Antibacterial Silver Core Spherical Nucleic Acids

Clayton H. Rische, Aakriti Goel, Aleksandar F. Radovic-Moreno, and Sergei M. Gryaznov

Excure, Inc., 8045 Lamon Ave Suite 410, Skokie, IL 60077 USA

Bacterial drug resistance (DR) has the potential to be one of the most significant medical challenges of the 21st century. Infections involving DR bacterial strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) or DR *Acinetobacter baumannii* spread in hospital and community settings and are associated with an increased risk of death. New therapeutic strategies and modalities are needed to treat these DR infections, especially for DR Gram-negative infections. Here, we describe silver-core spherical nucleic acids (Ag-SNAs) as a broad-spectrum antibacterial. Ag-SNAs leverage the broad spectrum antibacterial activity of silver nanoparticles (Ag-NPs), while exhibiting significantly lower (30-fold) minimum inhibitory concentration (MIC) values in comparison to Ag-NPs and reduced toxicity (14-fold) to mammalian cells. Ag-SNAs demonstrate low nanomolar MICs against both DR Gram-negative and DR Gram-positive bacteria *in vitro* and reduced *in vitro* toxicity toward human cells *vis-à-vis* non-derivatized silver nanoparticles. In a topical *in vivo* wound model involving a multi-DR, clinically-isolated, Gram-negative *A. baumannii* strain, Ag-SNAs significantly reduced bacterial load as compared to controls (1.51-fold log₁₀ reduction). Our findings open up possibilities for the further development of Ag-SNAs as new antibacterial agents, particularly against challenging DR Gram-negative infections.

1. Introduction

There has been relatively limited development in the field of antibiotics for the past 10 years, with new drugs often accompanied by new bacterial drug resistance (DR) [1]. Many current antibiotics have primarily been sourced from naturally occurring biochemicals, and if past naturally-sourced antibiotics serve as an example, resistance to new drugs appears inevitable, if only delayed [2–4].

Silver nanoparticles (Ag-NPs) [5] and silver ion [6] based treatments have been explored due to their broad spectrum antimicrobial activity, intrinsically low propensity for DR, and ability to synergize with existing antibiotics. However, successful clinical translation of silver-based antibiotics has often been hindered due to challenges with toxicity, formulation, biodistribution, and pharmacokinetics. Prior experiments have been devoted to addressing these challenges through the use of polymer coatings, functionalization with antibiotics, and as embedded constituents of materials such as bandages [7–9]. Due to the small size and relative ease of synthesis, the potential uses and applications of silver nanoparticles extend beyond antimicrobials and into areas such as plasmonic sensors, biological imaging, and microscopy [10,11].

Other approaches to antibiotics have begun to explore the use of antisense RNAs, synthetic oligonucleotides, and other nucleic acid-based molecules [12–14]. By targeting specific genes, essential biological functions in bacteria can be disrupted, leading to cell death or reduction of drug-resistance. These strategies also face difficulties, primarily when tackling delivery of the nucleic acid to the target within bacterial cells. Additionally, there are challenges posed by using compounds such as antisense RNAs, which may present unintentional off-target effects [15]. Despite the success of many of these approaches *in vitro*, there remains to be seen a successful clinical application of gene modulation in bacteria beyond animal models [16]. Other approaches, such as treatment with peptide nucleic acids, face challenges in uptake and biological half-life, despite resisting enzymatic degradation within cells [17]. An intended target of such therapies has been the acyl carrier protein (acpP), an essential gene in the synthesis of fatty acids for bacterial membranes and polyketides [18].

Spherical nucleic acids (SNAs) are oligonucleotides arranged radially around a core template. SNAs have applications in imaging [19] and gene regulation in eukaryotic cells [20,21], or as DNA probes capable of detecting a wide range of specific biological targets [22]. SNAs have many properties which make them useful in biological settings, including low toxicity, high solubility, protein binding, and intracellular delivery, and have found applications in intracellular mRNA detection and immunomodulation [23]. To date, SNA constructs have been formulated with templates consisting of gold nanoparticles [19,20], small unilamellar vesicles [24] and silver nanoparticles [25]. We hypothesized that the attractive biological properties of SNAs, together with their potential for clinical translation

including amenability to large-scale manufacturing, could be a way to address some of the hurdles to using silver-based antibacterials.

This work describes the design, synthesis and properties of antibacterial silver-core SNAs (Ag-SNAs, Scheme 1). These structures are a novel formulation of Ag-NPs that leverage the antibacterial properties of silver, but with significantly enhanced potency (up to 30-fold lower MIC) and reduced toxicity (14-fold lower) when compared to Ag-NPs. In addition, Ag-SNAs display broad-spectrum activity against DR Gram-positive and DR Gram-negative organisms, including a DR Gram-negative clinical isolate. We have evaluated Ag-SNA interactions with methicillin-resistant *Staphylococcus aureus* strain 252 (MRSA) and two strains of *Acinetobacter baumannii*, strain AYE and a multi-DR clinical isolate, termed UNT086-1, as a representative set of DR organisms. These three bacteria represent some of the more persistent Gram-positive and negative infections currently observed in the clinic [26,27]. Another useful property of silver is its ability to synergize with antibiotics [28]. Consequently, we also evaluated whether Ag-SNAs enhanced the activity of ampicillin, a commonly used systemic antibacterial. Individuals with drug-resistant superficial infections [1,29] or those at increased risk of infection (such as burn injury patients or the elderly) [30,31] may benefit from antibiotics with these properties.

2. Experimental Section

2.1. Bacterial Cell Culture

A. baumannii strain AYE and methicillin-resistant *Staphylococcus aureus* strain 252 (MRSA-252) were obtained from ATCC. The clinical strain, *A. baumannii* UNT086-1, was contributed from Pre-Clinical Services at the University of North Texas Health Science Center. Cell cultures were stored *via* Petri dish culture on trypticase soy agar (TSA (Teknova)) at 4°C. Mueller Hinton broth (MHB (Becton, Dickinson and Co.)) was used for all *in vitro* liquid cultures, which were grown at 37°C on a Thermo Scientific MaxQ 4000 orbital shaker (225 rpm) for overnight and intermediate time periods.

2.2. Oligonucleotide Selection, Synthesis, and Treatment

Oligonucleotide sequences were originally selected using a BLAST search to analyze matches for antisense sequences targeting the acyl carrier protein (acpP) gene in *A. baumannii* AYE. This gene was initially selected due to its importance in the synthesis of fatty acids for bacterial membranes. Additional screening was conducted using BLAST to ensure minimal off-target matches in humans. 3'-thiol-functionalized sequences were then synthesized using solid phase phosphodiester or phosphorothioate chemistry on a MerMade 48 (Bioautomation). Protected DNA bases and related modifications were acquired from ChemGenes. Synthesized oligonucleotide mass and quality was verified *via* MALDI-TOF and concentrations were verified using UV-visible spectroscopy. Each oligonucleotide was synthesized in the 3' to 5' direction, beginning with a 3' distal thiol group, followed with two successive 18-O-Dimethoxytritylhexaethyleneglycol,1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite ("spacer-18") molecules which linked to the oligonucleotide sequence. A 1 mL solution composed of approximately 150 μ M of oligonucleotide and 50 mM of DTT was prepared in phosphate buffer solution (Sigma Aldrich). After allowing the solution to incubate at room temperature for 20 minutes, the volume was washed with a 1:2 ratio of ethyl acetate, 99.5% (VWR). After washing three times, the solution was placed on a shaker table for 2 hours. The oligonucleotide was then purified using NAP desalting columns (GE Healthcare). The unconjugated thiol sample quality was evaluated using a 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB (Sigma Aldrich)) test. A solution of 30 μ M of oligonucleotide, 3.2 mM DTNB was created using 0.1 M phosphate buffer, pH 8. After allowing samples to sit for 15 minutes at room temperature, a DTNB blank was used to establish a baseline on UV-vis, prior to reading the samples between 300-600 nm. Sample concentration was determined using the extinction coefficient of ionized 2-nitro-5-thiobenzoate ($\epsilon=13,700 \text{ M}^{-1}\text{cm}^{-1}$). Oligonucleotides were mixed with silver nanoparticles to synthesize silver spherical nucleic acids. Several oligonucleotide sequences were initially synthesized, however due to later difficulties in SNA synthesis, this was later narrowed down to a scramble sequence and two target sequences, one of which presented superior loading on the surface of silver nanoparticles. The phosphorothioate (PS) DNA sequence which presented the best SNA loading was: 5'-

GCAAATCAAACGCCACAAG-3'. We also used a phosphodiester (PO) version of this sequence and a scramble sequence: 5'-TGACCCTACCCTAGTCCCA-3'.

2.3. Spherical Nucleic Acid Synthesis

Preparation of the Ag-SNAs was carried out using prior work with silver [25] and gold [19,20] cores as a basis. For PS-Ag-SNAs, methoxyl poly(ethylene glycol) thiol (PEG-SH, MW = 2 kD (Nanocs)) was added in 500-fold excess of 10 nM Ag-NPs (US Research Nanomaterials) in 0.1% Tween-20 and 10 mM phosphate buffer. This was necessary to prevent uncontrolled lateral association of PS oligonucleotides with the particle surface, and ensure that only the 3'-thiol group was the only sulfur atom that could interact with the particle surface. The particles were then incubated at 45°C on a shaker for 1.5 hours. After incubation, the thiol-oligonucleotide was added to the particles at 2000-fold excess of the 10 nM particle concentration, and incubated at 45°C for 1.5 hours prior to addition of sodium chloride (500 mM in solution). The particles were incubated overnight at 45°C. The following day, particles were centrifuged and washed in water 3 times, with the final suspension of particles in 0.0067 M phosphate buffered saline (PBS, Thermo Scientific). PO-Ag-SNAs did not utilize a PEG backfill, as it was not necessary to prevent the oligonucleotide from associating with the particle surface. The synthesis process for PO-Ag-SNAs was identical to that of PS-Ag-SNAs, however it did not include PEG-SH, was incubated after the addition of oligonucleotide for 3 hours, and stabilized with 250 mM of sodium chloride in solution. The above methods were also used to synthesize PS- and PO-Au-SNAs. Particle concentration was determined using UV-visible spectroscopy.

2.4. Growth Curves of *A. baumannii* AYE, UNT086-1, and MRSA

Cultures were grown in 96-well plates (Corning) on a rotary shaker (225 rpm) overnight at 37°C. Plates were prepared by placing a 1:2 serial dilution of Ag-SNAs across a span of wells (50 µL in each well), then adding 50 µL of bacterial cells at 10⁴ cells/mL. Several wells of media-only, an ampicillin standard curve, and some untreated wells containing only inoculated bacteria were included on each plate. Each plate tested also contained a dilution comparison of Ag-NPs and unconjugated oligonucleotides. After incubation, the optical density (OD₉₀₀) of bacterial growth was assessed using a plate reader. 900 nm readings were utilized due to interference on the order of 0.15-0.25 abs from the Ag-SNAs when reading samples at 600 nm. Two groups of Ag-SNAs were tested, one with PS backbone oligonucleotides, and one with PO backbone oligonucleotides. Wells which displayed no visible or detectable growth were verified with colony forming unit (CFU) counts on TSA. Dilutions were created using wells with detectable growth, no growth, untreated wells, and ampicillin-treated wells. The dilutions were 10⁻⁶, 10⁻⁷, 10⁻⁸, and 10⁻⁹ of the contents of each well sampled in CFU analysis. 5 µL of each dilution was plated on the agar and allowed to grow overnight at 37°C before counting. This evaluation was designed as a quick assessment and confirmation of apparent MIC concentrations of Ag-SNAs with the bacterial cells. These same methods were also employed to examine PS- and PO-Au-SNAs and multiple PS-Ag-SNAs used to compare different oligonucleotide sequences with *A. baumannii* AYE.

2.5. Ag-NP and PS-Ag-SNA Kill Curve with *A. baumannii* UNT086-1

A kill curve was determined from observing the growth of *A. baumannii* UNT086-1 challenged with 5 nM of PS-Ag-SNAs, 5 nM Ag-NPs, and a 26.2 µM gentamicin control. Bacteria were added to dilution curves of particles and antibiotic on a 96-well plate, with a final concentration being 10⁷ cells/mL after being plated. Cells were grown in MHB at 37°C. The effective kill curve of the pathogen was determined by obtaining CFU counts over an 8 hour period. Samples were diluted by a factor of 10³ in MHB and plated once every hour during the course of the experiment on TSA. After determining the CFU/mL, the silver particle data was plotted and compared to the antibiotic and untreated controls.

2.6. Membrane Integrity of *A. baumannii* AYE Treated with PS-Ag-SNAs

One nanomolar of PS-Ag-SNAs or Ag-NPs was incubated with *A. baumannii* AYE (10^5 cells/mL) in 10 mL of TSB for 4 hours. Samples were washed three times with 0.85% NaCl solution, with a final solution volume of 10 mL. A LIVE/DEAD BacLight kit (Invitrogen) was used to stain the washed cells using SYTO 9 and propidium iodide. Five microliters of each sample (included untreated control) was taken and placed on glass slides obtained from VRW. Samples were then heat-fixed and imaged with an Olympus IX71 inverted microscope using a Photometrics CoolSnap HQ2 camera.

2.7. TEM Images of *A. baumannii* AYE and PS-Ag-SNAs

Samples were prepared for TEM by inoculating 150 mL of trypticase soy broth with 10^4 cells/mL of *A. baumannii* AYE and adding 1 nM of PS-Ag-SNAs. After overnight incubation, the sample was centrifuged at 7000 rpm for 5 minutes and washed (repeated three times). For the final wash, samples were resuspended with a fixing solution of 1% glutaraldehyde, 4% formaldehyde in 0.15 M PIPES buffer, pH 7.3 (Electron Microscopy Sciences). After the final centrifugation cycle, the supernatant was removed and gently replaced with fresh fixing solution, so as not to disturb the pellet. For TEM imaging samples were shipped to Paragon Bioservices (Baltimore, MD), where samples were resuspended and centrifuged in 0.1 M PIPES. The samples were then resuspended and embedded in 5% low melt agarose in 0.2 M PIPES, and placed on ice to solidify. Each sample was then washed three times in 0.1 M PIPES (10 mins/wash) and fixed for 1 hour with osmium tetroxide in 0.1 M PIPES. After fixing, samples were washed three times with dH_2O (10 mins/wash). Solutions of 50%, 70% and 95% EtOH were then used to wash the samples (10 mins/wash). Two 100% EtOH washes followed. Next, samples were washed in 30 minute increments, starting with 2:1 100% EtOH/Spurr's, then 1:1 and 1:2, followed by 100% Spurr's for 1 hour, then 100% Spurr's O/N. Samples were then embedded in fresh Spurr's O/N in an oven at 65°C.

2.8. Fluorescence Images of Ag-SNAs with *A. baumannii* AYE

The same Ag-SNA synthesis outlined above was used to create fluorescently labeled PS-Ag-SNAs for treatment against *A. baumannii* AYE. The oligonucleotide produced from synthesis was identical to that of the previous experiment; however, the strand included a 5' Cy5 molecule (ex/em = 650/670 nm, Glen Research). A serial dilution starting at 50 nM PS-Ag-SNAs was prepared on a 96-well plate (50 μL per well) prior to addition of 50 μL of bacterial cells at 10^4 cells/mL. A row of untreated cells was also added for comparison. After overnight incubation, 100 μL was taken from the 5 nM PS-Ag-SNA or the untreated wells and placed in a microcentrifuge tube. Samples were washed three times in PBS. Five microliters of washed sample was taken and placed on glass slides obtained from VRW. Samples were then heat-fixed and imaged with an Olympus IX71 inverted microscope using a Photometrics CoolSnap HQ2 camera.

2.9. Synergy of PS-Ag-SNAs and Ampicillin

The checkerboard method for synergy [32] was used to evaluate MRSA and *A. baumannii* UNT086-1 growth. A serial dilution of ampicillin was conducted, starting at 673 μM with a final volume of 40 μL in each well. A concentrated serial dilution of PS-Ag-SNAs was added in 10 μL volumes to each well to bring the total volume up to 50 μL . Inoculum of 10^4 cells/mL of MRSA-252 was added at 50 μL volume to bring the total volume in each well to 100 μL . A strip of wells was reserved both for ampicillin-only and PS-Ag-SNA-only treatments to compare against the mixed wells and establish fractional inhibitory concentrations (FICs). When two therapeutic agents are used together, FIC is defined as the fractional value of an individual antimicrobial agent's MIC. In opposite corners of the plate, untreated controls were placed for growth comparison. Samples were read using the same settings from prior experiments (OD900) and evaluated based on optical growth density.

2.10. Toxicity Screen of Ag-SNAs, HEK293, and HFK cells

Flow cytometry was conducted using a Guava easyCyte 8HT from EMD Millipore. HEK293 cells were incubated with 3.75 nM of PS-Ag-SNAs or Ag-NPs for 4 hours before being treated with a propidium iodide and calcein AM stain. Each group contained at least 1,200 counted cells, and data was analyzed using FlowJo. An alamarBlue (Invitrogen) assay was used to evaluate the toxicity of PS-Ag-SNAs and Ag-NPs in the presence of human foreskin keratinocytes supplied by Northwestern University. Serial dilutions of the particles were created on a 96-well plate using cell culture media. Silver particle concentrations ranged from 10 nM to 0.3125 nM, and were incubated with cells for 24 hours at 37°C in a VWR Symphony CO₂ cell incubator. Following the treatment cells were washed with PBS and 10 µL volume of alamarBlue reagent was added directly to cells in culture medium in 96-well plate. Cells were then incubated for 1 to 4 hours at 37°C in the cell culture incubator, protected from light. Absorbance was then recorded at 570 nm using a plate reader. The cell viability data was expressed in terms of the percentage of cells that survived the treatment. Fluorescence microscopy images were obtained with HEK-293 cells incubated with 3.75 nM of fluorescent PS-Ag-SNAs for 4 hours in an 8-well chamber slide prepared with Geltrex basement matrix. Cells were washed 3 times with 0.85% NaCl solution before staining. Calcein AM and propidium iodide were used to track cell membrane integrity. NucBlue was utilized to stain each cell nucleus, and Cy5-functionalized oligonucleotides were used to formulate PS-Ag-SNAs in order to observe the relative locations of the particles. Cells were imaged with an Olympus IX71 inverted microscope using a Photometrics CoolSnap HQ2 camera.

2.11. Murine Superficial Skin Infection Model with A. baumannii UNT086-1

For the *in vivo* assessment of PS-Ag-SNA activity, three sample groups (n=5) and two control groups (colistin, n = 5; PBS control, n = 5) of CD-1 female mice were induced with partial neutropenia (cyclophosphamide, 150 mg/kg) 4 days prior to infection. The infection site was the back dorsal surface of each mouse, which was shaved prior to inoculation. The shaved area on each mouse was abraded with a +50 grit emory board, which resulted in a smooth, red, shiny surface with no bleeding. Five microliters of inoculum was then added to each mouse, with first topical application of PS-Ag-SNAs (5 nM), Ag-NPs (5 nM), unconjugated oligonucleotide (225 nM), or colistin (2.6 µM) occurring 4 hours post-infection. Mice were treated twice per day for 3 days. The mice were sacrificed 12 hours after the final application of antibiotics, and a tissue sample approximately 2 cm² in area was excised. Tissue samples were homogenized in 2 mL of PBS with a Polytron PT10-35 and placed on ice. CFU counts were executed after 18 hours of incubation at 37°C on brain heart infusion agar with charcoal. These experiments were conducted by the University of North Texas Health Science Center.

2.12. Statistical Analysis

One-way ANOVA with Tukey's HSD was used to determine the significance of the *in vivo* data, and a t-test was performed on the toxicity data.

3. RESULTS AND DISCUSSION

3.1. Design and Synthesis of Ag-SNAs

One of the challenges in using silver-based antimicrobials, and Ag-NPs in particular, is unwanted toxicity toward host cells [33]. Therefore, in order to increase the therapeutic index, pharmacokinetic properties, and stability of the Ag-NPs a common strategy has been to coat or functionalize Ag-NPs with various materials, such as chitosan [34]. While chitosan-coated silver nanoparticles demonstrate reduced toxicity, chitosan, as a positively-charged polymer, is subject to biofouling and low colloidal stability *in vivo* [35]. Ag-SNAs present an oligonucleotide coating on the nanoparticles, which can be co-functionalized with other molecules. As discussed earlier in the paper, our sequence was designed with antisense functionality in mind, however as we will detail in the following paragraphs, it became clear

that the sequence was irrelevant to the function of the Ag-SNAs, and that the oligonucleotide performed a far more structural role.

On our PS-Ag-SNAs we co-functionalized PEG strands on the colloidal silver in order to aid in overall oligonucleotide loading and SNA stability. As an added effect, the PEG strands may increase the biocompatibility of Ag-NPs and reduce protein biofouling *in vivo* [36]. PS-Ag-SNAs were synthesized through a simple stepwise addition of thiol-PEG followed by a large fold-excess of thiol-oligonucleotides to Ag-NPs. PO-Ag-SNAs underwent a similar process, minus PEG and at lower salt concentrations. Pre-treating the oligonucleotides to be loaded onto SNA with dithiothreitol (DTT) allowed us to increase the binding availability of oligonucleotides to Ag-NPs [37]. Subsequently, this method made more efficient use of oligonucleotides used to synthesize Ag-SNAs as compared with prior work [25]. Our synthesis utilizes 1 nM of Ag-NPs to every 2 μ M of oligonucleotides, whereas the work of Lee *et al* utilizes a ratio of 1 nM of Ag-NPs for every 4.7 μ M of oligonucleotides. This is a 57% reduction in synthesis materials which, even if a high loading of 200 oligonucleotides per particle is assumed for Lee *et al*, results in 4.5 μ M of wasted oligonucleotide for every 1 nM of Ag-NP material, whereas our particle synthesis results in about 1.95 μ M of waste. Additionally, our synthesis resulted in a 90% or greater yield of particles, with losses accounted for during purification. A 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) test was utilized to quantify free-thiol and ensure that excess DTT was removed from the oligonucleotides after desalting. We only utilized DTNB-tested oligonucleotides that were within 5% of the initial concentration of oligonucleotide, according to the reading on the UV-vis spectrometer. If samples presented a significantly higher reading, it indicated that DTT still remained in excess, and the samples needed to be purified by NAP columns once more. Any trace amounts of undetected DTT were likely removed during particle washing. This process is essential, as any toxic effects from DTT would have been undesirable in our experiments. In addition, our synthesis allowed for formulation of Ag-SNAs with variable oligonucleotide backbone chemistries while potentially improving particle stability (Supplemental Table S1). All oligonucleotide sequences were cross-referenced with a BLAST search to decrease the likelihood of off-target gene regulatory effects in human cells.

3.2. Particle Characterization

To verify the successful functionalization of Ag-NPs, we evaluated particle size and charge potential prior to *in vitro* and *in vivo* experimentation. Ag-NPs were not stabilized with capping agents and were suspended in H₂O. TEM analysis was used to determine the mean particle diameter and distribution of Ag-SNAs and Ag-NPs used in these experiments. ImageJ software analyzed particle diameter in the TEM images, and the mean size of the Ag-NPs was 25.8 ± 4.8 nm. The functionalized PS-Ag-SNAs had 45 ± 4 oligonucleotides per particle via OliGreen assay and were measured to have a mean diameter of 19.7 ± 4.1 nm. PO-Ag-SNAs contained 43 ± 5 oligonucleotides per particle, and our scramble PS-Ag-SNAs were loaded with 48 ± 3 oligonucleotides per particle. Based on past work, we expect that PS-Ag-SNAs are loaded with 200-400 PEG strands per particle [38,39].

In addition, our DLS analysis yielded diameter mean averages of 26.26 ± 3.49 nm (PS-Ag-SNAs) and 21.94 ± 0.54 nm (Ag-NPs). There are some inconsistencies between the TEM and DLS analyses, however this may be explained by sample variability or as an effect of the rigorous synthesis and purification process for Ag-SNAs. The increased DLS diameter of the PS-Ag-SNAs may be an artifact of the oligonucleotides present on the particle surface. Zeta potential measurements indicated the presence of negatively charged oligonucleotides on Ag-SNAs, with a shift in potential from -2.04 mV to -16 mV after particle functionalization. Additional data corresponding to the characterization of the particles are found in Supplemental Fig. S1. The characterization results indicated that our particles were functional, stable SNAs, possessing properties consistent with prior data [19,21].

3.3. Ag-SNAs Antibacterial Activity In Vitro

To evaluate the antimicrobial potency of the Ag-SNA variants relative to controls, 50 μ L cultures of *A. baumannii* AYE, *A. baumannii* UNT086-1, or MRSA at 10^4 cells/mL were each challenged with 50 μ L

variations of Ag-SNAs, Ag-NPs, or unconjugated oligonucleotides (100 μ L total). Nanoparticles formulated with PEG but without oligonucleotides were not stable, which was consistent with prior work indicating similar PEG-Ag-NP instability under various conditions.[40] We hypothesized that stable PEG-Ag-NPs require direct synthesis from AgNO₃ and capping with PEG-SH,[41] therefore we did not test PEG-Ag-NPs. Two groups of Ag-SNAs were utilized: particles with PS oligonucleotides and particles with PO oligonucleotides. The comparison of PS and PO allowed us to observe any potential influence of oligonucleotide backbone chemistry on particle efficacy. Fig. 1a-c shows the growth inhibition as a function of concentration of *A. baumannii* AYE, *A. baumannii* UNT086-1, and MRSA as detected *via* optical density using a 96-well plate reader at 900 nm. PS- and PO-Ag-SNAs generally showed a 10-16-fold reduction in MIC (0.3125 nM Ag-SNAs versus 5 nM Ag-NPs) and IC50 (0.41 Ag-SNAs versus 4.3 Ag-NPs), both for the Gram-positive MRSA and the Gram-negative *A. baumannii*. While all PS-Ag-SNAs used to challenge *A. baumannii* exhibited a 2-fold increase in efficacy over PO-Ag-SNAs, both SNA groups had comparably lower MICs as compared to bare Ag-NPs. Interestingly, PS- and PO-Ag-SNAs displayed similar activity against MRSA. Importantly, neither PS nor PO unconjugated oligonucleotide displayed any activity when used alone against the bacterial cells.

Another important implication was that PS- and PO-Ag-SNAs performed comparably while the PO-Ag-SNAs possessed no PEG, minimizing any potential impact made by including PEG molecules on silver activity. Additionally, while PEG-Ag-NPs were attempted by our group, we found that they could not be synthesized and purified in stable state, particularly under media or increased salt conditions. These observations indicated to us the decreased need and relevance of a PEG-Ag-NP comparison. We also conducted preliminary experiments varying the PEG length used to stabilize the PS-Ag-SNAs. Shorter PEG chains (2 kDa and 5 kDa) had higher potency than a longer PEG chain (15 kDa) (Supplemental Fig. S9). This is consistent with the view that direct interaction of the PS- and PO-Ag-SNAs with the bacteria is important to their enhanced activity. Additionally, the Ag-SNA variants resist dissolution of silver ions and aggregation more readily than Ag-NPs (Supplemental Table S1), which results in a more efficient and lethal exposure of silver ions to bacterial membranes, according to previous work.[42]

To evaluate the kinetics of antibacterial action, we performed a kill-curve experiment (Fig. 1d). PS-Ag-SNAs effectively eliminated the bacteria after 5 hours of incubation, with no detectable growth *via* CFU by the 7-hour mark. In contrast, Ag-NPs did not eliminate the bacteria by the end of the experiment (8 hours) and were slower to treat the infection than PS-Ag-SNAs. Overall, a marked gap in efficacy existed between both groups of Ag-SNAs and Ag-NPs, particularly in the case of the DR Gram-negative bacteria. This is a clear indication that the SNA construct is important to improving the functionality of silver nanoparticles as an antimicrobial.

We synthesized batches of gold-core (Au) SNAs utilizing the same methods as we employed for PS- and PO-Ag-SNAs. PS- and PO-Au-SNAs both contained between 90-100 oligonucleotides per particle. The Au-SNAs were used to challenge *A. baumannii* AYE along with an ampicillin positive control (Fig. 1e). The results indicate that without the silver core, the particles do not kill bacteria through any means related to the functionalized oligonucleotides. This, along with the lack of activity brought upon by the free oligonucleotides seen in Fig. 1a-c, indicates that there is no bacterial antisense activity attributable to using these oligonucleotides in SNA format.

Lastly, we confirmed whether different sequences used on the PS-Ag-SNAs would impact efficacy. In Figure 1f, we compared PS-Ag-SNAs synthesized with the sequence discussed previously and with a scrambled control shown not to match any significant targets in *A. baumannii* AYE. The results further support that hypothesis that the oligonucleotide sequence is irrelevant when considering the impact of antisense interactions.

3.4. PS-Ag-SNAs Interact with Bacterial Cell Membranes

We hypothesized that the differences in activity could be the result of differences in how the PS-Ag-SNAs interacted with bacteria. Accordingly, we sought to understand how the PS-Ag-SNAs interacted with bacteria through microscopy techniques.

A. baumannii AYE membrane integrity in the presence of PS-Ag-SNAs or Ag-NPs was evaluated using a propidium iodide and SYTO 9 stain via fluorescence microscopy (Fig. 2a). Images indicate that treatment with PS-Ag-SNAs resulted in a greater degree of bacterial membrane leakage as compared to Ag-NPs. It is important to note that both PS-Ag-SNAs and Ag-NPs permeabilized the cell membranes extensively enough to allow propidium iodide to stain the DNA inside the cells. However, only the PS-Ag-SNAs appeared to render cells completely inviable, indicated by the low number of cells effectively retaining the SYTO 9 stain. Conversely, the Ag-NP-treated cells appear to possess a greater amount SYTO 9 dye, and therefore greater membrane integrity.

We also used TEM to determine whether the PS-Ag-SNAs penetrated the cytosol or if the particles were purely membrane-associated (Fig. 2b). The TEM images suggest PS-Ag-SNAs associated with bacterial cell membranes. This would indicate that gene regulation was highly unlikely, as cell penetration is necessary to achieve this effect. Furthermore, fluorescence images indicated increased membrane damage by PS-Ag-SNAs, consistent with our current understanding of silver's antimicrobial properties.[43] The enhanced antimicrobial effects of PS-Ag-SNAs are thus potentially a result of improved particle-bacteria contact.[44] TEM images of Ag-NPs were not included in our study as prior work has already well-described these interactions and serves as a good comparison to our data.[45]

To track the association of PS-Ag-SNAs with bacterial cell populations, fluorescence microscopy was utilized to look at the association of particles and cells. PS-Ag-SNAs functionalized with 5'-Cy5 fluorescent oligonucleotides were mixed with bacteria *in vitro*. In Fig. 2c, untreated *A. baumannii* AYE cells are shown in comparison with PS-Ag-SNA treated cells. Treated cells demonstrated a strong fluorescence signal that colocalized with the bacteria. Previous works observed similar associations of Ag-NPs and bacteria, indicating a similar association of Ag-NPs and bacterial cells.[46]

3.5. PS-Ag-SNAs Synergize with Ampicillin

Silver is a unique antimicrobial in part because of its ability to synergize with commonly used antibiotics [28]. Ag-NPs have been functionalized with ampicillin to attack Gram-negative bacteria by way of metal depletion, which interferes with DNA unwinding [7]. Here, we sought to compare the synergistic effects of PS-Ag-SNAs or Ag-NPs with ampicillin against *A. baumannii* UNT086-1 and MRSA. Our results show that PS-Ag-SNAs are superior to Ag-NPs. Ampicillin alone, to which both bacteria displayed resistance, had MIC values of 10.8 mM and 168.3 μ M for *A. baumannii* UNT086-1 and MRSA, respectively. Alone, PS-Ag-SNAs displayed MIC values of 1 nM and 3.125 nM for *A. baumannii* UNT086-1 and MRSA, respectively. The fractional inhibitory concentration (FIC) is a method to analyze the degree of synergy between two agents. The FIC values of MRSA and *A. baumannii* UNT086-1 challenged by both groups of silver particles are shown in the graphs of Fig. 3a, b. The MIC values of both PS-Ag-SNAs and ampicillin decrease significantly when used together. Both bacterial groups tested demonstrated a significant 4-fold and 128-fold reduction in effective PS-Ag-SNA and ampicillin MIC values, respectively. We also assessed synergy with vancomycin and ciprofloxacin, showing a 4-fold reduction in MIC value of both antibiotics (Supplemental Fig. S4). The data reveal two things: support of prior work, which demonstrates synergy between antibiotics and silver [47], and demonstration of the enhanced synergistic effects of PS-Ag-SNAs as compared to conventional Ag-NPs in conjunction with antibiotics. This synergy is likely a product of the destabilization of the bacterial membrane by both silver particles and ampicillin, and may include the inhibition of DNA unwinding within damaged cells [42] via the interactions of the silver cores within the SNAs, however more comprehensive evidence is required to confirm this hypothesis.

3.6. Reduced Toxicity of Ag-SNAs as Compared to Ag-NPs in Human Cell Lines

Concerns over non-specific host toxicity of silver nanomaterials[33,48] prompted us to probe differences in toxicity more deeply. We evaluated cell membrane integrity with the use of flow cytometry and a propidium iodide stain, which bound to exposed DNA and RNA. Healthy or functional cells were stained with calcein AM. This allowed us to differentiate between silver-damaged cells and unaffected

cells *in vitro*. We performed an alamarBlue assay to establish a relative toxicity index for these particles *in vitro*. The graphs in Fig. 4 outline the difference in toxicity between Ag-SNA variants and Ag-NPs. Flow cytometry (Fig. 4a) measured three distinct HEK-293 cell populations (dead, damaged, or healthy cells) treated with 3.75 nM of PS-Ag-SNAs or Ag-NPs. Ag-NPs killed or damaged approximately 80% of the total cell population, in contrast to fewer than 40% for PS-Ag-SNAs (Fig. 4a). This is in contrast with bacterial cell interactions, which demonstrated higher membrane sensitivity to PS-Ag-SNAs.

The viability data (Fig. 4b) show a 14-fold difference between the therapeutic MIC of *A. baumannii* AYE and the concentration where a statistically significant loss in human foreskin keratinocyte (HFK) viability occurs (estimated at 4.375 nM of PS- and PO-Ag-SNAs). No statistical significance was observed between the two Ag-SNA populations. In comparison, polymyxin antibiotics are known to display some adverse nephrotoxic side-effects if delivered intravenously, with a therapeutic index less than 2.5-fold.[49] Importantly, PS- and PO-Ag-SNAs demonstrate a decrease in mammalian cell toxicity as compared to Ag-NPs, which is critical in any *in vivo* applications of the particles. We cannot solely attribute this decrease in toxicity as an effect of PEG functionalization, as the PO-Ag-SNAs do not contain this polymer. This indicates that the mechanisms for decreased toxicity must be primarily associated with another interaction between mammalian cells and Ag-SNAs. The eukaryotic cellular toxicity that is observed is likely due to the same mechanisms as bacterial toxicity: membrane permeabilization and metal depletion.

We theorize that the reduction in toxicity may also be due in part to cellular uptake mechanisms that are associated with SNAs [20,23] (Fig. 5). These images display a HEK-293 cell associated with fluorescently-labeled PS-Ag-SNAs. The SNAs appear to be concentrated in two areas, around the cell and within the cellular membrane, around the nucleus. These preliminary findings may indicate signs of endocytosis, which would be consistent with prior understanding of cellular uptake by SNAs [50], however further investigation is required to determine if this is the mechanism by which the Ag-SNAs exhibit decreased toxicity *in vitro*. Additionally, has been shown that ionic silver has a less toxic impact when compared to chelated silver [42]. This would be relevant in the case of endosomal uptake, where the Ag-SNAs would be dissolved in an acidic compartment. This process would effectively decrease the ability of the Ag-SNA to interfere with DNA unwinding inside cells.

3.7. *In Vivo* Topical Application of PS-Ag-SNAs

Once we determined *in vitro* toxicity of the NPs, our next step was to investigate the use of PS-Ag-SNAs *in vivo*. The treatment of an *A. baumannii* UNT086-1 infection in a topical wound was explored. Infections demonstrated a statistically significant response to PS-Ag-SNA treatment with a log₁₀ reduction of 1.51 when compared to the untreated controls (Fig. 6). Comparisons with Ag-NPs (log₁₀ reduction = 0.63) and unconjugated oligonucleotide (log₁₀ reduction = 0.58) further emphasize the improved efficacy of PS-Ag-SNAs. These results demonstrate that antibacterial activity of these PS-Ag-SNAs extend beyond *in vitro* examination and is transferable to *in vivo* examination, specifically for a clinically relevant species of DR Gram-negative bacteria.

4. Conclusions

In summary, the introduced PS- and PO-Ag-SNAs represent a new strategy in antibiotic design with applicability against DR Gram-positive and DR Gram-negative organisms. Our data demonstrate that Ag-NPs functionalized with PS- and PO-oligonucleotides offer a means for Ag-NPs to better interact with the surface of bacterial cells, while improving efficacy and decreasing the toxic effects for the host. The Ag-SNA also presents the possibility of using low-dose silver synergistically with existing antibiotics, such as ampicillin. Ag-SNAs exhibit a functional, stable, and broad spectrum approach to antimicrobials. Our data suggests that the polyvalent nature of the SNA construct combined with the biocidal nature of silver nanoparticles brings us closer to the possibility of safe and powerful silver-based topical antibiotics. These Ag-SNAs differentiate themselves through their enhanced antimicrobial efficacy, potent antibiotic synergy, and improved control over unwanted toxicity as compared to silver nanoparticles, and may find

applications in treating clinically challenging, multi-DR infections. We believe our data indicates that the unique properties of the SNA construct are instrumental in bringing about these differences in efficacy and stability.

Animal Care and Use Statement

The experimental protocols and standards of care for mice involved in the topical infection *in vivo* study were approved for use under the guidelines and supervision of the University of North Texas Health Science Center's Institutional Animal Care and Use Committee. Experiments were also in accordance with the regulatory guidelines of the U.S. Army Medical Department's Animal Care and Use Review Office. All experiments were conducted within local and federal laws.

Acknowledgements

We would like to extend thanks to B. Tutterow, A. Schook, V. Hilgenberg, and S. Mix for their excellent technical support. This work made use of the EPIC facility (NUANCE Center-Northwestern University), which has received support from the MRSEC program (NSF DMR-1121262) at the Materials Research Center; the Nanoscale Science and Engineering Center (NSF EEC-0647560) at the International Institute for Nanotechnology; and the State of Illinois, through the International Institute for Nanotechnology. We would also like to thank W. Weiss and the UNT Health Sciences Center for execution and analysis of the *in vivo* work, J. Goolsby and Paragon Bioservices for preparing and acquiring the TEM images of bacteria, and we are grateful for the support of the Defense Advanced Research Projects Agency under grant number W911QX-13-C-0070.

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Scheme 1 Silver spherical nucleic acid design and interaction. Using a silver nanoparticle core, a basic PS-Ag-SNA is formulated with the adsorption of 3'-thiol-oligonucleotide and methoxyl poly(ethylene glycol) thiol. SNAs are known to adhere to or transfect across various types of cell membranes, which serve as the driving force behind a silver-derived SNA. The unique properties of PS- and PO-Ag-SNAs enable the use of these particles as potent antimicrobials.

Fig. 1 Growth curves for treated bacteria. (a) *A. baumannii* AYE treated with PS-Ag-SNAs demonstrates an MIC value of 0.3125 nM. The corresponding PO-Ag-SNAs and Ag-NPs displayed MICs of 0.625 and 5 nM, respectively. (b) *A. baumannii* UNT086-1 demonstrated MICs of 0.625, 1.25, and 5 nM when comparing PS-Ag-SNAs, PO-Ag-SNAs, and Ag-NPs, respectively. (c) MRSA showed a similar reaction to the particles, with MICs of 2.5, 5, and 10 nM for PS-Ag-SNAs, PO-Ag-SNAs, and Ag-NPs, respectively. Unconjugated oligonucleotide did not display activity against any bacteria tested. (d) The time-dependent kill curve of Ag-SNAs and Ag-NPs shows that the SNAs were able to completely eliminate bacteria within 7 hours, whereas Ag-NPs were only able to reduce the bacterial population at the same Ag concentration. (e) Using the same oligonucleotide sequences and a scramble sequence, gold SNAs were formulated and tested against *A. baumannii* AYE. No growth inhibition was observed, indicating a lack of effective antisense activity on the part of the oligonucleotides. (f) Gene-targeted Ag-SNAs were compared with Ag-SNAs formulated with a randomized scramble control oligonucleotide. There were not any detectable differences stemming between the Ag-SNAs, further suggesting that gene regulation was not a factor in the efficacy of these particles.

Fig. 2 (a) *A. baumannii* AYE was stained with propidium iodide and SYTO 9 after treatment with 1 nM of PS-Ag-SNAs or Ag-NPs. Interestingly, PS-Ag-SNAs appeared to damage cell membranes more extensively compared with Ag-NPs. The low number of cells stained with SYTO 9 and large number of cells exclusively stained by propidium iodide indicate significant membrane leakage in the PS-Ag-SNA sample. In contrast, a significant number of cells treated with Ag-NPs retain SYTO 9 stain, and appear to be damaged but viable. (b) TEM images were taken at 30,000x (untreated and left Ag-SNA) and 65,000x (right PS-Ag-SNA) direct magnification. Images display the accumulation of PS-Ag-SNAs on the bacterial membrane. There are signs of membrane deterioration visible in the PS-Ag-SNA affected cell. No significant penetration into the cytosol was observed in the samples. (c) Fluorescence microscopy images of untreated (left) and Cy5-labeled PS-Ag-SNA treated (right) *A. baumannii* AYE demonstrate the association of particles with cells in a bacterial population. Interestingly, a universal distribution of particles is not observed.

Fig. 3 Ampicillin acts synergistically with PS-Ag-SNAs and Ag-NPs. (a) In MRSA, the best results were seen when PS-Ag-SNAs and ampicillin were dosed at 0.78 nM and 1.32 μ M, respectively. (b) The clinical baumannii strain, UNT086-1, displayed similar results. Only 0.25 nM of PS-Ag-SNAs and 84 μ M of ampicillin were used at the greatest reduction in MIC with respect to ampicillin. In both tested bacterial strains, these numbers represent 4-fold and 128 fold decreases in the respective dosage of PS-Ag-SNAs and ampicillin.

Fig. 4 (a) Flow cytometry was used to investigate cell membrane integrity in the presence of Ag-NPs and Ag-SNAs. HEK-293 cells were treated with 3.75 nM of PS-Ag-SNAs or Ag-NPs. Red stains (propidium iodide) indicate dead cells, whereas green (calcein AM) indicates healthy cells with intact membranes, and a mixture indicates membrane damage on living cells. PS-Ag-SNAs appear to be significantly less damaging to cell membranes. (b) To establish an in vitro toxicity index between the effective treatment of bacteria and toxicity to host cells, human foreskin keratinocytes were treated with various concentrations of Ag-SNA variants and Ag-NPs. A t-test determined that Ag-NPs appeared to have statistically significant (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) toxic effects on cells at several concentrations compared to PS- and PO-Ag-SNAs. This difference may be attributable to relative SNA stability and more freely available silver ions as compared to the Ag-NPs.

Fig. 5 Fluorescent PS-Ag-SNA interactions with HEK-293 cells. The same cell is displayed with a stain of the cell nucleus (a), membrane (b), and PS-Ag-SNAs (c). The merged image (d) displays all stains concurrently. The Cy5 image appears to indicate both membrane-associated particles as well as localized groups of particles around the nucleus. This may be an indicator of endocytosis.

Fig. 6 Topical Infection of *A. baumannii* UNT086-1 treated with Ag-SNAs. Ag-SNAs displayed a decrease in CFUs after treatment, with a significant $\log_{10}$ reduction of 1.51 ($p < 0.05$) compared to the untreated control. Ag-NPs and unconjugated oligonucleotide did not demonstrate significant activity, with minor $\log_{10}$ reductions of 0.63 and 0.58. This data appears consistent with our in vitro results, in which Ag-SNAs were the top performer.