HHS Public Access

Author manuscript

Analyst. Author manuscript; available in PMC 2017 August 15.

Published in final edited form as:

Analyst. 2016 August 15; 141(17): 5120–5126. doi:10.1039/c6an00540c.

High Throughput LSPR and SERS Analysis of Aminoglycoside Antibiotics

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Abstract

Aminoglycoside antibiotics are used in the treatment of infections caused by Gram-negative bacteria, and are often dispensed only in severe cases due to their adverse side effects. Patients undergoing treatment with these antibiotics are therefore commonly subjected to therapeutic drug monitoring (TDM) to ensure a safe and effective personalised dosage. The ability to detect these antibiotics in a rapid and sensitive manner in human fluids is therefore of the utmost importance in order to provide effective monitoring of these drugs, which could potentially allow for a more widespread use of this class of antibiotics. Herein, we report on the detection of various aminoglycosides, by exploiting their ability to aggregate gold nanoparticles. The number and position of the amino groups of aminoglycoside antibiotics controlled the aggregation process. We investigated the complementary techniques of surface enhanced Raman spectroscopy (SERS) and localized surface plasmon resonance (LSPR) for dual detection of these aminoglycoside antibiotics and performed an in-depth study of the feasibility of carrying out TDM of tobramycin using a platform amenable to high throughput analysis. Herein, we also demonstrate dual detection of tobramycin using both LSPR and SERS in a single platform and within the clinically relevant concentration range needed for TDM of this particular aminoglycoside. Additionally we provide evidence that tobramycin can be detected in spiked human serum using only functionalised nanoparticles and SERS analysis.

Graphical Abstract

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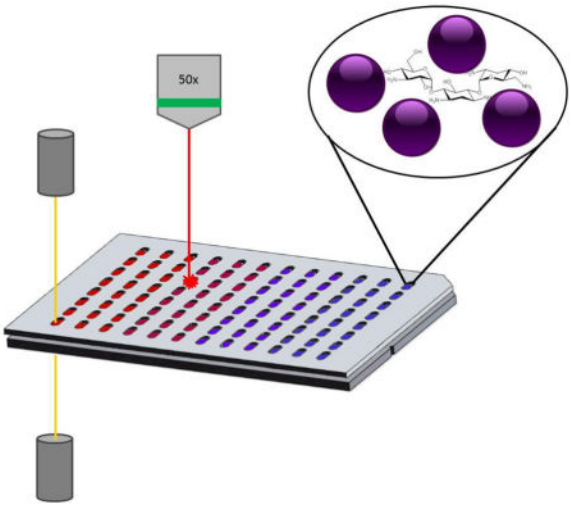

INTRODUCTION

Aminoglycosides are one of the earlier known classes of antibiotics beginning with the discovery of streptomycin in 1944, which became one of the first agents used in the treatment of tuberculosis.[1, 2] Today the aminoglycosides constitute a large class of antibiotics and include tobramycin, kanamycin, amikacin and neomycin B, and are often used in the treatment of life threatening infections resulting from Gram-negative bacilli such as *Escherichia coli*.[1, 3, 4] They share a general structure containing two or more aminosugars linked by glycosidic bonds to an aminocyclitol component,[1, 2] and their polycationic structure is able to inhibit peptide elongation at the 30S ribosomal subunit, thus disrupting protein synthesis. Although effective, there has been a decline in the use of aminoglycoside antibiotics over the years due to the occurrence of severe side effects including nephrotoxicity and ototoxicity.[1] As a result, less toxic antimicrobial agents have been favoured for widespread use, while the aminoglycosides are limited to the treatment of only the most serious infections.

In order to obtain maximum drug efficacy while minimising these side effects, therapeutic drug monitoring (TDM) can be carried out on patients undergoing therapy with aminoglycoside antibiotics to ensure correct and patient specific dosing.[5] This commonly involves analysing the concentration of these antibiotics within the blood stream at two different time intervals during treatment.[1] Since the side effects mentioned can be the result of accumulation of these antibiotics in certain organs, a trough concentration is measured before the next dose of antibiotics is administered to ensure that they have been properly metabolized and eliminated by the patient. Similarly, a peak concentration is measured between 30 minutes and 1 hour after the antibiotics have been administered to ensure that the concentration in the blood stream reaches that required to treat the infection, since these antibiotics have a concentration-dependent therapeutic response.[1] The use of TDM during treatment with aminoglycoside antibiotics not only has health benefits to the patient, it has also been shown to reduce hospital stay and overall costs of treatment.[5, 6]

Currently the most common methods used for the analysis of aminoglycoside antibiotics include liquid and gas chromatographic techniques, alongside mass spectrometry,[1, 7, 8] all of which require expensive instrumentation limiting their use to a centralized laboratory. Therefore, there is a need to develop simple and rapid methods of analysing aminoglycoside antibiotics in order to provide novel methods for TDM, which could essentially enable the more widespread use of these extremely effective antibiotics. Given their low resistance rates in comparison to other classes of antibiotics,[7, 9] the clinical importance of aminoglycosides is being reassessed and the use of novel assays and instrumentation is one way in which these antibiotics could be widely reintroduced into the healthcare system while maintaining patient safety.

To date, a vast amount of the literature pertaining to the development of novel methods of analyzing aminoglycosides focuses on the detection of these antibiotics in food and environmental samples.[7, 8, 10] Treatment of farm animals with antibiotics is a practice that has been used extensively in the food industry for many decades,[8] and is now known to be one of the contributing factors towards antibiotic resistance among humans, which is becoming a significant problem in the healthcare field.[11] Therefore, in order to minimise the intake of antibiotics it is essential to analyse certain products before they leave the farm, and this needs to be done using simple and effective instrumentation that can be carried out on site and in complex matrices. Thus, the results obtained within this research area can easily be used to help progress the detection of aminoglycoside antibiotics within the healthcare field, in particular due to the commonality of the required analysis in a complex matrix, be it milk or human serum.

One of the simplest methods of confirming the presence of an analyte is *via* colorimetric detection. Metallic nanoparticles, with their unique optical properties, have been used in many such applications.[12] It is well known that these nanoparticles exhibit a characteristic colour change upon aggregation, which can be induced in a number of different ways, and is often visible to the naked eye.[12, 13] Very recently there have been a number of publications that take advantage of this phenomenon, and exploit the localized surface plasmon resonance (LSPR) of metallic nanoparticles for the colorimetric detection of aminoglycoside antibiotics.[14–18] For example, Caglayan and Onur created an extremely simple colorimetric method of detection using silver nanoparticles.[15] By exploiting the interaction between the negatively-charged nanoparticles and the cationic aminoglycoside antibiotics, they were able to visually detect a colour change from yellow to red in the presence of gentamicin, tobramycin and amikacin. Due to the interaction between the amino groups on the antibiotics and the surface of the nanoparticle, a simple and rapid method of detection was realized that was applied to milk and pharmaceutical preparations. Additionally, Zhang *et al.* were able to utilize the charge influence caused by aminoglycosides to alter a synthetic protocol for gold nanoparticles, resulting in a red to blue colour change in the presence of four different antibiotics.[19] Ban and co-workers alternatively used a DNA aptamer for kanamycin and by absorbing this short strand of DNA onto the surface of gold nanoparticles they were able to stabilize them against salt-induced aggregation.[14] This stability was existent only in the absence of kanamycin and, upon the addition of this antibiotic, the aptamer was removed from the surface of the nanoparticle, binding to the aminoglycoside, and thus causing a red to blue colour change when salt was added to the nanoparticles.

Although colorimetric detection is an extremely simple method of detection, it can often lack the sensitivity and specificity required for analysis in complex matrices within the required concentration range. A spectroscopic technique compatible with colorimetric detection using metallic nanoparticles is surface enhanced Raman spectroscopy (SERS), which provides a vibrational fingerprint spectrum of a molecule present within the enhanced electromagnetic field between aggregated nanoparticles. Surprisingly, however, there are very few examples in the literature of SERS being used in the analysis of aminoglycoside antibiotics, although other classes of antibiotics have been reported.[20–24] In a recent example, Zengin *et al.* developed a sandwich assay for the detection of kanamycin using SERS analysis alongside magnetic nanoparticles as separating agents to enable antibiotic detection in spiked milk samples with an analysis time of less than 10 minutes.[25] This example, however, is once again designed for use in the food industry, and it appears there are no current reports on the use of SERS of aminoglycoside antibiotics for use in therapeutic drug monitoring. Although other optical spectroscopies have been employed for the analysis of these antibiotics in human fluids,[26–29] there are very few publications progressing SERS towards this purpose.

The work described herein exploits the previously reported ability of aminoglycoside antibiotics to aggregate metallic nanoparticles *via* surface charge interactions, and utilizes a 96-well plate setup to provide high throughput analysis at concentrations within the clinical range required for TDM. Additionally, we have shown that SERS can be employed for the detection of aminoglycoside antibiotics over the required concentration range and within the same platform as LSPR analysis providing a novel dual detection method for potential use in therapeutic drug monitoring. We report on the analysis of various aminoglycoside antibiotics before focussing on tobramycin to determine a linear concentration, and provide evidence that TDM can be performed for this antibiotic in human serum.

EXPERIMENTAL

Gold Nanoparticle Synthesis and Functionalization

Gold nanoparticles were synthesized according to a standard citrate reduction method. All glassware was cleaned with Aqua Regia before use and 50 mg $HAuCl_4$ (Sigma-Aldrich) in 10 mL distilled and deionised water (d.H_2O) was added to 500 mL d.H_2O and heated until boiling. 75 mg of sodium citrate (Fisher Scientific) in 10 mL d.H_2O was added and the solution was boiled for another 15 minutes before cooling to room temperature. Constant stirring was maintained throughout. Size and concentration of nanoparticles were calculated from the extinction spectrum to be 20 nm and 0.2 nM. For the conjugation of a Raman reporter molecule, 10 μL of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma-Aldrich) was added to 1 mL of 0.2 nM gold nanoparticles overnight with constant shaking on an orbital shaker. Nanoparticles were then cleaned using centrifugation and re-suspended in 50 mM Tris buffer at pH 8.0.

Extinction Spectroscopy of Various Antibiotics

A final concentration of 100 nM of amikacin, kanamycin, tobramycin (AK Scientific) neomycin B (TCI America) and ampicillin (Bio Basic Canada) was prepared in 50 mM Tris

buffer at pH 8.0. 20 nm Au-DTNB nanoparticles were added in a 1:1 ratio to a volume of 1 mL in a plastic cuvette. The extinction spectrum was analysed between 200 nm and 800 nm using a Cary UV Spectrophotometer every minute for a total of 20 minutes. A control sample was analysed by adding Tris buffer to the conjugated nanoparticles, and the spectrum was compared to the final spectra taken for the antibiotic samples.

SERS of Antibiotics

A final concentration of 50 nM of amikacin, kanamycin, neomycin B, tobramycin and ampicillin (along with a control containing no antibiotics) were prepared in 50 mM Tris buffer at pH 8.0. 20 nm Au-DTNB nanoparticles were added in a 1:1 ratio to a final volume of 100 μL in a custom built 96-well plate.[30] Each antibiotic was prepared in triplicate and analyzed using a 633 nm laser wavelength at 100% power and 50× long working range objective on a Renishaw InVia Raman system. The analysis consisted of 10 accumulations at 1 second, and each replicate well was scanned 5 times. The average spectra were calculated for each antibiotic. This same analysis procedure was used to carry out a limit of detection study for tobramycin with varying concentrations of the antibiotic.

LSPR Automated Analysis of 96-Well Plate

The design and use of the custom built 96-well plate and plate reader for high throughput plasmonic analysis, along with the data processing involved, has been extensively discussed in a previous publication.[30] The main difference with the setup described here is the use of plain glass coverslips as opposed to nanohole array surfaces, to create a simple multi-well setup for solution based sensing. Varying concentrations of tobramycin was added to 20 nm Au-DTNB in a 1:1 ratio to a final volume of 100 μL. 3 replicate wells were prepared for each concentration along with a control containing no antibiotics. After addition of the samples, a Plexiglas cover was placed atop the wells, and analysis carried out using the automated plate reader according to previously described protocols, with each well scanned 3 times. All results are displayed by taking the ratio of the absorbance at 700 nm and 550 nm and are the average of the replicates described.

Analysis of Tobramycin in Human Serum

Tobramycin was added to crude human serum (Sigma-Aldrich, Canada) at a final concentration of 4 μM and 20 μM within the typical clinical range and then diluted by a factor of 1000 with Tris buffer at pH 8.0 to fit the calibration range. Samples were combined with 20 nm Au-DTNB in a 1:1 ratio in a final volume of 100 μL within the 96-well plate. A control sample consisted of human serum with no antibiotics treated to the same dilution and analysis procedure. 3 replicate wells for each sample were prepared and analysed by SERS and LSPR.

RESULTS AND DISCUSSION

Aminoglycoside antibiotics, by their very mechanism of action, are required to be cationic in nature. Consisting of two or more aminosugars (Figure 1) these polycationic molecules can integrate into the 30S ribosomal unit of bacterial DNA, disrupting protein synthesis.

Although effective, these antibiotics can have extreme side effects and thus it is essential to find novel pathways to detect these molecules in a simple and rapid manner to ensure patient safety during treatment. Of the many novel methods of analysis proposed for these antibiotics in recent years, the simplest by far is that of colorimetric detection using metallic nanoparticles.[14–16, 18] This method exploits the polycationic nature of these antibiotics, alongside negatively-charged nanoparticles, commonly citrate-capped silver or gold. The complementary charges between aminoglycoside antibiotics and negatively-charged metallic nanoparticles will cause them to aggregate in solution, and this can be detected *via* a characteristic shift of the LSPR band of these nanoparticles. In the case of gold nanoparticles, this can be monitored visually *via* a very striking colour change from red to blue as the antibiotics bring the nanoparticles into close contact, allowing their plasmons to combine and causing a bathochromic shift in wavelength (Figure 2A). Although all of the antibiotics that come under the class of aminoglycosides carry a positive charge, interestingly, they do not all aggregate gold nanoparticles to the same extent. The ability of four different aminoglycosides to aggregate 20 nm gold nanoparticles was investigated and, as can be seen in Figure 2A, only neomycin B and tobramycin have noticeably caused aggregation at the chosen concentration. Kanamycin causes a very slight broadening of the plasmon band, while amikacin is indistinguishable from the control sample without antibiotics. Ampicillin, a beta-lactam antibiotic (Figure S1) was also tested as a way of confirming the specificity of analyzing aminoglycosides *via* nanoparticle aggregation and ampicillin caused no aggregation of the nanoparticles.

This experiment was carried out using a standard UV-Vis spectrophotometer, which has inherent drawbacks. For example, a large sample volume is needed, and instrumentation can often only analyze a maximum of six samples sequentially, at a relatively slow speed. Previously, our group reported on a method of high throughout plasmonic analysis using a custom built 96-well plate and plate reader.[30] Although, this was primarily designed to analyze gold nanohole arrays at optimum incident angles, it also provided a method of rapidly analysing the transmission spectra of gold nanoparticles in solution within a 96-well plate format. The custom multi-well plate reader can provide full visible spectra of a 96-well plate in a few minutes, compared to nearly 1 hour with commercial plate readers.

This multi-well plate was first used to carry out SERS analysis on the antibiotics initially analysed by extinction spectroscopy, allowing a sample size of 100 μL to be used, as well as the analysis of replicate samples in a timely manner. SERS serves as a complementary method of analysis to LSPR as upon aggregation, the plasmon band of the gold nanoparticles is centred at around 680 nm and therefore the use of a 633 nm laser wavelength will allow for enhanced Raman scattering. Unfortunately, scattering of the aminoglycoside antibiotics does not provide a distinct SERS spectrum (Figure S2), and perhaps this is the reason why there have not been many previous investigations into their analysis using SERS. However, by conjugating a common Raman reporter to the surface of the gold nanoparticles, in this case DTNB, an intense SERS spectrum was achieved, with the primary band at 1337 cm^{-1} representing the nitro groups on this Raman active molecule. The intensity of the SERS spectrum achieved was directly related to the extent of aggregation provided by the aminoglycoside antibiotics (Figure 2B). DNTB not only allowed a SERS spectrum to be elucidated upon interaction of the antibiotics with the

nanoparticles, it also helped to stabilise the nanoparticles in solution by modifying their surface using a simple thiol-gold bond interaction. With a pKa of 2.16, 2-nitobenzoic acid will have an overall negative charge under the assay conditions, thus electrostatically stabilising the nanoparticles against unwanted aggregation, while simultaneously providing an interaction site for the positive aminoglycoside antibiotics.

Similar to the results from extinction spectroscopy, both tobramycin and neomycin B provided the most enhanced SERS signal, which was to be expected given the absorbance of these nanoparticles at 680 nm. Interestingly, this difference in their ability to aggregate gold nanoparticles is primarily dependent on the structure of the antibiotic, and not solely on the number of charged amine groups present. Given that the interaction between the nanoparticles and antibiotic is based on charge, it seems reasonable to expect that antibiotics with a higher number of protonated amino groups will aggregate the nanoparticles to a greater extent, and indeed this appears to explain the poor results seen for kanamycin in both the extinction spectrum and SERS analysis. At our working pH of 8.0, this aminoglycoside will have the lowest number of charged amine groups (position 6′, Figure 1) compared to tobramycin (positions 2′ and 6′, Figure 1), amikacin (positions 3″′, 4″′ and 6′, Figure 1) and neomycin B (positions 1, 6′ and 6″′, Figure 1). However, given the vastly superior reactivity of tobramycin and neomycin, in comparison to not only kanamycin, but amikacin, it is unlikely that the number of charged amine groups is the only explanation with regards to the mechanism of aggregation, and since these functional groups are responsible for bridging between two nanoparticles, it is likely their position is equally important. While the aminoglycosides all have a similar general structure, the number and position of the amino groups differ between individual antibiotics (Figure 1). Taking the general structure into account, with tobramycin, there are three amino groups at positions 1, 3, and 6′ on one side of the molecule and two on the other side at positions 2′ and 3″ (Figure 1), providing a number of potential interaction sites between the nanoparticles and antibiotic on both sides of the molecule, essential when the very nature of aggregation requires a minimum of two nanoparticles to be brought into close contact. Kanamycin, by comparison, has three amino groups at positions 1, 3, and 6′ on one side of the molecule, and only one amino group at position 3″ on the other (Figure 1), which will likely be insufficient to bind another nanoparticle, in particular if this is not protonated. Neomycin B, like tobramycin, is very effective at aggregating the nanoparticles given the high number of amino groups and equal distribution on both sides of the molecule (amino groups at positions 1, 3, and 6′ on one side and at positions 2′, 2″ and 6″ on the other side, Figure 1). Although there is no difference in the extinction spectra between tobramycin and neomycin B, the SERS signal is greater for tobramycin. It is likely that this is a result of the larger size of neomycin B compared to tobramycin, which will create a larger space between the aggregated nanoparticles, enhancing the signal to a lesser extent. Finally, ampicillin, a beta-lactam antibiotic, has only one amino group (Figure S1) with a negatively-charged acid under the experimental conditions and, as such, does not aggregate the nanoparticles to any extent. Thus, it is clear that although these aminoglycoside antibiotics appear very similar, a number of slight changes in structure can lead to very different results, and consideration of both the working pH and the structure of the aminoglycoside in question is essential when carrying out detection of these antibiotics using nanoscale materials and surface spectroscopies.

Although both tobramycin and neomycin B provided visible aggregation and an intense SERS spectrum, given the prevalence of tobramycin in the healthcare industry, and the availability of a current clinical concentration range for use in therapeutic drug monitoring, this aminoglycoside antibiotic was chosen to be investigated further. Tobramycin, like many of the aminoglycosides, is often used in the treatment of life threatening bacterial infections, and not being able to pass through the gastrointestinal tract is often administered intravenously or intramuscularly. It is a prime candidate for therapeutic drug monitoring given its concentration-dependent therapy and toxic side effects. Although many factors can affect the ideal concentration range, including age and renal function, it is recommended that trough plasma concentrations, usually acquired before the next dosage, do not exceed 2 μg/mL, and peak plasma concentrations, usually acquired around 90 minutes after injection, do not exceed roughly 12 μg/mL (4 μM and 26 μM, respectively).[1] A limit of detection study for tobramycin in buffer was carried out using both transmission measurements on the automated plate reader and using SERS to determine the linear concentration range using these techniques.

Figure 3 depicts the results obtained from LSPR measurements of DTNB coated nanoparticles after the addition of varying concentrations of tobramycin. As this was carried out using the 96-well plate and customized plate reader, analysis of all concentrations and replicates could be carried out using a relatively small sample volume within 30 minutes, including sample preparation and analysis. As can be seen in Figure 3A, aggregation manifests itself as an increase in the absorbance at 700 nm relative to the peak at 550 nm, and the ratio of these two absorbance wavelengths was taken to provide a linear concentration plot between 5 and 75 nM final concentration of tobramycin. Above this concentration, the nanoparticles were all fully aggregated given the fixed concentration of nanoparticles used, and indeed at the higher end of the linear range we begin to see an increase in the error due to the lack of sufficient absorbance at 550 nm. Therefore, once concentrations of aminoglycosides are out with the linear range it would be impossible to predict an exact concentration. However, it is worth noting that high concentrations will provide an On/Off method of detection for aminoglycoside antibiotics that can be detected by the naked eye.

The linear concentration range obtained *via* LSPR was then used to prepare samples for analysis using SERS (Figure 4). This study using SERS analysis also resulted in a linear range based on the peak height of the DTNB molecules on the surface of the nanoparticles, confirming the complementary nature of both LSPR and SERS for use in this type of analysis. The sensitivity of these techniques, however, means the usable concentration range is almost 3 orders of magnitude below the desired concentration range for use in therapeutic drug monitoring. However, far from a problem, this allows the samples to be diluted before analysis, and thus could be analysed in more complex matrices which would commonly interfere with extinction measurements in particular.

As a proof of concept, human serum was spiked with tobramycin and diluted 1000 times taking the final concentration from the micromolar range to the nanomolar range which could then be detected by using both SERS (Figure 5) and LSPR (Table S1). The

concentrations chosen for analysis represent peak and trough concentrations commonly analysed in therapeutic drug monitoring of tobramycin, at 20 μM and 4 μM, respectively.

Analysis in opaque samples such as human serum is a common problem with colorimetric detection, and additionally the use of nanoparticles in physiological fluids can cause inaccurate measurements as a result of the protein corona.[31, 32] In the case of the assay described here, the formation of a layer of proteins onto the nanoparticle surface from a biological fluid could potentially stabilize these nanoparticles against aminoglycoside-induced aggregation, and indeed the addition of 20 μM tobramycin in crude human serum to 20 nm DTNB-Au resulted in no visible aggregation in the extinction spectrum (Figure S3). Although SERS is a technique amenable to analysis in crude biological fluids, dilution of the sample would be required in the analysis of aminoglycoside antibiotics due to the low sensitivity obtained, and this has the added benefit of reducing the effects of a potential protein corona which could skew the results. As can be seen in Figure 5, we can clearly detect the presence of tobramycin in diluted human serum at starting concentrations useful for therapeutic drug monitoring.

The results obtained from LSPR analysis of tobramycin in serum show discrimination over the background even at the lower concentration analysed, however, this does not appear to be concentration dependent, as both 4 μM and 20 μM gave similar results (Table S1). Alternatively, SERS provides an accurate analysis of tobramycin in human serum and provides a concentration dependent response for clinically relevant concentrations. Although the error bars are relatively large for the lower concentration analysed by SERS (Table S1), this data still serves to highlight that SERS has the potential to be used in the analysis of aminoglycoside antibiotics within human serum for therapeutic drug monitoring.

CONCLUSIONS

Herein, we have shown that both LSPR and SERS can be used in combination for the analysis of various aminoglycoside antibiotics. Additionally, we have shown that both techniques can cover a concentration range which would be amenable to therapeutic drug monitoring of tobramycin at 3 orders of magnitude lower sensitivity than required, allowing for analysis in diluted human serum. We report on an extremely simple method of analysing these antibiotics, which can be carried out in a matter of minutes with high throughput capabilities. Along with the easy to use and relatively compact instrumentation already provided for LSPR analysis, and the ever increasing miniaturisation and availability of hand-held Raman spectrometers, this could provide a simple and effective method of dual detection which would be of great benefit to therapeutic drug monitoring of aminoglycoside antibiotics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank financial support from the National Science and Engineering Research Council (NSERC) of Canada Grant 412305-2011 (J.-F.M.) and the National Institutes of Health (NIH) Grant AI090048 (S.G.-T.).

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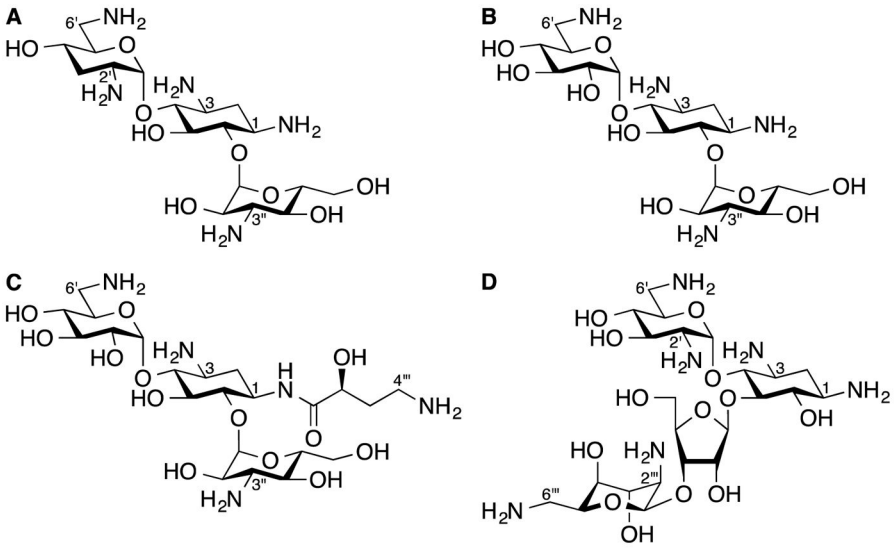

Figure 1.

The structure of various aminoglycoside antibiotics. (A) Tobramycin, (B) kanamycin, (C) amikacin, and (D) neomycin B. The positions of the different amino groups are labeled on the chemical structures.

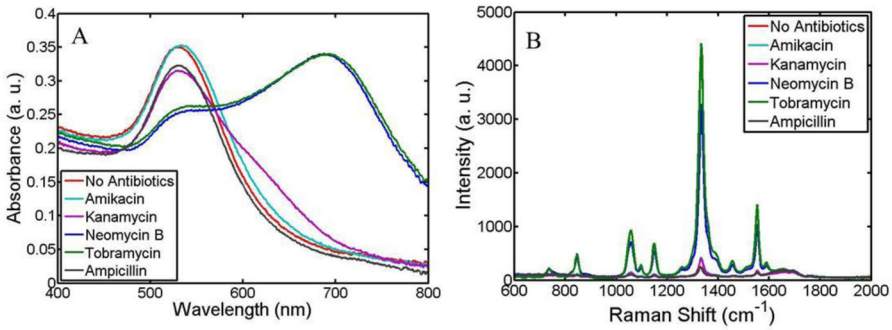

Figure 2.

(A) Extinction spectra of 20 nm DTNB coated nanoparticles after the addition of 100 nM of various aminoglycoside antibiotics. Two controls were included in the analysis, one with no antibiotics added, and one with a beta-lactam antibiotic added at 100 nM. (B) SERS spectra taken after the addition of 50 nM of various aminoglycosides to 20 nm DTNB coated nanoparticles along with the two control samples. A 633 nm laser wavelength was used for the analysis of 100 μL of each sample in a custom built 96-well plate. Spectra are an average of 3 replicate wells and 5 scans of each well.

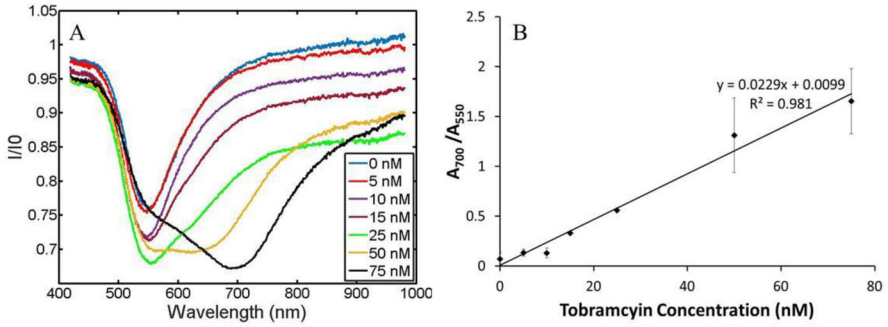

Figure 3.

Tobramycin limit of detection study analyzed using 96-well plate transmission measurements. Varying concentrations of tobramycin in buffer were added to 20 nm Au-DTNB at a 1:1 ratio in a final volume of 100 μL inside the 96-well plate. (A) The average transmission spectra obtained for each concentration and (B) the linear range observed. The standard deviation represents 3 replicate wells and 5 scans of each well.

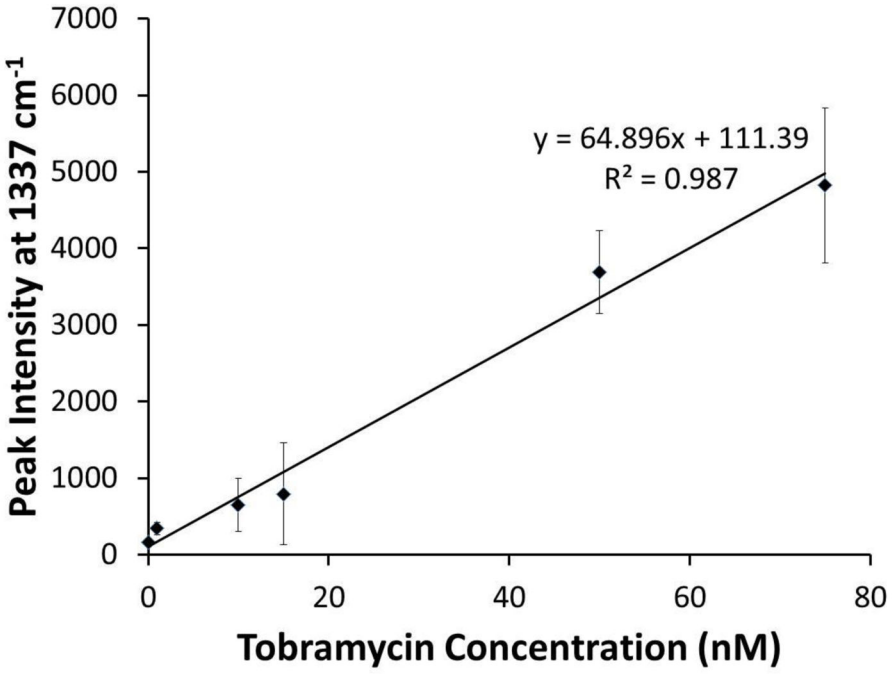

Figure 4.

Tobramycin limit of detection study analyzed using SERS. Varying concentrations of tobramycin in buffer were added to 20 nm Au-DTNB at a 1:1 ratio in a final volume of 100 μL inside the 96-well plate. The standard deviation represents 3 replicate wells and 5 scans of each well

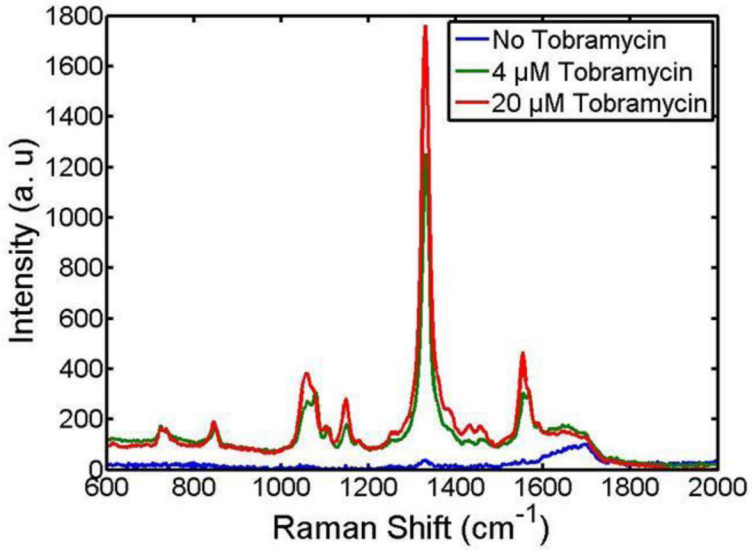

Figure 5.

SERS spectra of varying concentrations of tobramycin in diluted human serum corresponding to 4 μM and 20 μM, the extreme limits of the clinical range for which TDM is needed. Human serum was spiked with the chosen concentrations or no tobramycin, before being diluted by a factor of 1000 and added to 20 nm Au-DTNB. 3 replicate sample wells were analysed 5 times using a 633 nm laser wavelength at 100% with a 1 second accumulation time and 10 accumulations. Spectra are an average of all scans recorded.