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SPR sensing of small molecules with modified RNA aptamers: Detection of neomycin B

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ABSTRACT

We have studied how the modification of the RNA aptamer evolved against neomycin B at 2′ position of ribose with a methyl group influences the affinity of the interaction. Using surface plasmon resonance (SPR) and faradaic impedance spectroscopy (FIS) an affinity constant in the μM range was calculated. The results showed that the modification of the aptamer does not significantly alter the affinity of the aptamer for the antibiotic. This finding opens up the possibility of designing modified RNA aptamers resistant to endonucleases without variation of the analytical features. In addition to this, we propose a competitive assay for the detection of neomycin B using SPR as a transduction technique. A range of quantification between 10 nM and 100 μM was obtained, which shows the feasibility of detecting small molecules using aptamers with high sensitivity.

1. Introduction

The detection of small molecules using aptamers (short synthetic nucleic acid sequences selected to strongly bind a ligand with high affinity and specificity) as molecular recognition element is more challenging than the detection of proteins because of the weaker affinity shown (dissociation constant values are typically in the μM range). Besides, sandwich assay formats are not feasible because the small size of the ligand does not make the evolution of two aptamers against different binding sites possible.

Self-reporting strategies, which involve labelling of the aptamer, are often used with good results. However, sometimes this approach strongly depends on the conformational change experienced by the aptamer after target binding, which must cause a significant change in an analytically useful property of the label (e.g. electron transfer efficiency, fluorescence, etc.). Unfortunately, binding of small molecules often causes slight distortions in the native structure of the aptamer, making the self-reporting strategy useless. This is the case of aminoglycoside antibiotics neomycin B and tobramycin. Both antibiotics bind to RNA stem-loop structures with loops comprising 13–14 nucleobases. In the presence of the ligand, this relatively large loop is zippered up through formation of non-canonical base pairs leaving a reduced loop of 5 nucleotides (Schroeder et al., 2000). Antibiotics are sandwiched between the floor of the major groove of the aptamer and a purine base of the reduced loop that flaps over it (adenine for neomycin B (Schroeder et al., 2000), guanine for tobramycin (Jiang and Patel, 1998)). Therefore, the minor change induced in the aptamer tertiary structure is not sufficient to allow a self-reporting labelling strategy. In addition to this, the direct detection of these antibiotics is not straightforward because they lack useful spectroscopic and electrochemical properties. Methods proposed so far include derivatization steps (Agarwal, 1990; Apffel et al., 1985; Kim et al., 2003; Krzek et al., 2001; Posyniak et al., 2001; Roets et al., 1995; Serrano and Silva, 2006; Shaikh et al., 1991; Stead and Richards, 1997) or they are time-consuming immunoassays (Haasnoot et al., 1999; Jin et al., 2006; Knecht et al., 2004). Capillary zone electrophoresis (Ackermans et al., 1992) with detection at copper electrodes (Voegel and Baldwin, 1997), liquid chromatography with pulse electrochemical detection (Zawilla et al., 2006), and electrochemical catalysis at ruthenium dioxide composite electrodes (Leech et al., 1990) have also been reported. We have recently proposed a label-free method for the detection of neomycin B using a 2′O-methylated-modified RNA aptamer with impedimetric detection (de-los-Santos-Alvarez et al., 2007). This was the first example of an RNA aptamer applied to biological samples with no loss in stability and selectivity when compared to the native (unmodified) aptamer.

RNA modified-aptamers are being used for therapeutical/biomedical applications because of the extended lifetime in biological fluids. However, to the best of our knowledge 2′OMe RNA

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aptamers have not been thoroughly characterized for biosensing purposes. Therefore, the first part of the present work is devoted to acquiring further insights on the binding affinity of this modified RNA aptamer. Surface plasmon resonance spectroscopy (SPR) is a surface-sensitive label-free technique that provides detailed information about the affinity and the kinetics of biomolecular interactions with high sensitivity. The determination of kinetic and binding constants with SPR is advantageous compared with other techniques because of its rapidity and simplicity of handling. Real-time binding curves are displayed by monitoring the change in the angle at which the surface plasmons of a, typically, gold film, are excited by a laser source. This way, the dissociation constant and the stoichiometry of the neomycin–aptamer interaction can be measured.

In addition to kinetic studies, SPR can be used as a transduction technique in analytical devices. However, it has scarcely been used in aptasensors. To the best of our knowledge, only four SPR-based aptasensors have been reported (Bini et al., 2008; Lee et al., 2008; Ostatna et al., 2008; Tombelli et al., 2005), all of them for protein detection. In the second part of the work, we propose the use of SPR as a transduction technique for the detection of small molecules, specifically, neomycin B.

2. Materials and methods

2.1. Instrumentation

A double-channel cuvette-based SPR instrument with an autosampler incorporated (Autolab-ESPRIT, Ecochemie, The Netherlands) was used for all SPR experiments. The instrument is controlled by Data Adquisition Software (ESPRIT version 4.1.2). Data treatment (overlapping and alignment of sensorgrams, zeroing and baseline corrections) were done with the accompanying software provided with the instrument (ESPRIT kinetic evaluation version 4.1.0). Non-linear fitting of the resulting sensorgrams was carried out using OriginPro 7.5 (Northampton, MA). All SPR experiments were carried out at $24 \pm 1\,^{\circ}C$. The temperature was controlled with a thermostat HAAKE D1 (Germany).

The impedimetric measurements were conducted using a conventional three-electrode electrochemical cell driven by a computer-controlled AutoLab PGstat-12 potentiostat with GPES and FRA 4.9 software (EcoChemie, The Netherlands). A platinum wire and a 1.6 mm diameter gold electrode (Bioanalytical Systems, Inc., IN) acted as auxiliary and working electrodes, respectively. An Ag|AgCl|KCl saturated reference electrode was used.

2.2. Reagents

Fully 2′-O-methylated RNA aptamer 5′-GGCCUGGGCGAG-AAGUUUAGGCC-3′ (HPLC-purified) was purchased from Sigma-Proligo (Madrid, Spain). Neomycin B sulfate (N-1876), ethanolamine and 4-(2-hydroxyethyl)piperazine-1-ethanosulfonic acid (HEPES) were obtained from Sigma–Aldrich (Madrid, Spain). Salts for buffer solutions (NaCl, $MgCl_2$, KCl), 1 M TRIS/HCl pH 7.4 solution, and water were RNAse free for the preparation of the aptamer stock solution and were also obtained from Sigma–Aldrich. 3-mercaptopropionic acid, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), potassium ferricyanide and ferrocyanide were purchased from Fluka. All other reagents were of analytical grade.

2.3. Immobilization procedure

The gold-coated glass sensor was immersed in piranha solution (3 H_2SO_4:1 H_2O_2) (CAUTION piranha solution is strongly oxidizing and should be handled with care!) for 10 min and thoroughly rinsed with water and ethanol, and dried with a stream of N_2. Then the sensor was placed onto the clean hemi-cylinder lens previously coated with a drop of immersion oil. Afterwards, water was injected in both channels and the surface was equilibrated for at least 30 min.

The immobilization method was basically adapted from the previously reported procedure for impedimetric measurements (de-los-Santos-Alvarez et al., 2007). The sensor was modified with a self-assembled monolayer (SAM) using 40 mM mercaptopropionic acid ethanolic solution (75:25), manually injected in both channels (Fig. 1A). The channels were capped with parafilm (Sigma–Aldrich) to avoid evaporation during the overnight formation of SAM. Afterwards, the sensor surface was washed with ethanol and water, and equilibrated in water for 1 h before further modification. The covalent attachment of neomycin B (channel one, solid line in Fig. 1B) and ethanolamine (reference channel, dotted-dashed line in Fig. 1B) was automatically achieved using the ESPRIT autosampler and sequencer. The procedure is depicted in Fig. 1B and included three consecutive injections of a mixture of 0.2 mM EDC and 0.05 mM NHS in water (10 min each, activation step) followed by a brief washing step with water, and three consecutive injections of 25 mM neomycin B in 0.1 M HEPES pH 8.64 (20 min each, ligand binding step). After briefly washing with water, the inactive unreacted carboxylic groups were blocked with 1 M ethanolamine solution in HEPES for 30 min (blocking step). During each injection the solution was continuously stirred. In the reference channel 1 M ethanolamine was added instead of neomycin B using an identical procedure. The modified channels were equilibrated in binding buffer until a stable SPR baseline was obtained.

2.4. SPR measurements

The binding buffer used was 50 mM TRIS/HCl pH 7.4 containing 250 mM NaCl and 5 mM $MgCl_2$, which is very similar to the conditions under which the selection of the aptamer through SELEX (Wallis et al., 1995) was carried out.

The entire automatic procedure included baseline, association, dissociation, regeneration and back to baseline steps, and it is schematized in Fig. 1C. Triplicate 5 min injections of buffer were performed before each cycle to account for systematic drifts and shifts in baseline present even in the reference channel. The average signal generated from these blank injections was subtracted over each data set. For kinetic/affinity measurements varying concentrations of aptamer dissolved in binding buffer (35 μl) were injected into both channels from the 384-well microtiter plate. Then, the association phase was monitored for 5 min, after which the channels were drained, and 50 μl of binding buffer was injected. The dissociation was monitored for 5 min. After an initial decrease, a stable signal was achieved whose value was always higher than the baseline. Thus, a regeneration step was compulsory and comprised of 2–3 injections of 0.05 M HCl for 1 min. After that, the signal returned to baseline level, and a new measurement cycle was started. In all steps, the solution was agitated to minimize mass transport effects.

For the competitive assay, a mixture of 2.1 μM aptamer with varying concentrations of neomycin B was added during the association phase. All other steps were performed as indicated above.

2.5. Electrode cleaning, pre-treatment and immobilization procedure

The Au electrode was cleaned and pretreated as published elsewhere (de-los-Santos-Alvarez et al., 2007). A SAM of mercapto-