Aptamer-Based Inhibition Assay for the Electrochemical Detection of Tobramycin Using Magnetic Microparticles

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Abstract
The detection of small molecules using aptamers is not straightforward when the induced fit triggered by the analyte is also small. Herein a labeling strategy is explored for the voltammetric detection of the antibiotic tobramycin using magnetic microparticles (MPs). A competition between MPs-immobilized tobramycin and free tobramycin for the limited amount of the biotinylated-aptamer was established. The addition of the enzymatic conjugate and the substrate generated a product that was electrochemically oxidized on screen-printed carbon electrodes. The analytical signal was proportional to the antibiotic concentration from 5–500 μM and the reproducibility was 5.7%. The assay selectivity was also studied.

Keywords: Antibiotic tobramycin, Aptamer biosensor, Electrochemical detection, Magnetic microparticles

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1 Introduction

Electrochemical aptamer-based sensors are experiencing a rapid development [1,2], since 2005 when the first assay was published [3]. Most of them require the immobilization of the molecular recognition element (the aptamer) on the electrode surface through a variety of strategies previously developed for the immobilization of other biomolecules [4]. In general, immobilization facilitates washing steps to remove the unspecifically bound or unreacted compounds as well as regeneration steps. Besides, it is essential for the construction of array devices for high throughput analysis and integration into portable devices. The use of magnetic microparticles (MPs), also called beads, as a platform for immobilization of a capture molecule before the electrochemical transduction of the recognition event presents many advantages. Since MPs are in suspension, the kinetics of the affinity interaction between ligand and receptor is greatly accelerated and the number of capture biomolecules anchored increases because of the larger surface area of the beads in comparison with conventional electrode surfaces. Additionally, washing and separation steps are very simple because paramagnetic particles, since temporarily magnetized, are attracted by permanent magnets returning to the initial state when these are removed. Therefore, the elimination of unbound species (matrix separation) does not require filtration or centrifugation. Their ease of handling also allows performing several experiments in parallel (high throughput analysis).

MPs are commercialized with a wide variety of surface functional groups, which expands the diversity of molecules that can be attached. In combination with aptamers, MPs have been used for shortening and improving the evolution of aptamers by SELEX procedure [5–8], and for the isolation of pathogens from complex samples. In the latter cases the detection was achieved by means of fluorimetric sandwich assays [9] or RT-PCR after releasing of the target to improve the limit of detection [10].

As synthetic nucleic acid-based receptors, aptamers can be used in a similar manner than antibodies with important advantages such as improved thermal and chemical stability and tolerance to denaturation. Mascini group has explored several ELISA format assays for the detection of thrombin [11,12] and C reactive protein [13] by transferring the MPs, where the recognition event takes place, to a screen-printed electrode with a magnetic block in the bottom and further electrochemical detection of an enzymatic product.

Taking advantage of the intrinsic characteristics of nucleic acids such as hybridization, displacement assays have also been reported on magnetic beads using Au nanoparticles as electrochemical labels [14]. Recently, an

on-off switching electrochemical aptamer molecular beacon was used for the detection of thrombin. A hairpin aptamer carrying carminic acid at both ends was immobilized on MPs through hybridization with a short DNA probe. It exhibited a reduced electrochemical activity in comparison with its opened structure after interaction with the target. The magnetic enrichment greatly improved the detectability [15].

All these methods were developed for the detection of large molecules. However, the detection of small molecules using aptamers is a challenge because of the lower affinity interaction when compared with large molecules (dissociation constants in the μM range for the former and in the pM-nM for the latter [16]). Additionally, the transduction of the recognition event often relies on the induced fit experienced by the aptamer in the presence of the analyte, which may be very small for this kind of molecules leading to minute changes in the analytical signal. This is the case of the anti-tobramycin aptamer, a 27-mer RNA with a hairpin structure containing a hexanucleotide loop and a bulged adenine base [17], which widens when encapsulating the antibiotic [18]. Tobramycin is a broad-spectrum antibiotic belonging to the group of aminoglycosides, which exhibit bactericidal activity against some *Gram-positive* and many *Gram-negative* organisms. Similarly to other aminoglycosides, the use of tobramycin is limited by a narrow therapeutic range (2–12 μg/mL (4.3–25.7 μM) in serum)) [19] owing to potential side adverse effects such as oto- and nephrotoxicity that can appear. Therefore, careful monitoring of the drug levels in patients serum is required. Label-free detection is an attractive solution to address this weakness, as it was recently shown using faradaic impedance spectroscopy [20]. Nevertheless, labeling strategies deserve further investigations. Herein the use of a labeled aptamer for the detection of tobramycin, using MPs as a solid support for carrying out an inhibition assay is proposed. The voltammetric signal associated to the enzymatically generated product is measured at disposable screen-printed electrodes.

2 Experimental

2.1 Apparatus and Reagents

Electrochemical measurements were performed on disposable screen-printed carbon electrochemical cells (SPCE) (Dropsens, Oviedo, Spain) composed of a 4 mm diameter carbon working electrode, a carbon counter electrode and a Ag pseudo-reference electrode, driven by a computer-controlled μ-AutoLab type II PGstat-12 potentiostat with GPES 4.9 software (EcoChemie, The Netherlands).

The 12-tube mixing wheel (Dynal MX1) used for sample mixing in vials and the magnet (DynaMag-2) for magnetic separation were purchased from Invitrogen (Barcelona, Spain).

A 5′-biotinylated 27-mer anti-tobramycin RNA aptamer (BATA) was used throughout this work. It was modified with a methoxy group at 2′-OH position of the ribose moieties in all nucleobases except at U12 position. The BATA, with the following sequence, 5′ [Biotin] [mG] [mG] [mC] [mA] [mC] [mG] [mA] [mG] [mG] [mU] [mU] U [mA] [mG] [mC] [mU] [mA] [mC] [mA] [mC] [mU] [mC] [mG] [mU] [mG] [mC] [mC] 3′, was synthesized by Sigma-Genosys (Madrid, Spain) and purified by HPLC.

Carboxylated magnetic microparticles (MPs) Dynabeads MyOne Carboxylic Acid (1.05 μm in diameter) were from Invitrogen (Barcelona, Spain).

Tobramycin sulfate, streptomycin sulfate, kanamycin sulfate, *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), streptavidin-alkaline phosphatase conjugate ($Strep_2$-ALP) and 1-naphthyl phosphate (α-NP) were purchased from Sigma-Aldrich (Madrid, Spain). 4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), ethanolamine, Tween 20, bovine serum albumin (BSA), salts for buffer solutions (KCl, NaCl, $MgCl_2$, $CaCl_2$) and 1 M Tris/HCl pH 7.4 solution (all RNAse free) were also obtained from Sigma-Aldrich (Madrid, Spain). All other reagents were of analytical grade.

All glassware in direct contact with aptamer was previously cleaned with RNAseZAP (Sigma-Aldrich, Spain) and all aqueous solutions were prepared with RNAse free water purified by a Direct-Q system with a BioPack cartridge (Millipore).

The compositions of the buffers used for the experiments are as follows:

Immobilization solution: 0.1 M HEPES pH 8.64. (Buffer A)

Affinity solution: 20 mM Tris-HCl pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$. (Buffer B)

Washing solution: Buffer B + 0.01 % Tween 20. (Buffer C)

Detection solution: 0.5 M Tris-HCl pH 9.8, 1 mM $MgCl_2$. (Buffer D)

2.2 Procedures

2.2.1 Modification of MPs

For the preparation of the sensing phase tobramycin was covalently immobilized through the amino groups to the surface of the carboxylated MPs using the carbodiimide chemistry.

For this purpose, a suspension of 50 μL of the MPs stock solution (10 mg/mL) was washed twice with 500 μL of 0.01 % Tween 20 aqueous solution for 2 min. During each washing or incubation step the vials were positioned in the mixing wheel to allow a proper contact between the solution and the MPs. After incubation, the vials were placed in a magnet holder where MPs were trapped to the vial wall making the removal of the supernatant easy. MPs were re-suspended in 500 μL of a mixture of 0.2 M EDC and 0.05 M NHS for 30 min to activate the functional groups available on the MPs surface. After two wash-

ing steps with 500 μL of buffer A containing 0.01 % Tween 20, the MPs were re-suspended in a 40 mM tobramycin solution prepared in buffer A for 1 h (unless otherwise stated). Once the antibiotic was covalently attached to the surface of the MPs, and after washing twice with 500 μL of buffer A containing 0.01 % Tween 20, a 1 h incubation was carried out in 500 μL of a 1 M ethanolamine solution prepared in buffer A. The latter step allowed the inactivation of the remaining carboxylic groups that had not reacted with tobramycin. Finally, two washing steps were carried out with 500 μL of buffer C and the tobramycin-modified MPs (TMPs) were re-suspended in 500 μL of buffer B (final TMPs concentration, 1 mg/mL). This suspension must be stored at 4 °C and used within the following 24 h to guarantee the proper condition of the immobilized tobramycin.

2.2.2 Inhibition Assay Protocol

The TMPs were used for developing an aptamer-based inhibition assay for tobramycin detection in aqueous solution.

With this aim, several 10 μL aliquots of the TMPs stock solution were washed twice adding 990 μL of buffer C for 2 min each. After discarding the supernatant, the TMPs were re-suspended in 1 mL of a solution containing a constant concentration of BATA and different concentrations of tobramycin ranging from 0 to 500 μM prepared in buffer B containing 0.1 % BSA and incubated for 15 min. Then, two washing steps were carried out employing 1 mL of buffer D containing 1 % BSA and 0.01 % Tween 20 for 2 min each. After that, the MPs were re-suspended in 1 mL of 4.3×10^{-3} g/L $Strep_2$-ALP conjugate prepared in buffer D containing 1 % BSA and 0.01 % Tween 20. After 10 min of incubation time, the MPs were washed twice with 1 mL of buffer D and finally re-suspended in 100 μL of buffer B (final MPs concentration, 0.1 mg/mL).

Once the enzymatic conjugate was attached to the MPs, both the enzymatic reaction and the electrochemical measurements were carried out on SPCEs. For this purpose 15 μL of the modified MPs were deposited and trapped onto the working electrode by means of a magnet placed under it. Subsequently, the electrochemical cell was fully covered adding 25 μL of the enzymatic substrate (α-NP) prepared in buffer D to a final concentration of 4 mM. After 10 min of enzymatic reaction (unless otherwise stated), the product, α-naphthol (α-N) was detected by differential pulse voltammetry (DPV) measurements from 0 V to 0.55 V with the following parameters: modulation amplitude 0.05 V; step potential 0.0015 V; interval time: 0.15 s; modulation time 0.05 s.

3 Results and Discussion

Preliminary attempts of developing a voltammetric aptasensor for tobramycin detection taking advantage of the employment of MPs as solid support were based on a two-step impedimetric aptasensor previously reported by our group [20]. This assay relied on the displacement of the anti-tobramycin aptamer from its complex with an antibiotic-modified surface by free tobramycin in solution. It was carried out by first saturating the tobramycin-modified electrode surface with aptamer, and subsequent exposure of the sensing phase to a tobramycin solution (with no aptamer). Experimental results indicated that the displacement of the previously bound BATA to the TMPs towards free tobramycin in solution did not take place in a sufficient extent. An alteration in the affinity of the biotinylated aptamer with respect to the unlabeled counterpart is speculated to be the cause of this behavior. Ongoing work is currently under development to elucidate this finding.

A one-step format (inhibition assay) was then designed, and it is depicted in Figure 1. In this case, the TMPs were exposed to a mixture of a fixed concentration of BATA and varying concentrations of tobramycin (Figure 1A). This way, the free aptamer will bind to the tobramycin linked to the magnetic particles. Thus, the higher the tobramycin concentration in the sample, the lower the amount of BATA available for binding to TMPs. Then, the $Strep_2$-ALP conjugate was added (Figure 1B) and finally, both the enzymatic reaction and the voltammetric measurements were carried out on SPCEs (Figure 1C). For this purpose a small magnet was placed under the working electrode in order to assure that the MPs were placed only on the working electrode, which greatly increased the reproducibility of the measurement. The addition of the electrochemically inactive substrate α-NP, covering all three electrodes, allowed the electrochemical detection of the product enzymatically generated, α-N by DPV at 260 mV. The voltammetric signal constituted a measurement of the amount of $Strep_2$-ALP anchored to the modified MPs and, therefore, it was related to the tobramycin concentration in the sample. With this design a signal-off approach was expected because the decrease of the amount of BATA available for surface interaction when increasing the tobramycin concentration in the sample necessarily leads to a smaller amount of enzymatic conjugate linked to the MPs and, thus, to a smaller voltammetric current.

It is well-known that the short shelf life of RNA in solution complicates its use in biosensors. Fortunately, chemical modification of its phosphate-sugar backbone can successfully avoid its degradation by endonucleases. Recently, methoxylation of 2′ position of riboses has been probed to be effective to allow the use of RNA aptamers as recognition elements in biological fluids [21]. After this pioneered work, it was decided to modify the native antitobramycin aptamer with methoxy groups at all its 2′ positions of its ribose moieties except the corresponding to the base U12 because it was described that the 2′-OH group of the U12 plays some role in the molecular recognition event of tobramycin [18]. A biotin molecule was added to the 5′-end for conjugation with $Strep_2$-ALP. The

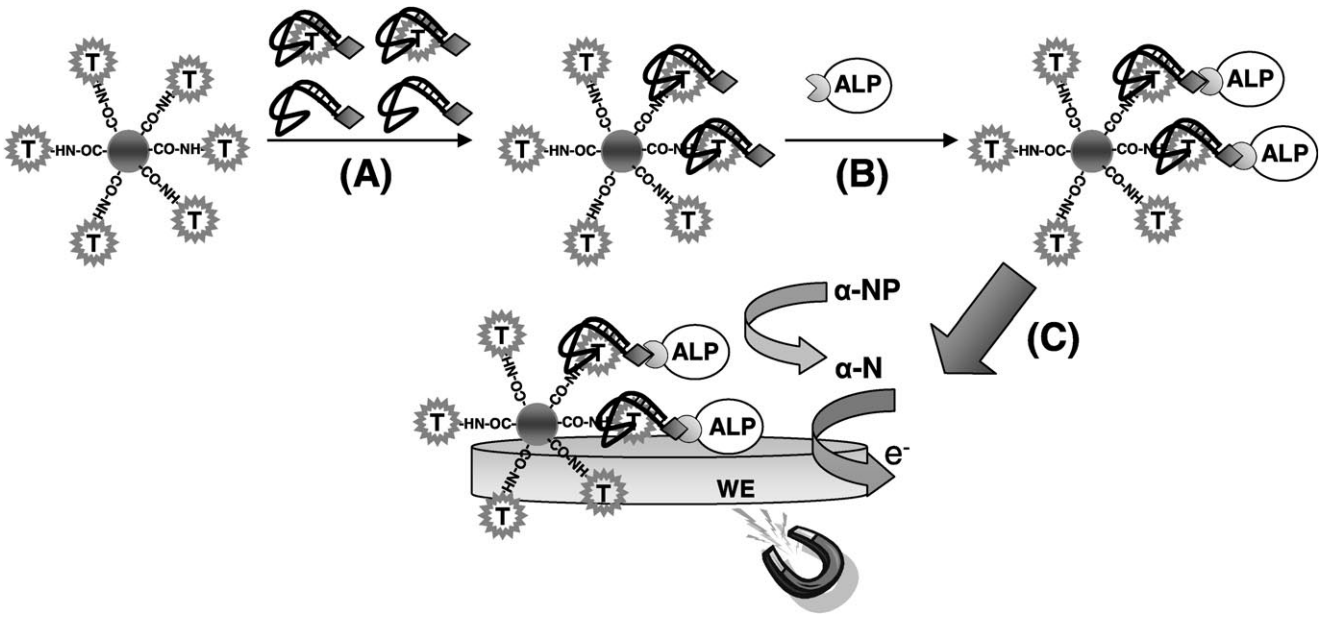

Fig. 1. Scheme of the proposed inhibition assay. (A) Incubation of TMPs with a fixed concentration of BATA and varying concentrations of tobramycin, (B) $Strep_2$-ALP labeling, (C) Enzymatic reaction and electrochemical measurement on SPCEs.

nonbiotinylated form of this modified aptamer was successfully applied to serum samples [20].

3.1 Optimization of the MPs Modification

The great enhancement in the effective surface area for molecular recognition when using MPs as a solid support compared with the typical area of solid electrodes requires a special control of the conditions during the MPs modification in order to ensure a maximum coverage of MPs surface and to minimize the unspecific binding.

Optimization of the tobramycin concentration used for its covalent attachment was carried out in order to obtain saturated tobramycin-modified MPs (TMPs). Following the protocol described above, MPs were modified with three concentrations of tobramycin (20, 40 and 80 mM) and the inhibition assay was carried out using 0.5 μM BATA in the absence of tobramycin (blank experiment) followed by the electrochemical measurements. This way, in the absence of tobramycin in solution, all BATA was allowed to recognize the immobilized antibiotic and this blank experiment represented the maximum signal achievable with the inhibition assay. The difference between the analytical signal measured for the blank and the corresponding nonspecific signal obtained after incubation without aptamer and tobramycin in solution was evaluated at each tobramycin coverage. This nonspecific signal was associated to the adsorption of the enzymatic conjugate to the sensing phase. It is worth noting that the nonspecific signal decreased when increasing the tobramycin concentration. In the highly covered MPs the nonspecific adsorption sites or/and the access of the bulky conjugate to the specific anchoring sites on the particle surface is more difficult. As a consequence, a decrease in the nonspecific signal which quantifies the nonspecific adsorption of the enzymatic conjugate ($Strep_2$-ALP) to the sensing phase was observed.

Figure 2 shows the blank current (grey columns) and nonspecific (white columns) values obtained at different tobramycin coverages. It is clear from the figure that the increase in tobramycin concentration from 20 to 40 mM led to a great enhancement in the measured signal while, at higher concentrations of tobramycin, the signal magnitude remained constant within the experimental error.

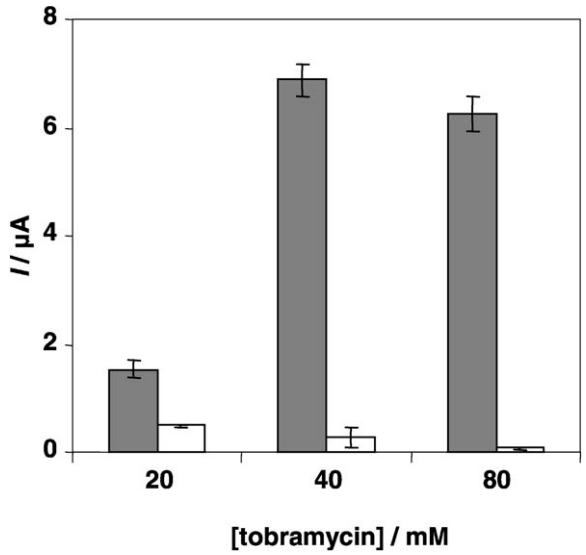

Fig. 2. Effect of the tobramycin coverage in terms of concentration of tobramycin used during the immobilization on the analytical signal. Grey columns correspond to the specific signal; white columns refer to the nonspecific signal (see text for details).

This result indicated that at the lowest concentration there was not sufficient tobramycin to react with all the surface active carboxylic groups that were subsequently deactivated by ethanolamine, which lacks specificity towards BATA. However, at concentrations above 40 mM of tobramycin a saturation of the active groups was reached. It is worth noting that the nonspecific adsorption of the enzymatic conjugate (white columns) decreased when increasing the tobramycin coverage and it was almost negligible when the MPs were saturated with tobramycin. Considering both specific and nonspecific signals, 40 mM of tobramycin was found to be the optimum antibiotic concentration for the MPs modification and was used in further experiments.

It is worth noting that the preparation of the sensing phase was much simpler and rapid using MPs as a solid support than in the case of polycrystalline Au electrode modification through SAMs [20]. The total modification time was reduced from two days to 3 hours.

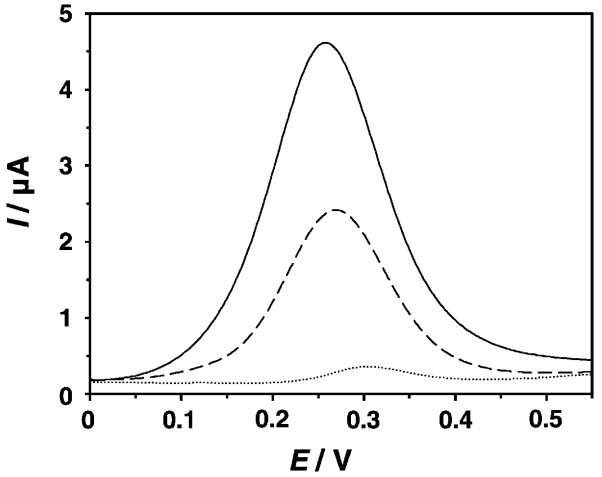

Fig. 3. DPV measurements recorded after an enzymatic reaction time of 10 min using: solid line: 0.5 μM BATA with no tobramycin (blank experiment); dashed line: 0.5 μM BATA +100 μM tobramycin solution and dotted line: in the absence of either BATA and tobramycin (nonspecific signal).

3.2 Inhibition Assay Development

The effect of the length of the enzymatic reaction was studied incubating the $Strep_2$-ALP conjugated TMPs with the substrate from 5 to 30 min. The analytical signals obtained with 0.5 μM BATA in the absence of analyte (blank) and in the presence of 100 μM of tobramycin were evaluated at each incubation time. The nonspecific signal was also checked (aqueous solution without analyte and aptamer). When the enzymatic time increased, the analytical signal measured also increased for both blank and sample solutions. However, this enhancement was not linear and less pronounced in the case of sample measurements. This way, after 30 min of enzymatic reaction the increase of the blank and 100 μM tobramycin solution current was 65% and 70%, respectively. Since a maximum difference between both signals was desirable, the optimum incubation time was found to be 10 min. At this time a decrease of 57% from the maximum current intensity (blank) was obtained for a tobramycin concentration of 100 μM. The corresponding DPV signals are depicted in figure 3. The nonspecific signal (Figure 3, dotted line) was found to be very low.

Selection of the optimum value of BATA is of paramount importance for a proper development of an inhibition assay. It is accepted that the limited reagent in reagent-limited assays (BATA in this particular case) corresponds to the reagent concentration providing about half of the binding sites occupied (50% of the maximum signal) [22]. For this purpose, several experiments were carried out entailing the incubation of different BATA concentration solutions (0.05–5 μM) with a constant amount of TMPs. The results are depicted in figure 4 and expressed as a percentage of the maximum current intensity measured. As expected, the analytical signal rapidly increased when increasing the concentration of BATA up to 0.5 μM. At higher concentrations the increase was less pronounced. A nonlinear data fitting allowed the estimation of the optimum BATA concentration, 0.1 μM, corresponding to the concentration that led to 50% of the maximum signal.

Once the experimental conditions were optimized, the inhibition assay was performed as explained above, testing tobramycin solutions ranging from 1 μM to 500 μM and employing the previously optimized BATA concentration of 0.1 μM. From Figure 5 it is apparent that the analytical signal (S % expressed as percentage of the maximum signal corresponding to the blank) decreased with a linear pattern with the tobramycin concentration because of the smaller amount of BATA available for interaction with TMPs. The calibration plot, $S\ \% = (-0.111 \pm 0.004)$ [tobramycin (μM)] $+ (84 \pm 1)$, covered a linear range from 5 μM to 500 μM of tobramycin. The proposed assay allowed the detection of 5 μM of tobramycin in aqueous solution. The reproducibility was found to be 5.7% ($n = 4$) for a tobramycin concentration of 300 μM.

It is worth noting that the analytical signal varied linearly with the concentration of the analyte instead of the logarithm of the concentration as it was found when using FIS measurements [20]. This behavior is probably related to the enzymatic amplification introduced in this inhibition assay.

The nonspecific adsorption to the sensing phase was evaluated by carrying out an incubation of the TMPs without any aptamer or sample (only buffer) and further labeling with the enzymatic conjugate. This contribution was found to be 10% of the maximum signal.

3.3 Selectivity of the Sensing Phase

The selectivity of the assay was also evaluated with respect to two different aminoglycoside antibiotics: kanamycin (which only differs from tobramycin in a single hy-

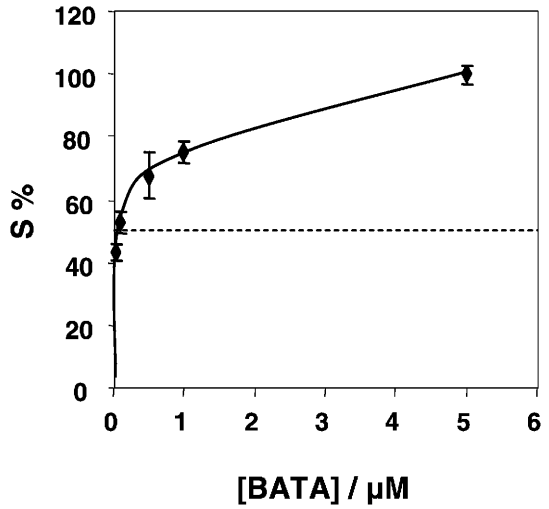

Fig. 4. Variation of the percentage of the maximum current intensity obtained by DPV as a function of the aptamer concentration.

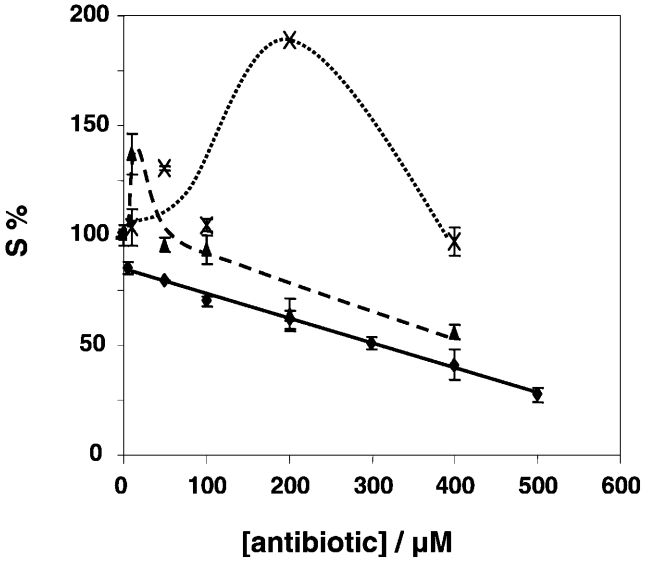

Fig. 5. Dose-response curves obtained for different aminoglycosides antibiotics after carrying out the inhibition assay; solid line: tobramycin; dashed line: kanamycin; and dotted line: streptomycin.

droxyl group) and streptomycin (whose hexose ring is streptidine instead of streptamine). Several concentrations of each antibiotic ranging from 1 to 400 µM were assayed employing the previously optimized BATA concentration of 0.1 µM and the protocol described for the tobramycin measurements. Both dose-response curves are depicted in Figure 5 obtained by plotting the percentage of the maximum signal (corresponding to the blank) vs. the antibiotic concentration. As it is clear from Figure 5, the sensing phase was completely selective to streptomycin according to the dose-response profile shown. The presence of this antibiotic drew to a great enhancement of the percentage of the maximum signal, being the highest value registered 90% higher than the value related to the blank, which corresponds to a streptomycin concentration of 200 µM.

On the other hand, selectivity towards kanamycin was not so distinct. From 5 to 50 µM, it seemed to show a good discrimination between kanamycin and tobramycin. However at concentrations above 50 µM, the dose-response curve showed a decrease associated to the increase in kanamycin concentration similar to that exhibited for tobramycin. It is worth noting that the percentage values of the signals for kanamycin were around 20% higher than those corresponding to tobramycin, but the presence of kanamycin could give rise to false positives when present in high concentrations. This result was surprising taking into account the excellent selectivity exhibited by the previously reported impedimetric aptasensor for tobramycin when tested in kanamycin solutions [20]. The only difference between both assays, in addition to the detection technique, was the use of the biotinylated anti-tobramycin aptamer as a biorecognition element. This result again pointed to an alteration of the affinity of the aptamer after labeling as mentioned above. Thus, the loss in selectivity might be ascribed to a lower affinity of the labeled aptamer for its cognate ligand and an increased affinity for structurally related compounds.

4 Conclusions

An inhibition assay with voltammetric detection for the determination of tobramycin using magnetic microparticles as a solid support was developed. A wide linear range was found with a limit of detection of 5 µM. Although this value was slightly higher than that obtained using FIS measurements, the preparation of the sensing phase was much shorter because of the easy handling of MPs that, additionally, allowed the parallel determination of several samples. A saturated coverage of the MPs with tobramycin as well as an efficient capture of the MPs on the electrode surface using a magnet were critical parameters to achieve a good reproducibility.

Acknowledgements

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