J|A|C|S
COMMUNICATIONS

Published on Web 03/14/2007

Modified-RNA Aptamer-Based Sensor for Competitive Impedimetric Assay of Neomycin B

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Received December 14, 2006; E-mail: ptb@uniovi.es

We are just beginning to exploit the fascinating potential of the structured RNA or DNA molecules, called aptamers[1] that can selectively recognize specific targets, as tools for drug screening, biosensing applications, or even therapeutics.[2]

In the field of biosensors, most methodologies are based on target-induced conformational changes[3] leading to not generalizable detection schemes. Thus, excellent electrochemical aptasensors[4] have been developed for high molecular-weight analytes but the detection of small molecules is more challenging.

The label-free aptamer sensors for small ligands described so far use structured DNA as recognition element.[5] However, many small molecules interact with structured RNA over DNA, disturbing the synthesis of the encoded-protein. Therefore, most aptamers evolved so far are mainly RNA sequences although their intrinsic lower endonuclease stability precludes their application in biological fluids. Substitution at the 2′ position of nucleobases with amino, fluoro, or O-methyl functional groups makes RNA nuclease-resistant and compatible with enzymes used in the SELEX process, although variations in the binding affinity have been reported.[6]

Aptamers possess functional characteristics in many cases similar to antibodies. Most immunoassays for small molecules are competitive assays relying on the displacement of surface-bound antibodies by the analyte in solution. A similar assay using aptamers was explored for the detection of large molecules leading to no useful signals.[7] Despite this, we herein examine the feasibility of such an approach for the impedimetric detection of a small molecule using a fully 2′-O-methylated RNA aptamer based on the previously evolved RNA motif[8,9] against neomycin B.

Detection of aminoglycoside antibiotics is a challenging problem because they lack spectroscopic and electrochemical useful properties. Time-consuming label-based immunoassays,[10,11] electrophoretic[12] and HPLC methods based on derivative fluorimetric[13] or pulsed amperometric[14] detection, have been developed. Neomycin B is an aminoglycoside antibiotic for which aptasensors have never been previously described with the exception of an acoustic wave device aimed to study the inhibitory effect of aminoglycosides over interactions between native RNA and peptides.[15]

In this work, faradaic impedance spectroscopy (FIS) was selected as transducer. Neomycin B was immobilized on a self-assembled monolayer (SAM) of mercaptopropionic acid (MPA) on Au electrodes through carbodiimide chemistry.[16] The aptamer was immobilized by affinity binding to the surface-linked neomycin B (Figure 1). This design was preferred to the conventional electrode-bound aptamers to magnify the impedance change associated to aptamer displacement by the solution-phase analyte.

Faradaic impedance spectra were recorded as a Nyquist plot and data were adjusted to a Randles equivalent circuit. The covalent attachment of neomycin B caused a dramatic decrease in the electron-transfer resistance (R_{et}) from about 2 kΩ to 350 Ω because

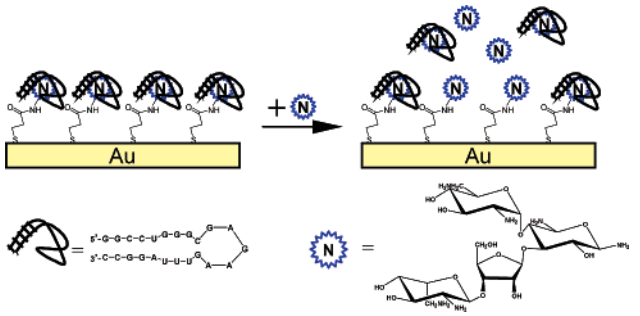

Figure 1. Schematic illustration of the modified electrode and the competitive assay

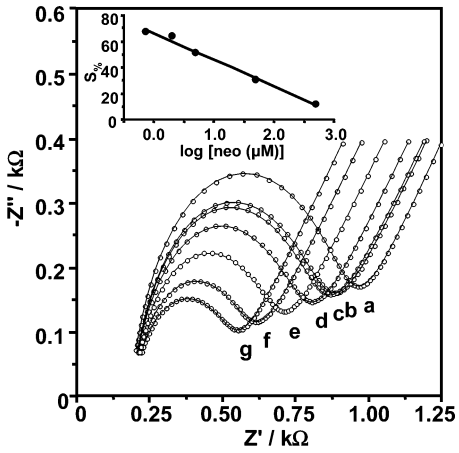

Figure 2. Nyquist plot of the MPA−neomycin−aptamer modified electrode after incubation with neomycin: (a) 0, (b) 0.75, (c) 2, (d) 5, (e) 50, and (f) 500 μM; curve g is obtained for the MPA−neomycin-modified electrode with no bond aptamer.[16] The inset shows the linear relationship between the logarithmic concentration of neomycin B and the percentage of R_{et} decrease.

the positive charged amino groups of neomycin attract the redox probe $[Fe(CN)_6]^{3-/4-}$ facilitating the electron-transfer reaction.

Afterward, the MPA−neomycin-modified electrode was exposed to the aptamer in the affinity solution until saturation.[16] As expected, the polyanionic phosphate backbone of the aptamer enhanced the R_{et} (Figure 2, curve a), although this increase is not as high as that exhibited by the MPA monolayer alone.

The MPA−neomycin−aptamer assembly was exposed to different concentrations of neomycin B in the affinity solution. The stoichiometry of the neomycin−aptamer complex depends on the ionic strength.[9] While a 3:1 complex is formed at low ionic strength, a 1:1 stoichiometry is observed at physiological conditions. Two low affinity sites probably associated to electrostatic interactions and one high affinity site involving hydrogen bonding were identified, the latter being responsible for the 1:1 complex. We have selected high ionic strength incubation conditions to ensure a 1:1

10.1021/ja0689482 CCC: $37.00

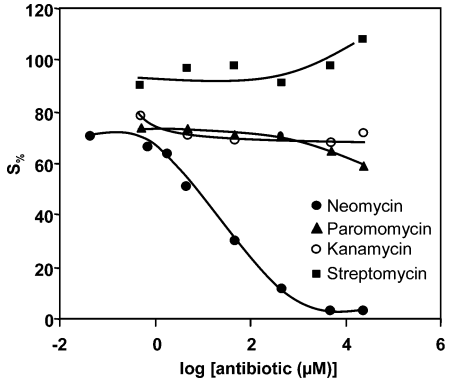

Figure 3. Calibration curves for different aminoglycoside antibiotics.

stoichiometry and avoid electrostatic interactions that can increase cross-reactivity with other antibiotics. As shown in Figure 2, the competitive displacement of surface-bound aptamer to form a complex in solution leads to a drop in the electron-transfer resistance, which is consistent with a reduction in the negative charge of the electrode surface.

The release of the aptamer from its complex on the surface is very fast, reaching the equilibrium in 5 min with a sharp decrease (more than 80% of the signal) within 1 min (Supporting Information, Figure S1), which dramatically reduces the assay time in comparison with immunoassays.[11] A sigmoidal dose−response curve (Figure 3) was obtained when the signal (S) is expressed as percentage of R_{et} decrease according to the following expression: $S_{\%} = (R_i - R_g)/(R_a - R_g) \times 100$, where R_i is the R_{et} after incubation with a concentration i of neomycin B, and R_a and R_g correspond to the R_{et} measured from curves a and g (Figure 2), respectively. The linear range covers more than 2 orders of magnitude (0.75−500 μM) with a limit of detection in the submicromolar range, a low value in comparison with other aptasensors for small targets.[5] The impedimetric aptasensor is completely recovered by a treatment in a concentrated solution of neomycin and further rebinding of the aptamer with a reproducibility of 2% ($n = 4$ cycles). The small amount of aptamer consumed in each round and the increased stability provided by the modified bases allows the reusability of the aptamer solution at least for a week, minimizing the cost of the assay. The device was employed at least for a week when stored in neomycin solutions, and more than 20 measurement cycles can be performed with no significant loss in sensitivity.

It has been shown that conserved rings A and B (neamine structure) of neomycin class-aminoglycosides are essential for binding to RNA, which causes a characteristic miscoding pattern. Additional rings play a secondary role increasing the binding affinity and conferring a directional specificity.[17] All the aptamers evolved against these aminoglycosides present a similar structure.[18] Therefore, cross reactivity to neamine-containing aminoglycosides (paromomycin and kanamycin) and other related antibiotics such as streptomycin was tested (Figure S2). The high specificity of the aptamer selected is apparent from Figure 3. A dramatic variation of binding affinity is observed because none of the compounds tested were able to significantly release antineomycin aptamer from its complex on the surface even at mM concentrations.

This finding is impressive in the case of paromomycin because it only differs from neomycin B in the substitution of a single NH_2 group with a OH group at C6 of ring A. Likewise it confirms that the affinity binding is not mainly electrostatic and hydrogen bonds are dominant. Besides, it seems that the previously reported 100-fold lower binding affinity between neomycin aptamer and paromomycin relative to neomycin[8] is not affected by the use of 2′-*O*-Me bases, which opens the possibility to the selective detection of neomycin B in biological samples.

To verify the compatibility of our device with biological fluids, neomycin-enriched whole milk was analyzed.[16] The only treatment needed was a dilution and ultracentrifugation. The milk was diluted with the affinity buffer to disrupt the potential protein−neomycin binding.[19] Although an increase in R_{et} was apparent in this matrix, probably because of the adsorption of the soluble fraction of proteins, this fact did not preclude the detection of neomycin B within a linear range between 25 and 2500 μM in milk (Figure S3). A recovery of 102% for 50 μM and 109% for 200 μM was obtained. The operational lifetime is not significantly reduced by the repetitive immersion in biological samples.

In summary, we present a novel aptamer-based assay for the detection of neomycin B in milk, whose format can be easily tailored to other small molecule compounds. We show that the use of modified-RNA sequences do not alter the exquisite selectivity of the aptamer, which is able to distinguish subtle changes in functionalities of the target molecule.

Acknowledgment. This work was supported by Gobierno del Principado de Asturias, (Spain) (Project FICYT IB05-048).

Supporting Information Available: Materials and reagents, experimental methods, kinetic data and chemical structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0689482