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Direct Observation of Aminoglycoside–RNA Binding by Localized Surface Plasmon Resonance (LSPR) Spectroscopy

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Abstract

RNA is involved in fundamental biological functions when bacterial pathogens replicate. Identifying and studying small molecules that can interact with bacterial RNA and interrupt cellular activities is a promising path for drug design. Aminoglycoside (AMG) antibiotics, prominent natural products that recognize RNA specifically, exert their biological functions by binding to prokaryotic ribosomal RNA and interfering with protein translation, ultimately resulting in bacterial cell death. The decoding site, a small internal loop within the 16S rRNA, is the molecular target for the AMG antibiotics. The specificity of neomycin B, a highly potent AMG antibiotic, to the ribosomal decoding RNA site, was previously studied by observing AMG–RNA complexes in solution. Here we study this interaction using localized surface plasmon resonance (LSPR) transducers comprising gold island films prepared by evaporation on glass and annealing. Small molecule AMG receptors were immobilized on the Au islands via PEG-thiol linkers, and the interaction with target RNA in solution was studied by monitoring the change in the LSPR optical response upon binding. The results show high-affinity binding of neomycin to 27-nucleotide model A-site and A-modified RNA sequences in the nanomolar range, while no specific binding is observed for synthetic RNA oligomers (e.g., poly-U). The impact of specific base substitutions in the A-site RNA constructs on binding affinity and selectivity is determined quantitatively. It is concluded that LSPR is a powerful tool for providing molecular insight into small molecule–RNA interactions and for the design and screening of selective antimicrobial drugs.

Keywords

Gold; island films; localized plasmon; biosensors; neomycin; antibiotics; RNA

INTRODUCTION

Owing to its critical role in numerous biological processes within the cell, RNA has emerged as a prime target for small molecule targeting.^{1,2} As with proteins, being therapeutic targets since the dawn of modern medicine, RNA structures show a rich functional diversity; the result of well-defined secondary structures and unique three-dimensional folds. This structural sophistication, combined with negatively-charged pockets generated by unique

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SUPPORTING INFORMATION

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RNA architectures, provides cavities with potential for small molecule binding.^{3–6} Indeed, an increasing number of small molecules of different structures have been discovered that bind to RNA.⁷ The number of potential RNA targets is quite large and includes RNA which is involved in cellular protein interactions such as transcription, splicing, and translation, and RNA which is involved in viral infection such as the human immunodeficiency virus (HIV) Rev response element (RRE),^{8,9} the trans-activation responsive element (Tar),^{10,11} and the hepatitis C-virus internal ribosome entry site (IRES) RNA.^{12,13}

Among the most promising RNA targets is the bacterial ribosome 16S decoding region aminoacyl-tRNA site (A-site).^{14–20} Given the fundamental importance of RNA to the ribosome and the centrality of protein biosynthesis to cellular function, it is not surprising that a host of natural and synthetic antibiotics target the ribosomal RNA in one or more steps of this pathway.

Aminoglycosides (AMGs) are effective drugs that are widely used in therapy against bacterial infections. The antibacterial activity of AMGs derives from their binding to the major groove of the prokaryotic ribosomal 16S aminoacyl A-site.^{14–19} AMG binding to this region causes mistranslation of mRNA as well as premature termination of message readout, ultimately resulting in bacterial cell death.²¹ AMGs are effective drugs, widely used in therapy against bacterial infections; however, they display high nephrotoxicity and ototoxicity in the clinic, that may lead to kidney failure, hearing loss and deafness.²² Furthermore, as is the case with virtually all classes of antibiotics, bacterial resistance has been widely developed against AMG antibiotics.^{23,24}

Rational design of selective RNA binders requires detailed knowledge of structure–affinity relationships between RNA and small molecules and analysis of the binding specificity. Various methods have been employed to the study of such interactions. Label-based immunoassays,^{25,26} electrophoretic²⁷ and HPLC methods based on derivative fluorimetric²⁸ and pulsed amperometric detection,²⁹ have been reported, in addition to fluorescence-based tools.^{30–37} These methods are rather low-throughput due to their time- and material-consuming nature. Methods based on radioactivity or fluorescently labeled AMGs introduce a modification into the molecules, which may affect their binding properties.

Label-free sensing of RNA–small molecule interactions has been shown using propagating surface plasmon resonance (SPR) and electrochemistry methods.^{38,39} The common approach to the preparation of optical⁴⁰ and electrochemical^{41,42} transducers is based on immobilization of RNA as the recognition element. In the case of the SPR-based transducer, several 5′-biotinylated RNA transcripts were synthesized and immobilized on the surface of a streptavidin-modified SPR chip, and the binding of AMG antibiotics to different RNA sequences on the surface was studied.⁴⁰ A major drawback of this approach is the intrinsically low RNA endonuclease stability, precluding application in biological fluids. An RNA-aptamer based electrochemical transducer for testing the interaction with AMG rapidly deteriorated even after methylation of all the 2′-hydroxyl groups outside of the aptamer's binding pocket.⁴² Moreover, immobilization of the RNA via 5′-phosphorothioate modification implies an additional synthetic step and the need to derivatize each tested RNA construct.

These drawbacks are overcome in the present work by immobilization of AMG ligand molecules on localized surface plasmon resonance (LSPR) transducers, serving as the recognition layer for detection of the interaction with diverse target RNA constructs in solution. The LSPR transducers comprise gold nanoisland films prepared by evaporation on glass slides and annealing.^{43,44} Such transducers present several desired properties, such as label-free assay, simple and cost-effective instrumentation and transducer production,

reproducibility, and convenient tuning of the surface plasmon (SP) resonance for system optimization.

The physics underlying analyte detection in LSPR spectroscopy are based on the sensitivity of the SP extinction band to the effective refractive index (RI) of the environment near a nanostructured metal surface.^{45–47} Change of the local RI upon analyte binding to a recognition layer immobilized on the metal nanostructures generates an optical signal, expressed as change in the intensity, wavelength or shape of the SP extinction band. LSPR spectroscopy allows label-free sensing of molecular interactions with a sensitivity similar to that of SPR, using a straightforward spectrophotometry based detection system and high-throughput array measurements.^{46,48–51}

The main motivation for the present work is the development of a biorecognition interface comprising a self-assembled monolayer (SAM) of functional receptors, namely, the AMG neomycin B derivatized with a PEG-thiol tail, denoted AMG-SH (Figure 1A). The monolayer is assembled on Au island films, providing label-free optical transducers capable of monitoring the interactions of AMG antibiotics with the ribosomal RNA decoding site and other potentially competing RNA constructs. Major advantages of covalent attachment of the ligands to the transducer surface (rather than immobilizing the RNA) are stability and robust response to RNA binding, the possibility of sensor recycling (as demonstrated in this work), and the ability to easily change the RNA sequence for analyzing selectivity. The results are expected to shed light on the interaction between neomycin B and different RNA constructs, as well as further the understanding of toxicity mechanisms and open the way to studies on other types of RNA targeting antibiotics.

EXPERIMENTAL SECTION

Chemicals and materials

Ethanol (Baker analyzed, J.T. Baker), methanol (anhydrous, Mallinckrodt chemicals), sulfuric acid (95–98%, BioLab), hydrogen peroxide (30%, Frutarom), ammonium hydroxide (Frutarom), 3-aminopropyl trimethoxysilane (APTS) (Aldrich), sodium chloride (Frutarom), and gold (99.99%, Holland-Moran, Israel), were used as received. Water was triply distilled. The inert gas used was household nitrogen (from liquid N₂).

Buffer solution

The buffer solution used in all the biochemical experiments was 50 mM HEPES/50 mM NaCl/5 mM MgCl₂, pH=6.5.

Gold island films

Gold island films were prepared by evaporation on glass slides followed by high-temperature annealing, as described previously.^{43,52} The Au nominal (mass) thickness (i.e., the evaporator QCM thickness monitor reading) was 5.0 nm or 7.5 nm (for preparation of transducers) and 15 nm (for experiments involving Ag nanoparticle (NP) binding). The annealing was carried out at 580 °C for 10 h. The transducer preparation procedure is highly reproducible: UV-vis spectral measurements of several batches (24 slides each) of 5.0 nm Au films taken after annealing show the following values (determined using the modified centroid procedure^{53,54}): a SP band wavelength of 525.0±0.9 nm and an extinction intensity of 0.196±0.006 a.u., with a full width at half maximum (fwhm) of 59.0±0.2 nm. The measured refractive index sensitivity (RIS) is typically 60 nm RIU⁻¹ and 0.55 a.u. RIU⁻¹ for 5.0 nm films, and 73 nm RIU⁻¹ and 0.61 a.u. RIU⁻¹ for 7.5 nm films.⁵⁵ The measured SP decay length of these transducers is 10–13 nm for 5 nm films and 19–24 nm for 7.5 nm films.⁵⁶

Continuous gold films

Continuous gold films were prepared by evaporation of 100 nm gold onto silanized glass slides followed by low-temperature annealing (20 h at 200 °C), according to our previously published procedure.⁴⁶

Synthesis of the modified aminoglycoside (AMG) antibiotic AMG-SH

The PEG-thiol derivatized aminoglycoside antibiotic neomycin B (AMG-SH, Figure 1A) was synthesized by modifying the primary hydroxyl group of the ribose core, known to be non-essential for RNA binding. A detailed synthetic procedure is given in the Supporting Information, together with all the analytical data.

RNA synthesis and purification

Transcripts of the A-site decoding region of rRNA and its variants were prepared *in vitro* with an Ampliscribe T7 High Yield Transcription (Epicentre Biotechnologies) production kit using synthetic oligonucleotide templates (Danyel Biotech), according to a procedure provided by the manufacturer. The RNA transcripts were purified by ethanol precipitation with ammonium acetate.

Direct preparation of self-assembled monolayers (SAMs) of AMG-SH

Gold films, either continuous or island-type, were treated 10 min in a UV-ozone cleaning system (UVOCS model T10×10/OES/E), dipped 20 min in ethanol with stirring, and dried under a nitrogen stream.⁵⁷ Self-assembly of AMG-SH was achieved by incubation of the cleaned Au substrates in 300 µM aqueous solution of AMG-SH either 30 min or overnight (ca. 12 h). After incubation the samples were washed 10 min in triply-distilled water with stirring and dried under a nitrogen stream.

Preparation of a SAM of AMG-SH using a preformed complex

100 µL of 0.5 µM RNA (16S A-site rRNA) solution were mixed with 100 µL of 0.5 µM AMG-SH solution for 1 h to obtain a preformed AMG-SH/RNA complex in solution. A cleaned Au transducer was incubated with the preformed complex solution for 2 h followed by washing with triply-distilled water and drying under a nitrogen stream, resulting in a SAM of AMG-SH/RNA on the Au surface. To dissociate the RNA, the sample was treated for 30 min with 8 M urea solution at 60 °C, followed by washing 10 min with triply-distilled water with stirring and drying under a nitrogen stream. The urea treatment leads to denaturation and desorption of the RNA molecules.

RNA binding, desorption and rebinding

For RNA binding a 100 µL drop of 0.5 µM RNA in buffer solution was placed on the antibiotic-modified Au substrate for 2 h at room temperature in a closed Petri dish kept at 100% relative humidity. The sample was then washed 10 min in triply-distilled water with stirring and dried under a nitrogen stream. In cases where RNA desorption and rebinding was demonstrated, the bound RNA was detached from the antibiotic SAM on the Au surface by 30 min incubation of the sample in 8 M urea solution at 60 °C. The slide was then washed 10 min in triply-distilled water with stirring and dried under a nitrogen stream. RNA rebinding was carried out in the same manner as RNA binding, using 4–5 h incubation.

UV-vis spectroscopy in air

Transmission UV-vis spectra were obtained with a Varian CARY 50 spectrophotometer. Spectra were recorded in the range 350–800 nm at a scan speed of 300 nm min⁻¹ using air as the baseline. Samples to be examined were dried from solvent under a stream of nitrogen

and placed in a special holder enabling transmission measurements of the same spot on the slide during all stages of the experiment.

Kinetics of AMG-SH assembly

The kinetics of assembly of a SAM of AMG-SH on 5 nm Au island films were recorded by immersion of the transducer in 300 μM aqueous solution of the antibiotic and recording the absorbance change *in situ* at a single wavelength of 560 nm in a quiescent solution.

Measurements of RNA binding kinetics

Kinetic data for RNA binding to a SAM of AMG-SH on Au island films were obtained using a flow system consisting of a controllable syringe-based pumping unit (Longer Precision Pump Co.), manual selection valves and a micro flow-cell.⁴⁸ The home-made micro flow-cell was designed to accommodate $8\times 8\text{ mm}^2$ samples while minimizing the solution volume (60 μL cell volume) required to perform the kinetic assay. Au island transducers (5.0 nm nominal thickness) were cut to $8\times 8\text{ mm}^2$ pieces and placed in the micro flow cell. The cell was filled with buffer solution and mounted in the Varian CARY 50 spectrophotometer. Solutions entered the flow-cell via a four-way valve fed by a set of motor-driven syringes. All the components were connected using Teflon tubing. The flow system was flushed with buffer before each experiment to purge air bubbles. Binding of RNA was measured by monitoring the extinction change at a constant wavelength of 560 nm. A 1 μM buffer solution of a given RNA was pumped at $50\text{ }\mu\text{L min}^{-1}$ through the cell, initially filled with pure buffer solution, and the extinction intensity at 560 nm measured in the transmission mode using 0.3 s integration time per point and stored at 1 s intervals.

Polarization modulation Fourier-transform infrared reflection-absorption spectroscopy (PM-IRRAS)

Spectra were recorded using a Fourier-transform infrared (FTIR) spectrometer (Bruker Tensor 27) with a polarization-modulation setup (PMA50) equipped with a photoelastic modulator (PEMTM 100, Hinds Instruments, USA). The spectra were recorded at a resolution of 4 cm^{-1} . The photoelastic modulator maximum efficiency was set for the half-wave retardation at 2600 cm^{-1} , tuned for nucleotide base pairs and PO_2 vibrations, observed at 1610 cm^{-1} (A–C) and 1698 cm^{-1} (G–U) pairs, and 1089 cm^{-1} and 1240 cm^{-1} (PO_2 symmetric and asymmetric stretching vibrations, respectively). Each spectrum represents the average of 1420 scans collected within a total of 20 min; the first 100 scans were acquired as background. The angle of incident light was 85° . The spectra were collected in air after purging the sample and detector compartment with pure nitrogen, and processed using the OPUS software (Bruker).

High-resolution scanning electron microscopy (HRSEM)

HRSEM images were obtained using an ULTRA 55 FEG ZEISS microscope. Measurements of island films were carried out at an applied voltage of 2 kV and a working distance of 3 mm using the Everhart-Thornley secondary electron (SE) detector. Measurements on continuous films were carried out at an applied voltage of 15 kV and a working distance of 3 mm using the high efficiency in-lens SE detector.

Synthesis of Ag nanoparticles (NPs)

One-step synthesis of aqueous negatively-charged phosphonate-stabilized Ag NPs of $9.3 \pm 1.0\text{ nm}$ mean diameter was carried out as described elsewhere.⁵⁸

Electrostatic binding of Ag NPs to Au surfaces

Au films (continuous and island-type) on glass (cleaned by UV-ozone, washed with ethanol and dried under a N₂ stream⁵⁷) were immersed in a 300 μ M aqueous solution of AMG-SH for 30 min or 12 h, rinsed with triply distilled water and dried under N₂. The slides were then covered with 50 μ L of $\sim$ 46 nM Ag NP solution for 2 h. Following NP adsorption the slides were washed with triply distilled water and dried under a N₂ stream.

RESULTS AND DISCUSSION

Label-free sensing of aminoglycoside (AMG)–RNA interactions

The PEG-thiol modified AMG antibiotic neomycin B (AMG-SH, Figure 1A) was custom-synthesized for this study. Choice of the 5' position of the AMG for derivatization and surface immobilization is based on biochemical and structural studies indicating that this position is not directly involved in the binding to 16S A-site rRNA,^{59–62} hence attachment to the Au via a linker at this position is expected to minimally interfere with binding specificity. Several prokaryotic A-site rRNA constructs were reported previously,^{63–65} for the present study we chose the simple construct shown in Figure 1B as the prokaryotic 16S A-site model.

A full SAM of the antibiotic on Au films was prepared by overnight incubation of the Au substrate in the AMG-SH solution. Two RNA sequences were used as targets: (i) a 27-nt 16S A-site rRNA (Figure 1B); and (ii) a 27-nt poly-U RNA as a negative control. Schematic representation of the sensing experiments is shown in Figure 2, steps (a) and (b); details on AMG-SH immobilization and RNA binding are found in the Experimental Section. Respective transmission spectra (using 5 nm Au island transducers) are presented in Figure 3A, C. The spectra in Figure 3A show a typical response of the LSPR transducer to immobilization of the ligand (AMG) followed by binding of a specific analyte (RNA), i.e., red shift of the SP band wavelength and increase of the extinction intensity. On the other hand, the unrelated RNA (Figure 3C) does not exhibit any binding to the AMG-modified transducer. Binding of the cognate RNA target to the AMG-modified LSPR transducer is supported by PM-IRRAS results, as described below (Transducer Regeneration section).

To determine the wavelength shift and extinction intensity change of the SP band, the modified centroid procedure was applied.^{53,54} This method improves significantly the signal-to-noise ratio and hence the accuracy of the measured parameters. Immobilization of AMG-SH on the Au island films causes an increase of ca. 0.017 a.u. in the SP intensity and a 3.8 nm red shift of the band maximum (Figure 3A), as qualitatively expected from the increase in the local effective RI.^{46,48} Subsequent binding of the specific 16S A-site RNA leads to further intensity increase of ca. 0.011 a.u. and 2.9 nm red shift (Figure 3A), while exposure to the nonspecific poly-U RNA results in essentially no change in the extinction band (Figure 3C). Importantly, when a bare Au transducer was exposed to a 0.5 μ M solution of the 16S A-site RNA, no notable change in the spectrum was observed (data not shown). These results establish the feasibility of studying the interactions between RNA and antibiotic molecules using Au island based LSPR spectroscopy.

Theoretical SP changes for a full monolayer of AMG-SH were calculated by means of a simple phenomenological model^{46,56,66,67} using the RIS and decay length values given in the Experimental section, an AMG-SH layer thickness of $\sim$ 1.15 nm, and a difference of 0.5 RIU between the RI of the organic layers and the medium. The calculated values are 4.6 nm (SP peak shift) and 0.036 a.u. (SP extinction intensity change). Subsequent binding of specific RNA molecules leads to a calculated overall SP wavelength shift of 12.5 nm and intensity increase of 0.059 a.u., assuming an overall thickness of the AMG-SH/RNA complex of $\sim$ 2.6 nm. The calculated changes are ca. twice the experimental values (overall

experimental changes: 6.7 nm peak shift, 0.028 a.u. intensity change, see above), attributed to the simplicity of model applied and the fact that the adsorbed layers may not be tightly packed.

System optimization

A full coverage of the transducer surface by receptors is not necessarily an optimal situation for high sensitivity to analyte binding, and often spacing of the receptor molecules may lead to enhanced response, as in the case of DNA hybridization.⁶⁸ In the present system it was hypothesized that spacing of the AMG-SH molecules on the Au surface may decrease the steric hindrance and improve the accessibility of target RNA to the immobilized ligands, thereby enhancing the response to the recognition event.

Transducers with different coverage of the AMG receptor were prepared by kinetic control of the AMG-SH assembly. The kinetics of assembly of a SAM of AMG-SH on a 5 nm Au island film were measured by recording the absorbance at a single wavelength of 560 nm, somewhat red-shifted from the SP peak (Figure 3).^{69–71} Binding of AMG-SH results in an increase of the absorption, gradually approaching a limiting coverage (Figure 4A). The data suggest that >50% of the maximum coverage is attained in 30 min of adsorption.

To visualize the difference in surface coverage between AMG-SH adsorbed 30 min and 12 h, negatively-charged Ag nanoparticles (NPs), 9.3 ± 1.0 nm in mean diameter,⁵⁸ were bound electrostatically to AMG-SH covered Au surfaces. Ag NPs were chosen for this purpose because they furnish easily detectable microscopic and spectroscopic signatures; use of metal NPs for mapping of organic layers immobilized on Au surfaces was previously reported.^{48,72,73} The substrates used in this experiment were continuous Au films and large, 15 nm (nominal thickness) Au island films;⁵⁵ while the former provide convenient assessment by HRSEM imaging, the latter enable measurement of the NPs' SP band using transmission spectroscopy.

Figure 4B shows the result of a control experiment, indicating no NP binding to a bare Au surface (continuous film or islands (inset)) exposed to Ag NPs. Figure 4C, D presents HRSEM images obtained after binding of Ag NPs to AMG-SH modified Au surfaces; the bright spots are Ag NPs. The images show a marked difference in the amount of NPs on the differently prepared AMG-SH coated Au substrates. Surfaces treated 12 h with a solution of AMG-SH show a considerably higher density of NPs than those treated for 30 min. This trend is also seen in the LSPR spectra recorded in the wavelength window characteristic of dried Ag NP films (Figure S2A, B, Supporting Information), particularly in the difference spectra (Figure S2C, D, Supporting Information), showing substantially increased intensity after overnight adsorption.

The response of a transducer with partial AMG-SH coverage (30 min adsorption) to 16S A-site RNA (Figure 3B) is considerably higher than that of a transducer with a full AMG-SH coverage (Figure 3A): The maximum SP intensity change for RNA binding is, respectively, 0.026 and 0.011 a.u., while the peak shift is, respectively, 4.9 and 1.9 nm. Control experiments with the unrelated and nonspecific poly-U RNA using both transducers resulted in essentially no change in the spectrum ($<10^{-4}$ a.u. and 0.04 nm, Figure 3C, D).

Another approach to spacing of the receptors on the Au surface for achieving better transducer response involves assembly on the bare Au islands of a preformed AMG-SH/RNA complex followed by dissociation of the RNA from the surface (see Experimental Section), to obtain a transducer with properly spaced ligands for RNA rebinding. Sequential UV-vis spectra taken after each step of this scheme are shown in Figure S3B (Supporting Information). Binding of the AMG-SH/RNA complex to the Au surface is indicated by a 6.9

nm red shift of the SP band and a significant increase of 0.034 a.u. in the extinction intensity. After RNA dissociation with urea solution the intensity and position of the resulting SP band are different by 0.002 a.u. and 1.8 nm, respectively, from those of the bare Au (spectrum 5 in Figure S3B, Supporting Information), pointing to the formation of a low-density AMG-SH layer (compare these values with 0.017 a.u. and 3.8 nm for a full AMG-SH layer, see above). A second incubation with RNA does not show a significant loss of signal (spectrum 6 in Figure S3B, Supporting Information). Rebinding of 16S A-site RNA molecules reveals a 0.030 a.u. intensity increase and a 4.7 nm red shift, a considerably better sensitivity than that obtained with a full AMG-SH SAM (0.011 a.u. and 2.9 nm red shift, Figure 3A). Use of a preformed complex (Figure S3B, Supporting Information) does not provide a clear advantage over 30 min AMG-SH adsorption (Figure S3A, Supporting Information) in terms of improving the sensitivity, and therefore the preformed complex approach was not pursued.

The results substantiate the notion that an optimal surface density of the antibiotic molecules is required for achieving maximal response to RNA binding, while with higher coverages the interaction of RNA and the immobilized AMG is significantly diminished.⁶⁸ Note that since no nonspecific RNA binding was detected on bare Au (see above), the present system does not require blocking of exposed Au areas in 30 min substrates. In cases where such blocking may be necessary, PEG-thiol⁴⁸ or BSA⁴⁹ may be used as blocking agents.

Transducer regeneration

Features that may be crucial for actual application of LSPR transducers include regeneration and stability. Transducer regeneration was demonstrated here using the scheme presented above for transducer preparation by means of a preformed complex, namely, heating to 60 °C in 8 M urea, leading to RNA dissociation (Figure S3B, Supporting Information).

Regeneration by 30 min immersion in 8 M urea at 60 °C results in RNA denaturation and release from the AMG layer (Figure 5A, spectra 1–4). Following RNA desorption the transducer is ready for the next assay, i.e., second RNA binding (Figure 5A, spectrum 5). The regeneration step appears not to damage the AMG recognition layer — after regeneration the RNA rebinding occurs with no loss of signal (Figure 5B, C and Table S1, supporting information). The slightly higher extinction change after RNA rebinding is assumed to reflect certain rearrangement of the AMG-SH SAM during RNA desorption.

The LSPR results are supported by PM-IRRAS data obtained with 100 nm continuous Au films (Figure 5D). The peak at 1529 cm⁻¹ in all spectra is assigned to the primary amine of the AMG. Binding of RNA molecules is evidenced by the appearance of the major RNA marker bands at 1089 cm⁻¹ and 1242 cm⁻¹ (PO₂ stretch) and 1698 cm⁻¹ (G,U),⁷⁴ which are absent before RNA binding and after RNA desorption. The characteristic RNA peaks are restored after the second incubation with RNA (Figure 5D).

RNA binding kinetics

Kinetic experiment were carried out to quantify the affinity of different RNA molecules to the AMG layer, i.e., to probe the relationship between the structure of the prokaryotic 16S A-site RNA and its binding to AMGs. Several RNA sequences (Figure 1B–E) were synthesized by the action of T7 RNA polymerase on appropriate DNA templates, as detailed in the Experimental Section. In the case of the prokaryotic construct studied here, AMG binding involves the pocket formed by the bulged A1492 residue.⁷⁵ An important difference between prokaryotic and eukaryotic A-site regions with respect to AMG binding is the substitution of G for A at position 1408 of the prokaryotic construct.⁷⁶ The importance of the A1492 residue for AMG binding was probed using A-modified and G-site RNA

(constructs C and D, respectively, Figure 1). The *H. Sapiens* 18S (A1408G) construct (Figure 1E) was also prepared and tested for AMG binding.

The kinetics of RNA binding to an AMG-SH SAM were monitored in a flow configuration using 5.0 nm Au island transducers, ensuring (by measurement at different flow rates) that under the applied conditions the RNA binding was not mass-transport limited. Binding of the different RNA constructs was measured by monitoring the extinction change at a constant wavelength of 560 nm (see Experimental Section); representative data are shown in Figure 6. Binding of all the RNA oligonucleotides to the AMG layer is observed, with different binding affinities.

The rates of RNA binding were fitted to a standard 1:1 first-order binding kinetics model.⁴⁸ The binding constants (k_{on}) were evaluated and are given in Table 1. The LSPR data confirm the high affinity of the bacterial RNA to the antibiotic. The prokaryotic A-site construct exhibits a binding constant of $4.9 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, close to the value of $3.26 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ obtained for the high-affinity complex of DNA-aptamer and daunomycin antibiotic.⁷⁷ Binding of the analogous 18S human RNA construct shows an order-of-magnitude smaller k_{on} (Table 1). AMG binding to the eukaryotic A-site construct is expected, as AMGs are believed to bind weakly to the 18S human A-site; under the present conditions we found a k_{on} value similar to that of the 18S human RNA. Despite their lower association constants with respect to the 16S bacterial A-site, the 18S human RNA and mutant eucariotic A-site show much higher k_{on} values compared to the binding of neomycin to *Leishmania* rRNA; the latter is known to be weak with a k_{on} of $3 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$.⁷⁸ The binding affinity of the G-site RNA was too low to be quantitatively evaluated (the binding time-constant is more than an order-of-magnitude higher than that of the A-site construct) (Figure 6).

CONCLUSIONS

We have presented an LSPR-based assay for detecting and quantifying the interaction between aminoglycoside (AMG) antibiotics and RNA, capable of distinguishing subtle changes in the functionality of the target RNA molecule. To address the issue of toxicity and development of resistance against AMG antibiotics, a PEG-thiol modified Neomycin B antibiotic was chosen as a target for screening with several RNA sequences.

AMGs are known to bind to the rRNA decoding region. This binding to the prokaryotic rRNA cannot be entirely specific inasmuch as AMGs can also cause mistranslation in eukaryotes, as is clear from the toxicity associated with clinical use of these drugs. The clinical utility of AMGs could depend to an extent on preferential binding to the prokaryotic A-site vs. the corresponding A-site of eukaryotes. The data shown here may therefore be relevant to deducing the molecular basis of side-effects observed during clinical treatment with AMG antibiotics.

Immobilization of the AMG layer on the LSPR transducer surface, rather than the RNA (as previously done), provides several advantages: (i) a stable and robust platform for label-free screening of RNA-antibiotic interactions; (ii) the possibility of convenient transducer regeneration; and (iii) a simple scheme that avoids the need to modify each tested RNA. The method is well suited for array configuration, allowing simultaneous detection of a large number of biomolecular interactions. Sizeable areas of reproducible Au island films can be prepared, on which numerous discrete measurements can be carried out concurrently in the transmission or reflection modes using CCD detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. (A) Chemical structure of the PEG-thiol modified aminoglycoside antibiotic neomycin B (AMG-SH) used in this study. (B–E) Secondary structures of the RNA molecules used in this study.

Figure 2. Schematic representation of the sensing scheme. (a) Self-assembly of a monolayer of the aminoglycoside antibiotic AMG-SH on the LSPR transducer; (b) RNA recognition; (c) RNA desorption; (d) RNA rebinding. Inset: HRSEM image (tilted projection, 70°) of a 5 nm (nominal thickness) Au island transducer.

Figure 3.

Transmission UV-vis spectra for specific recognition (A, B) and nonspecific binding (C, D) of RNA using 5 nm Au island transducers. (A, C) Full coverage of AMG-SH (12 h incubation); (B, D) partial coverage of AMG-SH (30 min incubation). Black lines: bare Au; blue lines: SAM of AMG-SH; red lines: binding of 16S RNA. Left panels: full spectra; right panels: magnification of the peak region.

Figure 4.

(A) Kinetics of adsorption of AMG-SH on a 5 nm Au island transducer, measured *in situ* by monitoring the increase of the extinction intensity at a constant wavelength of 560 nm. (B–D) Representative HRSEM images of Ag NPs bound electrostatically onto AMG-SH coated 100-nm-thick continuous Au films. (B) No AMG-SH layer; (C) AMG-SH layer adsorbed for 30 min; (D) AMG-SH layer adsorbed for 12 h. Insets: Corresponding HRSEM images of large Au islands in 15 nm (nominal thickness) Au island films. Scale bars: 100 nm.

Figure 5.

LSPR spectra (A) and changes in the peak wavelength (B) and maximum extinction intensity (C) for transducer regeneration. Sequential transmission UV-vis spectra for the binding of 16S A-site RNA to an AMG-SH SAM on a 7.5 nm Au island transducer; desorption of the RNA; and RNA rebinding. (D) Reflection-absorption FTIR (PM-IRRAS) spectra obtained with a continuous, 100 nm Au films at different stages of interface preparation and RNA binding and release. (1) Bare Au; (2) SAM of AMG-SH (30 min immersion); (3) binding of 16S RNA; (4) RNA desorption with urea; (5) rebinding of 16S RNA.

Figure 6. Representative LSPR binding curves for 5 nm Au island transducers coated with a layer of AMG-SH (30 min incubation). Kinetic data were obtained by measuring the change in the extinction intensity at a constant wavelength of 560 nm in a micro flow cell. Data were recorded by first flowing a buffer solution, then 1 μ M RNA solution. (A) bacterial 16SA-site RNA; (B) mutant bacterial site A-mod RNA; (C) G-site RNA; (D) *H. sapiens* 18S RNA.

Table 1

Estimated binding constants for the binding of different RNA molecules to AMG-SH antibiotic immobilized on a gold island transducer; data from Figure 6.

RNA	k_{on} [$M^{-1} s^{-1}$]
Bacterial 16S A-site	4.9×10^4
Homo Sapiens 18S	4.7×10^3
Mutant A-modified site	4.5×10^3