NANOCRYSTALLINE SILVER INHIBITS ANTIBIOTIC-, ANTISEPTIC-RESISTANT BACTERIA.

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Abstract (Boldfaced text indicates changes)

BACKGROUND/AIMS–Resistance to antibacterial agents is increasingly prevalent. This study evaluated the activity of nanocrystalline silver (NCS, crystallite size <50nm), produced by physical vapor deposition, against bacteria resistant to either antibiotics or the antiseptic benzalkonium chloride (BAC). METHODS– Minimal Inhibitory Concentration (MIC) assay: NCS was serially diluted; bacteria were added and incubated overnight at 37°C; growth of bacteria was assessed by optical density of the cultures. RESULTS–The MIC of NCS against multi-resistant isolates of *Burkholderia dolosa* was **7 ±0.8** (s.e.) µg/ml, and of *B. multivorans* was **7 ±1.0** µg/ml. This compared to **MICs of 3 to 6** µg/ml for other Gram-negatives tested. Additionally, the activity of NCS (MIC= 3 µg/ml) against *Pseudomonas aeruginosa* (PA) with artificially-introduced tetracycline resistance was comparable to its activity (MIC= 4 µg/ml) against the non-resistant parent strain. Lastly, NCS was effective (MIC=6 µg/ml) against a clinical isolate of PA resistant to BAC. CONCLUSIONS–NCS effectively inhibits the growth of bacteria with natural resistance and artificially-introduced resistance to antibiotics, and is also effective against antiseptic-resistant PA. NCS is a promising candidate for development of future antibacterial therapies.

Introduction

Bacterial infection is a major cause of morbidity and mortality in both developed and developing parts of the world. The emergence of antibiotic resistant strains and multiresistant strains has compounded this problem. More recently, bacterial strains with resistance to commonly used antiseptics such as quaternary ammonium compounds (QACs) have been reported (**1**,**2**), and have been isolated from patient samples (**3**). The emergence of antiseptic resistance among hospitalized individuals is particularly alarming since the universal application of antiseptics in the hospital setting can select for these resistance determinants. There is an urgent need for an ever-expanding repertoire of antimicrobial agents for clinical, as well as commercial, applications.

Silver has been known since ancient times (for review, see refs **4** and **5**) to possess antibacterial properties. The antibacterial mechanisms of silver may include modification of sulfhydryl-containing biomolecules such as proteins (**6**), the collapse of electrochemical gradients across the bacterial cell membrane (**7**), and the generation of reactive oxygen species (**8**). Given its potential to target multiple bacterial targets simultaneously, silver-based antimicrobials are expected to be less likely to select for resistant strains, compared to antibiotics which typically target a single bacterial enzyme or process.

Although silver possesses desirable antimicrobial properties, the solubility characteristics of silver metal and common silver salts (such as silver nitrate) render it impractical in many clinical scenarios. NUCRYST has developed a process of forming a thin film of silver nanocrystals utilizing a custom synthesis called *magnetron sputtering*, which is a type of physical vapor deposition. Nanocrystalline silver (NCS) produced by this method has both antibacterial properties (**9**) and anti-inflammatory properties (**10**). Because of its dual activity, NCS is an attractive candidate for the treatment of infectious diseases in which the pathology is attributable to both infection and the host's own inflammatory process. Here, we report on the spectrum of activity of NCS, and demonstrate that the antibacterial properties of NCS extend to antibiotic-resistant, multi-drug resistant, and antiseptic resistant bacteria. Additionally, we present evidence that NCS releases its active moiety(ies) at concentrations likely to be active *in vivo*, over an extended time frame (as long as one week).

Materials & Methods

Bacterial culture- Cultures were performed at 32-37°C in Mueller-Hinton Broth or Mueller-Hinton Agar (*P. aeruginosa* or *S. aureus*, and *Burkholderia spp*), or on tryptic soy agar containing 5% sheep blood (*S. pneumoniae* and *S. pyogenes*).

Nanocrystalline Silver Powder- Nanocrystalline silver (NCS) consists of 96.1% silver with a crystallite size <50 nm. For broth microdilution Minimal Inhibitory Concentration assays, nanocrystalline silver powder was suspended in 0.1 M Lactate buffer, pH 4.0, filtered at 0.2 microns, and stored in the dark at room temperature.

Microdilution MIC assay- Stock solutions of nanocrystalline silver, antibiotics, or antiseptics were serially diluted, and then inoculated with bacterial suspension in log-phase. Plates were incubated 16-24 hours at 32-37°C and growth was assessed by measuring the optical density of the cultures at 625 nm.

D-test for Inducible Macrolide-Lincosamide-Streptogramin Resistance- Overnight cultures were streaked onto Tryptic Soy Agar. Each plate received one erythromycin disk (15µg) and two clindamycin (2µg) disks placed 16mm and 25mm from the erythromycin disk. The plates were incubated overnight at 32°C.

Modified D-test for Silver Resistance- This was performed similar to the standard D-test with the following exceptions. Plates received erythromycin disks and nanocrystalline silver. The latter was added to the plate by cutting a 5-mm diameter well into the nutrient agar and placing the nanocrystalline silver (approximately 1-2 mg) into the well. The well containing the nanocrystalline silver was placed 25-, 16-, 9-, or 3mm from the erythromycin disk.

Time course dissolution antibacterial assay- 1 mg of either NCS, crystalline $AgNO_3$ (Sigma), nanosized silver metal (70 nm, Aldrich), or silver shot (1-3 mm, Aldrich) were placed into the bottom of a conical tube and 0.5 ml of bovine calf serum was added. The silver-containing solids were allowed to settle to the bottom of the tube. At various time points, 0.2 ml of supernatant was removed and replaced with fresh serum, gently to leave the pellet undisturbed. The 0.2 ml samples were reserved for microdilution MIC assays. Results were expressed as the relative amount of antibacterial activity (fold-MIC) present in the supernatant at each time point.

Table 1. Spectrum of NCS antibacterial activity. Values shown in ppm (µg/ml) measured by standard broth microdilution MIC assay.

		Median	MIC_{90}
G+	***S. aureus***	12	31
	***S. epidermidis* (TYPE)**	3	NT* Not tested
	S. pyogenes	8	NT
	S. pneumoniae	3 †	NT
G-	***P. aeruginosa***	3	6
	C. freundii	3 †	NT
	E. cloacae	3	NT
	K. pneumoniae	3	NT
	P. mirabilis	3 †	NT
	S. marcescens	3	NT
	E. coli	3 †	NT

† = only one isolate was tested

Conclusion: NCS inhibits the growth of a wide range of Gram-positive and Gram-negative bacterial species.

Table 2. Correlation of Methicillin sensitivity with NCS efficacy among 20 isolates of *S. aureus*. Methicillin sensitivity was determined by disk diffusion. MIC was by standard broth microdilution assay. N is the number of isolates. MIC in ppm (µg/ml) is shown as avg± st dev among isolates.

Methicillin sensitivity	N	NCS MIC
Sensitive	9	10±4
Resistant	11	10±4

P = 0.70 by two-tailed T-test

Conclusion: MRSA is inhibited by NCS as effectively as methicillin-sensitive SA.

Table 3. NCS antibacterial activity against multidrug resistant *Burkholderia* isolates. Values shown in ppm (µg/ml) measured by standard broth microdilution MIC assay.

Species	Isolate	Resistance	NCS MIC
B. multivorans	**BBM1**	To, P, Km, Gm, Am	7
B. multivorans	**BBM2**	NT (Not Tested)	7
B. multivorans	**BBM4**	NT	7
B. dolosa	**SLC6-3**	To, Te, P, C, Km, Cp, Gm, Am	7
B. dolosa	**SLC6-4**	NT	7

Conclusion: NCS inhibits the growth of multidrug resistant *Burkholderia isolates*

Figure 1. Identification of iMLS resistant *S aureus*. iMLS strains of *S. aureus* were identified by D-test. DA = clindamycin, E = erythromycin.

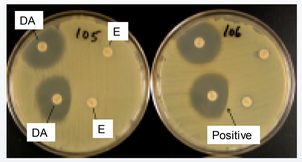


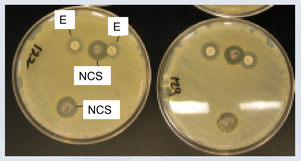
Table 4. Correlation of iMLS phenotype with NCS efficacy among 20 isolates of *S. aureus*. N is number of isolates tested. MIC in ppm (µg/ml) was measured by standard broth microdilution MIC assay. Value shown is avg± st dev.

D-test result	N	NCS MIC
Positive for iMLS	4	11±6
Negative	16	9±3

P = 0.41 by two-tailed T-test

Conclusion: iMLS *S. aureus* are inhibited by NCS as effectively as isolates lacking iMLS resistance.

Figure 2. Modified D-test of efficacy of NCS during induction of MLS resistance. *S. aureus* identified as iMLS by a standard D-test were tested in a modified test using erythromycin disks (E) and wells filled with NCS-containing cream (NCS).



Conclusion: The iMLS phenotype confers no resistance to NCS.

Table 5. Efficacy of NCS against a strain of *P. aeruginosa* with artificially introduced tetracycline resistance. MIC results are shown as ppm (µg/ml) measured by MIC assay.

Strain	Tet sensitivity	NCS MIC
PA232	**Resistant**	3
PA1026	**Sensitive (isogenic)**	4

Conclusion: NCS is as effective against a strain with artificially-introduced tetracycline resistance as it is against the non-resistant parent strain.

Table 6. Screening for BAC resistance. Results shown as ppm (µg/ml) measured by MIC assay.

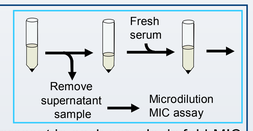
Isolate	Clinical source			BAC MIC
	Gender	Age	Site	
NP217	**M**	**91**	**Resp.**	125
NP218	**M**	**91**	**Urine**	63
NP219	**M**	**85**	**Urine**	63
NP220	**F**	**85**	**Derm.**	63
NP221	**M**	**42**	**Derm.**	4000
NP222	**M**	**74**	**Resp.**	63

Table 7. Efficacy of NCS against antiseptic- resistant isolate NP221. NP221 and several other isolates were screened by MIC assay for susceptibility to additional antiseptics, and to NCS. MIC results are shown as %(v/v), or as ppm (µg/ml) when not indicated.

Agent	NP217	NP218	NP219	NP220	NP221	NP222
Lysol				0.5%	1%	1%
Cetrimide				125	500	125
Roccal-D				0.008%	0.03%	0.008%
Betadyne				>1%	>1%	>1%
Virosan				0.03%	0.06%	0.06%
NCS	6	6	6	3	6	6

Conclusion: NCS is as effective against an isolate resistant to Quaternary Ammonium Compounds (QACs) as it is against QAC-sensitive isolates.

Table 8. Time course dissolution of NCS antibacterial activity. Pellets of various silver sources were incubated under a serum supernatant for 1 week. Supernatant samples were pulled at indicated times and assayed by MIC.



The table shows the amount of activity present in each sample, in fold-MIC.

	NUCRYST NCS	Sigma $AgNO_3$	Aldrich Nano-Silver metal	Aldrich Silver metal shot
Time 0	16	ND* None detected	ND	ND
16 hours	16	8	ND	ND
23 hours	16	8	ND	ND
40 hours	8	4	ND	ND
47 hours	8	2	ND	ND
112 hours	8	4	ND	ND
119 hours	8	2	ND	ND
136 hours	8	2	ND	ND
143 hours	4	ND	ND	ND
160 hours	4	ND	ND	ND

Conclusion: NCS and silver nitrate salt both release antibacterial activity in a sustained fashion, although NCS sustains release for a longer time. Silver metal [Ag(0)] does not release activity at a detectable level, and this is true whether it is used as nano-sized particles or as larger shot.

Discussion

The discovery of new classes of antibacterial agents is imperative given the continuous emergence and spread of drug-resistant clones of bacteria. Silver is a promising candidate given its established anti-infective properties, and its nearly 200-year history of safe use in the form of silver nitrate eye drops.

Resistance of bacteria to silver has been documented in several bacterial genera including *Pseudomonas* (**11**), *Escherichia* (**12**), and *Klebsiella* (**13**), and the genetic determinants of such resistance have been elucidated for some bacteria (**14**). Yet despite the widespread usage of silver in clinical, industrial, and nutritional settings silver resistance has yet to emerge as a common problem. The rarity of silver resistance may be attributable to silver's multiple modes of action against living cells (**6-8**).

The process of magnetron sputtering results in the formation of silver-containing nanocrystals which form various orders of nano-sized particles. The structural disorder inherent in these particles may confer differential solubility characteristics that ultimately result in sustained release of active, soluble moieties from the NCS. This could be a critical determinant of efficacy in a wound environment where exudate from the tissue has the potential to continuously dilute pharmaceuticals that are introduced to the site, and where proteins present in the exudate can bind to or otherwise inactivate the pharmaceutical. The simple model shown below can be used to illustrate potential differences in the antibacterial activity of different forms of silver, and may be useful for explaining the data presented in Table 8.

R1 represents the solution rate of active soluble silver from the solid, and R2 represents the rate at which the active silver is inactivated by binding to serum proteins (**6**). We propose that in the case of NCS, R1 remains sufficiently large to maintain the concentration of soluble active silver above the MIC for up to one week. Silver metal [Ag(0)] releases soluble silver ions at a very slow rate, such that the soluble silver is inactivated by serum faster than it is released (R1<<<R2). Silver nitrate releases active silver, but does not do so in a sustained fashion, so the activity is release quickly, and is diluted over the course of the experiment.

In summary, NCS is a promising candidate for development of future antibacterial therapies because of its wide spectrum of activity. Its unique structure may result in solubility characteristics, making NCS useful for a wide array of applications where sustained activity is vital for success.

Acknowledgments

The authors thank Dr. Gregory Priebe for clinical isolates of *Burkholderia multivorans* and *B. dolosa,* and Dr. George O'Toole for *P. aeruginosa* strains PA232 and PA1026. They also extend their gratitude to Katherine Thompson and Nicole Bellefeuille for expert technical and analytical assistance.

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Detailed Materials & Methods

Bacterial Strains and Isolates- Clinical isolates of *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae*, and *Streptococcus pyogenes* were obtained from the Beth Israel Deaconess Medical Center, Needham, MA. *P. aeruginosa* strains PA232 and PA1026 were a kind gift from Dr. George O'Toole, Dartmouth College. Clinical isolates of *Burkholderia dolosa* and *Burkholderia multivorans* were obtained from the Children's Hospital, Boston, MA.

Culture conditions- Isolates of *P. aeruginosa*, *S. aureus*, and *Burkholderia spp.* were cultured by either inoculation of 6ml portions of Mueller-Hinton Broth (MHB; Remel, Winsor, CT), or by streaking onto Mueller-Hinton Agar (MHA; BBL, Sparks, MD). Isolates of *S. pneumoniae* and *S. pyogenes* were cultured by streaking onto tryptic soy agar containing 5% sheep blood (BBL, Sparks, MD). All cultures were done at 32-37°C.

Nanocrystalline Silver Powder- Nanocrystalline silver (NCS) was produced by physical vapor deposition using a process of magnetron sputtering and consists of 96.1% silver with a crystallite size <50 nm. For broth microdilution Minimal Inhibitory Concentration Assays, NCS powder was added to 0.1 M Lactate buffer, pH 4.0 and was sonicated for five-minutes. Large particles were removed by centrifugation, and the supernatant further cleared of particulates by passage through a 0.2-micron cellulose acetate filter (Corning, Corning, NY). The resulting suspension was stored in the dark at room temperature until use.

Microdilution MIC assay- Stock solutions of nanocrystalline silver, antibiotics, or antiseptics, were added to wells in a 96-well microtiter plate (VWR, Bridgeport, NJ), and were serially diluted by transferring 100 µl of each well into another well containing 100 µl fresh MHB. Each serial dilution was then inoculated with 10 µl bacterial suspension containing 10^4 cfu in log-phase growth. Plates were incubated 16-24 hours at 32-37°C and growth was assessed by measuring the optical density of the cultures at 625 nm. Each assay was performed in quadruplicate, and the MIC was taken as the concentration of antimicrobial at which none of the four quadruplicate tests had an optical density greater than 0.100.

D-test for Inducible Macrolide-Lincosamide-Streptogramin Resistance- Overnight cultures of *S. aureus, S. pneumoniae*, or *S. pyogenes* were streaked onto two Tryptic Soy Agar. Antibiotic susceptibility disks (Remel, Lenexa, KS) containing 15 micrograms erythromycin or 2 micrograms clindamycin were placed onto the agar plates using sterile forceps. Each plate received one erythromycin disk and two clindamycin disks: the first clindamycin disk was placed 25mm from the erythromycin disk, and the second clindamycin disk was placed 16mm from the erythromycin disk. The plates were incubated overnight at 32°C. A positive result was scored when the clindamycin zone was flattened facing the erythromycin disk.

Modified D-test for Silver Resistance- This was performed similar to the standard D-test with the following exceptions. Plates received two erythromycin disks and nanocrystalline silver. The latter was added to the plate by cutting a 5-mm diameter well into the nutrient agar with a sterile cork bore, and placing the nanocrystalline silver (approximately 1-2 mg) into the well. The well containing the nanocrystalline silver was placed 25-, 16-, 9-, or 3mm from the erythromycin disk. The plates were incubated overnight at 32°C.

Time course dissolution antibacterial assay- 1 mg of either NCS, crystalline $AgNO_3$ (Sigma), nanosized silver metal (70 nm, Aldrich), or silver shot (1-3 mm, Aldrich) were placed into the bottom of a conical tube and 0.5 ml of bovine calf serum was added to the tubes. The silver-containing solids were allowed to settle to the bottom of the tube. At various time points, 0.2 ml of supernatant was removed and replaced with fresh serum, gently to leave the pellet undisturbed. The removed samples were reserved, and at the end of the experiment were used in microdilution MIC assays. Results were expressed as the relative amount of antibacterial activity (fold-MIC) present in the supernatant at each time point.