Bactericidal effect of 5-mercapto-2-nitrobenzoic acid coated silver nanoclusters against multidrug-resistant *Neisseria gonorrhoeae*

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ABSTRACT. *Neisseria gonorrhoeae* is among the most multidrug-resistant bacteria in circulation today and new treatments are urgently needed. In this work, we demonstrate the ability of 5-mercapto-2-nitrobenzoic acid-coated silver nanoclusters (MNBA-AgNCs) to kill strains of *Neisseria gonorrhoeae*. Using an *in vitro* bactericidal assay, MNBA-AgNCs showed significantly higher anti-gonococcal bioactivity than the antibiotics ceftriaxone and azithromycin, and silver nitrate. These nanoclusters were effective against both planktonic bacteria and a gonococcal infection of human cell cultures *in vitro*. Treatment of human cells *in* vitro with MNBA-AgNCs did not induce significant release of lactate dehydrogenase, suggesting minimal cytotoxicity to eukaryotic cells. Our results suggest that MNBA-AgNCs hold great potential for topical treatment of localized gonorrhoeae.

INTRODUCTION

Neisseria gonorrhoeae causes the sexually transmitted infection (STI) gonorrhoea, with ~ 87 million cases reported annually worldwide.[1] In men, gonococcal infection is manifested as a purulent urethritis, with discharge and painful micturition, and in women, infection of the endocervix and ectocervix leads to a purulent cervicitis. Infection can often be asymptomatic and in women, it can worryingly ascend into the upper reproductive tract to cause pelvic inflammatory disease (PID), which is a range of inflammatory conditions that include endometritis in the uterus and salpingitis in the Fallopian tubes. Untreated infection can lead to serious sequelae in women that include pelvic pain, abscess formation (tubal, ovarian), ectopic pregnancy and infertility.[2–4]

Although antibiotics have been successful for treating gonococcal infections, *N. gonorrhoeae* has developed multi-drug resistance[5] with recent cases of treatment failure confirmed worldwide. The World Health Organization (WHO) has classified *N. gonorrhoeae* as a high priority pathogen for research prompting the discovery and development of new antibiotics as

well as the repurposing of old antibiotics and new non-antibiotic treatments.[6]

Recently, the use of various types of nanoparticles (NPs) has been suggested as a strategy and/or complementary tool to fight Gram-negative and Gram-positive bacterial infections for multiple reasons.[7,8] Nanoparticles' size, ligand coating and the chemical composition of their core can be easily modified to generate NPs with tunable properties (e.g. optical, magnetic, redox, and thermal etc.). Furthermore, they demonstrate high reactivity at their local microenvironment and designed functionalities (e.g. target specificity, and drug delivery). The antibacterial efficacy of NPs can also be tuned, as it is strongly dependent on the aforementioned attributes.[9–11] NPs can kill bacteria by a combination of different mechanisms of action, which depend on the composition of the NP core, their morphology and ligand coating that could minimize or slow down the potential development of resistance.[12] These different mechanisms include: 1) direct interaction and disruption of the bacterial walls, 2) generation of oxidative stress induced by the formation of reactive oxygen species (ROS) and 3) interaction of metal ions with intracellular molecules (DNA, enzymes or other proteins).[13] For example, gold NPs synthesized in the presence of 4, 6-diamino-2-pyrimidinethiol have been reported to induce bacterial death by depleting intracellular ATP and inhibiting the binding of ribosomal RNA.[14] Rotello and coworkers have demonstrated that hydrophobic and positively charged gold NPs (2 and 6 nm) coated with ligands of varying chain lengths with non-aromatic and aromatic characteristics kill multi-drug resistant-bacteria by interacting with the bacterial wall.[15,16] Furthermore, these type of nanoparticles in combination with antibiotics block bacterial efflux pumps thus provoking a synergistic effect that permits treatment with lower doses of drugs.[17] On the other hand, gelatin stabilized copper NPs (56 nm) have been shown to induce bacterial death by altering membrane potential[18] and they have also been used for lipid peroxidation and protein oxidation.[19]

The bactericidal effect of silver and its compounds has attracted strong research interest.[20,21]

Silver nanoparticles (AgNPs) of different sizes and ligand coatings have been shown to have antibacterial properties deriving from various mechanisms of action.[22] Li *et al.* suggested that in Gram-negative bacteria, AgNPs with a 5 nm size firstly enter the outer bacterial membrane, which results in the leaking of the cellular material. This is followed by entry into the inner membrane that leads to inhibition of the respiratory chain and simultaneous Ag^+ ion release from the AgNPs, which bind to certain proteins and collapse the membrane.[23] This hypothesis is widely accepted by the scientific community and there are several studies that have discussed how the uptake pathway affects the mechanism of action of various types of AgNPs.[24–27] As with other metal NPs, it was shown that the antibacterial activity of silver nanoparticles strongly depends on both the NP size and ligand coating.[26]

The strong dependence of antibacterial activity on NP size has been studied and it has been demonstrated that decreasing the size of NPs can enhance their killing efficacy.[28–31] Silver nanoclusters (AgNCs) are usually named nanoparticles with a size below 2 nm and can be functionalized with a number of different stabilizing agents.[32–35] Recently, several methods have been reported to synthesize robust water soluble silver nanoclusters, which enables their broader applicability.[35, 36] For example, Xie *et al.* used glutathione-stabilized AgNCs to kill *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Bacilus subtilis*.[37] These AgNCs showed antibacterial activity similar to that observed with the antibiotic ampicillin and displayed low toxicity for mammalian cells. Setyawati *et al.* suggested that increasing the stability of AgNCs in lysosomes by choosing an appropriate ligand could reduce the toxicity to human cells.[38] Recently, it was shown that polyethylenimine-functionalized AgNCs have a broad-spectrum activity against multi-drug-resistant bacteria, whilst also showing low toxicity towards mammalian cells.[39] Their high antibacterial activity was attributed to their small size, surface chemistry and cationic nature. Zheng *et al.* showed that the combinatorial use of AgNCs with antibiotics had a synergistic effect and induced severe

DNA damage,[40] whilst Javani *et al.* reported the effective growth inhibition of Gram-positive and Gram-negative bacteria when incubating with DNA coated AgNCs.[41]

In this work we take advantage of the small size of silver nanoclusters to tackle *N. gonorrhoeae,* a multi-antibiotic resistant pathogen in need of new treatments. By coating AgNCs with 5-mercapto-2-nitrobenzoic acid (MNBA), a ligand that has not previously been tested in conjunction with AgNCs as an antibacterial agent, we show that an enhanced antibacterial activity is observed against *N. gonorrhoeae*. In comparison to the currently recommended antibiotics, ceftriaxone and azithromycin, MNBA-AgNCs were able to kill both planktonic bacteria and bacteria attached to human cells whilst showing no toxicity towards mammalian cells.

RESULTS AND DISCUSSION

Synthesis and characterization of MNBA-AgNCs

We chose 5-mercapto-2-nitrobenzoic acid as the stabilizing ligand for AgNCs to yield MNBA-AgNCs (**Figure 1A**). The MNBA ligand was chosen as it facilitates the synthesis of atomically monodisperse nanocrystals. Furthermore, this ligand binds strongly to silver through the sulfur atom due to the presence of both the -NO_2 and -COOH electron withdrawing groups on the aromatic ring and provides the AgNCs with high stability in aqueous media.[36] Prior to the synthesis of the nanoparticles, 5,5'-dithiobis(2-nitrobenzoic acid) was stirred in a basic solution in order to cleave the disulfide bond and yield 5-mercapto-2-nitrobenzoic acid. Then, silver nitrate ($AgNO_3$) was added, and an Ag-S complex was formed after stirring. The mixture was then reduced with $NaBH_4$, followed by washing to produce MNBA-AgNCs (**Scheme S1**). This experimental protocol has been shown to produce MNBA-AgNCs in a large scale.[36] The synthesized MNBA-AgNCs were characterized using different techniques. **Figure 1B** shows a representative UV-Vis spectrum of the MNBA-AgNCs in Dulbecco B Phosphate-

Buffered Saline (PBSB). The spectrum showed four different optical bands at ~400, ~480, ~550, and ~650 nm, representative of the small size of the MNBA-AgNCs ($Ag_{44}(MNBA)_{30}$) as previously reported.[36,42,43,44] The multiple peaks observed are due to the strong splitting in the lower unoccupied molecular orbitals (LUMOs) of the sp band and they are characteristic for AgNCs stabilized by thiolated ligands. Due to the presence of the 5-mercapto-2-nitrobenzoic acid ligand on their surface, MNBA-AgNCs were negatively charged with a zeta potential of -13.0 ± 1.0 mV in PBSB.

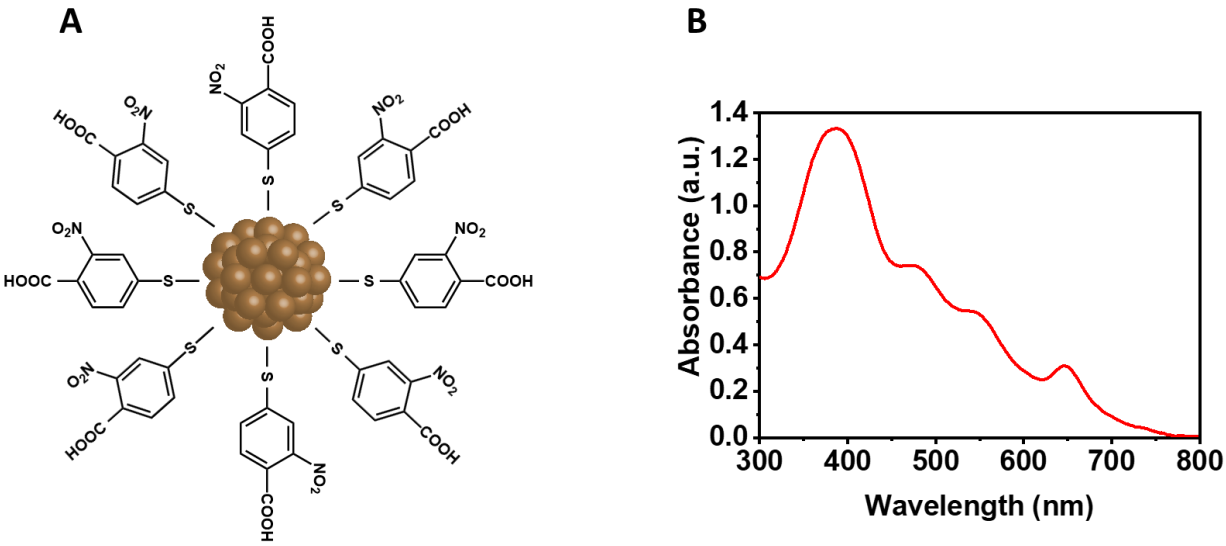

Figure 1. A) A schematic illustration of MNBA-AgNCs: Silver atoms (small brown spheres) comprise a nanocluster which is surrounded by 5-mercapto-2-nitrobenzoic acid ligands bound to the cluster via a thiol group. B) Representative UV-Vis spectrum of AgNCs stabilized by MNBA showing multiple optical transitions.

MNBA-AgNCs kill *N. gonorrhoeae*

The ability of MNBA-AgNCs to kill gonococci was assessed using an *in vitro* bactericidal assay in comparison to ceftriaxone, a well-known antibiotic used for treating gonococcal infections (see **Figure S1 A** for antibiotic structure). We also compared the MNBA-AgNCs and ceftriaxone against $AgNO_3$, since the use of Ag salts for treating gonorrhoea has a history dating back to Phillippe Ricord in 1838 and Carl Credé in 1881, who recommended the

application of $AgNO_3$ to the eyes of patients infected with gonorrhea.[45,46] However, the use of $AgNO_3$ is associated with chemical conjunctivitis, toxicity and treatment failures.[47] Although the use of $AgNO_3$ is generally no longer recommended, there are occasional reports of its use for treating neonatal conjunctivitis.[48] **Figure 2** shows the percentage of *N. gonorrhoeae* (strain P9-17) killed by MNBA-AgNCs, $AgNO_3$ and ceftriaxone, at increasing concentrations and different incubation times. MNBA-AgNCs, $AgNO_3$ and ceftriaxone treatments showed dose- and time-dependent bactericidal effects. A dose of 0.019 µM and 0.467 µM of MNBA-AgNCs was needed to kill 50 % and 100 % of the bacteria by 1 h, respectively. Lower doses of 0.001 µM and 0.005 µM silver nanoclusters were also effective after an incubation time of 3h. In comparison, when treated with $AgNO_3$, higher doses of 0.467 µM and 2.34 µM were needed to kill 50 % and 100 % of the bacteria by 1 h, whereas for a longer incubation time (3 h) the same effect was achieved with doses of 0.009 µM and 0.093 µM. The bactericidal effect of MNBA-AgNCs was significantly greater than that observed with ceftriaxone ($P<0.05$), which needed a minimum of 2.34 µM to kill 50 % of the bacteria following a 1h treatment and 0.467 µM for a 3h treatment. Notably, the highest concentration of ceftriaxone tested (11.7 µM) was unable to kill 100 % of the bacteria even after a 3h incubation period.

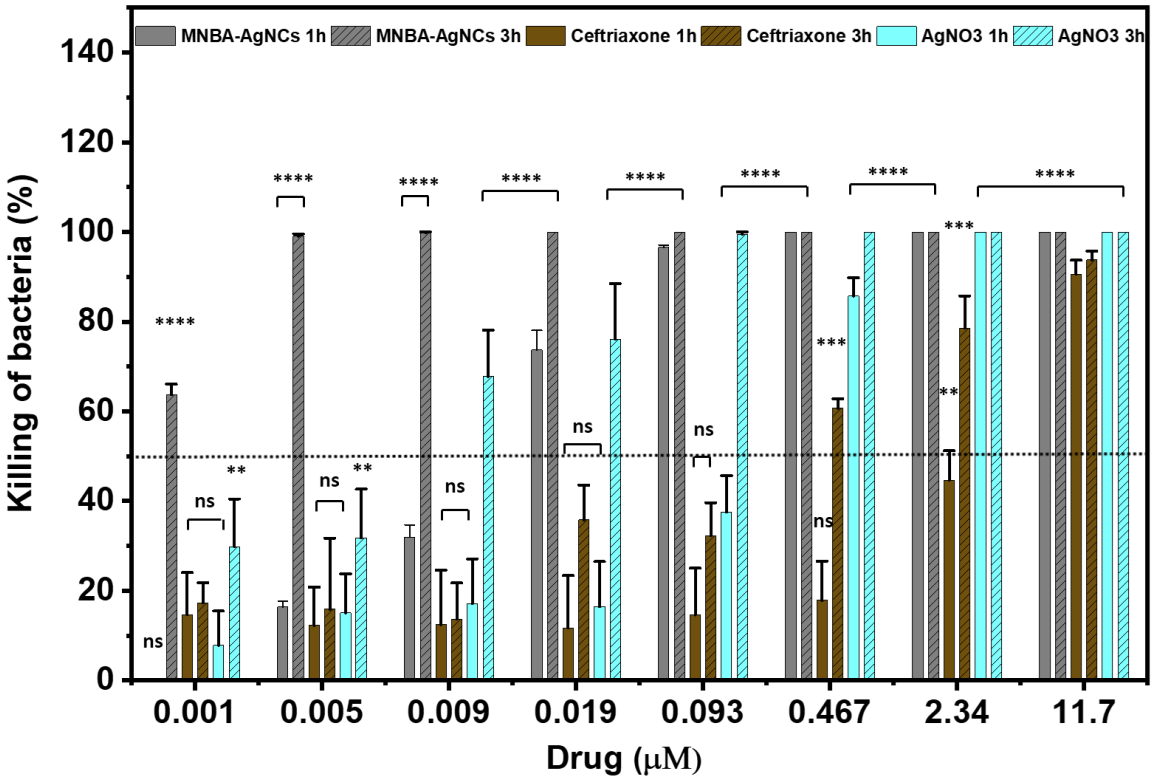

Figure 2. Killing of *N. gonorrhoeae* strain P9-17 with increasing concentrations of MNBA-AgNCs, ceftriaxone and $AgNO_3$ at different incubation times. The columns represent the mean % killing and the error bars the standard error of the means for at least n=3 independent experiments. Statistical significances between values and control (no treatment, 0% killing) were determined by one-way ANOVA and Tukey's Post-hoc test: ****p<0.0001, ***p<0.005, **p<0.05, ns= no significant differences.

Then we examined what was the minimum time of exposure to MNBA-AgNCs required to kill gonococci. In **Figure 3A**, P9-17 bacteria were exposed to 0.467 – 11.7 µM of MNBA-AgNCs in a bactericidal assay for 1, 5, 15, 30 and 60 min. Compared to the no treatment (control experiment), treatment with 11.7 µM MNBA-AgNCs killed 100 % of P9-17 within 15 min, whereas 30 min were required with a concentration of 2.34 µM and 1 h with 0.467 µM of MNBA-AgNCs. Our standard bactericidal assay contained ~10^3 Colony Forming Units (CFU)/well (**Figures 2** and **3A**), however, the number of organisms in mucosal surface infections may be considerably higher.[49,50] In order to assess the total number of gonococci that MNBA-AgNCs could kill within 1 h, we incubated increasing concentrations of bacteria with

0.467 µM of MNBA-AgNCs and compared the bactericidal effect against untreated cultures after 1 h. **Figure 3B** shows that MNBA-AgNCs killed 100 % of gonococci at concentrations ≤ 10^5 CFU/mL and ~90 % of bacteria of 10^6 CFU/mL. The bactericidal activity of the MNBA-AgNCs was also tested against strain P9-17 following storage of MNBA - AgNCs at 4 °C in PBSB over an extended period in order to examine the long-term effectiveness of the nanoclusters. **Figure S5** shows that there was no reduction in bactericidal activity of the MNBA-AgNCs on storage for up to 180 days when tested at doses of 0.467 - 11.7 µM, demonstrating that the preparation has a significant shelf-life when stored at 4ºC.

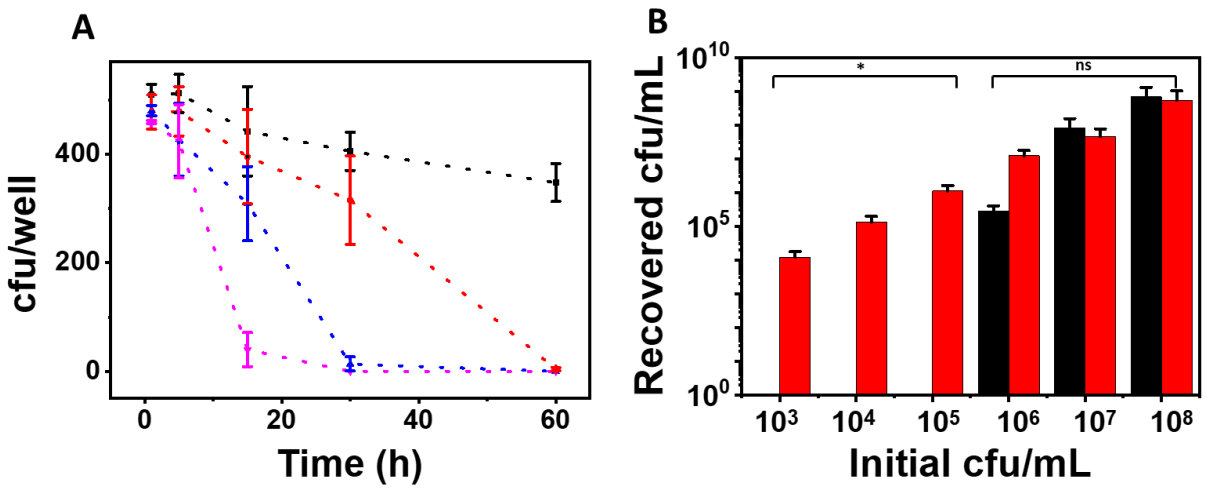

Figure 3. A) Minimum time required to kill *N. gonorrhoeae* strain P9-17 with 0.467 – 11.7 µM MNBA-AgNCs. The symbols represent the mean CFU/mL and the error bars the standard error of the means for n=3 independent experiments. Color guide: black – 0 µM, red – 0.467 µM, blue – 2.34 µM, pink – 11.7 µM B) Killing effect of MNBA-AgNCs (0.467 µM) on increasing concentrations of *N. gonorrhoeae* strain P9-17 at 1h. Color guide: black – MNBA-AgNCs, red – no MNBA-AgNCs. The columns represent the mean CFU/mL and the error bars the standard error of the means deviations for n=3 independent experiments. Statistical significances were calculated by Student's t-test: *$p < 0.05$, ns= no significant differences.

At present, resistance to antibiotics previously used to treat gonorrhea is high worldwide. This is the case, for example, for sulfonamides, penicillin and its derivatives, tetracyclines,

fluoroquinolones and some macrolides and cephalosporins.[51] Ceftriaxone and azithromycin are the only treatments currently recommended by the Centers for Disease Control and Prevention (CDC) for treatment of uncomplicated gonorrhea in the US.[52] In 2017, only 0.2 % of US gonococcal isolates had elevated ceftriaxone minimum inhibitory concentrations (MICs), whereas during 2014–2017, there was an increase from 2.5 % to 4.4 % in the number of isolates with elevated azithromycin MICs (https://www.cdc.gov/std/stats17/gonorrhea.htm). In our current study, we tested the ability of our MNBA-AgNCs to kill *N. gonorrhoeae* isolates available in the CDC Antimicrobial Resistance Panel that are reported to show the most resistance to ceftriaxone and azithromycin (**Figure S1**). **Table 1** shows the concentrations of MNBA-AgNCs that could kill 50 % and 100 % of these isolates after 1 h and 3 h of treatment. The MNBA-AgNCs showed high levels of bactericidal activity, with doses ranging from 0.019 – 1.87 μM and 0.002 – 0.093 μM capable of killing 50-100 % of these gonococcal isolates within 1 h and 3 h of treatment, respectively. Thus, our bactericidal assay data show that MNBA-AgNCs can rapidly kill gonococcal isolates with reported resistance to azithromycin and to ceftriaxone. This is an important observation, since resistance to ceftriaxone and reports of antibiotic-treatment failures are now being reported regularly in many countries.[53] In order to confirm if the MNBA-AgNCs were significantly better than antibiotic treatments, future research studies could compare their bioactivity alongside ceftriaxone, azithromycin and the dual antibiotic therapy, as well as other antibiotics, e.g. doxycycline, spectinomycin or ertapenem that have been used to treat antibiotic-treatment failures.[54] These comparisons should be done using the internationally recommended Clinical and Laboratory Standards Institute (CLSI) accepted agar dilution assay for *N. gonorrhoeae*.[55] Notably, the significant benefit of using the MNBA-AgNCs is to avoid the problem of gonococci developing antibiotic resistance, which hampers effective treatment.

Table 1. Concentrations of MNBA-AgNCs that kill 50% and 100% of *N. gonorrhoeae* isolates

Neisseria gonorrhoeae strain (AR Bank No)	MNBA-AgNCs, 1h (μM)		MNBA-AgNCs, 3h (μM)		Ceftriaxone, 24 h (μM)[b]	Azithromycin, 24 h (μM)[b]
	50%[a]	100%[a]	50%[a]	100%[a]	MIC	MIC
166	0.467	0.935	0.009	0.093	0.209	1.34
167	0.093	0.468	0.019	0.093	0.013	10.7
173	0.093	0.468	0.009	0.093	0.209	0.668
174	0.093	0.468	0.005	0.009	0.209	1.34
175	0.019	0.468	0.002	0.019	0.013	21.4
179	0.093	0.468	0.009	0.093	0.013	10.7
181	0.093	0.468	0.005	0.093	0.050	341
190	0.468	0.468	0.093	0.093	0.209	1.34
194	0.093	1.870	0.009	0.093	0.835	0.668
202	0.093	0.468	0.002	0.093	0.013	21.4

[a] Concentrations (μM) of MNBA-AgNCs that kill 50% and 100% of gonococcal strains in the *in vitro* bactericidal assay. Determinations are mean values from n=3 independent experiments. [b] The reported MIC data are included for isolates with the highest levels of resistance to ceftriaxone and azithromycin present in the Centers for Disease Control and Prevention (CDC) and Food and Drug Administration (FDA) Antibiotic Resistance (AR) Isolate Bank (https://www.cdc.gov/ARIsolateBank/Panel/PanelDetail?ID=11).

The activity of the MNBA-AgNCs prepared in our study can also be compared with the activity of other antimicrobial silver nanomaterials published in literature. However, certain factors have to be considered. For example, the bacterial cultivation methods[56] and the selection of the specific strains used for different studies,[57,58] probably influences bacterial susceptibility to the nanomaterials. From a synthetic point of view, different synthetic procedures will yield nanomaterials with different stability and surface features, which could in turn influence their interaction with bacteria. Additionally, using specific buffers or surfactants to enhance the solubility of the nanomaterials could play a role in their interplay with the microorganism. Some studies and patents have focused on the use of AgNP composites to achieve successful bacterial death.[59–61] However, to the best of our knowledge, there is only one study that discusses the use of AgNPs to kill gonococci, where a range of nanomaterials were tested.[62] In that study, large AgNPs were reported as effective with a MIC50 of 12.5 µg/mL at 24 h against *N. gonorrhoeae* strain ATCC 49226 and between 25-50 µg/mL against five other clinical isolates. However, the particles had a broad size distribution and showed colloidal aggregation. Our data show that MNBA-AgNCs are colloidally robust and significantly more bactericidal including at shorter treatment times as we observed that the dose of MNBA-AgNCs that killed 50% of *N. gonorrhoeae* strain P9-17 was 0.019 µM (0.2 µg/mL) after 1 h of treatment and 0.001 µM (0.0125 µg/mL) after 3 h (**Figure 2**).

It is probable that MNBA-AgNCs function *via* multiple pathways, including by damaging the bacterial cell wall. We examined this hypothesis using a LIVE/DEAD™ *Bac*Light™ Bacterial Viability kit, in which live bacteria take up the dye SYTO® 9 and stain green, whereas dead bacteria take up propidium iodide through their damaged membranes and stain red. **Figure 4** shows that few gonococci (~25%) died after treatment with a non-bactericidal dose of MNBA-AgNCs (0.004 µM), whereas treatment with bactericidal doses (0.467 – 58.4 µM) led to increased killing (~75 – 90 % of bacteria). Thus, rapid bacterial cell death occurring within 1h

of exposure to MNBA-AgNCs probably suggests that there is membrane damage occurring, since other intracellular events that could cause cell death, e.g. oxidative stress and the interactions with intracellular molecules, , may take longer time. Comprehensive studies of the possible mechanisms of action of these MNBA-AgNCs constitute downstream requirements for further product development.

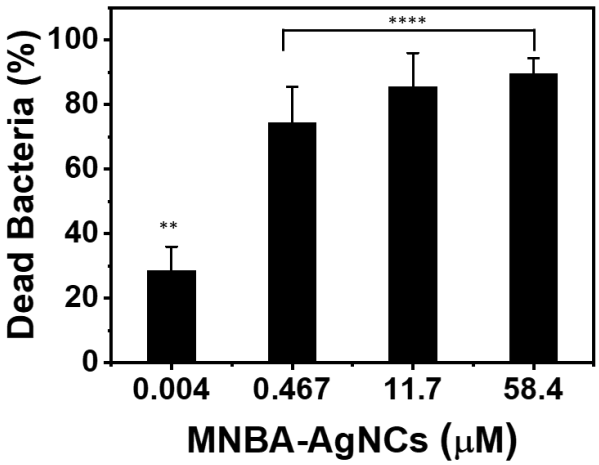

Figure 4. Assessment of gonococcal viability in the presence of MNBA-AgNCs using the LIVE/DEAD™ *Bac*Light™ Bacterial Viability kit. The columns represent the mean percentages of dead bacteria in a suspension treated with MNBA-AgNCs normalized to control bacteria that were not treated with MNBA-AgNCs. Statistical significances between values and control (no treatment, 0 μM) were determined by one-way ANOVA: ****$p<0.0001$, **$p=0.005$

Bactericidal activity of MNBA-AgNCs during infection of human cells *in vitro*

The observation of MNBA-AgNCs bactericidal activity *in vitro* would not necessarily indicate that the nanoclusters would be bactericidal during gonococcal infection that involves attachment to human host cells. Initially, we determined whether MNBA-AgNCs were cytotoxic to human cells by treating Chang conjunctival epithelial cells, HEC-1B endometrial adenocarcinoma and JEG-3 placenta choriocarcinoma cells in culture with increasing concentrations of MNBA-AgNCs (0.468 – 58.4 μM). Cell viability was then assessed at 1 h, 3 h and 24 h post incubation by measuring the release of lactate dehydrogenase (LDH).[63] No

significant release of LDH was observed, even after treatment of the three cell cultures for 24 h (**Figure S2**), suggesting that the cells remained viable with intact membranes.[38]

Assessing membrane integrity by measuring the release of intracellular enzymes is one useful preliminary method to examine cell viability. Indeed, for drug discovery studies, cell-based MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and LDH assays are useful for initial cytotoxicity screening. However, a more comprehensive analysis of cytotoxicity is required for downstream product development. Further preliminary experiments did show that treatment with 11.7 μM MNBA-AgNCs, which was the highest concentration tested in **Figure 2** that killed 100% of gonococci within 3h, had no effect on the morphology of Chang epithelial cells (**Figure S3**). However, longer time exposure studies are probably warranted. Other *in vitro* tests for cytotoxicity could include a standard haemolysis test, cell proliferation assays, detecting apoptosis with annexin V and DNA fragmentation, and determining caspase-3 and caspase-8 activity.[64] Importantly, these cellular responses would need to be assessed in non-MNBA-AgNC-treated epithelial cells.[65]

However, future studies of MNBA-AgNC bioactivity and cytotoxicity would require *in vivo* testing with mouse models, which is outside the scope of the current manuscript. These are the adult and neonatal murine models of gonococcal conjunctivitis[66] and the mouse gonorrhoea model of intravaginal gonococcal infection.[67] These models would be important for testing whether topically applied MNBA-AgNCs clear gonococci from both the conjunctiva and the lower reproductive tract of gonococcal-infected mice, and for studying mammalian host toxicity and drug metabolism and pharmacokinetics (DMPK).

Since the MNBA-AgNCs did not appear to be cytotoxic to human cells *in vitro*, we were able to test their ability to kill gonococci during active infection of human Chang conjunctival cells. These cells were chosen for our experiments as they have been widely used as an *in vitro* model for studying gonococcal interactions with human cells.[68–71] In these experiments, we infected

Chang cell monolayers with 10^6 CFU of P9-17 for 3 h to allow association of bacteria to the cells and, after washing to remove non-adherent bacteria, the cultures were treated with various concentrations of MNBA-AgNCs. **Figure 5A** shows that after 3 h of infection, ~20 % of bacteria associated with the cells and when left untreated, CFU numbers increased with time. Bacterial survival was then examined after 1 h, 3 h and 24 h of treatment with MNBA-AgNCs. During infection, treatment with 58.4 µM MNBA-AgNCs for 1 h killed ~80 % of P9-17 bacteria, which increased to >99 % after 3 h of treatment, when compared to untreated cultures ($P<0.05$). Treatment with 11.7 µM MNBA-AgNCs for 1 h was also effective, killing >80 % of bacteria, with the bacterial numbers contained up to 24 h later ($P<0.05$). By contrast, concentrations of 0.467 – 2.34 µM MNBA-AgNCs were non-bactericidal.

The infections described in **Figure 5A** were for a short-term infection of human cells. However, studies have shown that longer-term infection of human cells with gonococci result in the formation of multi-aggregate biofilm-like colonies of bacteria on cell surfaces *in vitro*.[72] The accumulation of such biofilm-like growth has been reported to reduce the efficacy of antimicrobials against different bacteria.[73] Thus, we infected Chang cells with ~10^6 CFU of P9-17/monolayer for 24 h to encourage higher levels of bacterial association. **Figure 5B** shows that after a prolonged infection, $1.17 \times 10^6 \pm 0.3 \times 10^6$ CFU of P9-17 were associated per monolayer and treatment with varying concentrations of MNBA-AgNCs (0.467 – 11.7 µM) was non-bactericidal ($P>0.05$). By contrast, treatment was effective only with the highest concentration of 58.4 µM, with only 935 ± 313 CFU of P9-17 recovered per monolayer, a reduction in bacterial count of 99.9%, compared to the non-treated monolayers ($P<0.05$). In addition, treatment with 58.4 µM for 24h was the only dose and condition that killed gonococci (>99%) in cell cultures that had been infected for 48h ($P<0.05$).

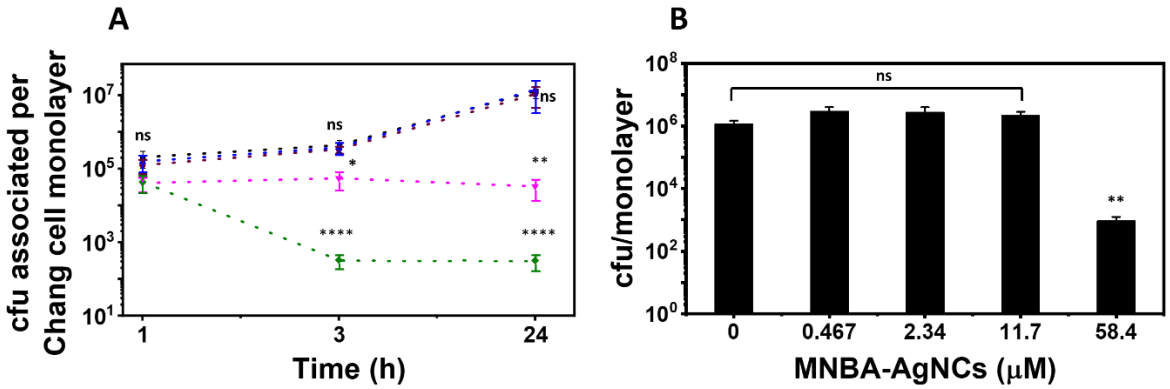

Figure 5. A) Effect of MNBA-AgNCs during short-term infection of human Chang conjunctival cells with *Neisseria gonorrhoeae* P9-17. Color guide: Black – 0 μM, Brown – 0.467 μM, Blue – 2.34 μM, Pink – 11.7 μM, Green – 58.4 μM B) Effect of 24h treatment with various doses of MNBA-AgNCs on 24h infected Chang cell monolayers. Statistical significances between values and control (no treatment with MNBA-AgNCs) were determined by one-way ANOVA and Student's t-test: ****p<0.0001, **p=0.01, * p <0.05 ns= no significant differences.

Data from **Figure 3B** showed that a concentration of 0.467 μM of MNBA-AgNCs killed >90% of ~10^6 CFU of gonococci in PBSB within 1 h. By contrast, in **Figure 5** data showed that higher concentrations of MNBA-AgNCs were required to kill gonococci associated with human cells after both short and long-term infections. Whereas gonococcal numbers remain static in PBSB, they increase over time in the cell infection medium. It is therefore possible that higher MNBA-AgNCs concentrations are required simply because the bacterial numbers are increasing in the infection experiments and not in the bactericidal assays. To investigate this, we performed control experiments in which ~10^6 planktonic P9-17 bacteria were maintained in cell culture infection medium alone and treated with MNBA-AgNCs for 1 h and 24 h. In these control experiments, concentrations of 11.7 - 58.4 μM MNBA-AgNCs were bactericidal after 1 h and 24 h (>98% reduction in CFU compared to control), whereas concentrations of 0.467 – 2.34 μM were not (**Figure S4**). In particular, the lack of bactericidal activity of 11.7 μM MNBA-AgNCs on biofilm-infected cell cultures in comparison to

bactericidal activity in cell infection medium alone suggests possibly that gonococci can resist the effect of MNBA-AgNCs when forming multi-aggregate, adherent colonies. An alternative hypothesis is that the eukaryotic cells also adsorb and/or absorb the MNBA-AgNCs, thereby reducing the concentration of available MNBA-AgNCs. Moreover, both possibilities may not be mutually exclusive and higher concentrations of MNBA-AgNCs may be needed to eliminate long-term adherent bacteria compared to bacteria in planktonic growth.

CONCLUSIONS

MNBA-AgNCs were synthesized and their antibacterial activity was assessed against strains of *Neisseria gonorrhoeae* resistant to ceftriaxone and azithromycin. Using an *in vitro* bactericidal assay, MNBA-AgNCs showed higher efficacy than ceftriaxone and azithromycin, antibiotics that are recommended for treating gonococcal infections. In addition, preliminary studies of cytotoxicity using a LDH release assay showed that the bactericidal doses MNBA-AgNCs were not significantly cytotoxic towards mammalian cells *in vitro*. MNBA-AgNCs were also effective in killing planktonic and multi-aggregates of bacteria during infection of human cells *in vitro.* This study demonstrates that MNBA-AgNCs could be exploited for treatment of gonococcal infections, possibly adjunctive to antibiotic treatment for intractable infections. It is probable that MNBA-AgNCs would be useful for topical treatment of exposed mucosae, e.g. in treating neonatal conjunctivitis and potentially for uncomplicated lower genital tract gonorrhoea. Systemic use is unclear and would require *in vivo* studies in animal models for safety and efficacy. In addition to treating gonococcal infections, MNBA-AgNCs could have other applications, e.g. for treating mucosal and skin infections caused by other multi-antibiotic resistant bacteria.

EXPERIMENTAL PROCEDURES

General reagents. All purchased chemicals were used without further purification. Silver nitrate ($AgNO_3$, 99.9 %) was purchased from Fisher Scientific (Loughborough, UK). Sodium hydroxide, methanol, sodium borohydride (99 %) and 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNBA, 99 %) were purchased from Sigma-Aldrich (St. Louis, MO). All reactions were carried out using Milli-Q water unless stated otherwise.

Bacteria. *Neisseria gonorrhoeae* strain P9-17, a 1B-26 serovar isolate (ND: P1.18-10,43: F1-26: ST-1926), was isolated originally from a patient with gonococcal prostatitis.[74] The panel of 50 *N. gonorrhoeae* isolates assembled by the Centers for Disease Control and Prevention (CDC) in collaboration with the Food and Drug Administration (FDA) was also obtained. These isolates represent a diversity of antimicrobial susceptibility to drugs that are used to treat infections and their genomes have been sequenced (https://www.cdc.gov/drugresistance/resistance-bank/currently-available.html). Gonococci were grown for ~16 h on supplemented GC-agar plates[75,76] incubated at 37 °C in an atmosphere containing 5 % (v/v) CO_2.

Synthesis of silver nanoclusters. The synthesis of MNBA-AgNCs was done following a previously reported protocol with minor alterations (Scheme S1 in supplementary material).[36] DTNBA (9.9 mg, 25 mmol) was dissolved in 25 mL of an aqueous solution of NaOH (20 mL, 1 M) in a 25 mL Erlenmeyer. The resulting solution was stirred for 45 min at room temperature until its color changed from orange to dark yellow. Then, a solution of $AgNO_3$ in water (5 mL, 10 mM) was added to the mixture and it was stirred for 45 min until it turned greenish. In the last step, a fresh solution of $NaBH_4$ (2 mL, 13.2 mM) was quickly added while the mix was

vigorously stirring which resulted in a crude dark brown color. The reaction mixture was vigorously stirred for 4h. After the reaction time, a dark burgundy color was appreciated. Finally, the silver nanoclusters were precipitated by adding 20 mL of MeOH:H_2O 1:1 and centrifuged at 9000 rpm for 10 min. The supernatant was discharged and the solid was washed with MeOH:H_2O 1:1 through centrifugal filtration (cut off 10 kDa) at 9,000 rpm for 10 min. The washing step was repeated until the filtrate became colorless. The silver nanoclusters were dried at room temperature and stored in the dark until further use.

Characterization of silver nanoclusters. The Z-potential measurements were performed using a Zetasizer Nano ZS instrument (Malvern Instruments, UK). The acquired data was processed using the software provided by Malvern (Zetasizer software v7.03). The UV−vis spectroscopy was carried out with a DeNovix DS-11 microvolume Spectrophotometer (Wilmington, USA).

Bactericidal assay to calculate the concentrations of MNBA-AgNCs, ceftriaxone and $AgNO_3$ that kill 50% and 100% of *Neisseria gonorrhoeae*. Concentrations of MNBA-AgNCs, ceftriaxone and $AgNO_3$ required to kill 50 % and 100 % of *N. gonorrhoeae* strain P9-17 were estimated by a 96-well microdilution method. From an overnight culture of *N. gonorrhoeae* P9-17 bacteria grown for ~16h, bacteria were removed and suspended in 1 mL of Dulbecco's Phosphate Buffered Saline (PBSB), pH 7.4 to a concentration of ~1.25 $\times 10^4$ Colony Forming Units (CFU)/mL. MNBA-AgNCs, ceftriaxone and $AgNO_3$ were diluted independently in PBSB at different concentrations (0.001, 0.005, 0.009, 0.019, 0.093, 0.467, 2.34 and 11.7 µM). Then, 20 µl of increasing concentrations of MNBA-AgNCs, ceftriaxone or $AgNO_3$ were added to 80 µl of bacteria solution (final concentration 10^3 CFU/mL) in triplicate wells of a 96-well plate. The plate was incubated at 37 ºC with 5 % (v/v) CO_2 for 1h and 3h.

Additionally, a control experiment with 20 µl of PBSB (i.e. no treatment) and 80 µl of bacteria was prepared. After incubation, 15 µl aliquots of each MNBA-AgNCs, ceftriaxone or $AgNO_3$-bacteria culture were spread onto supplemented GC-agar plates in triplicate and incubated for 18 h. The surviving CFU/mL were counted and the percentage of killing for every concentration of MNBA-AgNCs, ceftriaxone or $AgNO_3$ was calculated by comparison with the control experiment [i.e. 100 - (CFU/mL with treatment ÷ CFU/mL control) × 100]. The concentrations of MNBA-AgNCs, ceftriaxone and $AgNO_3$ that killed 50 % and 100 % of the bacteria at given time points were determined from the titration curves. Bactericidal assays with MNBA-AgNCs were also done with 10 different strains of *N. gonorrhoeae* obtained from the CDC/FDA strain bank (AR-181, 194,166, 175, 174, 202, 167, 190, 179 and 173) that showed different reported Minimum Inhibitory Concentration (MIC) values for ceftriaxone and azithromycin.

Determination of the minimum time required for MNBA-AgNCs to kill gonococci *in vitro.* In these experiments, 200 µL of MNBA-AgNC (0.467, 2.34 and 11.7 µM solutions in PBSB) were added to 800 µL of *N. gonorrhoeae* P9-17 (1.25×10^4 CFU/mL) and incubated for 1 h at 37 ºC and 5 % (v/v) CO_2. Control was 200 µl of PBSB with 800 µl of bacteria alone (0 µM MNBA-AgNC). Bacterial viability was sampled after 1, 5, 15, 30 and 60 min, by plating 15 µL onto triplicate GC-agar plates. The surviving CFU/mL were counted and the percentage of killing for every concentration of MNBA-AgNCs at the different time points was calculated by comparison with the control experiment [i.e. 100 - (CFU/mL with treatment ÷ CFU/mL control) × 100]. The experiment was done independently three times.

Determination of number of bacteria killed by MNBA-AgNCs *in vitro*. In these experiments, 20 µL of a concentration of 0.467 µM MNBA-AgNCs (100% bactericidal value

at 1h) in PBSB were added to 80 µl of various concentrations of *N. gonorrhoeae* P9-17 ($\sim10^3$, $\sim10^4$, $\sim10^5$, $\sim10^6$, $\sim10^7$, and $\sim10^8$ CFU/mL) on a 96-well plate. Control wells contained bacteria alone and PBSB. The plate was incubated for 1h at 37 ºC and 5 % (v/v) CO_2. After the incubation time, 15 µL aliquots of each bacteria/MNBA-AgNCs culture were spread in triplicate on supplemented GC-agar plates and incubated for 18 h (serial dilutions of the samples in PBSB were done as required prior to plating). The surviving CFU/mL were counted and the percentage of killing for MNBA-AgNCs and the different concentrations of bacteria was calculated by comparison with the control experiment [i.e. 100 - (CFU/mL with treatment ÷ CFU/mL control) × 100]. The experiment was done in triplicate.

Effect of storage at 4ºC in PBSB on the bactericidal activity of MNBA-AgNCs. A preparation of MNBA-AgNCs was suspended in PBSB and stored at 4 ºC for several months. Determinations of bactericidal activity of concentrations of 0.467, 2.34 and 11.7 µM against *N. gonorrhoeae* P9-17 were done at intervals over time as described in the bactericidal assay method above.

Cytotoxicity of MNBA-AgNCs for human cell culture lines. Human Chang conjunctival epithelial cells, HEC-1B endometrial adenocarcinoma and JEG-3 placenta choriocarcinoma cells were grown in DMEM medium containing 2 % (v/v) heat-inactivated fetal calf serum (dFCS) to confluence in sterile 96-well cell culture plates. The cytotoxicity of MNBA-AgNCs towards the different cell types was quantified by measuring the release of Lactate DeHydrogenase (LDH) enzyme using the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega, UK). The percentage cytotoxicity was calculated following the manufacturers' instructions. For these experiments, cultured cells were plated in a 96-well plate at $\sim10^5$ cells/well in 80 µl of DMEM containing 2 % (v/v) dFCS and incubated with 20 µL of

increasing concentrations of MNBA-AgNCs (0.0003, 0.0006, 0.001, 0.002, 0.005, 0.009, 0.018, 0.093, 0.467, 2.34, 11.7 and 58.4 µM) at 37 ºC and 5 % (v/v) CO_2 for 1 h, 3 h or 24 h. A control experiment with 20 µL of PBSB was additionally done. The experiment was done independently three times.

Morphological analysis of human Chang conjunctival epithelial cells treated with MNBA-AgNCs. Human Chang conjunctival epithelial cells were grown in DMEM medium supplemented with 10 % (v/v) heat-inactivated fetal calf serum (dFCS) to 100% confluence on sterile NUNC Lab Tek II 8 well chamber slides at 37 ºC with 5% (v/v) CO_2. Cell monolayers were treated with 11.68 µM MNBA-AgNCs in supplemented DMEM for 2-3 h at 37 ºC with 5 % (v/v) CO_2. An untreated control (supplemented DMEM only) was also done. After 2 h incubation, cell supernatants were removed, and cells were immediately stained with LIVE/DEAD™ Viability kit reagents (ThermoFisher) in DMEM for 15 min at 37 ºC with 5% (v/v) CO_2. The integrity of the cell monolayers, cellular morphology and cell viability were visualized by confocal laser scanning microscopy (Leica SP8). Three biological replicates of the experiment were done.

Bacterial viability measured using the LIVE/DEAD™ *Bac*Light™ Bacterial Viability kit. *Neisseria gonorrhoeae* P9-17 bacteria (~10^3 CFU) were treated with 0.004 – 58.4 µM MNBA-AgNCs for 1 h in PBSB, using the standard bactericidal assay, with untreated bacteria as controls. After 1 h, the LIVE/DEAD™ *Bac*Light™ Bacterial Viability kit reagents (ThermoFisher) were mixed and added to the suspensions for 15 min, following the manufacturer's instructions. The numbers of live (green fluorescent) and dead (red fluorescent) bacteria were counted by fluorescent microscopy (Leitz). The experiment was done independently three times.

Effect of MNBA-AgNCs on short- and long-term gonococcal infection of Chang conjunctival cells. Human Chang conjunctival cells were grown as described in the cytotoxicity measurement experiments. Prior to infection, the cells were washed four times with sterile PBS and then infected in triplicate with ~10^6 CFU/monolayer in DMEM containing 1 % (v/v) dFCS and without any antibiotic present. For a short-term infection, the bacteria were left in contact with the cells for 3h, whereas for the long-term infections, they were kept in contact for 24h and 48h. After infection at 3h, 24h and 48h, the cells were washed gently three times with PBSB to remove any unattached bacteria and then 80 µL of DMEM medium alone was added to each well. Next, 20 µL of 0.467, 2.34, 11.7 and 58.4 µM MNBA-AgNCs prepared in DMEM were added to triplicate wells. Control wells received 20 µL of DMEM alone. The plates were then incubated for 1 h, 3 h and 24 h at 37 ºC with 5 % (v/v) CO_2. To determine the total number of associated bacteria per monolayer, at each time-point after treatment with the MNBA-AgNCs, the extracellular medium was removed and 50 µL of a lysis solution of 1 % (w/v) saponin in PBSB containing 1% (v/v) dFCS, sterilized by filtration (0.22 µm filter), was then added to each individual well. The plate was incubated for 15-20 min at 37 ºC with 5 % (v/v) CO_2. Next, pools of the triplicate wells containing saponin were made (final volume 150 µL) and sampled by viable counting on GC-agar plates as described in the bactericidal assay method above.

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REFERENCES

(1) Rowley, J.; Hoorn, S. V.; Korenromp, E.; Low, N.; Unemo, M.; Abu-Raddad, L. J.; Chico, R. M.; Smolak, A.; Newman, L.; Gottlieb, S.; et *al*. Chlamydia, Gonorrhoea, Trichomoniasis and Syphilis: Global Prevalence and Incidence Estimates, 2016. *Bull. World Health Organ.* **2019**, *97*, 548-562.

(2) Rice, P. A.; Shafer, W. M.; Ram, S.; Jerse, A. E. *Neisseria Gonorrhoeae*: Drug Resistance, Mouse Models, and Vaccine Development. *Annu. Rev. Microbiol.* **2017**, *71*, 665–686.

(3) Mayor, M. T.; Roett, M. A.; Uduhiri, K. A. Diagnosis and Management of Gonococcal Infections. *Am. Fam. Physician* **2012**, *86*, 931–938.

(4) Mitchell, C.; Prabhu, M. Infectious Disease Clinics of North America. *Infect. Dis. Clin. North Am.* **2013**, *27*, 793–809.

(5) Unemo, M.; Shafer, W. M. Antimicrobial Resistance in *Neisseria Gonorrhoeae* in the 21st Century: Past, Evolution, and Future. *Clin. Microbiol. Rev.* **2014**, *27*, 587–613.

(6) Tacconelli, E.; Carrara, E.; Savoldi, A.; Harbarth, S.; Mendelson, M.; Monnet, D. L.; Pulcini, C.; Kahlmeter, G.; Kluytmans, J.; Carmeli, Y.; et *al.* Discovery, Research, and Development of New Antibiotics: The WHO Priority List of Antibiotic-Resistant Bacteria and Tuberculosis. *Lancet Infect. Dis.* **2018**, *18*, 318–327.

(7) Wang, L.; Hu, C.; Shao, L. The Antimicrobial Activity of Nanoparticles: Present Situation and Prospects for the Future. *Int. J. Nanomedicine* **2017**, *12*, 1227–1249.

(8) Gupta, A.; Mumtaz, S.; Li, C. H.; Hussain, I.; Rotello, V. M. Combatting Antibiotic-Resistant Bacteria Using Nanomaterials. *Chem. Soc. Rev.* **2019**, *48*, 415–427.

(9) Vimbela, G. V.; Ngo, S. M.; Fraze, C.; Yang, L.; Stout, D. A. Antibacterial Properties and Toxicity from Metallic Nanomaterials. *Int. J. Nanomedicine* **2017**, *12*, 3941–3965.

(10) Sadeghi, B.; Garmaroudi, F. S.; Hashemi, M.; Nezhad, H. R.; Nasrollahi, A.; Ardalan, S.; Ardalan, S. Comparison of the Anti-Bacterial Activity on the Nanosilver Shapes: Nanoparticles, Nanorods and Nanoplates. *Adv. Powder Technol. J.* **2012**, *23*, 22–26.

(11) Huo, S.; Jiang, Y.; Gupta, A.; Jiang, Z.; Landis, R. F.; Hou, S.; Liang, X. J.; Rotello, V. M. Fully Zwitterionic Nanoparticle Antimicrobial Agents through Tuning of Core Size and Ligand Structure. *ACS Nano* **2016**, *10*, 8732–8737.

(12) Pelgrift, R. Y.; Friedman, A. J. Nanotechnology as a Therapeutic Tool to Combat Microbial Resistance. *Adv. Drug Deliv. Rev.* **2013**, *65*, 1803–1815.

(13) Baptista, P. V.; McCusker, M. P.; Carvalho, A.; Ferreira, D. A.; Mohan, N. M.; Martins, M.; Fernandes, A. R. Nano-Strategies to Fight Multidrug Resistant Bacteria-"A Battle of the Titans". *Front. Microbiol.* **2018**, *9*, 1–26.

(14) Cui, Y.; Zhao, Y.; Tian, Y.; Zhang, W.; Lü, X.; Jiang, X. The Molecular Mechanism of Action of Bactericidal Gold Nanoparticles on Escherichia Coli. *Biomaterials* **2012**, *33*, 2327–2333.

(15) Li, X.; Robinson, S. M.; Gupta, A.; Saha, K.; Jiang, Z.; Moyano, D. F.; Sahar, A.; Riley, M. A.; Rotello, V. M. Functional Gold Nanoparticles as Potent Antimicrobial Agents against Multi-Drug-Resistant Bacteria. *ACS Nano* **2014**, *8*, 10682–10686.

(16) Hayden, S. C.; Zhao, G.; Saha, K.; Phillips, R. L.; Li, X.; Miranda, O. R.; Rotello, V. M.; El-Sayed, M. A.; Schmidt-Krey, I.; Bunz, U. H. F. Aggregation and Interaction of Cationic Nanoparticles on Bacterial Surfaces. *J. Am. Chem. Soc.* **2012**, *134*, 6920–6923.

(17) Gupta, A.; Saleh, N. M.; Das, R.; Landis, R. F.; Bigdeli, A.; Motamedchaboki, K.;

Campos, A. R.; Pomeroy, K.; Mahmoudi, M.; Rotello, V. M. Synergistic Antimicrobial Therapy Using Nanoparticles and Antibiotics for the Treatment of Multidrug-Resistant Bacterial Infection Related Content. *Nano Futur.* **2017**, *1*, 015004.

(18) Wang, Y.; Yang, F.; Zhang, H.-X.; Zi, X.-Y.; Pan, X.-H.; Chen, F.; Luo, W.-D.; Li, J.-X.; Zhu, H.-Y.; Hu, Y.-P. Cuprous Oxide Nanoparticles Inhibit the Growth and Metastasis of Melanoma by Targeting Mitochondria. *Cell Death Dis.* **2013**, *4*, e783.

(19) Chatterjee, A. K.; Chakraborty, R.; Basu, T. Mechanism of Antibacterial Activity of Copper Nanoparticles. *Nanotechnology* **2014**, *25*, 135101.

(20) Chernousova, S.; Epple, M. Silver as Antibacterial Agent: Ion, Nanoparticle, and Metal. *Angew. Chemie - Int. Ed.* **2013**, *52*, 1636–1653.

(21) Alexander, J. W. History of the Medical Use of Silver. *Surg. Infect.* **2009**, *10*, 289–294.

(22) Rai, M. K.; Deshmukh, S. D.; Ingle, A. P.; Gade, A. K. Silver Nanoparticles: The Powerful Nanoweapon against Multidrug-Resistant Bacteria. *J. Appl. Microbiol.* **2012**, *112*, 841–852.

(23) Li, W. R.; Xie, X. B.; Shi, Q. S.; Zeng, H. Y.; Ou-Yang, Y. S.; Chen, Y. Ben. Antibacterial Activity and Mechanism of Silver Nanoparticles on Escherichia Coli. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1115–1122.

(24) Hwang, E. T.; Lee, J. H.; Chae, Y. J.; Kim, Y. S.; Kim, B. C.; Sang, B. I.; Gu, M. B. Analysis of the Toxic Mode of Action of Silver Nanoparticles Using Stress-Specific Bioluminescent Bacteria. *Small* **2008**, *4*, 746–750.

(25) Reidy, B.; Haase, A.; Luch, A.; Dawson, K. A.; Lynch, I. Mechanisms of Silver Nanoparticle Release, Transformation and Toxicity: A Critical Review of Current Knowledge and Recommendations for Future Studies and Applications. *Materials* **2013**, *6*, 2295–2350.

(26) Durán, N.; Durán, M.; de Jesus, M. B.; Seabra, A. B.; Fávaro, W. J.; Nakazato, G. Silver

Nanoparticles: A New View on Mechanistic Aspects on Antimicrobial Activity. *Nanomedicine Nanotechnology, Biol. Med.* **2016**, *12*, 789–799.

(27) Sánchez-López, E.; Gomes, D.; Esteruelas, G.; Bonilla, L.; Lopez-Machado, A. L.; Galindo, R.; Cano, A.; Espina, M.; Ettcheto, M.; Camins, A.; et *al.* Metal-Based Nanoparticles as Antimicrobial Agents: An Overview. *Nanomaterials* **2020**, *10*, 292.

(28) Baker, C.; Pradhan, A.; Pakstis, L.; Pochan, D. J.; Shah, S. I. Synthesis and Antibacterial Properties of Silver Nanoparticles. *J. Nanosci. Nanotechnol.* **2005**, *5*, 244–249.

(29) Zheng, K.; Setyawati, M. I.; Lim, T.-P.; Leong, D. T.; Xie, J. Antimicrobial Cluster Bombs: Silver Nanoclusters Packed with Daptomycin. *ACS Nano* **2016**, *10*, 7934–7942.

(30) Lemire, J. A.; Harrison, J. J.; Turner, R. J. Antimicrobial Activity of Metals: Mechanisms, Molecular Targets and Applications. *Nat. Rev. Microbiol.* **2013**, *11*, 371–384.

(31) Agnihotri, S.; Mukherji, S.; Mukherji, S. Size-Controlled Silver Nanoparticles Synthesized over the Range 5-100 nm Using the Same Protocol and Their Antibacterial Efficacy. *RSC Adv.* **2014**, *4*, 3974–3983.

(32) Goswami, N.; Zheng, K.; Xie, J. Bio-NCs-the Marriage of Ultrasmall Metal Nanoclusters with Biomolecules. *Nanoscale* **2014**, *6*, 13328–13347.

(33) Zheng, K.; Yuan, X.; Goswami, N.; Zhang, Q.; Xie, J. Recent Advances in the Synthesis, Characterization, and Biomedical Applications of Ultrasmall Thiolated Silver Nanoclusters. *RSC Adv.* **2014**, *4*, 60581–60596.

(34) Luo, Z.; Zheng, K.; Xie, J. Engineering Ultrasmall Water-Soluble Gold and Silver Nanoclusters for Biomedical Applications. *Chem. Commun.* **2014**, *50*, 5143–5155.

(35) Desireddy, A.; Conn, B. E.; Guo, J.; Yoon, B.; Barnett, R. N.; Monahan, B. M.; Kirschbaum, K.; Griffith, W. P.; Whetten, R. L.; Landman, U.; et *al.* Ultrastable Silver Nanoparticles. *Nature* **2013**, *501*, 399–402.

(36) AbdulHalim, L. G.; Ashraf, S.; Katsiev, K.; Kirmani, A. R.; Kothalawala, N.; Anjum, D. H.; Abbas, S.; Amassian, A.; Stellacci, F.; Dass, A.; et *al.* Scalable Synthesis of Highly Stable and Water Dispersible $Ag_{44}(SR)_{30}$ Nanoclusters. *J. Mater. Chem. A* **2013**, *1*, 10148 –10154.

(37) Yuan, X.; Setyawati, M. I.; Leong, D. T.; Xie, J. Ultrasmall Ag^{+}-Rich Nanoclusters as Highly Efficient Nanoreservoirs for Bacterial Killing. *Nano Res.* **2014**, *7*, 301–307.

(38) Setyawati, M. I.; Yuan, X.; Xie, J.; Leong, D. T. The Influence of Lysosomal Stability of Silver Nanomaterials on Their Toxicity to Human Cells. *Biomaterials* **2014**, *35*, 6707–6715.

(39) Huma, Z.; Gupta, A.; Javed, I.; Das, R.; Zajif Hussain, S.; Mumtaz, S.; Hussain, I.; Rotello, V. M. Cationic Silver Nanoclusters as Potent Antimicrobials against Multidrug-Resistant Bacteria. *ACS Omega* **2018**, *3*, 16721–16727.

(40) Zheng, K.; Setyawati, M. I.; Lim, T. P.; Leong, D. T.; Xie, J. Antimicrobial Cluster Bombs: Silver Nanoclusters Packed with Daptomycin. *ACS Nano* **2016**, *10*, 7934–7942.

(41) Javani, S.; Lorca, R.; Latorre, A.; Flors, C.; Cortajarena, A. L.; Somoza, Á. Antibacterial Activity of DNA-Stabilized Silver Nanoclusters Tuned by Oligonucleotide Sequence. *ACS Appl. Mater. Interfaces* **2016**, *8*, 10147–10154.

(42) Bakr, O. M.; Amendola, V.; Aikens, C. M.; Wenseleers, W.; Li, R.; Negro, L. D.; Schatz, G. C.; Stellacci, F. Silver Nanoparticles with Broad Multiband Linear Optical Absorption. *Angew. Chem. Int. Ed.* **2009**, *48*, 5921–5926.

(43) Zhu, M.; Aikens, C. M.; Hollander, F. J.; Schatz, G. C.; Jin, R. Correlating the Crystal Structure of A Thiol-Protected Au_{25} Cluster and Optical Properties. *J. Am. Chem. Soc.* **2008**, *130*, 5883–5885.

(44) Russier-Antoine, I.; Bertorelle, F.; Vojkovic, M.; Rayane, D.; Salmon, E.; Jonin, C.; Dugourd, P.; Antoine, R.; Brevet, P. F. Non-Linear Optical Properties of Gold Quantum

Clusters. The Smaller the Better. *Nanoscale* **2014**, *6*, 13572–13578.

(45) Crede, C. *Die Verhütung Der Augenentzuüdung Bei Neugeborenen*; Hirschwald: Berlin, **1884**.

(46) Ricord, P. *Traité Pratique Des Maladies Vénériennes [A Practical Treatise on Venereal Diseases]*; Rouvier, J., Bouvier, E. L., Eds.; Librairie des Sciences médicales: Paris, **1938**.

(47) Nishida, H.; Risemberg, H. M. Silver Nitrate Ophthalmic Solution and Chemical Conjunctivitis. *Pediatrics* **1975**, *56*, 368–373.

(48) Kaštelan, S.; Jurica, S. A.; Orešković, S.; Župić, T.; Herman, M.; Antunica, A. G.; Marković, I.; Bakija, I. A Survey of Current Prophylactic Treatment for Ophthalmia Neonatorum in Croatia and a Review of International Preventive Practices. *Med. Sci. Monit.* **2018**, 24, 8042–8047.

(49) Lowe, T. L.; Kraus, S. J. Quantitation of *Neisseria Gonorrhoeae* from Women with Gonorrhea. *J. Infect. Dis.* **1976**, *133*, 621–626.

(50) Isbey, S. F.; Alcom, T. M.; Davis, R. H.; Haizlip, J.; Leone, P. A.; Cohen, M. S. Characterisation of *Neisseria Gonorrhoeae* in Semen during Urethral Infection in Men. *Genitourin Med.* **1997**, *73*, 378–382.

(51) Unemo, M.; Golparian, D.; Eyre, D. W. Antimicrobial Resistance in *Neisseria Gonorrhoeae* and Treatment of Gonorrhea. In *Neisseria gonorrhoeae: Methods and Protocols*; Christodoulides, M., Ed.; Humana Press/Springer: Totowa, **2019**, 37–59.

(52) Workowski, K. A.; Gail A. Bolan. Centers for Disease Control and Prevention. Sexually Transmitted Diseases Treatment Guidelines, 2015. *Morb. Mortal. Wkly. Rep.* **2015**, *64*, 1–137.

(53) Fifer, H.; Natarajan, U.; Jones, L.; Alexander, S.; Hughes, G.; Golparian, D.; Unemo, M. Failure of Dual Antimicrobial Therapy in Treatment of Gonorrhea. *N. Engl. J. Med.*

2016, *374*, 2504–2506.

(54) Eyre, D. W.; Sanderson, N. D.; Lord, E.; Regisford-Reimmer, N.; Chau, K.; Barker, L.; Morgan, M.; Newnham, R.; Golparian, D.; Unemo, M.; et *al.* Gonorrhoea Treatment Failure Caused by a *Neisseria Gonorrhoeae* Strain with Combined Ceftriaxone and High-Level Azithromycin Resistance. *Eurosurveillance* **2018**, *23*, 1800323.

(55) Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Susceptibility Testing, 28th ed. CLSI supplement M100.* Wayne, P.A. Ed.; Clinical and Laboratory Standards Institute (CLSI): USA, **2018**.

(56) Balouiri, M.; Sadiki, M.; Ibnsouda, S. K. Methods for in Vitro Evaluating Antimicrobial Activity: A Review. *J. Pharm. Anal.* **2016**, *6*, 71–79.

(57) Ruparelia, J. P.; Chatterjee, A. K.; Duttagupta, S. P.; Mukherji, S. Strain Specificity in Antimicrobial Activity of Silver and Copper Nanoparticles. *Acta Biomater.* **2008**, *4*, 707–716.

(58) Kotakadi, V. S.; Gaddam, S. A.; Venkata, S. K.; Sai Gopal, D. V. R. New Generation of Bactericidal Silver Nanoparticles against Different Antibiotic Resistant Escherichia Coli Strains. *Appl. Nanosci.* **2015**, *5*, 847–855.

(59) Mohamed, R. R.; Sabaa, M. W. Synthesis and Characterization of Antimicrobial Crosslinked Carboxymethyl Chitosan Nanoparticles Loaded with Silver. *Int. J. Biol. Macromol.* **2014**, *69*, 35–99.

(60) Qian, A. Antimicrobial Gel of Nanosize Ag for Treating Female External Reproductive Tract Infection and Its Preparation Method. CN 101305983, **2008**.

(61) El-Sayed, A. A.; Khalil, A. M.; El-Shahat, M.; Khaireldin, N. Y.; Rabie, S. T. Antimicrobial Activity of PVC-Pyrazolone-Silver Nanocomposites. *J. Macromol. Sci. Part A Pure Appl. Chem.* **2016**, *53*, 346–353.

(62) Li, L. H.; Yen, M. Y.; Ho, C. C.; Wu, P.; Wang, C. C.; Maurya, P. K.; Chen, P. S.; Chen,

W.; Hsieh, W. Y.; Chen, H. W. Non-Cytotoxic Nanomaterials Enhance Antimicrobial Activities of Cefmetazole against Multidrug-Resistant *Neisseria Gonorrhoeae. PLoS One* **2013**, *8*, 2–11.

(63) Decker, T.; Lohmann-Matthes, M. L. A Quick and Simple Method for the Quantitation of Lactate Dehydrogenase Release in Measurements of Cellular Cytotoxicity and Tumor Necrosis Factor (TNF) Activity. *J. Immunol. Methods* **1988**, *115*, 61–69.

(64) Fowler, M. I.; Ho Wang Yin, K. Y.; Humphries, H. E.; Heckels, J. E.; Christodoulides, M. Comparison of the Inflammatory Responses of Human Meningeal Cells Following Challenge with *Neisseria Lactamica* and with *Neisseria Meningitidis*. *Infect. Immun.* **2006**, *74*, 6467–6478.

(65) Suliman Y, A. O.; Ali, D.; Alarifi, S.; Harrath, A. H.; Mansour, L.; Alwasel, S. H. Evaluation of Cytotoxic, Oxidative Stress, Proinflammatory and Genotoxic Effect of Silver Nanoparticles in Human Lung Epithelial Cells. *Environ. Toxicol.* **2015**, *30*, 149–160.

(66) Zaidi, T. S.; Cywes-Bentley, C.; Roberts, C.; Vinacur, M.; Pier, G. B.; Development of a Murine Model of *Neisseria Gonorrhoeae* Conjunctivitis and Evaluation of the Protective Efficacy of Antibody to the Surface Polysaccharide/Capsule, Poly-N-acetyl glucosamine (PNAG). *American Society for Microbiology*, February 12-14, Baltimore, Maryland, **2018**.

(67) Jerse, A. E. Experimental Gonococcal Genital Tract Infection and Opacity Protein Expression in Estradiol-Treated Mice. *Infect. Immun.* **1999**, 67, 5699–5708.

(68) Hung, M. C.; Heckels, J. E.; Christodoulides, M. The Adhesin Complex Protein (ACP) of *Neisseria Meningitidis* Is a New Adhesin with Vaccine Potential. *MBio* **2013**, *4*, e00041-13.

(69) Minor, S. Y.; Banerjee, A.; Gotschlich, E. C. Effect of α-Oligosaccharide Phenotype of

Neisseria Gonorrhoeae Strain MS11 on Invasion of Chang Conjunctival, HEC-1-B Endometrial, and ME-180 Cervical Cells. *Infect. Immun.* **2000**, *68*, 6526–6534.

(70) Makino, S.; van Putten, J. P.; Meyer, T. F. Phase Variation of the Opacity Outer Membrane Protein Controls Invasion by *Neisseria Gonorrhoeae* into Human Epithelial Cells. *EMBO J.* **1991**, *10*, 1307–1315.

(71) Virji, M.; Everson, J. S. Comparative Virulence of Opacity Variants of *Neisseria Gonorrhoeae* Strain P9. *Infect. Immun.* **1981**, *31*, 965–970.

(72) Christodoulides, M.; Everson, J. S.; Liu, B. L.; Lambden, P. R.; Watt, P. J.; Thomas, E. J.; Heckels, J. E. Interaction of Primary Human Endometrial Cells with *Neisseria Gonorrhoeae* Expressing Green Fluorescent Protein. *Mol. Microbiol.* **2000**, *35*, 32–43.

(73) Mah, T. F. C.; O'Toole, G. A. Mechanisms of Biofilm Resistance to Antimicrobial Agents. *Trends Microbiol.* **2001**, *9*, 34–39.

(74) Ward, M. E.; Watt, P. J.; Glynn, A. A. Gonococci in Urethral Exudates Possess a Virulence Factor Lost on Subculture. *Nature* **1970**, *228*, 227–231.

(75) Zak, K.; Diaz, J. L.; Jackson, D.; Heckels, J. E. Antigenic Variation during Infection with *Neisseria Gonorrhoeae*: Detection of Antibodies to Surface Proteins in Sera of Patients with Gonorrhea. *J. Infect. Dis.* **1984**, *149*, 166–174.

(76) Kellogg, D. S. J.; Peacock, W. L. J.; Deacon, W. E.; Brown, L.; Pirkle, C. I. *Neisseria Gonorrhoeae*. I. Virulence Genetically Linked to Clonal Variation. *J. Bacteriol.* **1963**, *85*, 1274–1279.