Investigation of Aminoglycoside Induced Nanoparticle Self-Assemblies

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INVESTIGATION OF AMINOGLYCOSIDE INDUCED NANOPARTICLE SELF-ASSEMBLIES

By

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A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Biomedical Sciences in the College of Medicine and in the Burnett Honors College at the University of Central Florida Orlando, Florida

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ABSTRACT

Aminoglycosides are a group of broad-spectrum antibiotics that, under neutral pH conditions, carry a positive charge. The net cationic charge arises from the high number of amino groups in the core structure of aminoglycosides. Previous studies performed have shown that negatively charged citrate ligand-capped gold nanoparticles (AuNPs) can interact with various biomolecules such as aminoglycosides. AuNPs bound to biomolecules have been used in conjugation with various assaying techniques to detect and study compounds in vitro and in vivo. AuNPs also have strong light scattering properties that can be used with a wide variety of imaging and assaying techniques.

Our laboratory has previously performed experiments on the aminoglycoside antibiotic ribostamycin sulfate. During this experiment, the concentration dependent rod-like assembly of ribostamycin sulfate was characterized. This experiment used three analytical techniques in conjunction with AuNPs: (1) dynamic light scattering (DLS), (2) UV-Vis absorption spectroscopy, and (3) dark field optical microscope imaging (DFM). This suite of techniques was used to analyze mixtures of ribostamycin sulfate at different concentration with different sized AuNPs.

The primary objective of this research was to determine if the techniques used to characterize the self-assembly of ribostamycin sulfate could be generalized and applied to other aminoglycoside antibiotics. The secondary objective of this research was to determine if other aminoglycoside antibiotics formed rod-like assemblies.
This study demonstrated that AuNPs can be used to detect self-assembled oligomers for different aminoglycoside antibiotics. In addition, this study also revealed that not all aminoglycoside antibiotics will self assemble into rod-like oligomers similar to ribostamycin. It was observed that the aminoglycoside antibiotic amikacin self assembled into rod-like aggregates similar to ribostamycin sulfate but the aminoglycoside antibiotics neomycin sulfate and streptomycin sulfate did not.
ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS

AuNP: Gold Nanoparticle

AAC: Aminoglycoside acetyltransferase

ANT: Aminoglycoside nucleotidyltransferase

APH: Aminoglycoside phosphotransferase

BCL-2: B-cell lymphoma-2

DFM: Dark Field Optical Microscope Imaging

DOS: Deoxystreptamine

DLS: Dynamic Light Scattering

EPR: Enhanced permeability and retention effect

ETC: Electron transport chain

EDPI: Energy dependent phase I

EDPII: Energy dependent phase II

JNK: c-jun N-terminal kinases

LSPR: Localized surface plasmon resonance

Ribo: Ribostamycin sulfate

ROS: Reactive oxygen species
SPR: Surface plasmon resonance

$\Delta \Psi$: Membrane potential
INTRODUCTION

Aminoglycosides are a group of antibiotics which are primarily used to treat Gram-negative bacterial infections. For example, aminoglycosides are commonly used to treat a wide range of bacterial respiratory infections such as Mycobacterium tuberculosis. Aminoglycosides act by irreversibly binding the 16S subunit of the bacterial ribosome to inhibit protein translation and by creating breaks within the outer membrane of the target bacteria to cause the membrane to lyse.\(^1\) Despite the reversible nephrotoxic and permanent ototoxic side effects of aminoglycosides, aminoglycosides have maintained widespread clinical use to this day.\(^2\)\(^-\)\(^3\) However, an increasing number of bacterial strains have developed resistance to aminoglycoside treatment such as methicillin resistant Staphylococcus aureus.\(^4\) Development of aminoglycosides that can overcome these resistant strains requires an understanding of the structure of aminoglycosides and the mechanism of action to inhibit protein synthesis.

AuNPs have a wide range of applications for biomedical research and therapeutics such as compound analysis and drug delivery.\(^5\) The wide range of applications for AuNPs arises from their strong light scattering properties that can be measured by different techniques.\(^6\) For example, citrate ligand-capped AuNPs have been used to analyze aggregation of proteins with dynamic light scattering assay, UV-Vis absorption spectroscopy and dark field optical microscope imaging.\(^7\)
Our laboratory has previously characterized the concentration of dependent rod-like assembly of ribostamycin sulfate using AuNPs. The characterization was observed by analyzing ribostamycin-AuNP mixtures with DLS, UV-Vis absorption spectroscopy, and DFM. Three antibiotic agents were analyzed in this study: amikacin, streptomycin sulfate, and neomycin sulfate. Ribostamycin sulfate was analyzed in an additional experiment. Amikacin is a semi-synthetic aminoglycoside derived from kanamycin and has a molecular weight of 585.603 g/mol. Streptomycin sulfate is an aminoglycoside sulfate salt that forms electrostatic interactions with 1.5 sulfate ions per streptomycin and has a molecular weight of 728.69 g/mol. Neomycin sulfate is an aminoglycoside sulfate salt that forms electrostatic interactions with three sulfate ions per neomycin and has a molecular weight of 908.866 g/mol. Ribostamycin sulfate is an aminoglycoside sulfate salt that forms electrostatic interactions with one sulfate ion and has a molecular weight of 454.5 g/mol. All four aminoglycosides at neutral pH have a positive charge which allows them to form a sulfate salt.
BACKGROUND

Aminoglycoside Antibiotics Overview

In 1943, streptomycin was isolated from *Streptomyces griseus*. This was the first aminoglycoside that had been isolated. Streptomycin was the first antibiotic to successfully treat *Mycobacterium tuberculosis*, and it remains a common treatment for bacterial respiratory infections.9 Other naturally occurring aminoglycosides have been isolated and include: (A) neomycin was isolated from *Streptomyces fradiae* in 1949; (B) kanamycin was isolated from *Streptomyces kanamyceticus* in 1957; (C) gentamicin was isolated from *Micromonospora purpurea* in 1963; and (D) tobramycin was isolated from *Streptomyces tenebrarius* in 1967.10-13 To overcome bacterial resistance, previously discovered aminoglycosides have been modified to yield semi-synthetic antibiotics such as amikacin, a kanamycin derivative.14

The structure of aminoglycosides contains aminated sugars that form glycosidic bonds with a dibasic cyclitol. The core structure of an aminoglycoside is a neamine moiety which contains Ring I that is connected to the fourth position of Ring II, the dibasic cyclitol. Other sugars are attached to the core structure at position 5 or position 6 of Ring II. Common dibasic cyclitols include streptidine, the dibasic cyclitol in streptomycin, and 2-deoxystreptamine (2-DOS), the dibasic cyclitol for most aminoglycosides (Figure 1).

The two most common sub-classes of aminoglycosides are 4 and 5 disubstituted 2-DOS (4,5-2-DOS), and 4 and 6 disubstituted 2-DOS (4,6-2-DOS).15 An example of a 4,5-2-DOS aminoglycoside is neomycin. Examples of 4,6-2-DOS aminoglycosides are amikacin and
tobramycin. In 2-DOS aminoglycosides, the 2-DOS structure in combination with Ring I is necessary to bind to the A site of the 16S rRNA subunit.

Figure 1. Chemical structure of the aminoglycosides analyzed in this study.

Aminoglycoside Uptake and Mechanism of Action

Aminoglycosides are most effective in treating common strains of Gram-negative bacilli and can also be used to treat certain gram-positive bacteria. Gram-negative bacilli that are treated with aminoglycosides include *Mycobacterium Tuberculosis* and *Pseudomonas aeruginosa*.¹

Aminoglycoside uptake occurs in three phases: ionic binding, energy-dependent phase I (EDPI), and energy-dependent phase II (EDPII).¹⁸
The ionic binding phase is unique as it is the only energy-independent phase of aminoglycoside uptake. Aminoglycosides bind to anionic structures or sites on the surface of the bacteria through reversible and concentration-dependent electrostatic interactions. Aminoglycosides contain multiple positively charged amino groups on different rings allowing aminoglycosides to electrostatically bind negatively charged structures located on the bacterial envelope. Binding targets include lipopolysaccharides, phospholipids heads, teichoic acids, and negatively charged membrane proteins. After binding, the aminoglycoside is transferred into the cell in a process referred to as “self-promoted uptake.” During this process, Mg\(^{2+}\) bridges are disrupted when aminoglycosides bind to Mg\(^{2+}\) binding sites on the bacterial cell membrane. Aminoglycoside are able to enter the bacteria through these breaks in the membrane.

The next phase of aminoglycoside uptake is EDPI. This phase is initiated after ionic binding is completed. EDPI is the slow phase of aminoglycoside uptake and requires a functioning electron transport chain (ETC) for the uptake of aminoglycosides. This phase of aminoglycoside uptake requires a membrane potential (\(\Delta \Psi\)). It is currently unknown whether the \(\Delta \Psi\) is generated directly by the ETC. It has been demonstrated in antibiotic resistant bacteria that inducing a proton motive force will facilitate aminoglycoside uptake. For \(E. coli\), another factor that affects aminoglycoside uptake during EDPI is the growth rate of the \(E. coli\) culture. A higher initial growth rate of \(E. coli\) has been shown to increase aminoglycoside effectiveness. A higher initial growth rate will result in two main events. First, increased growth rates will lead to higher ribosomal activity rates. Second, a higher initial growth rate will cause an increase in aminoglycoside uptake with no change in aminoglycoside efflux. Both events contribute to higher aminoglycoside accumulation within the bacteria. This results in a
higher number of aminoglycoside compounds that can interact with a higher number of ribosomes.\textsuperscript{22}

The final phase of aminoglycoside uptake is EDPII. EDPII is the fast phase in aminoglycoside uptake. During EDPII, aminoglycosides undergo linear transport across the bacterial envelope. This process requires energy derived from the ETC, protein synthesis, and ATP hydrolysis.\textsuperscript{23} Even though aminoglycosides ultimately inhibit protein synthesis, this final phase requires aminoglycoside-sensitive ribosomes that are actively synthesizing proteins. If active aminoglycoside-sensitive ribosomes are not present, EDPII will not occur.\textsuperscript{24}

After aminoglycosides enter the bacteria, cell death is caused by the inhibition of protein synthesis. Aminoglycosides accomplish this by targeting the ribosomes and by compromising the integrity of the cytoplasmic membrane. After entering the bacteria, aminoglycosides bind to the 16S rRNA subunit. The aminoglycoside can bind to the A site of the polysome with differing conformations based on Ring II (dibasic cyclitol) interactions. Aminoglycoside binding requires high affinity bonds with adenine 1492 (A1492) and guanine 1494 (G1494) of the 16S rRNA subunit.\textsuperscript{25} Aminoglycosides then form Watson-Crick base pairs with the highly conserved adenine 1408 (A1408) and adenine 1493 (A1493). The Watson-Crick base pairing creates a pocket in the polysome. The pocket causes the polysome to misread the mRNA template and ultimately causes the termination of protein synthesis.\textsuperscript{26}

Another possible pathway for aminoglycoside-induced protein synthesis inhibition may be the stabilization of mRNA and tRNA in the A site. The tRNA will then be unable to move to the P site and no other amino acids can be added to the polypeptide chain. This is caused by changing codon-anticodon interactions.\textsuperscript{27} Although aminoglycosides primarily bind to the A
site, aminoglycosides have been observed to bind to other parts of the polysome. For example, it has been observed that neomycin and kanamycin form dimers which will bind to the ribosome in two spots with high affinity.\textsuperscript{28}

The bactericidal properties of aminoglycosides also are derived from the ability of aminoglycosides to cause breaks in a bacteria’s cytoplasmic membrane. This occurs during the ionic binding phase of aminoglycoside uptake. The “self-promoted uptake” increases membrane permeability in a fashion similar to cationic detergents. The increased membrane permeability ultimately allows more aminoglycosides to enter the cell. Compromising the membrane allows free flow of smaller molecules which can disrupt the ion balance and membrane potential. This results in the disruption of the bacterial cell’s metabolic processes.\textsuperscript{29}

**Aminoglycoside Toxicity**

Aminoglycosides exhibit ototoxic and nephrotoxic side effects. Aminoglycoside-induced damage to the inner ear is irreversible, but aminoglycoside-induced renal damage may be reversed over time. The type and extent of damage depends on the aminoglycoside used. Neomycin is one of the most toxic common aminoglycosides that can be administered to a patient. Systematic administration of neomycin can potentially lead to permanent hearing loss.\textsuperscript{1,30}

Nephrotoxicity is caused by aminoglycoside accumulation within the kidney’s epithelial cells. This can result in impaired reabsorption of fluid, protein and solutes which may ultimately result in nonoliguric renal failure. Upon administration, aminoglycosides will bind to the brush border through electrostatic interactions with negatively charged phosphatidylserine.
Next, megalin facilitates aminoglycoside uptake into the epithelial cells. Inside the epithelial cell, aminoglycoside compounds start to localize in the Golgi apparatus, endosomes, and lysosomes of epithelial cells in the proximal tubules.\textsuperscript{3} It has been observed that localization of aminoglycosides within lysosomes causes an increase in the volume of lysosomes within hours of aminoglycoside administration. Aminoglycosides inhibit enzymes such as lysosomal phospholipases and inhibit aggregation of phospholipids through electrostatic interactions. These two factors are believed to be associated with necrosis of epithelial cells.\textsuperscript{31} The actual mechanism for necrosis is unknown. It is believed that aminoglycoside-induced necrosis is a major contributor to nephrotoxicity. In most cases, however, renal damage is reversible and patients are able to recover.\textsuperscript{32}

Ototoxicity is caused by aminoglycoside-induced damage to either the auditory or vestibular organs. Aminoglycosides are categorized based on which part of the inner ear they primarily affect. Streptomycin is mostly vestibulotoxic with some selective cochleotoxic effects. Neomycin, kanamycin, and amikacin are mostly cochleotoxic.\textsuperscript{2} Aminoglycoside uptake into hair cells is most likely mediated by ion channels.\textsuperscript{33} After uptake, aminoglycosides induce hair cells to undergo apoptosis through the intrinsic apoptotic pathway. The entire pathway is unclear but it has been observed that aminoglycosides increase the synthesis of reactive oxygen species (ROS) which activate c-jun N-terminal kinases (JNK). JNK promotes pro-apoptotic B-cell lymphoma-2 (BCL-2) proteins such as Bax and Bak. BCL-2 proteins will translocate into the mitochondria and permeabilize the mitochondria resulting in the release of cytochrome c. Cytochrome c initiates the caspase cascade that results in the death of the hair cell.\textsuperscript{2}
Bacterial Resistance of Aminoglycosides

Bacteria have several methods of resisting aminoglycoside activity. A common method of resistance is to synthesize enzymes that modify the aminoglycoside’s structure. The three classes of enzymes which affect all aminoglycosides are aminoglycoside acetyltransferases (AACs), aminoglycoside nucleotidyltransferases (ANTs), and aminoglycoside phosphotransferases (APHs). These enzymes recognize amino groups on an aminoglycoside and modify the aminoglycoside using group transfers. Without the positively charged amino groups, aminoglycosides are unable to properly bind to the 16S rRNA subunit. APHs add a negatively charged phosphate group to carbon three which will form unfavorable interactions with the negatively-charged phosphate backbone of the rRNA. ANT and AACs catalyze group transfers that prevent polysome binding due to steric hindrance.

Another method of resistance is to decrease aminoglycoside uptake and develop a mechanism for increased aminoglycoside eflux. By increasing the expression of anaerobic respiratory pathway genes, the bacteria’s ATP yield decreases. This in turn reduces aminoglycoside uptake during the energy dependent phases. Certain E. coli strains have demonstrated active efflux of aminoglycosides in order to decrease accumulation.

There are many different methods for aminoglycosides to subvert antibiotic resistance. The most common method is the creation of semi-synthetic aminoglycosides such as amikacin. Semi-synthetic aminoglycoside are able to avoid targeting by aminoglycoside modifying enzymes. Another method of resistance is to increase the permeability of the bacterial envelope. This can be accomplished by using other molecules that compromise bacterial membrane integrity such as β-lactam antibiotics or plectasin.
**Gold Nanoparticles**

AuNPs are one of the most widely used metal nanoparticles in research. While AuNPs have become more widely used within the past decade, knowledge of AuNPs and their potential uses have been developed over the last 150 years. In 1857, Michael Faraday synthesized the first colloidal AuNP solution by reducing gold chloride in the presence of a phosphorus carbon disulfide solution. Currently, there are many different methods for synthesizing AuNPs with varying sizes and shapes. The most common method is the Turkevich method in which a gold salt undergoes citrate reduction to yield citrate-stabilized AuNPs.

AuNPs can be used with various analytical techniques due to their optical properties. The optical properties of AuNPs arise from their localized surface plasmon resonance (LSPR) range. The LSPR of AuNPs is located within the visible spectrum of light (520-550 nm). Exposing AuNPs to light from this range will result in extinction. The two main optical properties of AuNPs are strong light scattering properties and strong light absorbance. AuNPs demonstrate light scattering and absorption properties that are orders of magnitude higher than conventional fluorescent and absorbing dyes. In addition, AuNPs exhibit light scattering properties that are hundreds to thousands times stronger than polystyrene beads.

The strong light scattering exhibited by AuNPs is one of its most important properties. AuNPs’ light scattering and absorption properties are dependent on the size, shape and aggregation of the nanoparticle. AuNPs can vary in size from 2 nm to 100 nm. Commonly used AuNP shapes are rods and spheres. AuNPs can be synthesized in other shapes such as cuboidal or triangular plane AuNPs. Nanorods have demonstrated higher absorption and scattering properties than spherical nanoparticles of equivalent size. Spherical AuNPs have demonstrated
higher uptake and clearance from cells than nanorods.\textsuperscript{,6,43} These properties can be changed to fit the needs of the assay.

AuNPs have a wide variety of applications for assaying biomolecules. AuNPs have been utilized in protein studies, aggregation studies and colorimetric assays.\textsuperscript{44-46} Most applications are linked to the ability of AuNPs to bind to a variety of compounds, their low reactivity, and their various optical properties.\textsuperscript{47} An AuNP is commonly bound to a ligand to stabilize the particle. This ligand determines the properties of the AuNP and what solvent it can be used in. For example, a common ligand used is citrate or another molecule with a carboxylic acid functional group that allows the AuNP to be water soluble.\textsuperscript{48} The unique surface properties of AuNPs allow formation of ionic and hydrophobic interactions with various compounds. This allows the AuNP to bind to numerous biomolecules such as antibodies, DNA, RNA and various proteins.\textsuperscript{49}

A major application of AuNPs is the delivery of molecules and compounds to targets within the human body. AuNPs can be conjugated to small drug compounds which normally have poor uptake to cells. The AuNPs aid in the uptake of the drug into a target cell by a process known as “enhanced permeability and retention effect” (EPR effect).\textsuperscript{50} AuNPs can form a monolayer around DNA and RNA to deliver the polynucleotide to target cells. AuNPs form electrostatic interactions with the negatively charged DNA or RNA backbone and protect the molecule from enzymes.\textsuperscript{48}

AuNPs show promise for use in cancer therapeutics by aiding in drug delivery and radiotherapy treatments.\textsuperscript{51} In addition, AuNPs are being used to develop new cancer treatments such as siRNA and miRNA delivery to cut up mRNA for cancerous genes.\textsuperscript{52-53}
One currently debated topic is whether AuNPs are toxic to humans. Many studies report that most AuNPs are non-toxic. However some studies have found that AuNPs of a certain size combined with specific ligands can be toxic. The mechanism or reason for toxicity remains unknown in many of these studies. While AuNPs appear to be usable in vivo, more research needs to be done in this area.\textsuperscript{41, 54-55}

**Analytical Techniques Using AuNPs**

The DLS assay utilizes the strong light scattering properties and Brownian motion of AuNPs to detect the shape of the particle. AuNPs have light scattering properties that are significantly higher than conventional tags such as fluorescent and absorbing dyes.\textsuperscript{6}

The DLS assay shoots a laser through a sample and analyzes the scattered light. The laser should be within the wavelength range that AuNPs exhibit LSPR. Once a laser hits the sample, the light is scattered and picked up by a detector. The detector transforms the signal and plots the intensity of the scattering as a function of time. This data is then analyzed by a correlator which uses an autocorrelation function to determine various sized particles. Based on the principles of Brownian motion, the larger and denser a particle is, the slower it will move. Lighter and smaller particles will move through solution faster. After collecting the data of the largest particles, the DLS software employs various fitting algorithms to determine the size of the particle and graph this on an intensity distribution curve.

During this last step of the DLS assay, the hydrodynamic radius is calculated.\textsuperscript{56} The hydrodynamic radius is the radius of a hard particle which has the same diffusion rate. This is recorded by the DLS assay as the Z average diameter. The hydrodynamic radius is based on the
dominating or largest light scattering source in the solution. The Stokes-Einstein equation is used to calculate the hydrodynamic radius $R_H$. The variables that can be manipulated are Boltzmann’s constant $k$, absolute temperature $T$, dynamic viscosity $\eta$, pi $\pi$, and the diffusion coefficient $D$. For our experiment, all variables are kept constant except the diffusion coefficient. This allows manipulation of just the diffusion coefficient in order to yield the hydrodynamic radius.\(^{57}\) The optical properties of an AuNP change when the AuNP interacts with another molecule. This change in the optical properties results in a change in the hydrodynamic radius.\(^{58}\)

\begin{equation}
R_H = \frac{kT}{6\pi\eta D}
\end{equation}

*Equation 1. Stokes-Einstein equation.*

The presence of AuNPs and AuNP-aminoglycoside interactions affects the hydrodynamic diameter because pure aminoglycosides have little to no light scattering properties. DLS analysis of pure aminoglycoside dissolved in nanopore water, at experimental concentrations and up to 50 mM, gave photon count rates barely above background noise. UV-Vis absorption spectroscopy analysis of pure aminoglycosides dissolved in nanopore water failed to detect the contents of the solution. Both observations demonstrate that any recorded measurements were due to AuNPs or AuNP-aminoglycoside interactions.

DLS assay data is displayed in a distribution curve. One peak is characteristic of a spherical molecule. The Z average diameter represents the average particle size. Two peaks are characteristic of rod-like aggregation. The smaller peak located at a smaller Z average
diameter on the intensity distribution curve is a result of the rotational diffusion of the particle. The larger peak located at a larger Z average diameter on the intensity distribution curve is a result of the translational diffusion of the particle.\textsuperscript{59-60}

UV-Vis absorption spectroscopy measures the absorption spectra of the AuNP or the AuNP-aminoglycoside sample. UV-Vis absorption data is displayed in an absorption spectra. One surface plasmon resonance (SPR) peak is characteristic of a sphere or spherical particle. Rod-like particles or aggregates will display two peaks. There will be one peak within the SPR range of the AuNP and a second, smaller, peak at a longer wavelength.\textsuperscript{60}

Dark field optical microscope imaging uses an optical microscope combined with a dark field imaging accessory to visualize AuNPs. The strong light scattering properties of the AuNPs combined with their resistance to photobleaching allows visualization with DFM. Capturing the AuNP-aminoglycoside mixture solution on the microscope slide allows the viewing of AuNPs suspended in solution. This allows the viewer to see the Brownian motion of the AuNPs.\textsuperscript{61}

**Previous Research on Ribostamycin Sulfate**

Our laboratory previously performed research on ribostamycin sulfate. The goal of this study was to analyze ribostamycin sulfate using AuNPs. During this experiment it was observed that ribostamycin sulfate self assembled into rod-like aggregates in a concentration dependent manner. DLS analysis of ribostamycin sulfate with 40 nm and 60 nm AuNPs observed rod-like aggregation at 1 mM and 500 µM concentrations. All other ribostamycin-AuNP mixtures did not exhibit any peak shifts. UV-Vis absorption spectroscopy analysis revealed that 1 mM and 500 µM concentrations ribostamycin sulfate in the presence of 40 nm and 60 nm AuNPs
displayed two peaks. All other mixtures displayed peaks resembling the control. The two peaks showed that ribostamycin sulfate self-assembled into rod-like aggregates. These results were confirmed by DFM. DFM pictures of ribostamycin sulfate showed rod-like aggregation. In addition, even samples that did not have any detectable aggregation showed some kind of aggregate formation. These structures did not have any definite shape.

Figure 2. DLS results of previous ribostamycin sulfate study.
Figure 3. UV-Vis absorption spectroscopy of previous ribostamycin sulfate study.
Figure 4. Dark field imaging results from previous ribostamycin study.
METHODS

Antibiotics and Chemicals

The aminoglycosides used in this experiment were neomycin sulfate, amikacin, streptomycin sulfate and ribostamycin sulfate. The size and concentration of the following nanoparticle suspensions were used: 40 nm (9.00 x 10^{10} particle/mL), 60 nm (2.60 x 10^{10} particle/mL), 80 nm (1.10 x 10^{10} particle/mL) and 100 nm (5.60 x 10^{10} particle/mL).

The four different citrate ligand-capped AuNP suspensions were purchased from Ted Pella Inc. and were manufactured by British Biocell International. The antibiotics used in this experiment were purchased from MP Biomedicals. The antibiotics were stored in solid powder form in a refrigerator at 4 °C. The PB buffer was diluted down to a 100 µM with nanopure water from a 1 M stock and was stored in a refrigerator at 4 °C.

Dynamic Light Scattering Assay.

The analysis of each AuNP-aminoglycoside mixture solution was initiated with the DLS assay. The DLS Assay was performed with a Zetasizer Nano ZS90 DLS system with a green laser (532 nm, 4 mW) and an avalanche photodiode detector (quantum efficiency >50% at 532 nm). The Malvern DTS 5.10 software and 7.10 software were utilized for data processing and analysis. The detector was set at a 90 ° angle.

The scattering light intensity was measured and quantified as photons counted per second. This was displayed by the software in kilo counts per second (kcps). The incident power of the laser was changed based on the size of the nanoparticle so that the scattering
light intensity readings were maintained between 100 kcps and 1000 kcps. The laser was
adjusted by an attenuator that is built into the Zetasizer. The level of attenuation was displayed
as an attenuation number and each number corresponded with a specific laser power. The
attenuation numbers used in this experiment were 10 (1.2 mW), 9 (0.4 mW), 8 (0.12 mW), and
7 (0.04 mW). Attenuation numbers 10 and 9 were used when analyzing antibiotic-AuNP
mixtures that contained 40 nm AuNPs and 60 nm AuNPs, respectively. Attenuation numbers 8
and 7 were used when analyzing antibiotic-AuNP mixtures that contained 80 nm AuNPs and
100 nm AuNPs, respectively. When taking a sample measurement, two separate
measurements were taken with a 20 second fixed run time.

Each aminoglycoside was dissolved in either nanopure water or PB buffer. The
dissolved antibiotic was then diluted to 1 mM, 500 µM, 250 µM, 100 µM, 50 µM, 20 µM, or 10
µM concentrations. For each experiment, 6 µL of the aminoglycoside dilution was mixed with
100 µL of a AuNP suspension in an Eppendorf microtube. This mixture solution was then
incubated for ten minutes. Shortly before the ten minute mark, the mixture solution was
transferred to a cuvette (catalog number 67.758, Sarstedt, Germany). Immediately after 10
minutes of incubation had transpired, four DLS measurements were taken at 2 minute intervals.
This was repeated for each sample.

**UV-Vis Absorption Spectroscopy**

The second step in the analysis of each AuNP-aminoglycoside mixture solution utilized
UV-Vis absorption spectroscopy. The UV-Vis spectroscopy analysis was performed with a
NanoDrop 2000 UV-Vis spectrophotometer purchased from Thermo Scientific. For each
experiment, 2 µL of each AuNP-aminoglycoside mixture solution was pipetted onto the pedestal. Two measurements were recorded for each sample and the average of those measurements was recorded for results.

**Dark Field Optical Microscope Imaging**

The final step for analysis of each AuNP-aminoglycoside mixture solution utilized dark field optical microscope imaging (DFM). DFM was performed using an Olympus BX51 microscope with a dark field condenser, oil dispersed objective lens (100x/1.3) and a Soft Imaging System’s Colorview III camera with AnalySIS Life Sciences software. Slides were prepared by pipetting 8 µL of the aminoglycoside-AuNP mixture solution onto a glass microscope slide. The sample was covered with a glass cover, sealed, and mounted on the stage. An image was then taken with the camera and exported to a computer.

**Controls**

Each of the four aminoglycoside antibiotics (ribostamycin sulfate, amikacin, neomycin sulfate and streptomycin sulfate) were analyzed against a pure AuNP solution that acted as a control. Each control presented a single peak indicating that each control was comprised of only spherical nanoparticles. The AuNP control solutions were prepared for nanoparticles with average Z diameters of 40 nm, 60 nm, 80 nm or 100 nm. These controls were performed for every tested set of AuNP-aminoglycoside mixture solutions (Figures 5, 7, 9, 11, 13, 15).

It was observed that the UV-Vis absorption spectra of the pure AuNP controls exhibited a single SPR peak of approximately 530 nm for 40 nm AuNPs, 540 nm for 60 nm AuNPs, 550 nm
for 80 nm AuNPs and 580 nm for 100 nm AuNPs. These controls were performed for every tested set of AuNP-aminoglycoside mixture solutions (Figures 6, 8, 10, 12, 14, 16). It should be noted that after the amikacin 80 nm trial, the 80 nm control sometime presented two peaks. This anomaly may have been caused by contamination of the 80 nm GNP solution. During the amikacin-100 nm GNP trials, there was some contamination of the 100 nm GNP solution but this was corrected in later trials. Additional trials were not repeated to account for the anomalies due to time restrictions.
RESULTS AND DISCUSSION

The hypothesis was AuNPs could be used to analyze aminoglycosides in order to characterize rod-like aggregation in a concentration dependent manner. It was proposed that citrate ligand-capped AuNPs could form electrostatic charges with any aminoglycoside because the positively charged amino groups would be attracted to the negatively charged AuNPs. The formation of a complex between the aminoglycosides and AuNPs would allow the analysis of the aminoglycoside. This hypothesis was derived from previous research performed by our laboratory though the analysis of ribostamycin sulfate using DLS assay, UV-Vis absorption spectroscopy and dark field optical microscope imaging. To test this hypothesis, the same protocol used to analyze ribostamycin sulfate was performed on several other aminoglycosides.

**Dark Field Imaging**

DFM images were recorded for amikacin in order to confirm rod-like aggregates. The DFM confirmed the rod-like behavior of amikacin. The images were similar to those of ribostamycin sulfate (Figure 4). Unfortunately due to university computer issues, the DFM images that had been taken were lost. Additional trials to collect these images were not performed due to time restrictions.
Amikacin in Nanopure Water

Figure 5 contains the DLS data that is displayed as an intensity-averaged size distribution curve. When amikacin is mixed with 40 nm and 60 nm AuNP solutions, two strong peaks were observed (Figure 5-A, B). The 40 AuNP nm mixture displayed a second peak around the 8 nm mark and the 60 nm AuNP mixture displayed a second peak that ranged from the 7 nm to 12 nm mark. The 80 nm and 100 nm AuNP solutions mixed with amikacin sometimes displayed a smaller second peak but this may have been caused by contamination and not by amikacin-AuNP interactions. Amikacin mixed with 80 nm and 100 nm AuNP solutions each exhibited one strong peak which was almost identical to the peaks exhibited by the pure AuNP solution (Figure 5-C, D). This may have been caused by the larger sized AuNPs mixed with amikacin not being able to form significant sized anisotropic aggregates.

The UV-Vis absorption spectra data followed the same trend as the DLS data. It was observed that 40 nm and 60 nm AuNPs mixed with amikacin generated two peaks (Figure 6-A, B). The larger of the two peaks presented itself at around 540 nm. The second peak was observed at longer wavelengths that ranged between 600 nm to 700 nm. Similar to the DLS assay results, amikacin mixed with the 80 nm and 100 nm AuNPs presented one peak that was similar to the peak displayed by the pure AuNP solution (Figure 6-C, D).

DFM provided further evidence of the anisotropic assembly of amikacin. There were multiple self-assembled linear anisotropic particles. It was observed that 40 nm AuNP-amikacin and 60 nm AuNP-amikacin mixtures contained visible aggregates at all concentrations. The
AuNP-amikacin complexes displayed stronger light scattering than the pure AuNP solutions under dark field imaging (see Figure 4 for reference).

The results of the DLS assay, UV-Vis absorption spectroscopy and DFM suggest that amikacin displays concentration-dependent linear self assembly. Amikacin behaved similar to ribostamycin sulfate. Our observations further support the proposal that amikacin can self assemble as a linear rod-like aggregate.

There were some observed differences between the behavior of amikacin and ribostamycin sulfate. Unlike amikacin, only 1 mM or 500 µM concentrations of ribostamycin sulfate solution mixed with 40 nm or 60 nm AuNPs presented two peaks when analyzed with the DLS assay and UV-Vis spectroscopy. In contrast to ribostamycin sulfate, amikacin displayed two peaks at all tested concentrations when mixed with 40 nm and 60 nm AuNPs. This observation suggests that amikacin can form aggregates at significantly lower concentrations than ribostamycin sulfate. One possible explanation is that amikacin has one more amino group than ribostamycin sulfate (Figure 1). This would allow amikacin to form more electrostatic interactions with other molecules.
Figure 5. Intensity-averaged size distribution curves of AuNP-amikacin mixtures in nanopure water solvent. The 40 nm (A) and 60 nm (B) AuNP mixtures with amikacin exhibited two peaks at all tested concentrations indicating rod-like self assembly. The 80 (C) nm and 100 (D) nm AuNP mixtures with amikacin did not exhibit any peak shifts.
Figure 6. UV-Vis absorption spectra of AuNP-amikacin mixtures in nanopure water solvent. This supported the DLS measurements. The 40 nm (A) and 60 nm (B) AuNP mixtures displayed two peaks. The 80 nm (C) and 100 nm (D) AuNP mixtures displayed peaks similar to the control.
Streptomycin Sulfate in Nanopure Water

Figure 7 contains the intensity-averaged size distribution curves of streptomycin sulfate mixture solutions. AuNP-streptomycin sulfate mixtures consistently presented one strong peak during DLS assay analysis. It was often observed that streptomycin sulfate presented peaks with a Z average diameter much larger than pure AuNP. It was observed that 40 nm AuNP-streptomycin sulfate mixtures exhibited peaks at around 710 nm (Figure 7-A), 60 nm AuNP-streptomycin sulfate mixtures exhibited peaks at around 530 nm (Figure 7-B), 80 nm AuNP-streptomycin sulfate mixtures exhibited peaks at around 400 nm (Figure 7-C), and 100 nm AuNP-streptomycin sulfate mixtures exhibited peaks at around 300 nm (Figure 7-D). It was also observed that 100 µM streptomycin sulfate in the presence of 60 nm AuNPs and 80 nm AuNPs presented a peak almost identical to pure AuNPs (Figure 7-B, C). This is likely due to the formation of aggregates that precipitated out of solution.

It was observed that the 40 nm AuNP-100 µM streptomycin sulfate mixture exhibited three peaks (Figure 7-A). The exact cause of this is unknown. One possibility is that random aggregation occurred. A more likely explanation is that an aggregate was formed that was similar to rod-like aggregates.

The UV-Vis absorption data displayed no peaks for most samples of streptomycin sulfate (Figure 8). The absence of peaks was caused by the formation of aggregates which then precipitated out of solution. Similar to the DLS measurements, it was observed that 100 µM concentrations of streptomycin sulfate mixed with 60 nm and 80 nm AuNPs displayed peaks almost identical to pure AuNP (Figure 8-B, C). The DLS measurements and the UV-Vis absorption spectra data suggests that streptomycin sulfate forms a spherical or sphere like
aggregate in water. These aggregates were able to consistently form and be detected at concentrations above 100 µM.
Figure 7. Intensity-averaged size distribution curves of AuNP-streptomycin sulfate mixtures in nanopure water solvent. Most AuNP-streptomycin sulfate mixtures exhibited one peak at all concentrations above 100 µM. 100 µM streptomycin sulfate didn’t form any detectable aggregates in the presence of 60 nm (B) and 80 nm (C) AuNPs. The 40 nm AuNP-100 µM streptomycin sulfate mixture (A) displayed three peaks. The 100 nm AuNPs (D) detected peaks similar to other concentrations.
Figure 8. UV-Vis absorption spectra of AuNP-streptomycin sulfate mixtures in nanopure water solvent. Most samples did not display any peaks indicating that aggregates formed but then precipitated out of the solution. The 60 nm AuNP (B) and 80 nm AuNPs (C) with 100 µM streptomycin sulfate formed peaks similar to the control.
Neomycin Sulfate in Nanopure Water

Analysis of neomycin sulfate, mixed with different sized AuNPs at different concentrations of neomycin, using the DLS assay consistently presented one strong peak (Figure 9). It was observed that this single peak varied in size depending on the size of the AuNP solution mixed with the neomycin sulfate: (A) 40 nm AuNP-neomycin sulfate solutions had Z average diameters close to 700 nm (Figure 9-A), (B) 60 nm AuNP-neomycin sulfate mixtures had Z average diameters of approximately 600 nm (Figure 9-B), (C) 80 nm AuNP-neomycin sulfate AuNP mixtures had Z average diameters ranging between 400 nm and 500 nm (Figure 9-C), (D) 100 nm AuNP-neomycin sulfate mixtures had Z average diameters approximately 300 nm (Figure 9-D). These findings were supported by the UV-Vis absorption data (Figure 10). No meaningful peaks were detected at any neomycin sulfate concentration with any AuNP solutions. The absence of peaks was caused by aggregates forming and then precipitating out of the solution. The DLS assay data and UV-Vis spectra data indicate that neomycin sulfate forms spherical aggregates in nanopure water at all tested concentrations.
Figure 9. Intensity-averaged size distribution curves of AuNP-neomycin sulfate mixtures in nanopure water solvent. Neomycin sulfate exhibited one peak at all concentrations with all AuNP sizes used.
Figure 10. UV-Vis absorption spectra of AuNP-neomycin sulfate mixtures in nanopure water. Results are consistent with DLS data. No peaks were observed due to aggregates forming and then precipitating out of the solution.
Neomycin Sulfate at Lower Concentrations in Nanopure Water

It was observed that neomycin sulfate normally displayed one peak, indicating that it formed a spherical aggregate at concentrations between 1 mM and 100 µM (Figure 11). Testing was done at lower neomycin sulfate concentrations to see if neomycin sulfate was able to form aggregates at lower concentrations that could be detected by the DLS assay and UV-Vis absorption spectroscopy. It was observed 40 nm AuNP-neomycin mixtures exhibited one peak at around 632 nm. This indicated that neomycin sulfate retained the ability to form spherical aggregates that could be detected by the DLS at concentrations as low as 10 µM (Figure 11-A).

Some anomalies were observed. DLS analysis of the 40 nm AuNP-10 µM neomycin displayed a weaker second peak that only appeared in two trials.

All mixtures of 60 nm AuNPs and neomycin sulfate exhibited two peaks (Figure 11-B). It was observed that the 60 nm AuNP-50 µM neomycin sulfate mixture weakly exhibited two peaks. The 60 nm AuNP-20 µM neomycin sulfate and 60 nm AuNP-10 µM neomycin sulfate mixtures displayed two prominent peaks. The 80 nm and 100 nm AuNP mixtures did not exhibit any peak shifts except for the 50 µM neomycin sulfate mixtures. DLS analysis of the 80 nm and 100 nm AuNP solutions mixed with 50 µM neomycin sulfate exhibited one peak with a Z average diameter that was approximately 390 nm (Figure 11-C and 11-D).

In the UV-Vis absorption spectra data (Figure 12), the AuNP-40 nm and 20 µM neomycin sulfate mixture solution displayed a prominent second peak. This is in contrast to the DLS results which did not detect any second peak. This inconsistency may have been caused by the DLS laser not hitting any AuNP-neomycin sulfate complexes because only a few complexes were
able to form. All 60 nm AuNP mixtures displayed two peaks (Figure 12-B). Of the 60 nm AuNP-neomycin sulfate mixtures, the 60 nm AuNP-10 μM neomycin sulfate mixture displayed a stronger first peak. This suggests that neomycin sulfate can self assemble into rod-like aggregates. The 80 nm AuNP-neomycin sulfate and 100 nm AuNP-neomycin sulfate mixtures exhibited peaks similar to the pure control outside of the 50 μM neomycin samples (Figure 12-C, D). The 100 nm AuNP-20 μM neomycin sulfate mixture exhibited a weak second peak (Figure 12-D).

The UV-Vis absorption spectra data supports the findings of the DLS assay. At lower concentrations, neomycin sulfate can still form spherical aggregates. At low concentrations in the presence of 60 nm AuNPs, it was observed that neomycin sulfate was able to form rod-like self assemblies.
Figure 11. Intensity-averaged size distribution curves of AuNP-neomycin sulfate mixtures in nanopure water solvent. The 40 nm AuNP-20 µM neomycin sulfate mixture (A) exhibited a weak second peak. The 60 nm AuNP-20 µM neomycin sulfate and 60 nm AuNP-10 µM neomycin sulfate AuNP mixtures (B) exhibited strong second peaks.
Figure 12. Intensity-averaged size distribution curves of AuNP-neomycin sulfate mixtures in nanopure water solvent. The second peak for the 40 nm AuNP-20 µM mixture (A) is much more prominent than exhibited in DLS. All 60 nm AuNP-neomycin sulfate mixtures (B) displayed a second peak. All 80 nm and 100 nm AuNP mixtures with neomycin sulfate (C, D) displayed peaks similar to the control except for the 50 µM neomycin sulfate mixture.
Amikacin in 100 mM PB Buffer

In an additional experiment, amikacin was dissolved in 100 mM PB buffer to see if it would self assemble into rod-like aggregates in physiological conditions. DLS data revealed that two peaks were detected in various sample trials (Figure 13). The 40 nm AuNPs-500 µM amikacin mixture and 60 nm AuNP-100 µM amikacin mixtures both displayed two peaks (Figure 13-A, B). The majority of sample measurements only exhibited only one peak. This observation may have been due to fewer rod-like aggregates forming in the mixture solution. As a result, the particles intercepted by the DLS laser had a higher number of spherical aggregates and fewer rod-like aggregates. The 40 nm AuNP mixtures exhibited a single peak at approximately 615 nm (Figure 13-A). The single peak exhibited by the 60 nm AuNP mixtures was at approximately 600 nm (Figure 13-B). The single peak exhibited by the 80 nm AuNP mixtures ranged from approximately 350 nm to 540 nm (Figure 13-C). The single peak exhibited by the 100 nm AuNP mixtures was at approximately 340 nm (Figure 13-D).

The DLS data is further supported by the UV-Vis absorption spectra data (Figure 14). It was observed that AuNP-60 nm with 100 µM amikacin mixture displayed two low peaks (Figure 14-B). For the other samples, a weak second peak was observed. These observations support the proposal that although some rod-like aggregation occurred, more spherical aggregates were formed. These spherical aggregates, however, precipitated out of solution. It may be concluded that under physiological conditions amikacin forms concentration dependent rod-
like assemblies. It was also observed that amikacin dissolved in PB buffer compared to amikacin dissolved in nanopure water exhibited decreased formation of rod-like aggregates.
Figure 13. Intensity-averaged size distribution curves of AuNP-amikacin mixtures in PB buffer solvent. Overall one peak was observed with all amikacin-AuNP mixtures. Occasionally two peaks were observed indicating weak rod-like self assembly behavior. Unlike nanopure water, the 80 nm and 100 nm AuNP mixtures were able to detect some aggregation (C, D).
Figure 14. UV-Vis absorption spectra of AuNP-amikacin mixtures in PB buffer solvent. This data supported the results of the DLS assay. Few peaks were observed due to the aggregates precipitating out of solution. There were some weak second peaks observed in the 60 nm AuNP mixture (B).
Ribostamycin Sulfate in 100 mM PB Buffer

The results of experiments with amikacin resemble some of the results of prior research done by our laboratory with ribostamycin sulfate. Our past research established that ribostamycin sulfate self-assembled into rod-like aggregates in nanopure water. In an additional experiment, ribostamycin sulfate was analyzed in 100 µM Buffer to determine whether it would exhibit similar activity in physiological conditions. Comparing the results of ribostamycin sulfate dissolved in PB buffer to amikacin dissolved in PB buffer, it was observed that ribostamycin sulfate displayed a greater tendency to form rod-like assemblies in physiological conditions. Analysis of multiple ribostamycin sulfate samples using the DLS assay presented two peaks (Figure 15). It was observed that the 40 nm AuNP-250 µM ribostamycin sulfate mixture, 40 nm AuNP-100 µM ribostamycin sulfate mixture, 60 nm AuNP-1 mM ribostamycin sulfate mixture, 60 nm AuNP-500 µM ribostamycin sulfate mixture, and 60 nm AuNP-250 µM ribostamycin sulfate mixture displayed two peaks (Figure 15-A, B). The second peaks were not as strong as those displayed by ribostamycin sulfate-AuNP mixture solutions in nanopure water.

It was further observed that 80 nm AuNP and 100 nm AuNP mixture solutions mixed with ribostamycin sulfate displayed peaks that were almost identical to the peaks exhibited by their respective pure AuNP controls (Figure 15-C, D). The 60 nm AuNP with 100 µM ribostamycin sulfate sample also displayed peaks that were almost identical to the peaks exhibited by the pure 60 nm AuNP control (Figure 15-B).
The UV-Vis spectra data supports the DLS data (Figure 16). The 40 nm AuNP with 100 µM ribostamycin sulfate mixture solution exhibited two strong peaks (Figure 16-A). The 60 nm AuNP with 500 µM and 250 µM ribostamycin sulfate solutions exhibited two peaks (Figure 16-B). As further support of the DLS assay results, UV-Vis absorption spectra of the AuNP-60 nm-100 µM ribostamycin sulfate mixture, the 80 nm AuNP-ribostamycin sulfate mixtures, and the 100 nm AuNP-ribostamycin sulfate mixtures were almost identical to the pure AuNP control (Figure 16-B, C, D). The previous experiment performed by our lab analyzed ribostamycin sulfate with AuNPs and found that the results for the AuNP-80 nm and AuNP-100 nm mixture solutions were almost identical to the results for the pure AuNP suspensions (Figure 16-C, D).

These results strongly suggest that ribostamycin sulfate forms concentration dependent rod-like assemblies in physiological conditions. Compared to amikacin, ribostamycin sulfate is more able to form rod-like assemblies at different concentrations. Three more tested samples of ribostamycin sulfate exhibited two peaks. It was observed that the 40 nm AuNP with 100 µM and 250 µM ribostamycin sulfate consistently displayed two peaks unlike the AuNP-amikacin mixture solutions.
Figure 15. Intensity-averaged size distribution curves for AuNP-ribostamycin sulfate mixtures in PB Buffer solvent. 40 nm AuNP mixture (A) exhibited two peaks at 250 µM and 100 µM ribostamycin sulfate concentrations. It was observed that the 60 nm AuNP mixture (B) exhibited two peaks at all concentrations except for at the 100 µM ribostamycin sulfate concentration.
Figure 16. UV-Vis absorption spectra of AuNP-ribostamycin sulfate mixtures in PB buffer solvent. There is a strong second peak displayed by the 40 nm AuNP-100 µM ribostamycin sulfate mixture (A). There are weak second peaks displayed by the 60 nm AuNP-ribostamycin sulfate mixture (B). Most 80 nm and 100 nm AuNP mixtures with ribostamycin (C, D) resembled the controls.
CONCLUSION

The three aminoglycosides (amikacin, streptomycin sulfate, and neomycin sulfate) were successfully analyzed using AuNPs and the shapes of the three aminoglycosides were characterized. Of the three, amikacin was readily able to form concentration-dependent rod-like self assemblies in nanopure water. Neomycin sulfate was found to form spherical aggregates at concentrations between 1 mM and 100 µM. At 50 µM, 20 µM, and 10 µM concentrations, neomycin sulfate was able to form concentration dependent rod-like self assemblies in nanopure water. These rod-like assemblies were only detected using 60 nm AuNPs. Streptomycin sulfate was found to form spherical aggregates. It was observed that the spherical aggregates were not consistently formed at 100 µM concentrations.

It was observed that both amikacin and ribostamycin sulfate retained the ability to self assemble into rod-like aggregates in PB buffer. Compared to nanopure water, amikacin had a lower tendency to form rod-like aggregates and was more inclined to self assemble into spherical aggregates. Ribostamycin sulfate was able to consistently form rod-like self assemblies at different concentrations. One observed key difference between the observations using PB buffer and nanopure water as a solvent was the concentrations in which rod-like aggregates formed. In nanopure water, rod-like self assemblies tended to form at higher concentrations such as 1 mM or 500 µM. In PB buffer, rod-like self assemblies formed at concentrations below 500 µM.

It is important to study the behavior of aminoglycosides and other antibiotics in order to develop better therapeutic drugs. Increasing numbers of bacteria strains are developing
resistance to aminoglycosides. Understanding aminoglycoside behavior can aid in further understanding aminoglycoside pathways for uptake and the mechanism of action within the bacteria. This in turn can help circumvent antibiotic resistance. Comprehensive knowledge of aminoglycosides behavior can aid in understanding aminoglycoside interactions with the human body. Gaining further insight into these interactions could aid in synthesizing less toxic aminoglycosides.
LIST OF REFERENCES


