reference
Immobilization of PBP by adsorption over SPE-RC surface. The assay consists of a competitive binding assay format using PENG and 7-ACA-GOD. A 2-peptide was covalently bounded to the sensor surface.	Amperometry (+350 mV vs. Ag/AgCl)	—	5 (PENG)	Milk (50% diluted and undiluted)	6 min	[14]
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	[18]
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 (AMPI) 8 (AMOX) b) 2 (PENG)	Milk (previously defatted) and raw milk (previously heated)	40 min	[12]
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	[15]
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	[20]
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	[16]
Competitive affinity assay using PENG-HRP on PBP-modified MBs	Amperometry ($E_{app} = -0.20$ V vs. Ag pseudo ref. electrode)	34.0-255.1 (PENG)	4.3 (PENG)	Milk (1:1 PBST diluted)	30 min	This work

Abbreviations: 7-ACA-GOD: 7-aminocephalosporanic acid modified with glucose oxidase, AMOX: amoxicillin; AMPI: 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: DD-carboxypeptidase from *Actinomadura* R39; R39*: DD-carboxypeptidase from *Streptomyces* R39, SPE-RC: screen printed electrode with a rhodinated-carbon working electrode, SPR: surface Plasmon resonance.

Fig. 1

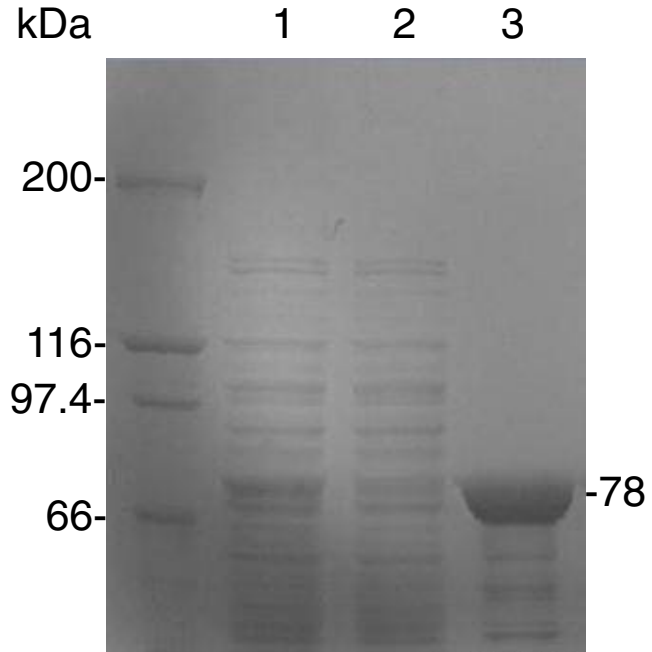


Fig. 2

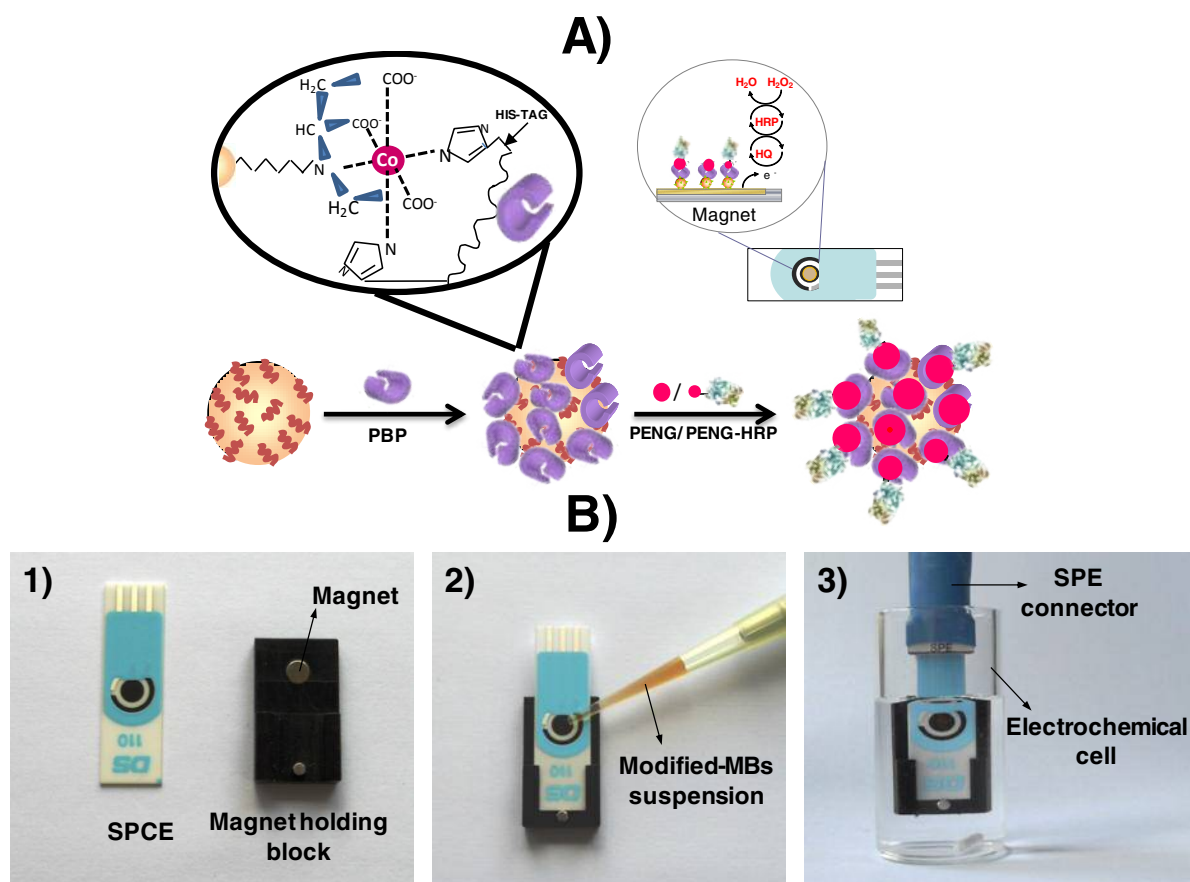


Fig. 3

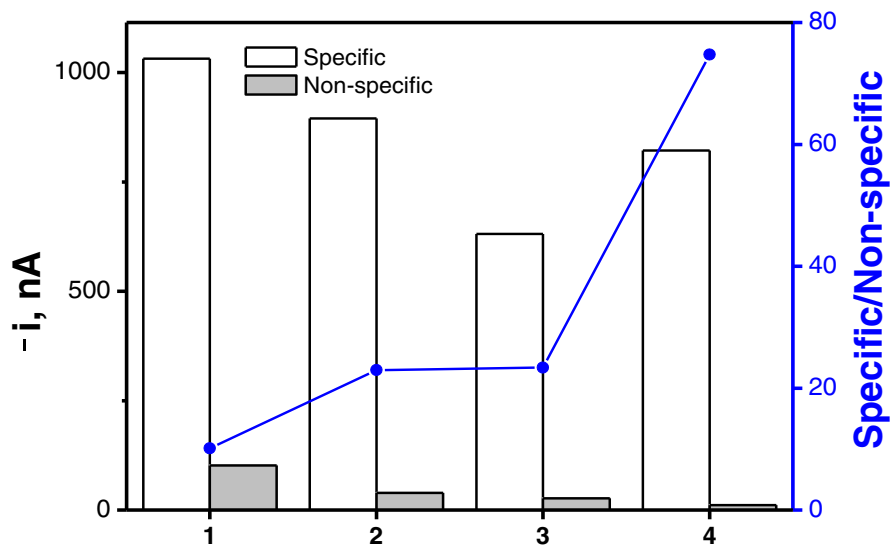


Fig. 4

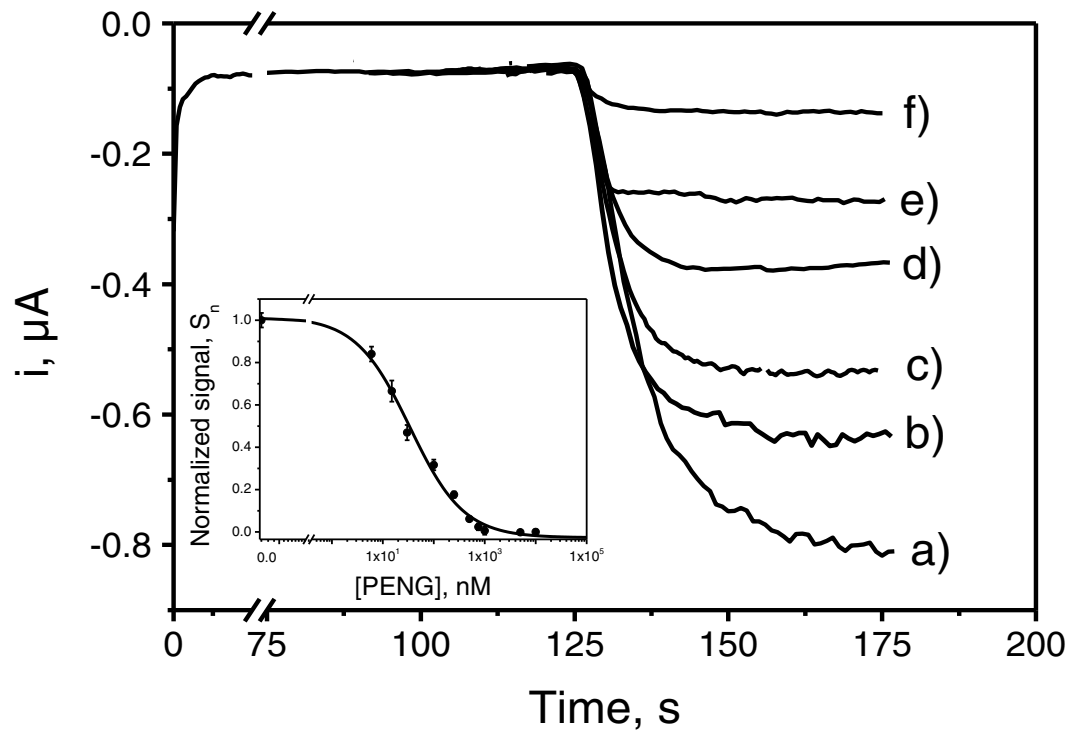


Fig. 5

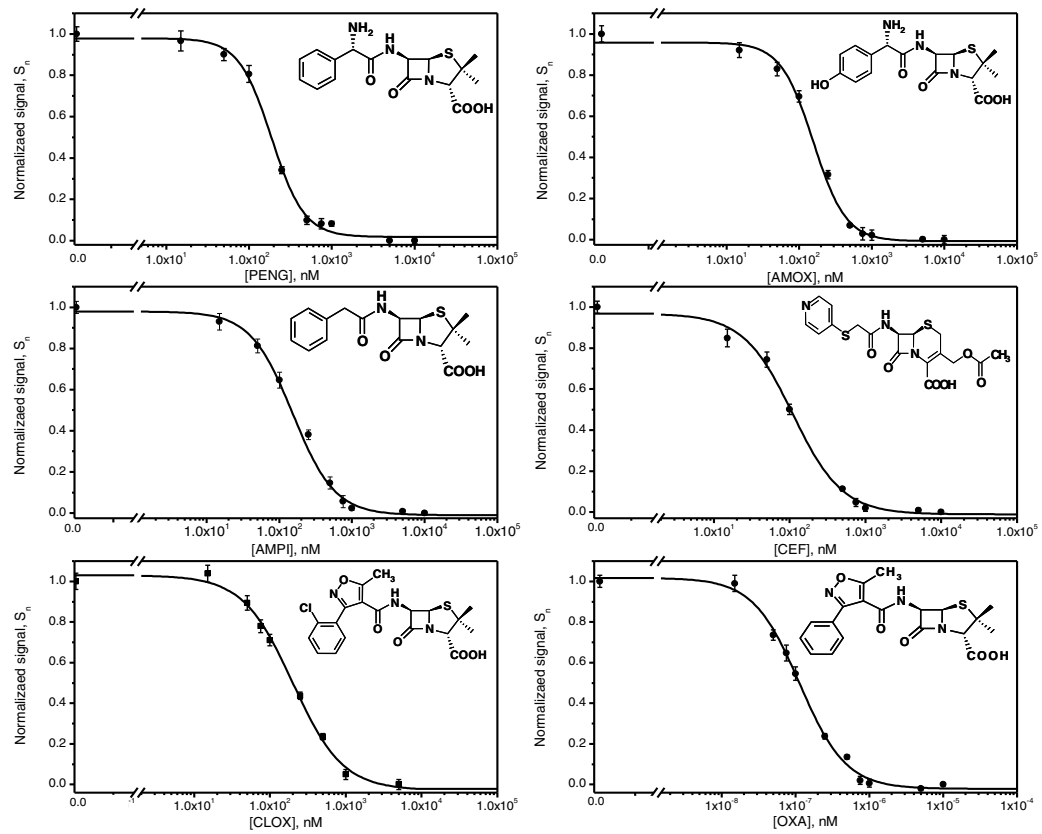
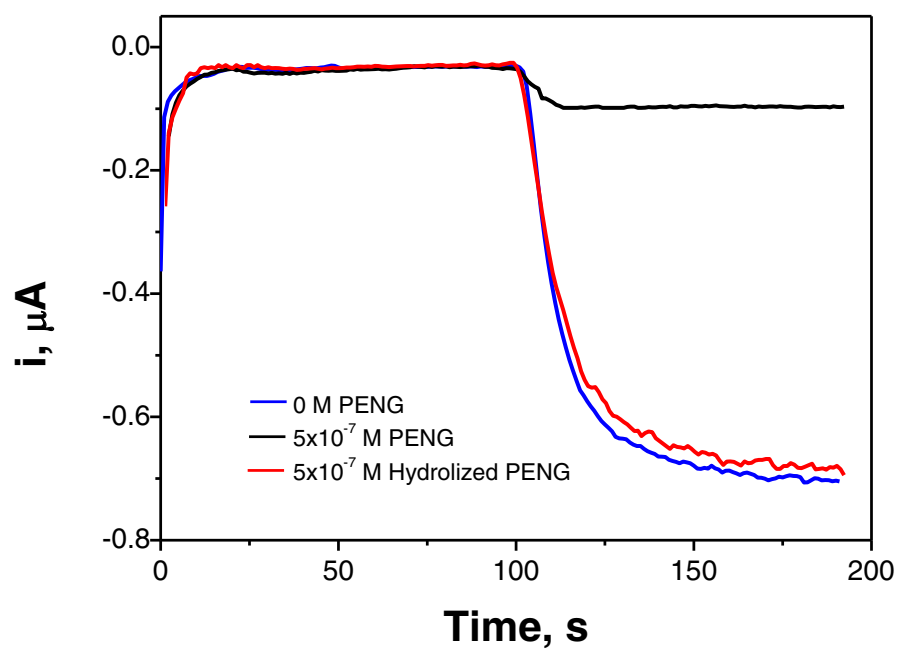
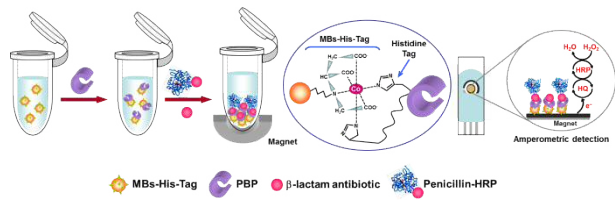


Fig. 6



TOC



TOC. Detection of β -lactam Antibiotics in Milk based on an Amperometric Affinity Penicillin-Binding Protein Magnetosensor