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Broad Spectrum Antimicrobial Nanoparticles with Low Toxicity to Prevent Biofilm Formation on Urologic Devices

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ABSTRACT

Antimicrobial coatings for medical implants are critical in preventing device failures and infections. Antibiotics are often used as prophylactic or coatings but fail to prevent biofilm formation and drive antibiotic resistance. Herein, the antibacterial and antibiofilm activities of different polyhydroxy fullerene-based metal nanoparticle coatings on polyurethane discs were quantified after exposure to *Escherichia coli*. Gold-silver nanoparticles (GSNP) exhibited superior antibacterial activity compared to other silver-containing nanoparticles. GSNPs were evaluated against *Escherichia coli, Enterococcus faecalis, Enterobacter hormaechei, Klebsiella oxytoca, Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from ureteral stents and inflatable penile prostheses and achieved 100% reduction of all tested urologic pathogens at physiological relevant bacterial loads ($p<0.0005$). GSNPs inactivate bacteria by reactive oxygen species production with the estimated minimum inhibitory concentrations slightly higher for Gram-positive than Gram-negative bacteria with highest observed for *S. epidermidis* at 2.23 μg mL^{-1}. Safety studies with fibroblasts demonstrate that GSNPs at estimated minimum inhibitory concentrations have minimal effect (<20%) on cell viability. Further, the GSNPs were able to reduce bacteria by six logs more than commercial nanoparticles. GSNPs represent a promising strategy for preventing biofilm formation on medical devices and implants due to their broad antibacterial activity and low toxicity.

Keywords: Antimicrobial, Biofilm, Coatings, Nanoparticles, Urologic Implants

INTRODUCTION

Device-associated infections account for over 25% of hospital-acquired infections.[1] This includes catheter-associated urinary tract infections, with estimated treatment costs ranging from $340-450 million per year when adjusted for inflation.[1-3] Implantable devices, such as penile prostheses, carry a major risk of severe infection including sepsis due to bacterial attachment and biofilm formation on their surfaces.[4, 5] Additionally, biofilms promote ureteral stent encrustation and associated urinary tract infections. [6] Due to biofilm-associated pathologies, devices often require removal or frequent exchanges, which impact morbidity, quality of life, and costs.[6, 7] Biofilms are a combination of microorganism and extracellular polymeric substances composed of proteins, polysaccharides, lipids, and other substances in complex three-dimensional structures adherent to surfaces. Importantly, they provide natural defense mechanisms against host immune responses and antibiotics.[8] In particular, biofilms exhibit resistance to antibiotic concentrations markedly higher than unattached, planktonic forms of the same bacterial species, which can then render these antibiotics ineffective due to prohibitive toxicity.[8]

Proposed strategies to prevent biofilm formation include polymeric surfaces (in addition to the polymers used in clinical practice), antibiotic and silver (Ag) coatings, antimicrobial nanoparticle coatings, and physical-mechanical properties that inhibit biofilm proliferation.[9-11] While current strategies have

achieved some clinical success, their effectiveness is limited after revision procedures, and the overall practical effectiveness has been questioned.[12, 13] Moreover, antibiotic-based coatings carry the concern of promoting resistance.[14]

One emerging strategy for bacterial control includes using polyhydroxy fullerenes (PHF), a non-toxic nanomaterial.[15] Previous studies have found that PHF enhances the catalytic and antimicrobial activity of titanium dioxide (TiO_2) and silver (Ag).[16, 17] Other metals such as gold (Au) have also shown to augment Ag antimicrobial activity in nanoparticles (NPs), possibly through Ag membrane reactivity enhancement.[18] However, PHF-based bimetallic NPs have not been evaluated. The objective of this study was to evaluate PHF-based metal NPs (MNP) in biofilm formation prevention, antibacterial activity, and toxicity.

RESULTS

Metal Nanoparticles

Different metal nanoparticles were synthesized by polyhydroxy fullerene (PHF) mediated synthesis method at room temperature. The formation of bimetallic nanoparticles, such as gold-silver nanoparticles, was validated through multiple imaging modalities, and are approximately 5 nm in size with a thin PHF coating (Figure 1A). The energy dispersive X-ray (Figure 1B) suggests that gold and silver are present as an alloy. The metal

nanoparticles were coated on polyurethane discs (Figure 1C) by drop casting, which appears to be non-uniform (Figure 1D). Since the focus of this study is evaluate the antibacterial and antibiofilm activity, the coatings were used without further optimization.

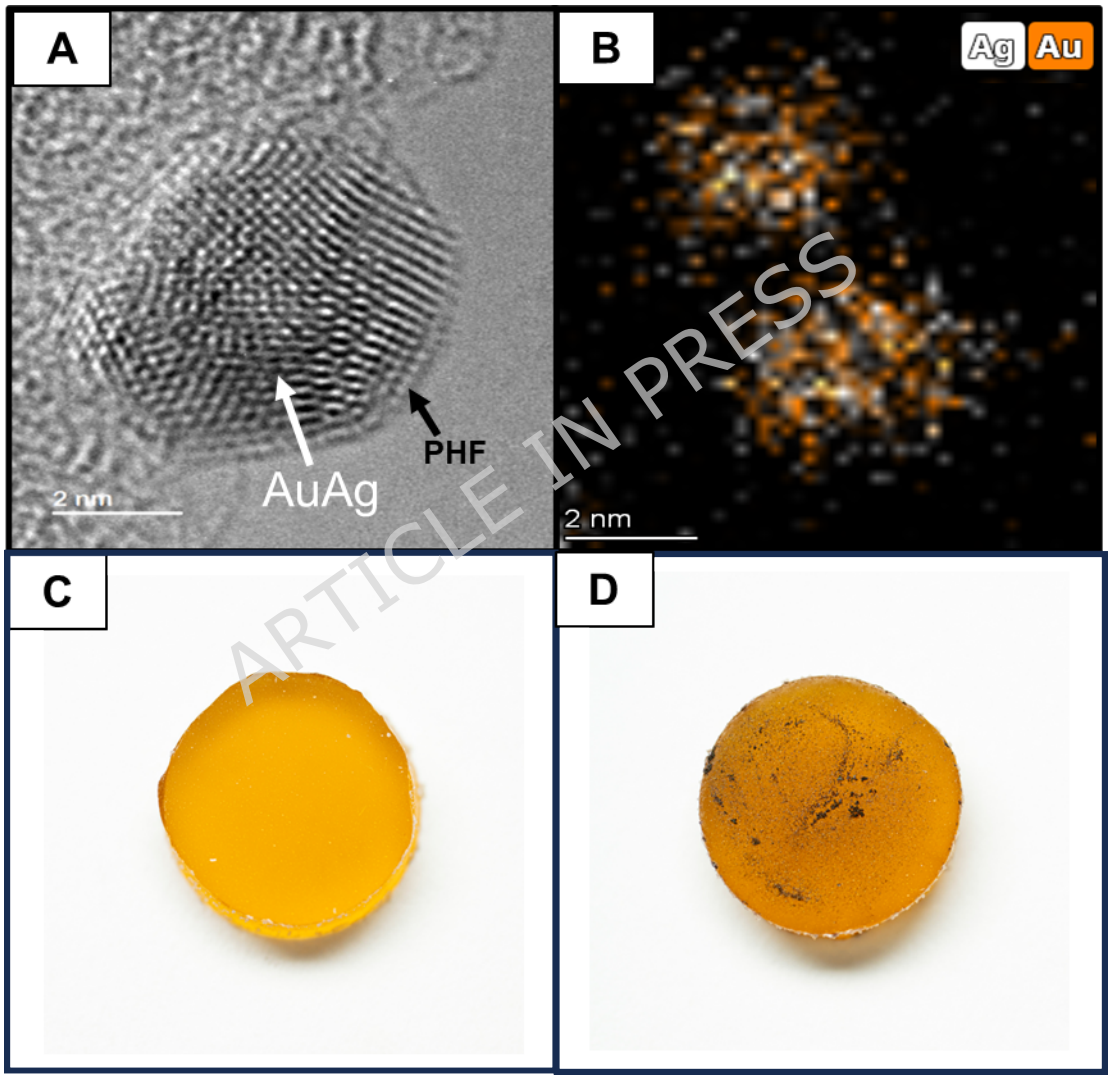


Figure 1: GSNP coatings and characterization. **(A)** aberration corrected-transmission electron micrograph of GSNP that confirms size and ultrastructure with a monolayer of polyhydroxy fullerene (PHF) coating (black arrow) over gold (Au) and silver (Ag) (white arrow). **(B)** energy-dispersive X-ray spectroscopy elemental mapping of GSNPs where orange and white pixels represent Au and Ag, respectively. White scale bars illustrate 2 nm. **(C)** uncoated polyurethane (PU) disc. **(D)** GSNP coated PU disc. *GSNPs*: PHF-based gold-silver nanoparticles

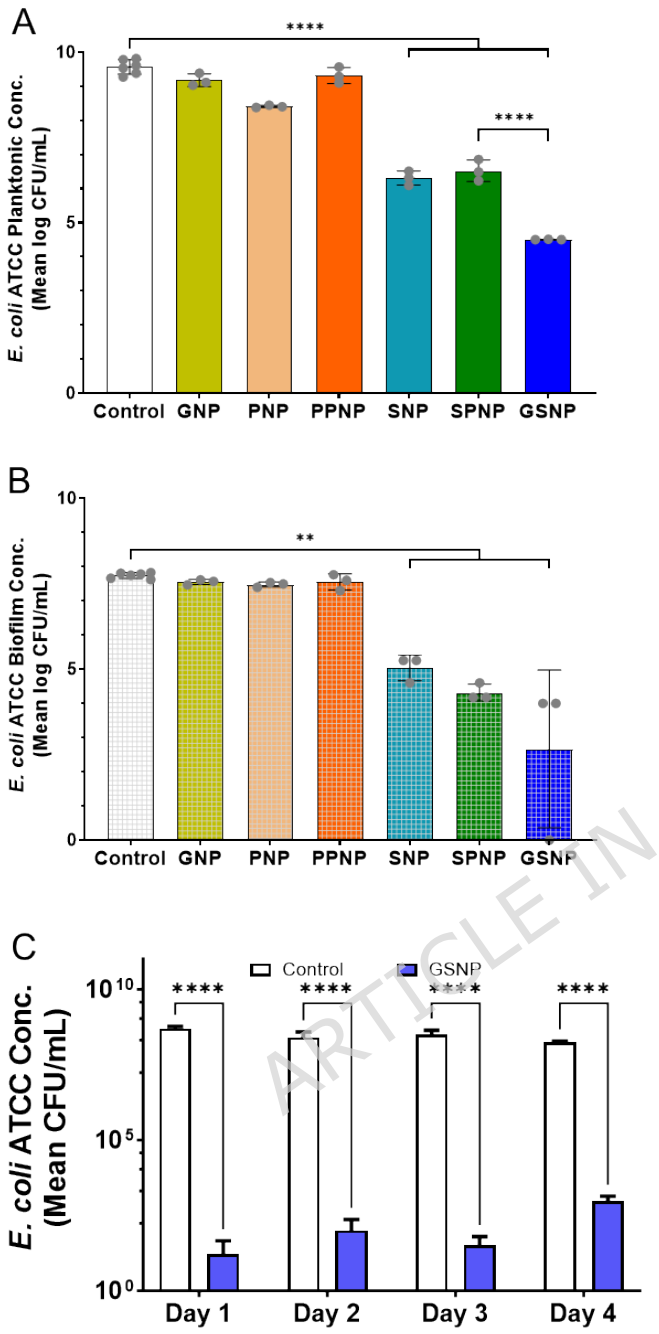


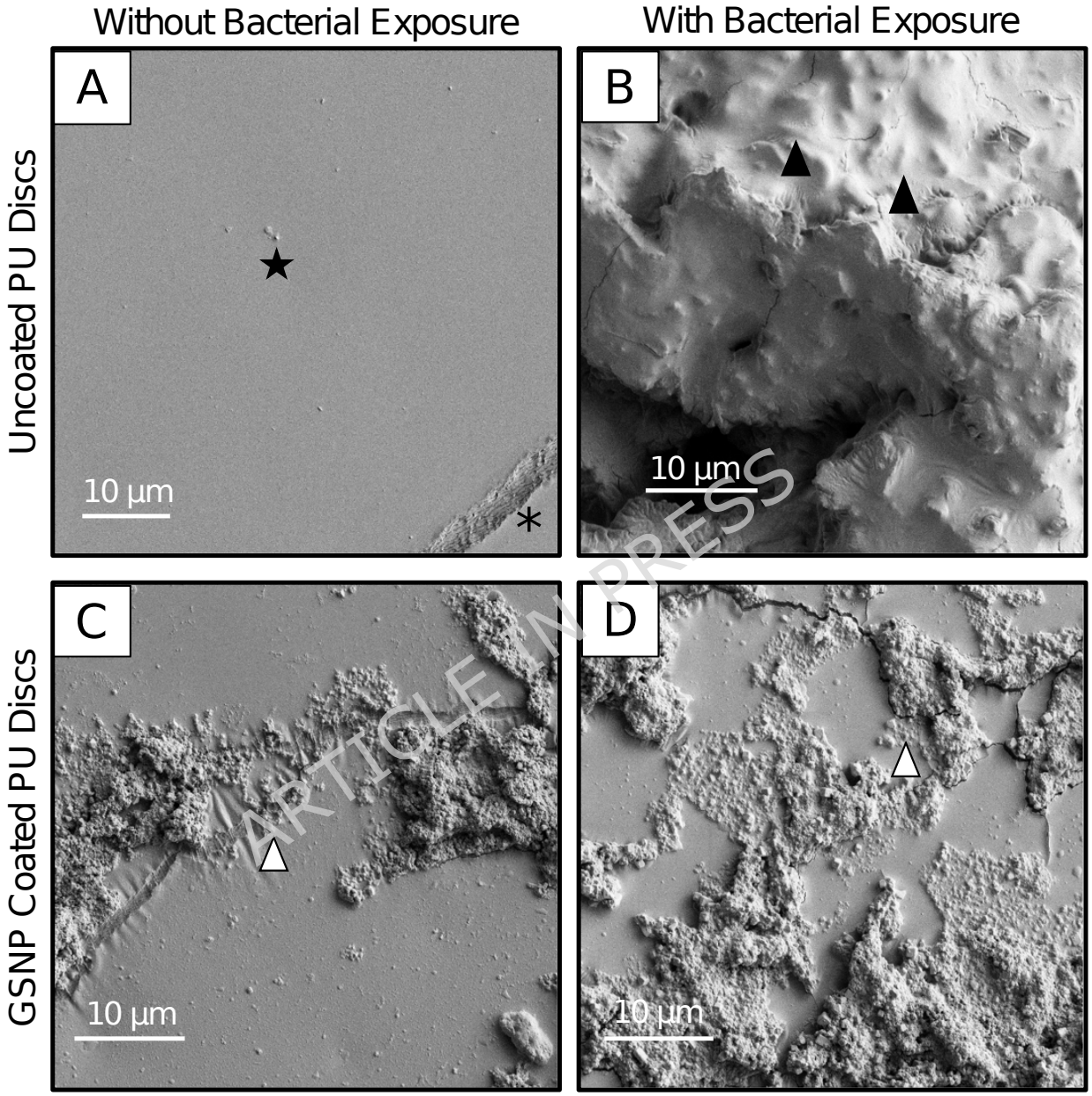
Figure 2: (A) Antibacterial, and **(B)** antibiofilm activity of different metal nanoparticles measured against *E. coli* ATCC 43886. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. *PHF*: polyhydroxy fullerenes; *GNP*: PHF-gold nanoparticles; *PNP*: PHF-palladium nanoparticles; *PPNP*: PHF-palladium-platinum nanoparticles; *SNP*: PHF-silver nanoparticles; *SPNP*: PHF-silver-platinum nanoparticles; *GSNP*: PHF-gold-silver nanoparticles. **(C)** Longitudinal experiment of GSNP drop-casted PU discs over 4 days.

Antibacterial and Antibiofilm Activity of MNPs

Consistent with the fact that gold, platinum, or palladium are not antibacterial, we did not observe antibacterial or antibiofilm activity for GNPs, PNPs and PPNPs compared to uncoated discs (Figure 2). Significant antibacterial and antibiofilm activities were observed for silver containing nanoparticles: SNPs, SPNPs, and GSNPs. (Figure 2A and 2B). GSNPs showed a significantly higher ($p<0.0001$) antibacterial activity than SNPs, achieving up to 5 log (99.999%) reductions. In contrast, there were no statistically significant differences between SPNPs and SNPs for antibacterial and antibiofilm

activities, suggesting gold as a key synergistic element.

The drop-casted GSNP coatings were evaluated for their stability in a



longitudinal experiment where the media containing *E. coli* (ATCC 43886) was replaced every 24 hours. The GSNP coating maintained high antibacterial activity for 4 days (Figure 2C). The antibacterial activity started declining from day 4 with no observable activity on fifth day due to loss of

nanoparticles and suggesting need for a better coating design for long-term activity. Scanning electron microscopy (SEM) of uncoated or GSNP-coated discs, with or without bacterial exposure, showed that *E. coli* (ATCC 43886) bacteria used in this study can easily form biofilm on polyurethane (PU) discs (Figure 3A and 3B). The nanoparticle drop-casting method creates a non-uniform coating (Figure 3C), however this GSNP coating was sufficient to prevent biofilm growth (Figure 3D).

GSNPs are Broad Spectrum Antibacterial

The antibacterial activity of GSNP was evaluated against three Gram positive and three Gram negative urologic pathogens. Initial testing at supraphysiologic bacterial concentrations demonstrated a significant antibacterial and antibiofilm activity across bacterial species (Supplementary Figure S1A and S1B), with a significantly higher effectiveness against *S. aureus.* (Supplementary Tables 1 and 2). Importantly, experiments at physiologically relevant bacterial loads (10^6 – 10^7 CFU mL^{-1}) displayed no bacterial growth (i.e., 100% reduction) in both planktonic and biofilm bacteria for all strains exposed to GSNPs coating ($p<0.0005$ for all species) (Figure 4A and 4B). Next, estimated minimum inhibitory concentration (eMIC) for GSNP nanoparticles was determined for each strain by exposing free

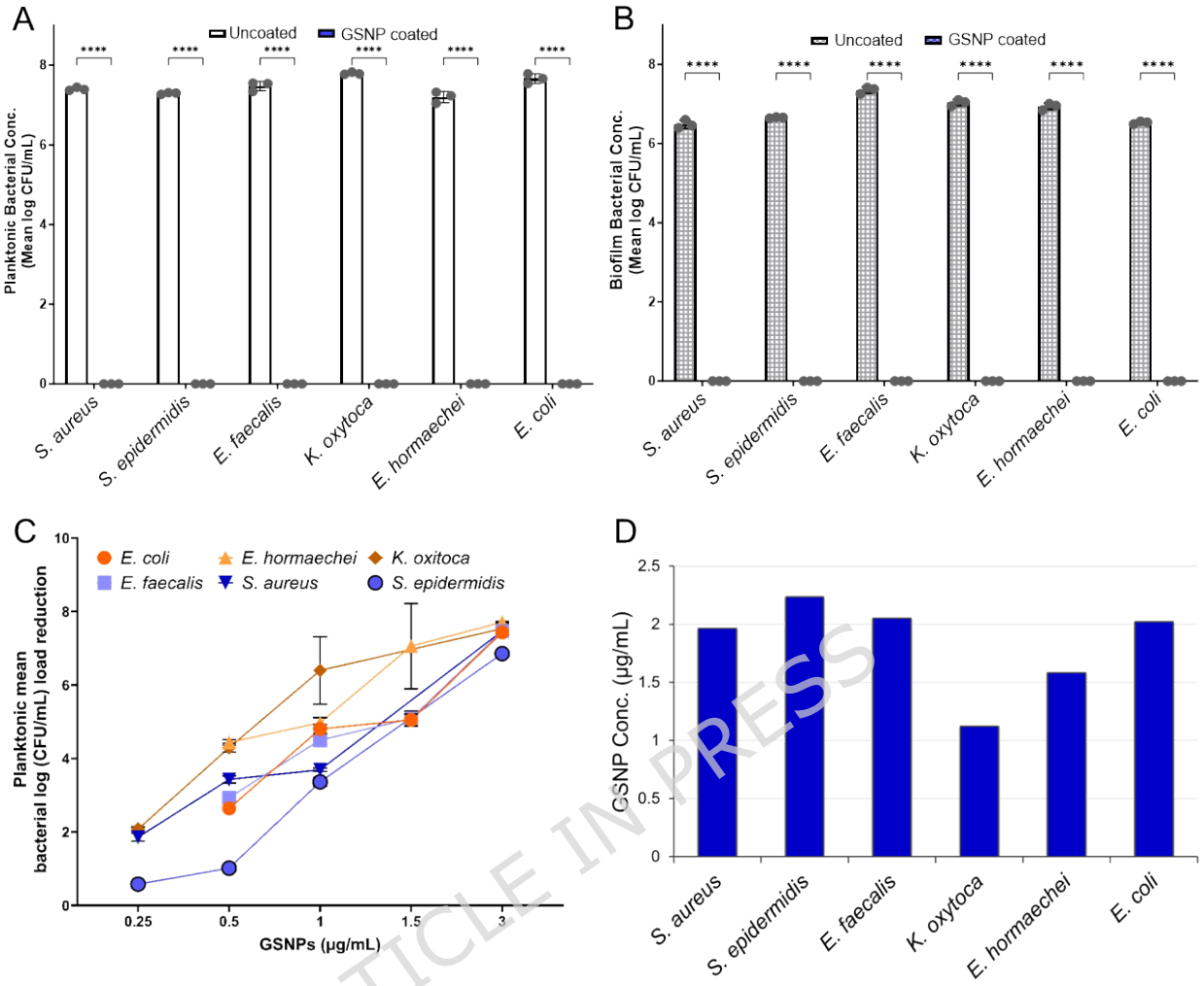


Figure 3: (A) Antibacterial and **(B)** antibiofilm activity of polyhydroxy fullerene coated gold-silver nanoparticles (GSNPs) on multiple Gram positive and Gram-negative bacterial strains. GSNP-coated polyurethane (PU) discs were exposed to physiologically relevant bacterial loads ($1.2x10^6$ - $5.9x10^7$ CFU/mL). Solid white bars represent planktonic bacterial growth in uncoated (control) discs, solid blue bars represent planktonic bacterial growth in GSNP-coated discs. Patterned white bars represent biofilm bacterial growth in control discs, and patterned blue bars represent bacterial growth in GSNP-coated discs. Scattered dots display individual samples log (CFU/mL) bacterial concentration. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.0005$ between samples. **(C)** Planktonic bacterial reduction (log CFU/mL) of different urologic pathogens as a function of increasing GSNP concentrations. **(D)** Estimated minimum inhibitory concentrations of GSNPs for different urologic pathogens. No error bars are displayed for calculated values.

floating bacteria to different concentrations of GSNPs (Figure 4C).

Importantly, no growth was observed at 3 µg mL^{-1} GSNP concentration for any strain, indicating complete inhibition at the highest tested concentration under these conditions and the MIC < 3 µg mL^{-1}. The eMIC for Gram positive

bacteria was slightly higher than Gram negative with the highest eMIC obtained for *S. epidermidis* at 2.23 µg mL^{-1} (Figure 4D).

Kinetics and Mechanism

The kinetics and preliminary mechanistic studies were carried out with *E. coli* ATCC43886 strain, quantifying bacterial load reduction following the same direct counting method used for the assays described above. A significant bactericidal activity ($p<0.0001$) was observed at 614 minutes when compared to other time points (Figure 5A). The estimated 100% bacterial load reduction period was calculated as 643 minutes. Preliminary studies suggest that the mechanism of action involves production of reactive oxygen species (ROS). GSNPs, by itself, did not produce any ROS (Figure 5B). While *E. coli* ATCC 43886 strain exhibited endogenous ROS formation, the ROS production measured was significantly higher when bacteria was exposed to GSNPs ($p<0.01$).

Safety and Comparison with Commercial NPs

The safety of GSNP was assessed with 3T3 murine fibroblasts and XTT assay. When fibroblasts were exposed to increasing concentrations of GSNPs, a 50% reduction in cell viability was observed at a calculated GSNP concentration of 3.48 µg/mL. The highest calculated eMIC was 2.23 µg mL^{-1} for *S. epidermidis* (Table 1) with a corresponding fibroblast viability of 83.22% at this concentration of GSNP. The eMICs for *K. oxytoca* and *E. hormaechei* did not

decrease calculated fibroblast viability, calculated through the equation of the line produced from Figure 5C (Table 1), therefore suggesting GSNPs are

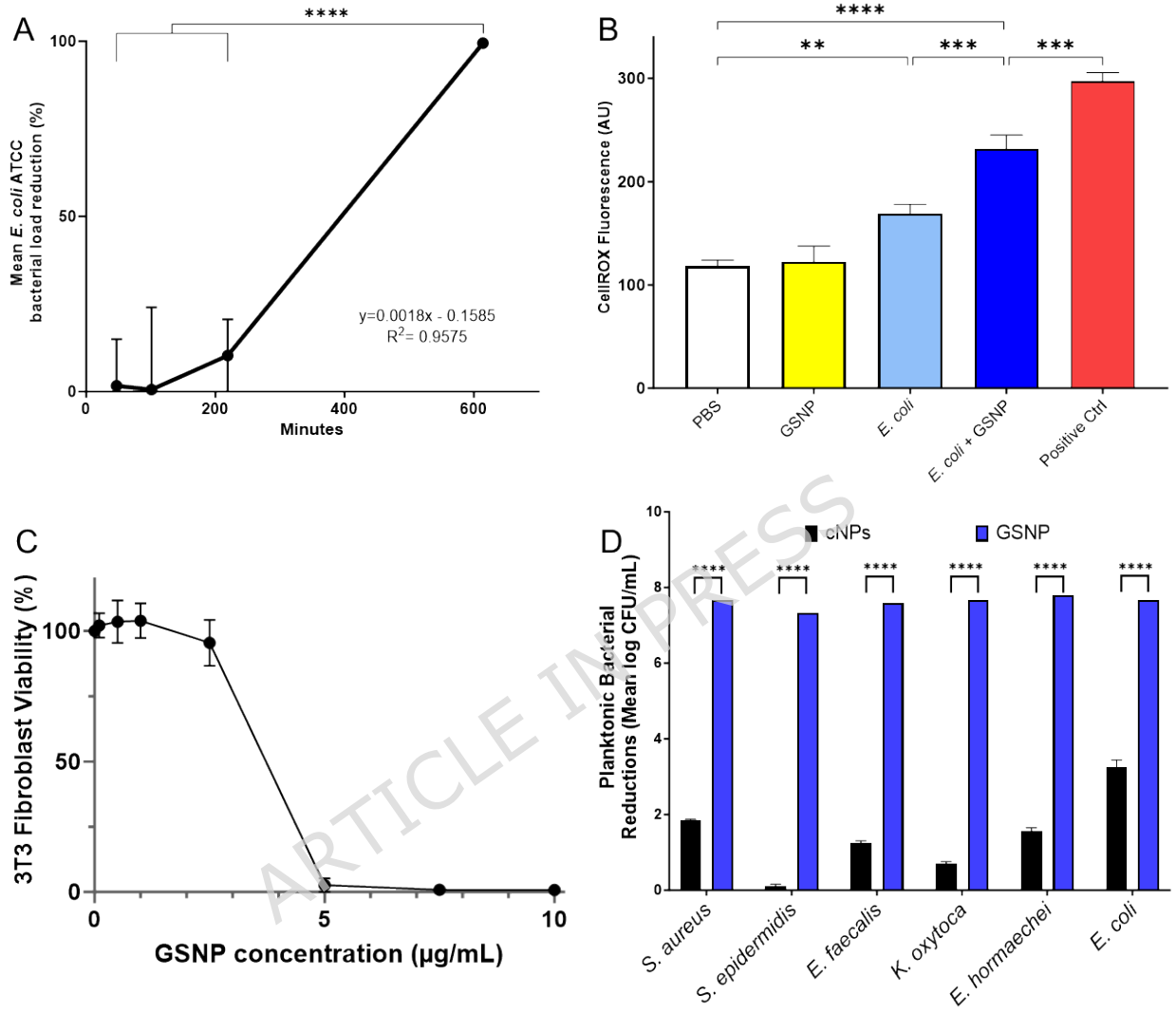


Figure 4: (A) E. coli ATCC bacterial percentage reduction when exposed to a 1 µg/mL concentration of GSNPs at different time points. **(B)** Reactive oxygen species formation in *E. coli* ATCC exposed to 1 µg/mL GSNPs for 1 hour evaluated by CellROX fluorescence. **(C)** Percent murine 3T3 fibroblast viability after exposure to different concentrations of GSNPs. **(D)** Planktonic bacterial log CFU/mL reductions of different strains exposed to commercial gold-silver nanoparticles (cNPs) and the calculated reduction when using the same concentration of GSNPs. No error bars are displayed for calculated values. **:$p < 0.01$; ***: $p < 0.005$; ****: $p < 0.0005$.

safe when used at antibacterial concentrations for diverse species. The antibacterial activity of GSNPs was compared with commercial nanoparticles (cNPs) at eMIC values, where cNPs achieved significant bacterial reduction

across species (p<0.0005 except *S. epidermidis* where p=0.01). However, the quantified reductions achieved with cNPs (tested in triplicate) were 6-7 times lower than the calculated reductions for GSNPs (p<0.0005 for all), suggesting GSNPs are a million times more effective than cNPs (Figure 5D). In Figure 5D, bacterial reductions at strain-specific estimated minimum inhibitory concentrations were based on quantitative data in Figure 4C.

Bacterial strain	**Estimated MIC (μg mL^{-1})**	**Regression fits (R^2)**	**Calculated fibroblast survival**
S. aureus	1.963	0.7883	90.41%
S. epidermidis	2.234	0.942	83.22%
E. faecalis	2.052	0.9065	88.04%
K. oxytoca	1.123	0.9693	100%
E. hormaechei	1.582	0.8971	100%
E. coli	2.022	0.9164	88.84%

Table 1: Estimated \minimum inhibitory concentrations (eMICs) for different bacterial strains are derived from dose-response CFU trends and are not CLSI/ISO MICs.

DISCUSSION

Biofilm formation on material surfaces has hindered the use of devices across medical fields. In this study, GSNPs were found to be a broad spectrum, highly effective antibacterial and antibiofilm coating for PU, a material commonly used in urology and other disciplines.[19, 20] The benefits of

developing a material capable of preventing or effectively eliminating biofilms without device removal can be practice-changing within and beyond urology. [4]

Silver nanoparticles kill bacteria mainly by binding to membrane proteins causing cell wall disruption. Silver nanoparticles have also been proposed to inactivate bacteria by producing ROS. PHF has been shown to enhance antibacterial activity of silver nanoparticles by enhancing ROS production. Gold has also been proposed to enhance antibacterial activity of silver nanoparticles by increasing ROS production. In this study, silver-containing nanoparticles possessed significant antibacterial and antibiofilm activity; while gold, by itself, did not exhibit antibacterial activity. However, only the GSNPs demonstrated a strong synergistic effect (Figure 2). Inactivation by ROS is preferred over binding to membrane proteins as the bacteria can develop resistance against the latter mechanism. Further studies are needed to determine the ability of uropathogens to develop resistance against GSNPs.

Combining gold with silver has been previously proposed to enhance antibacterial activity of silver nanoparticles. [18, 21] For example, gold-silver nanocages were evaluated against commercial strains of *E. coli, E. faecalis, S. mutans* and *S. aureus*. While these nanocages exhibited high antibacterial activity at very high concentrations (10 µg/mL) against some strains,

additional sulfadiazine was required to achieve complete bacterial eradication.[18] In contrast, our GSNPs showed 100% kill rate at low concentrations (3 μg/mL) for all urologic pathogens tested at physiological levels. To the best of our knowledge, the current study is the first description of antibacterial properties of gold-silver nanoparticles with the largest number of clinically isolated bacterial strains to date. It should be noted that the main advantage of testing material coatings with clinically isolated vs. commercial strains is the prior antibiotic exposure for isolates, which increases the validity of our results. Importantly, our GSNPs performed several fold better than commercial gold-silver nanoparticles (Figure 5D).

A limiting factor for the use of metals in medical devices is the potential for toxicity. Reported ionic silver toxic serum concentrations are variable; however, given the silver content in GSNPs and different blood absorption barriers, the likelihood of reaching such serum concentrations is low. [22] As for gold, it is considered clinically inert; nonetheless, metallic toxicity profiles for both metals can change when in nanoparticles depending on surface coating, size, and shape.[23] PHF has been previously reported to be non-toxic.[15] To gain further insight into GSNP toxicity, we directly evaluated bactericidal concentrations of GSNP on mammalian fibroblasts. We demonstrated that the eMIC across a broad spectrum of species is below the calculated levels of 50% reduction in cell viability (Figure 4D). The average computed viability loss in the highest eMIC was <20%. The low toxicity of

GSNPs is encouraging for further biomaterial development and could expand the potential application of these NPs as stable surface coatings with long-term antimicrobial efficacy in minimal release or GSNP-eluting coatings, using similar concepts already used in practice or *in vivo* under ongoing research.[10, 24]

Current antibiotic coatings for devices such as IPPs have successfully decreased associated infections, potentially by a bactericidal effect tailored to an initial sterility breach.[10] However, resistance to the coating antibiotics has been demonstrated, which may account for the reduced effectiveness in some revision procedures.[13, 25] Furthermore, the sterility breach source hypothesis has been recently questioned as subcutaneous tissue bacteria has been found to be one of the primary sources of bacteria in IPP biofilms, suggesting a more constant source of bacterial exposure.[25] The GSNPs in this manuscript carry several strengths for this purpose: 1) the potential to create a device-tailored coating that might provide longer-lasting protection; 2) the lower likelihood of developing resistant bacterial strains and 3) the broad spectrum antimicrobial and antibiofilm capability.

Limitations

Potential limitations of this study include the lack of *in-vivo* data and toxicity testing in a broader set of human cell lines, using additional assays such as the lactate dehydrogenase assay. Furthermore, the bacterial exposure

period did not evaluate extended timeframes. Nonetheless, given its potential in a wide-range of applications, *in-vivo* studies should be tailored to the specific tissues or devices to be targeted with appropriate models and coating types. The tested durations for antibiofilm activity should be adjusted to a given device and its potential indwelling period. Furthermore, only one growth media was used. Future studies will evaluate more physiologically relevant bacterial growth media. Finally, additional testing is needed, with more timepoints to evaluate the effective time for the GSNP-mediated inhibition of bacterial attachment.

CONCLUSION

This study characterized novel polyhydroxy fullerene coated mono- and bi-metallic nanoparticles for their antibacterial and antibiofilm effects and found that GSNPs was most effective. GSNPs was also demonstrated to achieve broad spectrum antibacterial and antibiofilm activities against human uropathogens. Importantly, at the minimum inhibitory concentrations, GSNPs had minimal effect on fibroblast viability. GSNPs are proposed as a material coating with broad-spectrum antimicrobial and antibiofilm activity that are easy to synthesize and have low toxicity. GSNPs may provide a valuable strategy as a coating in numerous applications to reduce device-related infections not limited to the urological practice.

METHODS

Nanoparticle synthesis

The metal nanoparticles (MNPs) were synthesized using modified methods previously described.[17] Briefly, different concentrations of silver (Ag), palladium (Pd), platinum (Pt) and gold (Au) were added to 2 mg/mL solution of PHF in deionized water to create PHF-based Au (GNP), Pd (PNP), Ag (SNP), Ag-Pt (SPNP), Pd-Pt (PPNP) and Au-Ag nanoparticles (GSNP). Resulting solutions were stirred and centrifuged (Table S1). The hydrodynamic size and absorption spectrum of GSNPs were measured using dynamic light scattering and UV-visible spectrophotometry. GSNP nanostructure and elemental composition were confirmed through aberration-corrected transmission electron microscopy (AC-TEM) and X-ray energy-dispersive spectroscopy (XEDS). (Figure 1)

MNP antibacterial-antibiofilm activity and visual confirmation

MNPs were coated on sterile 12.7 mm polyurethane (PU) discs by drop-casting method (Table S1). This results in surface adhesion predominantly through van der Waals interactions, suitable for rapid screening, but not final device-ready coating. Deposition was confirmed through both macro- and microscopic imaging (Figures 1&3). Control (uncoated) discs were added a 90% ethanol solution without MNPs. We chose PU due to its wide presence in

urology and other disciplines.[19, 20] Coated and uncoated discs were exposed to a brain heart infusion (BHI) broth incubated with a commercial, uropathogenic *Escherichia coli* strain (ATCC® 43886) diluted to a bacterial load of 5.5-7.1x10^7 colony-forming units (CFU) mL^{-1}. The MNP coating with the highest antibacterial and antibiofilm activity, GSNP, was selected for further experiments. In subsequent experiments, GSNPs were dispersed in ethanol to a 10 µg mL^{-1} concentration and drop-casted on PU discs as described above (Figure 1). To visually confirm biofilm and GSNPs surface presence, discs were inoculated with our *E. coli* strain and incubated at 37ºC overnight in BHI. Subsequently, discs were removed, rinsed with sterile phosphate-buffered saline (PBS) to remove unadhered bacteria and immersed into a 10% glutaraldehyde and followed a previously described protocol.[11] They were further air dried and sputter-coated using palladium for SEM imaging (ZEISS® Evo 15). SEM was carried out at 2.3kV accelerating voltage and images were acquired at 14000-18000x magnification.

Evaluation of GSNP spectrum

To determine the breadth of GSNP coatings, diverse pathogenic bacteria isolated from inflatable penile prosthesis (IPP) or ureteral stents (US) obtained in a clinical setting were used to characterize antibacterial and antibiofilm properties of coatings. Isolates included Gram positive and Gram negative species that spanned both aerobic and facultative anaerobic

bacteria. Species used included the ATCC *E. coli* strain, *Enterococcus faecalis, Enterobacter hormaechei, Staphylococcus aureus, Klebsiella oxytoca* and *Staphylococcus epidermidis*. Details on the collection and isolation of bacteria are described elsewhere (IRB approval No. 22-294 and 20-415 to Dr. Miller).[14, 26] Briefly, the surfaces of explanted devices were swabbed, and streaked onto Blood agar, CNA agar, Chocolate agar, and MacConkey agar plates prior to incubation at 37ºC for variable time periods. Unique CFU's were struck for isolation on the same media used for initial enrichment. Isolates were stored in 15% glycerol stocks at -80ºC and were identified through DNA extraction and Sanger sequencing of the full length 16S rRNA gene, using the 27F and 1492R primers. All studies were carried out in accordance with relevant guidelines and regulations. To preliminarily evaluate the efficacy of GSNPs, coatings were exposed to BHI or lysogeny broth to bacterial density ranging from $7.2x10^7$ to $5.9x10^8$ CFU mL^{-1}. Further evaluation was conducted at more physiologically-relevant bacterial loads of $1.2x10^6$ – $5.1x10^7$ CFU mL^{-1}.[14, 26] Coated and uncoated discs were immersed in 600µL of bacteria-containing media and incubated at 37°C overnight.

Antibacterial and antibiofilm properties determination

To quantify the antibacterial activity of NPs, free-floating (planktonic) bacteria were sampled from media after incubation and drop-plated onto BHI agar plates to quantify CFU/ml after overnight incubation at 37ºC. To quantify the antibiofilm activity, the discs were removed from media after

incubation and rinsed to remove non-adherent, planktonic bacteria. Biofilm bacteria were detached into tubes containing sterile PBS, then sonicated in sterile PBS at 160W at 20kHz for 2 minutes (Ultrasonic processer XL, Heat Systems). The resulting solution was drop-plated onto BHI plates for CFU/mL quantification after overnight incubation. For both planktonic and biofilm bacteria, all samples were serially diluted to 10^{-6} and all dilutions were plated in triplicate with 30 µL prior to incubation. All CFU's for all dilutions were quantified, even if below 30 and CFU values for dilutions producing <300 CFU's total were used for analysis. This method put a limit of detection of approximately 33 CFU's/ml. Thus, 100% inhibition is defined here as <33 CFU's/ml.

To quantify the long-term efficacy of nanoparticles, control and coated discs were used in assays as above. Subsequently, media was removed for CFU quantification and fresh media with *E. coli* was added back to wells, for a total of four days.

Toxicity, minimum inhibitory concentration (MIC), and efficacy comparison

Toxicity was evaluated by comparing toxic concentrations to effective antibacterial concentrations calculated as MICs. Murine 3T3 fibroblasts were employed to determine the potential for toxicity. The fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. The fibroblasts

were treated in cultured DMEM media containing serial concentrations of GSNPs (10, 7.5, 5, 2.5, 1, 0.5 and 0.1 µg/mL). Cell viability was assessed using the CyQUANT XTT Cell Viability Assay (Thermo Fisher Scientific) following manufacturer's protocol with specific modifications to prevent optical interference from the GSNP. After co-culturing with GSNP, cells were washed with PBS to remove extracellular particles, and fresh DMEM was added. Following the 4-hour incubation with the XTT working solution, the supernatant was transferred to a fresh 96 well plate. This transfer step was essential to eliminate background noise caused by cell debris or internalized GSNP. Complete DMEM culture medium without the cells was used as blank controls, and fibroblasts without NPs were used as live controls.

To quantify the estimated MIC (eMIC) of GSNPs, bacterial strains diluted to physiologically relevant bacterial densities were exposed to increasing concentrations of GSNPs. Bacteria without exposure to GSNPs were used as controls. Samples were incubated overnight and further plated for quantification. We defined eMIC as the calculated lowest necessary concentration of GSNPs to reduce the highest bacterial load observed in controls. GSNP efficacy was compared to 20nm Ag-shelled Au citrate-based commercial NPs (cNPs) (nanoComposix, Inc.). Using the calculated eMIC concentration for GSNPs, bacterial strains were exposed to cNP concentrations equivalent to the GSNP eMIC and incubated overnight along controls incubated without cNP. Because GSNPs were evaluated using CFU

enumeration across a limited set of discrete concentrations, we report the highest tested concentration producing complete inhibition and a regression-based estimated inhibitory concentration (eMIC) for comparative purposes. eMIC values are not equivalent to CLSI/ISO broth microdilution MICs and should be interpreted as descriptive estimates under the conditions of this assay.

Mechanism of action and kinetics evaluation

We evaluated production of reactive oxygen species (ROS) as a potential synergistic mechanism of action to infer a lower potential to develop resistance to GSNPs.[27] To evaluate ROS production, we used CellROX® Deep Red Reagent (Life Technologies, Inc.), which increases fluorescence when exposed to ROS. Broth containing *E. coli* 43886 was exposed to 1µg/mL GSNPs and incubated for 1h. Samples were then used in the CellROX® assay following manufacturer's protocol. Fluorescence at 665 nm was quantified on a UV-2450 plate reader (SHIMADZU, Japan). Differences in fluorescence emission were recorded and the emissions of the separate components (bacterial media, GSNPs and PBS) with CellROX® were used as controls. Fluorescence of the individual components without CellROX® was assessed to rule out interference. Bactericidal kinetics were evaluated to gain insight into a minimal implant indwelling time. To quantify the bactericidal kinetics of GSNPs, a separate set of samples were incubated for up to twelve hours.

Data were used to calculate an estimated inhibitory period to achieve a 100% bacterial concentration reduction.

Statistical analysis

All pertinent experiments were carried out in triplicate for statistical analysis. Parametric variables were analyzed using independent samples *t*-test or ANOVA and Tukey post-hoc analysis, as appropriate. Statistical analysis was conducted using IBM® SPSS v. 26, and p values < 0.05 were considered statistically significant.

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Authors' Contributions

JGA, SD, AM and VK conceived and designed the study. YX and VK synthesized and characterized the nanomaterials. JSRA and AM performed microbiological assays. All authors read and approved of the final manuscript.

Competing interests

V.K. has a patent on the synthesis of metal nanoparticles used in this study. The authors declare that they have no competing interests. All other authors declare no financial competing interests.

Data Availability

All data analyzed during this study are included in this published article and its supplementary information files.

Ethics Approval

This study received Institutional Review Board (Cleveland Clinic) approval No. 22-294 and 20-415. Informed consent was obtained from participants via Institutional Review Board-approved study protocol No. 20-415.

SUPPORTING INFORMATION

Supplementary figure for antibacterial and antibiofilm activity at supra-physiological bacterial concentrations. Supplementary tables showing statistical analyses and nanoparticle synthesis conditions.

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