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Monovalent labeling system improves the sensitivity of aptamer-based inhibition assays for small molecule detection

Eva González-Fernández, Noemí de-los-Santos-Álvarez, Arturo José Miranda-Ordieres, María Jesús Lobo-Castañón*

Dpto. Química Física y Analítica, Universidad de Oviedo, Av. Julián Clavería 8, 33006, Oviedo, Spain

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ABSTRACT

The design and performance of an inhibition assay for the electrochemical detection of tobramycin is reported. This platform uses a monovalent system for introducing the enzyme conjugate on a tagged-aptamer that specifically recognizes the aminoglycoside antibiotic tobramycin. Compared with multivalent systems such as biotin–streptavidin, the sensitivity is greatly improved reducing the limit of detection from 5 μM to 0.1 μM in aqueous solution. This labeling strategy along with the use of a nuclease-resistant RNA aptamer allowed the determination of tobramycin in pre-treated human serum samples within the therapeutic range, using magnetic microparticles as a solid support for the aptassay, and differential pulse voltammetry as detection technique.

1. Introduction

More than two decades after the first description of aptamers [1,2], the detection of small molecules using these fascinating promising recognition elements is still a challenge. On one hand, macromolecules possesses multiple interaction sites, so strong affinities are displayed (dissociation constants, K_D, in the (sub)nM range), whereas most hapten-like molecules exhibit K_D in the μM [3]. A few exceptions have been reported for tobramycin [4] and ochratoxin A aptamers [5]. On the other hand, the implementation of well-established strategies for protein detection is not straightforward when not unfeasible. From the electroanalytical point of view, their small size makes the use of direct detection strategies difficult because the formation of the aptamer-small molecule complex is not expected to change an analytically useful property in a large extent. In fact, only a few examples of electrochemical direct label-free detection have been reported by exploiting the blocking electron-transfer of a redox probe after the recognition event [6–9]. Recently an unexpected electroactivity of kanamycin on self-assembled monolayers (SAMs) of thiolated aptamer has been employed for direct detection [10].

Alternatively self-reporting strategies that rely on ligand-induced conformational changes of redox-labeled aptamers have been developed [11,12]. However, they also lack of general applicability because structural rearrangements of small-binding aptamers can become insignificant, which would oblige to a high-cost re-engineering of the aptamer sequence to obtain an efficient detection [13]. Self-reporting displacement assays based on the hybridization capacity of aptamers can mitigate this problem [14–17] but, it still requires a previous knowledge of the tridimensional conformation of the aptamer and/or a rational design of the duplex to cause the desired structure-switching activity. Refilling strategies where the aptamer sequence is displaced from the duplex and the free sites are subsequent occupied by a shorter or partially complementary labeled strand are also possible but they provide longer assay times due to the extra step and a careful design of the reporter strand is also needed in order not to displace the remaining aptamers from the duplex [18–20].

Sandwich assays are not possible either. Although fragmentation of the aptamer (split strategy), the analog approach for small molecules, has been successfully carried out [21–24], this is still a non-universal strategy. Aptamers with strong secondary structures such as a hairpin can reassemble in the absence of the ligand leading to high blank signals. Therefore, single-site binding approaches are the only option when assaying small molecules. Direct competitive assays are rare for small molecules due to the need for labeling the ligand [25,26]. We recently proposed an indirect competitive assay also called inhibition assay, to detect an aminoglycoside antibiotic [27]. This approach only requires the

* Corresponding author. Tel.: +34 985106235; fax: +34 985103125.
E-mail addresses: gfernandezeva@hotmail.com (E. González-Fernández), santosnoemi@uniovi.es (N. de-los-Santos-Álvarez), amir@uniovi.es (A.J. Miranda-Ordieres), mjlc@uniovi.es, mjloboc@gmail.com (M.J. Lobo-Castañón).

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use of a commercially available labeled aptamer in combination with enzymatic amplification. Although aptamers can be directly conjugated to enzymes [25], indirect attachment through affinity binding after the recognition event is more convenient because the large size of the enzyme might hinder the recognition ability of the smaller aptamer. Coupling enzymes can be achieved by means of (strept)avidin–biotin or hapten–antibody affinity reactions. Herein, we proposed the introduction of the enzyme through a fluorescein isothiocyanate (FITC)-anti-FITC Fab fragment as a way to improve the sensitivity of inhibition assays, when compared with the well-established streptavidin-biotin coupling system, for the detection of tobramycin. The levels of this broad-spectrum aminoglycoside have to be controlled during treatment because of adverse effects of oto- and nephro-toxicity. The aptamer-based assay was successfully applied to human serum samples using a partially 2′-OMe RNA antitobramycin aptamer designed to resist the endonuclease degradation in biological fluids [28].

2. Experimental

2.1. Instrumentation

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) and the magnet (DynaMag-2) for magnetic separation were purchased from Life Technologies (Madrid, Spain).

A Genesys 10S UV–vis spectrophotometer (Thermo Scientific, Spain) was used for kinetic measurements of the enzymatic reaction.

2.2. Reagents

A fluorescein isothiocyanate labeled 27-mer RNA anti-tobramycin aptamer (FITC-ATA) was used throughout this work. The sequence was modified at 2′-OH position of its ribose moieties with a methoxy group in all nucleobases except at U12 position due to its implication in the recognition event [28,29]. It was synthesized by Sigma–Aldrich and purified by HPLC. Its sequence is the following:

5′[Fluorescein][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′

Carboxylated magnetic microparticles (MPs) Dynabeads® MyOne™ Carboxylic Acid (1.05 μm in diameter) were from Life Technologies.

Tobramycin sulfate, streptomycin sulfate, kanamycin sulfate, N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for ELISA, 1-naphthyl phosphate (α-NP) and human serum were purchased from Sigma-Aldrich (Madrid, Spain). Anti-fluorescein isothiocyanate-alkaline phosphatase Fab fragment (anti-FITC-ALP-Fab) and anti-fluorescein isothiocyanate–peroxidase Fab fragment (anti-FITC-POD-Fab) conjugates were acquired from Roche. 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), ethanolamine, Tween 20®, bovine serum albumin (BSA), salts for buffer solutions (KCl, NaCl, $MgCl_2$, $CaCl_2$), phosphate buffered saline (PBS) 10× pH 7.4, casein blocking buffer (1% casein, 1% PBS) pH 7.4 and 1 M Tris/HCl pH 7.4 solution (all RNAse free) were also obtained from Sigma–Aldrich. All other reagents were of analytical grade and used as received.

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, Spain). Amicon-ultra 4 mL-10,000 Da filters (Millipore) were used for human serum samples ultracentrifugation.

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

Immobilization solution: 0.1 M HEPES pH 8.64.
Affinity solution: 20 mM Tris–HCl pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$.
Washing solution: affinity solution + 0.01% Tween 20
Conjugate solution: 0.5% casein, PBS 1×, 0.025% Tween 20.
Detection solution: 0.5 M Tris–HCl pH 9.8, 1 mM $MgCl_2$.

2.3. Experimental procedures

2.3.1. Modification of carboxylated MPs with tobramycin

For the preparation of the sensing phase, tobramycin was covalently immobilized through the amino groups to the surface of carboxylated MPs employing a protocol previously described by our group [27].

Briefly, 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. After magnetic separation, they were re-suspended in 500 μL of a mixture of 0.2 M EDC and 0.05 M NHS for 30 min. After two washing steps with 500 μL of immobilization solution containing 0.01% Tween 20, the MPs were re-suspended in a 40 mM tobramycin solution prepared in the immobilization solution for 1 h. Once the antibiotic was covalently attached to the surface of the MPs, and after washing twice with 500 μL of immobilization solution containing 0.01% Tween 20, a 1 h blocking step with 500 μL of a 1 M ethanolamine solution also prepared in the immobilization solution was carried out. Finally, two washing steps were carried out with 500 μL of washing solution and the tobramycin-modified MPs (TMPs) were re-suspended in the affinity solution to a final TMPs concentration of 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.

2.3.2. Inhibition assay protocol

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

With this aim, 10 μL of the TMPs stock solution were washed twice adding 1 mL of washing solution for 2 min each. After discarding the supernatant, the TMPs were re-suspended in 1 mL of affinity solution containing a constant concentration of FITC-ATA and different concentrations of tobramycin ranging from 0 to 1000 μM and 0.1% BSA, and incubated for 15 min. Then, two washing steps with conjugate solution for 2 min each were carried out. After that, the MPs were re-suspended in 1 mL of anti-FITC-enzyme-Fab conjugate diluted from the commercial stock in conjugate solution. After 30 min of incubation time, the MPs were washed twice with 1 mL of detection solution containing 0.01% Tween 20 and, finally, re-suspended in 100 μL of detection solution to a final MPs concentration of 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. Two different labeling conjugates