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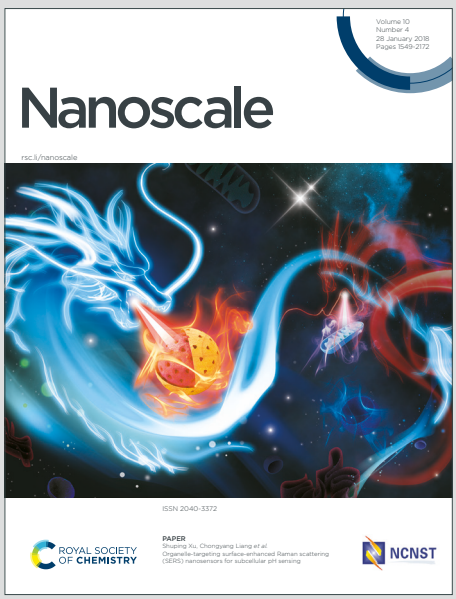

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ARTICLE

Persistent, Broad-Spectrum Antimicrobial Activity by Multi-Metal Surface Phase Modified Ceria Nanozymes

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With the growing threat of emergent microbes, such as novel viruses and drug-resistant bacteria, more robust and broad-spectrum antimicrobial technologies are of critical importance. Nanomaterials have the potential to deliver a broad-spectrum antimicrobial effect while maintaining resistance to degradation in various application environments. In the presented study, a multi-metal oxide nanoparticle formulation with substantial antimicrobial activity was developed: outperforming a previous bimetallic composition. The formulation was produced through a variation of an established method wherein the multi-metal oxide is formed through precursor ageing in a peroxide solution environment (room temperature, 8 week ageing period). Silver/zinc-modified cerium oxide nanoparticles exhibited substantial antiviral (0.05 mg/mL reduced virus titer to beyond detectable ranges for RV14 rhinovirus, ~3-log reduction for vesicular stomatitis virus on treatment with 0.15 mg/mL in $TCID_{50}$) and antibacterial (MIC values of ~4, 6, and 6 µg/mL, respectively, for *P. aeruginosa*; *S. aureus*, and methicillin-resistant *S. aureus* (MRSA)) activities. Of further interest, this nano-formulation evidenced persistent antibacterial activity towards bacterial (*P. aeruginosa*, *S. aureus*) and viral (RV14 rhinovirus; Vesicular stomatitis virus,VSV) lab strains resistant to previously developed silver-modified cerium oxide nanoparticles. Nanomaterial character and potential antimicrobial mechanisms were also investigated, with enzyme-mimetic *nanozyme* generation of reactive oxygen species suggested by density functional theory calculations.

1. Introduction

Human infection by pathogenic microbes continues to be a prominent societal issue, with hospital-acquired infections, emergence of novel viral variants, and growth of drug-resistant bacterial strains heightening the general health threat(5). Central to these issues is the ability of microbes to circumvent or surmount traditional antimicrobial interventions and the inherent difficulties in optimally deploying these methods in certain formats, such as high-touch surfaces in public spaces(3-5).

The use of nanomaterials has been seen as an attractive approach to address these challenges, given their versatility as functional materials and their greater physicochemical stability over organic antibiotics and antivirals and other conventional antimicrobial materials (6) (7) (8) (9) (10) (11). This stability for inorganic nanomaterials translates to robust activity across temperature ranges and a continuous, sustained activity over technologically relevant time periods (*i.e.*, mechanisms which do not rely on material consumption or irreversible alteration; potential for prolonged systemic dispersion, potential for catalytic reaction mechanisms, *etc*.) (12) (13) (14). Among these nanomaterials, metal oxide-based compositions are particularly attractive for biomedical applications because of their stability towards oxidation by the biological environment (15) (16) (17) (18, 19).

Cerium oxide is a metal oxide with substantial utility across numerous industrial sectors, due largely to its surface redox chemistry facilitated by the interconversion of cerium sites between Ce^{3+} and Ce^{4+} accompanied by the formation or healing of oxygen vacancies. In biomedical studies, surface redox reactions by cerium oxide nanoparticles (CNPs) have been identified as mimetic of reactions produced by enzymes (20) (21) (22). The class of materials which produce these catalytic reactions are known as *nanozymes*. These materials, including CNPs, demonstrate the above-mentioned stability and thereby show a strong advantage over biological enzymes. Indeed, multiple studies have been published highlighting the utility of pure CNPs, produced by assorted wet chemical synthetic approaches, in antimicrobial applications. Among these studies, smaller particle sizes and surface densities of Ce^{3+} states have been reported as key metrics for the observed activities (14). Understanding of essential nanomaterial

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Supplementary Information available: Figures S1, S2 and S3, Tables S1 and S2

structure-property relations has progressed such that production of designer nanomaterials for specific applications, *e.g.* as high-efficacy antimicrobial technologies, is becoming more feasible and practical (23) (24) (7) (25) (26).

In many studies, surface redox processes have been coupled with other chemical processes to facilitate catalytic reactions of industrial relevance (*i.e.*, soot reduction by silver-modified cerium oxide surfaces) (27) (28) . In these reactions, such as the case of nanoscale silver-cerium oxide, interface formation between phases allows for coupling of charge and/or mass transfer processes to produce complex reactions (29) (30). In biomedical applications, similarly structured nanomaterials have been produced to accomplish a larger set of relevant reactions, including as antimicrobial compositions. Antimicrobial activity for these materials is often attributed to an increase in reactive oxygen species (ROS) generation and/or the release of metal ions. Therefore, noble and transition metal compositions have seen substantial investigation; *e.g.*, Ag/Cu, Au/Pt, Cu/Zn, CuO/Ag, Ag/ZnO (11) (31) (32). (33) (34).

The presented work is built from several of our earlier investigations which demonstrated the use of leeching-stable AgCNP formulations for antiviral and antibacterial applications (1, 35, 36). In these studies, material properties including particle size, morphology, and chemical state distributions (*i.e.*, cerium redox states, Ce^{3+}/Ce^{4+}; Ag^0, Ag^+), were defined for different silver-cerium oxide nanoparticle formulations and related to virus (rhinovirus, RV14; parainfluenza virus, PIV5; coronaviruses, OC43 and SARS-CoV2) or bacteria (*Pseudomonas aeruginosa*; *Staphylococcus aureus*; methicillin-resistant *S. aureus* (MRSA)) interactions. In the current work, we consider the efficacy of a nanomaterial formulation with greater compositional complexity, cerium oxide nanoparticles surface modified with silver- and zinc- rich phases, as a multi-metal material for antimicrobial applications. In these studies, we hypothesize that inclusion of both transition metals would alter the material's surface redox reaction profile through introduction of additional metal (oxide) domains and/or alteration in silver charge transfer by alloying or complex formation. An initial material characterization and analysis was performed using, primarily, high-resolution transmission electron microscopy, energy-dispersive spectroscopy, and x-ray photoelectron spectroscopy. To facilitate comparison against previously reported silver-modified cerium oxide nanoparticles, studies were performed to assess the material's antiviral/microbials efficacy towards the viruses vesicular stomatitis virus (VSV) and rhinovirus (RV14) and to the bacterial species *P. aeruginosa*, *S. aureus*, and methicillin-resistant *S. aureus* (MRSA). Results and analyses from these and related density functional theory-based studies were then used to identify possible mechanisms of observed substantial antimicrobial activities for the silver/zinc-modified cerium oxide nanoparticle formulation.

2. Materials and Methods

2.1 Materials Synthesis and Characterization

2.1.1 Synthesis A nanoparticle formulation was produced incorporating silver and zinc with cerium oxide nanoparticle through a one-pot synthesis, similar to a previously reported method(36). A quiescent aqueous solution of 1.0 mM zinc nitrate (99.5% purity), 1.0 mM silver nitrate (99.9% purity), and 5.0 mM cerium nitrate hexahydrate (99.999% purity) (final molar concentrations) in a borosilicate storage bottle was treated with 2 mL of 3% hydrogen peroxide solution for a final solution volume of 50 mL. The solution was then homogenized *via* vortex mixer continuously for 2 minutes, the bottle's cap was loosened to allow release of evolved gases, and the solution was stored, without perturbation, in dark conditions for up to 8 weeks prior to characterization. All chemical manipulations were performed at standard temperature and pressure. All chemicals were purchased from *Sigma Aldrich* and were used without further modification.

2.1.2 X-ray Photoelectron Spectroscopy Samples were prepared by drying from solution onto clean gold foil substrates inside of a chemical fume-hood at room temperature. Measurements were performed using an ESCALAB-250 Xi spectrometer with a monochromatic Al-Kα radiation source under a vacuum < 1x10-9 mbar, operating power of 300 W (150 kV, 20 mA), pass energy of 20 eV, step size of 0.1 eV, and a spot size of 650 µm. All spectra were referenced to the C-C/C-H binding energy, within the C1s region, assigned a value of 284.6 eV. Peak assignment was performed based on reported values from relevant published literature.

2.1.3 High-resolution Transmission Electron Microscopy Samples for the transmission electron microscopy (TEM) studies were prepared according to the following procedure: a droplet of the aqueous suspension containing nanoparticles was poured onto the copper grid covered with a thin carbon layer and dried on air. Detailed analysis of nanoparticles was performed including bright field imaging (BF), selected area diffraction patterns (SADP), high resolution imaging in TEM (HRTEM) and the Scanning Transmission Electron Microscopy High-Angle Annular Dark Field mode (STEM) including mapping of elemental distribution in the selected areas using Themis G2 200 Probe Cs-corrected transmission electron microscope operating at 200 kV and equipped with X-FEG, HAADF-STEM and Super-X EDX System.

2.1.4 Computational Methodology Spin-polarized density functional calculations were run using the VASP code (37-39). The frozen-core projector-augmented wave (PAW) method was utilized with a plane-wave basis set with a cutoff of 500 eV (39, 40). 3D boundary conditions were implemented. The cut-off energy and k-point mesh have been checked for convergence as in our previous calculations. The exchange correlation functional was the Perdew-Burke-Ernzerhof (PBE) GGA (1, 2, 41, 42). An effective U = 5 eV was used on the Ce f orbitals using the Dudarev method (43) to enable localization of Ce^{3+} states as in our previous studies (44-46). METADISE code was used to

generate all models (47). The conventional 12-atom bulk CeO_2 unit cell was simulated with the electronic and ionic convergence criteria of $1x10^{-5}$ eV and $1x10^{-3}$ eV $Å^{-1}$, respectively, and the Brillouin zone sampled using a Γ-centered 5 x 5 x 5 k-point mesh. The simulated lattice constant (5.498 Å, space group 225) is overestimated as in previous computational studies due to the usage of the PBE+U methodology (48-54). The CeO_2 surface models were simulated using the slab method, where the top and the bottom of the slab are allowed to relax. The {100}, {110} and {111} surfaces are √2 x √2, a √2 x 2 and √2 x √2 expansions with 7 (28 CeO_2 units), 7 (28 CeO_2 units) and 5 (20 CeO_2 units) surface layers, respectively. To remove the dipole of the {100} slab, half of the surface oxygen atoms were moved from one side to the other of the slab. A vacuum gap of 15 Å perpendicular to the surface was introduced to minimize the interaction between images. The surfaces were simulated using the electronic and ionic convergence criteria of $1x10^{-5}$ eV and $1x10^{-2}$ eV $Å^{-1}$, respectively, with the Brillouin zone sampled using a Γ-centered 2 x 2 x 1 k-point mesh, with the third vector perpendicular to the surface plane. Oxygen vacancies (on the surface and subsurface layer), Zn dopant atoms and adsorbates were introduced symmetrically on both sides. To reduce the exploration of the configurational space for the adsorption of species, only models based on previous studies were used (1, 2). The adsorbate species (*i.e.* isolated H_2O_2 and O_2 molecules, and Ag_3 cluster) were geometry optimized in a cubic cell of side 10 Å at the Γ point with the electronic and ionic convergence criteria of $1x10^{-5}$ eV and $1x10^{-2}$ eV $Å^{-1}$, respectively. The energies of adsorption was calculated as

$$E_{Ads} = \left(E_{Surf+adsorbate} - E_{Surf} - E_{adsorbate}\right)/2$$

where $E_{Surf+adsorbate}$, E_{Surf}, and $E_{adsorbate}$ are the energies of the adsorbates adsorbed onto the surface, of the bare surface, and of the isolated adsorbate, respectively, and 2 accounts for the adsorption of the adsorbates on both sides of the slab.

2.2. Virus Studies

2.2.1 Cells and Viruses Cultures of HeLa cells were grown in Dulbecco modified Eagle medium (DMEM, Gibco, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal calf serum (HI FBS, Gibco, Thermo Fisher Scientific).
Human Rhinovirus 14 (RV14, ATCC, catalog number VR-284) was grown in HeLa cells at 33°C. RV14 stocks were determined by a $TCID_{50}$ assay using confluent HeLa cells as described previously(36). Briefly, virus stock was serially diluted in DMEM containing 10% Bovine Serum Albumin (BSA 7.5% solution, Gibco, Thermo Fisher Scientific) as carrier protein. Cells were incubated with diluted virus solutions for one hour at 33°C. Cells were then washed, replaced with DMEM containing 2% HI FBS, and incubated at 33°C for 3 days. Cells were stained with a crystal violet solution and $TCID_{50}$ units were calculated by the Spearman & Kärber algorithm as previously described(55). The Orsay strain of vesicular stomatitis virus (VSV) was grown in Vero cells at 37°C for 2 days and VSV stocks were determined by $TCID_{50}$ assay using confluent HeLa cells as described above.

2.2.2 AgCNP-Resistant Rhinovirus Selection. AgCNP-resistant (AgCNP2 from previous study (36), AgCNPctrl hereafter) RV14 was generated as described previously (35). Briefly, ~5x105 TCID50 units of parental RV14 was incubated with 0.1 mg/mL AgCNPctrl for 1 hr. Reactions were then diluted in DMEM containing 0.38% BSA and used to generate a virus stock in HeLa cells. These cycles of treatment and expansion were repeated for a total of 3 selection rounds with a nanoparticle concentration of 0.1 mg/mL. AgCNPctrl concentration was then adjusted to 0.2, 0.1, and 0.15 mg/mL for the 4th, 5th, and 6th selection rounds, respectively. After the 6th round, RV14 was harvested and pelleted as described above.

2.2.3 Nanoparticle Virus Inactivation Assays Nanoparticles were prepared and studies in liquid were performed as previously reported(36). Briefly, nanoparticles were ultrasonicated, diluted in inactivation buffer to the desired concentration as indicated in the figure legends, and incubated with virus at room temperature. After indicated incubation times, remaining infectious virus was quantified by TCID50 as described above.

2.2.4 Statistical Analyses. Unless otherwise indicated, values are the mean of three independent samples, with error bars representing the standard deviation. Statistical analysis was performed using GraphPad ordinary one-way ANOVA comparing untreated 1hr samples to the nanoparticle treated samples. In all figures, * indicates a p-value of <0.05, ** indicates p-value of <0.01, and *** indicates a p-value of <0.001.

2.3. Bacterial Studies

2.3.1 Bacterial Studies Minimum inhibitory concentration (MIC) determination for Ag/Zn-CNP The minimum inhibitory concentration (MIC) of Ag/Zn-CNP was determined in Tris buffer (25 mM Tris/75 mM NaCl; Tris-NaCl) and in Luria–Bertani (LB) broth. The lowest concentration of Ag/Zn-CNP that inhibits bacterial growth was measured for *P. aeruginosa* ATCC 15442 (Gram negative), *S. aureus* ATCC 6538 (Gram positive), and methicillin-resistant *S. aureus* ATCC 33592 (MRSA) in Tris-NaCl and LB media at pH 7.2. Bacterial inoculums were adjusted to 10^8 CFU/mL. Tris-NaCl and LB media inoculated only with 10^8 CFU/mL bacteria were used as controls. MICs were determined by broth dilution using a 96-well flat-bottom microtiter plate. Final Ag/Zn-CNP concentrations of 2, 4, 6, 8, and 10 µg/mL were prepared in Tris-NaCl medium for all three bacterial strains. In LB medium, final concentrations of 10, 20, 30, 40, 50, 60, 70, 80, and 100 µg/mL were tested for P. aeruginosa, while 10, 20, 30, 40, 50, 60, 70, 80, 100, 200, 300, and 400 µg/mL were tested for S. aureus and MRSA. Cultures were incubated at 37 °C with shaking at 210 rpm for 24 h, and changes in optical density at 600 nm were used to assess bacterial growth in LB medium. Gentamicin was included as a study control group.

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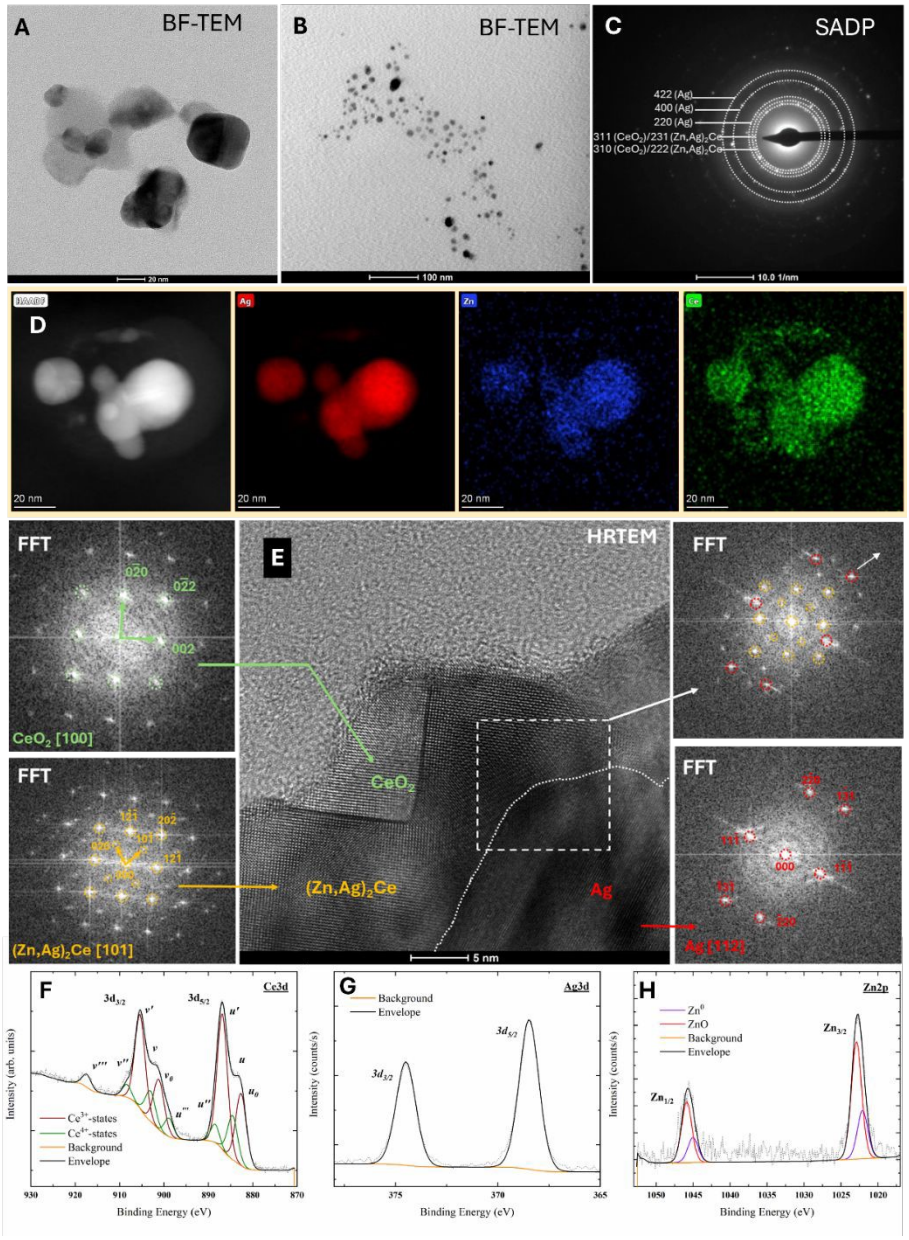

Figure 1. Materials Characterizations of Silver-Zinc Cerium Oxide Nanoparticles. A bright-field transmission electron microscopy (BF-TEM) image taken at higher magnification showing a morphology of some of the observed nanoparticles (A) together with a micrograph recorded at lower magnification (B) showing a distribution of the observed nanoparticles. The selected area diffraction pattern (SADP) taken from larger area indicating presence of different types of nanoparticles (C) together with the Scanning Transmission Electron Microscopy High-Angle Annular Dark Field (STEM-HAADF) image with results of elemental mapping indicated intermixing of cerium, silver and zinc in the observed area (D). High-Resolution Transmission Electron Microscopy (HRTEM) image with Fast Fourier Transforms (FFT) from selected regions showing the details of structure and symmetry of the observed nanoparticles. The d – values obtained from HRTEM images (E) suggest the formation of Zn-rich compounds. X-ray photoelectron spectroscopy (XPS) measurements with binding energy regions for Ce3d (F), Ag3d (G), and Zn2p (H) chemical states. Peak fittings to Ce3+ and Ce4+ states provide insight into the density of oxygen vacancies at the material surface and thereby their unique redox chemistry. Interestingly, silver (G) content in the nanoparticle formulation is observed in a metallic state while zinc (H) occurs in both metallic and oxide states. The range of component valencies may also contribute to observed antimicrobial activities via associated redox reactions such as the generation of free radicals by engineered nanoceria formulations.

2.3.2 Time scale study To carry out a time course assay, bacterial inoculums were adjusted to 10^8 CFU/mL in protein-free medium, Tris-NaCl. The MIC of Ag/Zn-CNP (4 µg/mL for P. aeruginosa and 6 µg/mL for S. aureus and MRSA) was evaluated in a final volume of 25 mL. Control groups included bacteria with (i) no treatment and (ii) 20 µg/mL gentamicin. The cultures were then incubated at 37 °C under agitation (210 rpm). 50 µL from each of the treated samples were spread onto LB agar plates, and growth was assessed at 15, 30, 45, 60, and 75 minutes for *P. aeruginosa*, and at 60, 120, 180, and 240 minutes for *S. aureus* and MRSA. Colony-forming units (CFU/mL) were quantified after incubation at 37 °C for 16 h.

2.3.3 Effect of temperature on Ag/Zn-CNP activity Bacterial inoculums were adjusted to 10^8 CFU/mL, and a time-course study was carried out in Tris-NaCl medium. The MIC of Ag/Zn-CNP (4 µg/mL for *P. aeruginosa* and 6 µg/mL for *S. aureus* and MRSA) was evaluated at a final volume of 10 mL. Cultures

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without Ag/Zn-CNP served as the control group and all cultures were incubated at 4 °C under agitation at 210 rpm. Aliquots (50 µL) were spread onto LB agar plates, and growth was assessed after 1 and 24 h for *P. aeruginosa*, and after 3 and 24 h for *S. aureus* and MRSA. Colony-forming units (CFU/mL) were quantified following incubation at 37 °C for 16 h.

2.3.4 Resistance-development towards Ag/Zn-CNPs Approximately 10^8 CFU/mL of *P. aeruginosa* and *S. aureus* were incubated in LB medium at their sublethal (IC_{50}) concentrations of Ag/Zn-CNP, corresponding to 20 µg/mL and 200 µg/mL, respectively. Culture grew at 37°C at 210 rpm for 2 hr. Then 50 µL of culture was plated onto an LB agar plate and incubated overnight at 37 °C. A single colony from each plate was then reinoculated into LB medium and exposed again to the IC_{50} concentration of Ag/Zn-CNP. This treatment cycle was repeated eight times. After the 6th and 8th cycles, *P. aeruginosa* and *S. aureus* were tested for resistance development by exposing them to their MIC values of Ag/Zn-CNP (30 µg/mL and 400 µg/mL, respectively) under the culture conditions of 37°C and 210 rpm for 16 hours. Cultures were consequently plated onto LB agar and examined for resistant colonies.

2.3.5 Bactericidal effect of Ag/Zn-CNP on resistant strains The effect of Ag/Zn-CNP was studied against the AgCNP-resistant strains *P. aeruginosa* and *S. aureus* (laboratory strain). 10^8 CFU/mL of bacteria were treated with increasing concentrations of Ag/Zn-CNP in Tris-NaCl (10, 20, 30, 40, 50, and 60 µg/mL) at 37 °C for 24 hours. 50 µL of the tested samples were plated onto the LB agar and incubated overnight at 37 °C. Grown colonies were counted and recalculated to the number of CFU/mL.

2.3.6 Effect of Ag/Zn-CNP on cell metabolic activity on macrophage cells The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, USA), 1% (v/v) penicillin–streptomycin (P/S; Thermo Fisher Scientific, USA), and 2 mM L-glutamine. Cultures were kept in a humidified incubator at 37 °C with 5% CO_2. Cell metabolic activity was assessed on days 1 and 3 using an MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (M2128, Millipore Sigma, USA). RAW 264.7 macrophages were seeded in 96-well plates at a density of $2.5x10^4$ cells/well and cultured at 37 °C with or without Ag/Zn-CNP at concentrations of 4, 6, 30, 100, or 400 µg/mL. On the designated culture days, 20 µL of a 5 mg/mL MTT solution was added to each well and incubated for 4 h. The supernatant was then carefully removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the resulting formazan crystals. Absorbance was measured using a SpectraMax iD3 multimode microplate reader (Molecular Devices, CA, USA).

2.3.7. Statistical Analyses. All bacterial experiments were carried out in triplicate. Data is presented as mean±standard error. Statistical analysis was performed using two-way ANOVA with a post-hoc Tukey's test for comparison between groups (GraphPAD Prism, v8, USA). *p* values <0.05 were considered significant.

3. Results and Discussion

3.1 Synthesis of Ag/Zn-CNP formulation

Silver and zinc surface phase modified cerium oxide nanoparticles (Ag/Zn-CNPs) were synthesized by treating an aqueous solution of Ag^+, Zn^{2+}, and Ce^{3+} nitrates with hydrogen peroxide, followed by ageing for 8-weeks. During this ageing period, similar to our observations for pure cerium oxide and silver-cerium oxide nanoparticle formulations produced by comparable methods, the solution coloration went from dark yellow-orange to pale yellow and finally to colorless. This cycling from clear to orange and back to a clear solution has been ascribed to redox cycling of Ce^{3+}/Ce^{4+} and to the formation/degradation of cerium peroxy, as well as oxyhydroxy, complexes, as previously reported for pure cerium nitrate-hydrogen peroxide systems. Additionally, a precipitate formation was observed, with a spontaneous re-dispersion occurring over the ageing period: also observed for comparable pure cerium and cerium-silver nitrate systems. The extended ageing period is ascribed to the slow kinetics of peroxide oxidation of cerium at the low pH of the synthesis (~3.5) and the absence of observable (*i.e.*, *via* black/brown coloration), or measured (*vida infra*; Fig. 1I), silver oxide tentatively to the presence of free peroxide ($Ag_2O + H_2O_2 \rightarrow 2Ag + H_2O + O_2$).

3.2 Materials Characterization of Silver/Zinc-Cerium Oxide Nanoparticles

3.2.1 Microscopy and Phase composition TEM observations (Fig. 1) confirmed the presence of small, aggregated clusters of spherical-shaped nanoparticles with a mean diameter of 11 nm (Fig. 1A-B). The EDX elemental mapping indicated intermixing of cerium, silver and zinc within them (Fig. 1D). Literature data on the thermodynamic equilibrium in the Ag-Ce-Zn system are very scarce, and, as expected, even less is known regarding the oxide combinations of these metals. However, in the study of Long et al. on the thermodynamics of Ag-Ce-Zn, the isothermal cross-sections have been calculated and experimentally verified for 450 and 600 °C (56). It has been shown that Ag_2Ce and Zn_2Ce phases can be combined as $(Ag,Zn)_2Ce$ because of their mutual solubility. Ag_2Ce phase (Pearson symbol of oI12, space group Imma), possess the lattice parameters of a=0.48nm, b=0.709 nm, c= 0.82 nm (57), while Zn_2Ce phase lattice parameters (and the same space group as Ag_2Ce) are very similar and as follows: a=0.4633 nm, b=0.7538 nm, c=0.749 nm) (58). Therefore, for the electron diffraction pattern taken from the larger area, beside the presence of silver nanoparticles (AgNPs) also $(Zn,Ag)_2Ce$ phase has been identified (Fig. 1C). Cerium oxide has a cubic structure with $Fm\bar{3}m$ space group (lattice parameter: a=0.547 nm), where Ce^{4+} is bonded to eight equivalent O_2^- atoms, while O_2^- is bonded to four equivalent Ce^{4+}. On the other hand, silver has the same structure as CeO_2 (cubic with $Fm\bar{3}m$ space group) with a lower lattice parameter a=0.41 nm. It is also

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worthy of mention that silver is able to dissolve relatively high amounts of zinc (up to 20 at.%) without changing its structure

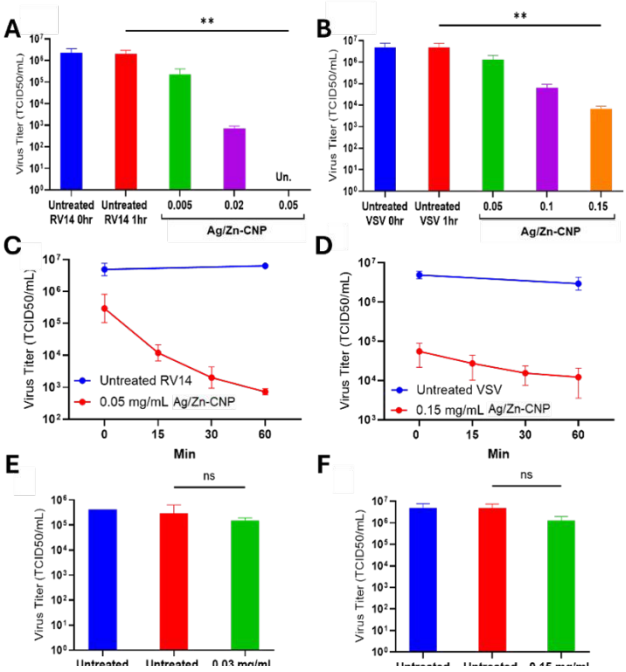

Figure 2. Effect of 77WC-1 and AgCNP3 Nanoparticles on RV14 and VSV Infectivity. Liquid reactions of virus and nanoparticles were incubated at room temperature for 1hr. Nanoparticle and virus reactions were collected at 1hr post nanoparticle addition while untreated virus controls were collected at 0hr and 1hr. Remaining infectious virus titer was determined by $TCID_{50}$ assay. A and B) increasing concentrations of 77WC-1 nanoparticles or buffer alone were incubated with RV14 (A) or VSV (B) then assayed for remaining infectious virus. C and D) 0.05 mg/mL of 0.15 mg/mL of 77WC-1 was incubated with RV14 (C) or VSV (D), respectively. Samples were collected at 0, 15, 30, and 60 min and TCID50 assay was used to quantify infectious virus in each sample. E and F) AgCNP3 nanoparticles were incubated with RV14 (E) or VSV (F) at concentrations of 0.03 mg/mL or 0.15 mg/mL, respectively, for 1hr at room temp then collected to quantify infectious viruses. Values in all panels are the mean of three replicates with error bars representing standard deviation.

(59). During the TEM studies it was found that Zn-containing particles $(Zn,Ag)_2Ce$ (see example in Fig. 1E) are not stable under an electron beam causing difficulties in data acquisition.

Kourouklis *et al*. have studied ceria pressure phase transformation up to 35 GPa and by registering the Raman spectra they observed this phenomenon near 31 GPa, when the structure of CeO_2 has changed from the above-mentioned fluorite-type to the so-called cottunite ($PbCl_2$ type, orthorhombic *Pnma*) (60). However, in our case, phase parameters fitted to the cubic structure of ceria (Fig. 1C); therefore, both ceria and $(Zn,Ag)_2Ce$ are marked as possible phases. High-Resolution Transmission Electron Microscopy (HRTEM) observations evidenced the presence of silver, $(Zn,Ag)_2Ce$ and CeO_2 compounds (Fig. 1E). The structure and symmetry of the observed nanoparticles were confirmed by Fast Fourier Transforms (FFT) collected from the selected regions showing ceria NP of cubic symmetry oriented along [001] axis, AgNPs along [112] and $(Zn,Ag)_2Ce$ located in between them and oriented along the [101] axis. Chemical state analysis was also performed for particle samples to further assess the formulation's phase composition and provide potential insight into later detailed biomedical properties/behaviors.

3.2.2 Chemical State Composition Analysis of Silver/Zinc-Cerium Oxide Nanoparticles X-ray photoelectron spectroscopy (XPS) was performed (Fig. 1) with analysis focused primarily on the Ce3d, Ag3d, and Zn2p binding energy regions (Figs. 1B, C, and D, respectively). Curve fitting was performed for all spectra over measured signal envelopes using peaks chosen by reference to relevant literature and database (*Thermo Scientific Avantage Data System*). The fitted spectra in the Ce3d region are reflective of the mixed valence state (Ce^{3+}/Ce^{4+}) typically observed for cerium oxide. Peaks attributed to each of the formal oxidation states are presented in Fig. 1G. Semi-quantitative analysis, performed through summation over area-under-the-curve calculations for specific chemical states

$$\%Ce^{3+} = 100 * \sum\int\{u_0,v_0,u',v'\}/\sum\int\{All\ Fitted\ Peaks\}$$

where u_0, v_0, u', and v' represent Ce^{3+}-associated states and the calculated percentage is with respect to the total measured population of cerium state related signal peaks (*All Fitted Peaks*), suggests a significant (calculated $\%Ce^{3+}$ = 51.18) population of Ce^{3+}-associated states for the Ag/Zn-CNPs formulation. This relatively high percentage of reduced chemical state cerium sites is characteristic of this synthesis: with previous investigations of pure and of silver-modified cerium oxide nanoparticle formulations being similarly rich in Ce^{3+} (61) (62) (63). For silver-modified cerium oxide materials, Ce^{3+} states have been observed in published experimental studies at higher densities at silver-cerium oxide interfaces, with related theoretical investigations noting a greater stability for silver ions at surface Ce^{3+}-sites. Similar to our previous findings for a silver-modified cerium oxide nanoparticle formulation (previously reported as AgCNP2), signal in the Ag3d region is well-described by fitting to a metallic silver (Ag^0)-associated doublet (Fig. 1H). Interestingly, signal envelope peaks in the Zn2p binding energy region show an asymmetry, with fitted doublets related to metallic (Zn^0) and cationic (Zn^{2+}) states approximating the measurement curve (Fig. 1I). The determined chemical state distributions for silver and zinc appear to corroborate findings from HRTEM analysis; specifically, the presence of larger silver nanoparticles and more complex $(Ag,Zn)_2Ce$ compound phases. However, it should also be noted that other studies have identified silver oxide interphase regions directly at silver-ceria interfaces; therefore, we should consider that these XPS measurements do not capture the total chemical state distribution of material components in complete detail. It is possible that oxide states for silver exist in minority populations, are not discernible in our collected XPS spectra, though they may contribute to catalytic behaviors.

We next considered the antimicrobial efficacy of this synthesized Ag/Zn-CNP formulation towards assorted virus (enveloped vesicular stomatitis virus, non-enveloped RV14 rhinovirus) and bacteria (Gram-positive *S. aureus*, methicillin-resistant *S. aureus*; Gram-negative *P. aeruginosa*) species.

3.3 Virus Studies

3.3.1 Silver/Zinc Cerium Oxide Nanoparticles Display Potent Antiviral Activity Against Rhinovirus and Vesicular Stomatitis Virus. To determine the extent to which silver-modified CNPs, prepared by a comparable synthesis approach and reported previously, (AgCNPctrl) or Ag/Zn-CNPs inactivate rhinovirus (RV14) or vesicular stomatitis virus (VSV), aqueous solutions of nanoparticles and virus were incubated together for 1hr. Remaining infectious virus was determined by 50% tissue culture infection dose (TCID50) assay. Here, we show that Ag/Zn-CNPs inactivate RV14 in a dose-dependent manner, with 0.05 mg/mL reducing the remaining virus titer to beyond detectable ranges (Fig. 2A). Ag/Zn-CNPs also demonstrate dose-dependent inactivation of VSV (Fig. 2B). However, it is notable that higher concentrations of Ag/Zn-CNPs are necessary to achieve the same log reduction in virus titer in VSV versus RV14. Time-course studies show rapid anti-viral activity of AgZn-CNPs against RV14 (Fig. 2C) and VSV (Fig. 2D), with greater than 50% total virus inactivation occurring within 15 min. The effective concentrations of Ag/Zn-CNPs were used to compare the antiviral activity of Ag/Zn-CNPs versus AgCNPctrl. The above finding that Ag/Zn-CNPs concentrations of 0.02 mg/mL and 0.15 mg/mL reduced > 50% infectivity of RV14 and VSV, respectively, contrasts with results in Fig. 2E and 2F showing treatment with concentrations of 0.03 mg/mL and 0.15 mg/mL of AgCNPctrl being ineffective at inactivating RV14 and VSV, respectively. Taken together, these data demonstrate that Ag/Zn-CNPs rapidly inactivate RV14 and VSV at lower concentrations than the previous nanoparticle formation, AgCNPctrl.

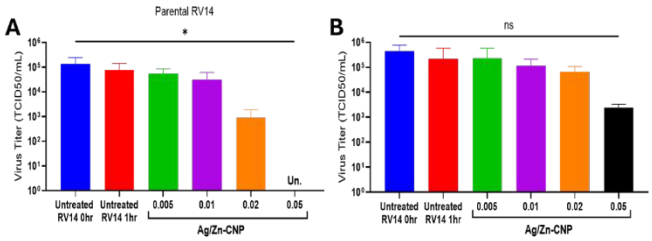

Figure 3. Antiviral Activity of Ag/Zn-CNPs against AgCNP-Resistant RV14. AgCNP-Resistant RV14 was generated as described previously(35). Liquid reactions of parental or AgCNP-resistant RV14 and nanoparticles were incubated at room temperature for 1hr. Nanoparticle and virus reactions were collected at 1hr post nanoparticle addition while untreated virus controls were collected at 0hr and 1hr. Remaining infectious virus titer was determined by TCID50 assay. A and B) increasing concentrations of Ag/Zn-CNP nanoparticles or buffer alone were incubated with parental RV14 (A) or AgCNP-Resistant RV14 (B) then assayed for remaining infectious virus. Values in all panels are the mean of three replicates with error bars representing standard deviation.

3.3.2 Ag/Zn-CNPs Demonstrate Antiviral Activity Against AgCNP-Resistant RV14. AgCNP-resistant RV14 was previously generated via sequential rounds of exposure to suboptimal AgCNP and was shown to gain resistance to AgCNP inactivation (35). To determine the extent to which Ag/Zn-CNPs inactivate AgCNP-resistant RV14, aqueous solutions of nanoparticles and virus were incubated together for 1hr. Remaining infectious virus was determined by 50% tissue culture infection dose (TCID50) assay. Here, we show that Ag/Zn-CNPs inactivate parental RV14 in a dose-dependent manner, with 0.05 mg/mL reducing the remaining virus titer to beyond detectable ranges (Fig. 3A). Ag/Zn-CNPs also demonstrate dose-dependent inactivation of AgCNP-Resistant RV14 (Fig. 3B), with 0.05 mg/mL resulting in a 2-log reduction in virus titer. Notably, the log reduction in resistant-RV14 is considerably less than that observed in the parental virus, suggesting that AgCNP-resistant RV14 might be partially resistant to Ag/Zn-CNPs inactivation. Together, these data demonstrate that Ag/Zn-CNPs display antiviral activity against AgCNP-resistant RV14, although higher doses of nanoparticle might be necessary to inactive resistant virus.

3.4 Bacterial Studies

3.4.1 Silver/Zinc-Cerium Oxide Nanoparticles in Absence of Protein. *P. aeruginosa*, *S. aureus*, and MRSA when treated with Ag/Zn-CNP in a simple 25 mM Tris/75 mM NaCl buffer medium, containing no potentially surface-fouling biomolecules, caused a concentration-dependent reduction in bacterial viability (Fig. 4A-C, respectively). When all three bacterial species were exposed to Ag/Zn-CNP, this resulted in a significant decrease in CFU/mL compared with the untreated controls with complete eradication measured at a dose >2 µg/mL in the *P. aeruginosa* group (p<0.0001) and >4 µg/mL in the *S. aureus* and MRSA groups (p<0.0001). The MIC for *P. aeruginosa*, was found to be ~4 µg/mL, and treatment with 2 µg/mL resulted in an ~3-log reduction in CFU/mL relative to the control. *S. aureus* showed a similar trend, with an MIC of approximately 6 µg/mL. Notably, MRSA exhibited the greatest susceptibility to Ag/Zn-CNP when compared to gentamicin with an MIC of approximately 6 µg/mL. Even in the presence of gentamicin (20 µg/mL), only a ~3-log reduction in CFU/mL was observed for MRSA when compared with the untreated group. The results indicate that Ag/Zn-CNPs have a potent bactericidal effect against both Gram-negative and Gram-positive bacteria, including antibiotic-resistant MRSA.

3.4.2 Silver/Zinc-Cerium Oxide Nanoparticles in Presence of Protein. The antibacterial efficacy of Ag/Zn-CNP was investigated against *P. aeruginosa*, *S. aureus*, and MRSA when cultured in protein-rich LB medium (Fig. 4D-F, respectively). A dose-dependent decrease in bacterial growth was observed in all three strains. *P. aeruginosa* growth was significantly reduced at Ag/Zn-CNP concentrations >20 µg/mL (p<0.0001). Similarly, *S. aureus* and MRSA growth were significantly reduced at concentrations >10 µg/mL and >20 µg/mL (p<0.05) respectively. However, it is notable that MIC values measured in LB broth were significantly lower than those measured for protein-free buffer medium. It is proposed that this difference is partly ascribable to fouling of the material surface by biomolecule adsorption. Modification of the material surface can alter surface chemistry, including enzyme-mimetic redox reactions shown previously to contribute to antimicrobial performance, as well as function as a physical barrier between nanoparticle and environment. The greatest observed efficacy was towards

the Gram-negative *P. aeruginosa* viability, with an MIC of around 30 µg/mL, while MIC values for the Gram-positive *Staphylococcus* strains were markedly higher at ~400 µg/mL. Interestingly, this trend in Gram positive versus negative bacteria inactivation was previously observed, for the same bacterial strains, for AgCNPs produced by a comparable synthesis (1).

3.4.3 Bacterial Inactivation Kinetics A time-dependent study of bacteria inactivation by Ag/Zn-CNPs was performed for all three bacterial strains at their respective MIC values (4 µg/mL for *P. aeruginosa*; 6 µg/mL for *S. aureus* and MRSA) in Tris-NaCl medium. A progressive and rapid reduction in viable bacteria was seen for all three bacterial species when treated with Ag/Zn-CNP; whereas the effects of gentamicin occurred at a significantly slower rate for all tested strains (only reaching 4, and 3 -log reductions for *P. aeruginosa*, *S. aureus*, and MRSA, respectively, by times of complete inactivation for Ag/Zn-CNPs). For *P. aeruginosa* (Fig. 4G), a complete inhibition of bacteria by Ag/Zn-CNP was observed by 75 minutes, whereas cells treated with gentamycin saw only a 5-log reduction within the same time period. Interestingly, the observed trend of greater sensitivity for *P. aeruginosa* over the *Staphylococcus* strains held for these kinetic studies: with complete bacterial elimination occurring by 180 minutes (*S. aureus*, Fig. 4H; MRSA, Fig. 4I). Ag/Zn-CNP nanoparticles demonstrated rapid and potent bactericidal activity across all tested species in Tris-NaCl medium, outperforming gentamicin. To further evaluate Ag/Zn-CNPs' utility as a potent antimicrobial agent, we next examined the potential for a resistant strain to be developed.

3.4.4 Probe of Resistance-development following repeated-use testing towards Ag/Zn-CNPs Repeated exposures of *P. aeruginosa* and *S. aureus* to sub-lethal concentrations (IC_{50}) of Ag/Zn-CNPs was performed and their susceptibilities at respective, previously determined, MIC values analyzed. Bacteria were exposed to eight sequential sub-lethal exposure and re-culture cycles, with potential resistance development evaluated by treating sub-cultures at the 6th (Fig. 5A,C) and 8th (Fig. 5B,D) cycles at their respective MIC values in LB medium. Across the studied bacterial species, no substantial increase in tolerance or survived cells were detected in the presence of Ag/Zn-CNP (Fig. 5): suggesting no appreciable tendencies towards development of phenotypic resistance, even after

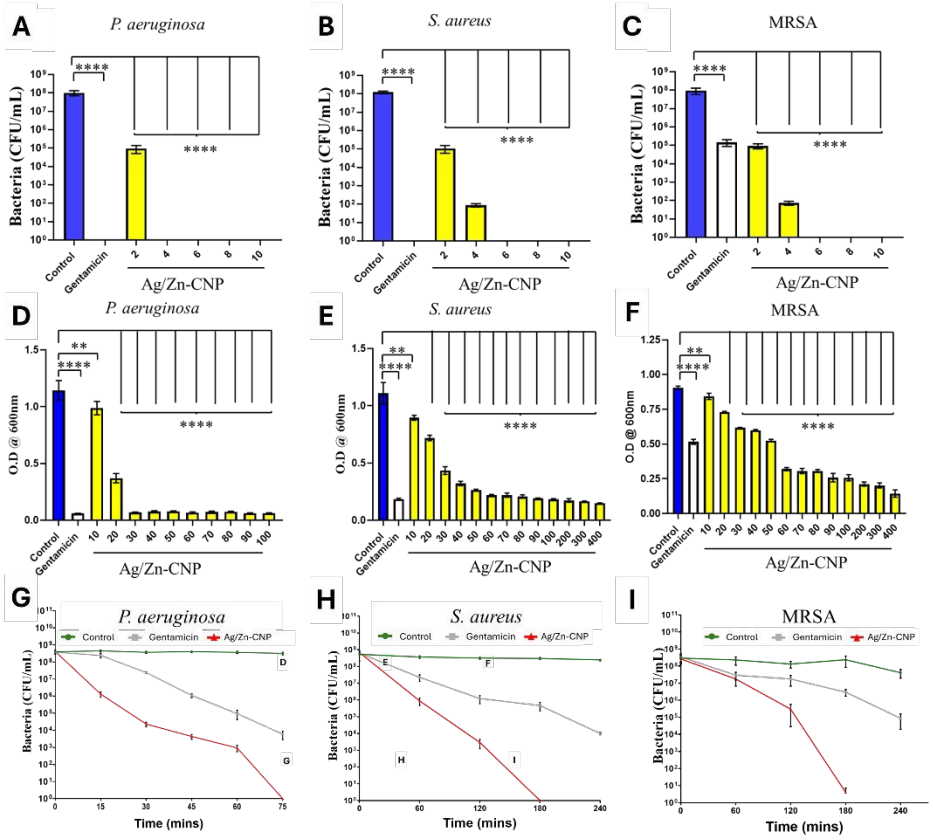

Figure 4. Bactericidal Efficacies of Ag/Zn-CNP against *P. aeruginosa*, *S. aureus* and, MRSA and Time-dependent Studies. Each bacterial strain was suspended in Tris-NaCl, pH 7.5 and treated with increasing concentrations of Ag/Zn-CNP and gentamicin (20 µg/mL) in a 96-well microtiter plate before being incubated for 24 hours at 37 °C (A-C). The MIC values of Ag/Zn-CNP were found to be 4 µg/mL for *P. aeruginosa* (A), 6 µg/mL for *S. aureus* (B), and 6 µg/mL for MRSA (C). Nanoparticle efficacy towards these strains at ~10^8 CFU/mL were further tested in Luria-Bertini (LB) broth and compared against gentamicin (20 µg/mL) in a 96-well microtiter plate, followed by incubation at 37 °C for 24 h. The MIC values of Ag/Zn-CNP were determined to be 30 µg/mL for *P. aeruginosa* (D), 400 µg/mL for *S. aureus* (E), and for MRSA (F). Experiments for both sets of studies were carried out in triplicate and data is presented as mean ± standard error. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Growth curves for control, gentamicin (20 µg/ mL), and Ag/Zn-CNP at respective MIC concentration for each bacteria. When tested at the MIC of Ag/Zn-CNP, an ~7-log reduction was seen in each group: exceeding the EPA minimum requirement for an antibacterial product.

multiple cycles of exposure. However, it was found in performing a similar study that lab strains of AgCNP-resistant *P. aeruginosa* and *S. aureus* could be produced. In a follow-on study, we examined the susceptibility of these AgCNP-resistant strains towards Ag/Zn-CNPs.

3.4.5 Susceptibility of AgCNP-resistant strain to Ag/Zn-CNP nanoparticle in Tris-NaCl. On treatment with Ag/Zn-CNPs, AgCNP-resistant *P. aeruginosa* and *S. aureus* (laboratory) strains experienced dose-dependent decreases in viability. For both bacteria species, the measured MIC values for parental strains (Figs. 5E and G, ~4 and 6 µg/mL, for *P. aeruginosa* and *S. aureus*, respectively) were significantly lower (p<0.0001) than those

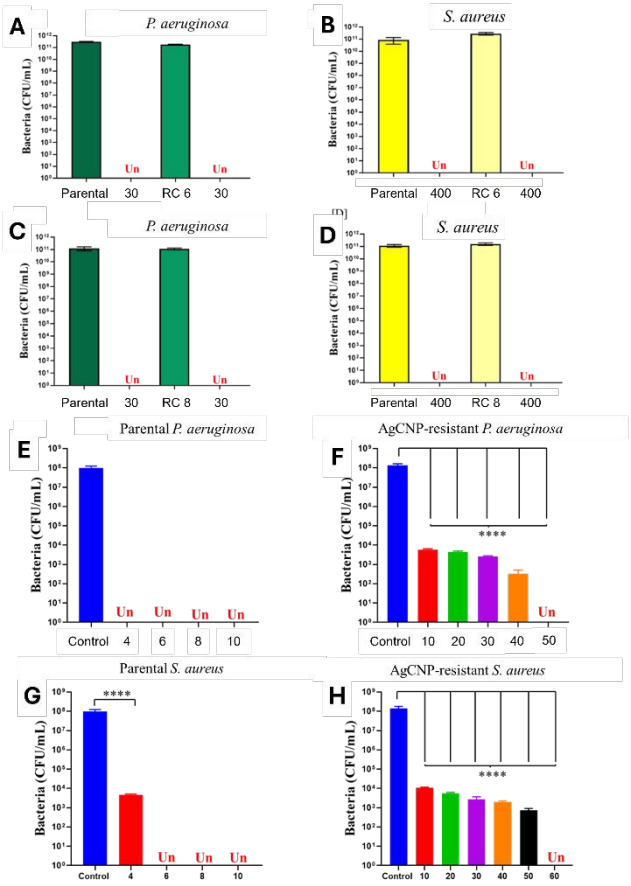

Figure 5. Resistance development to Ag/Zn-CNP NP assessed after repeated exposure rounds in LB medium**.** (A, C) Parental *P. aeruginosa* and *P. aeruginosa* cells are isolated after 6th (RC 6) and 8th (RC 8) cycle were treated with Ag/Zn-CNP of 30 µg/mL (MIC). Cells are undetected (Un) after 24 hours of treatment in both phenotypes. (B,D) Parental *S. aureus* and *S. aureus* cells are isolated after 6th (RC 6) and 8th (RC 8) cycle were treated with Ag/Zn-CNP of 400 µg/mL (MIC). Cells are undetected (Un) after 24 hours of treatment in both phenotypes. Thus, demonstrating that when cells are repeatedly exposed to Ag/Zn-CNP it did not induce resistance development. Antibacterial activity of Ag/Zn-CNP against parental and AgCNP resistant *P. aeruginosa* and *S. aureus*. (A, C) Parental *P. aeruginosa* and *S. aureus* strains treated with Ag/Zn-CNP (4, 6, 8 and 10 µg/mL). Cells are undetected (Un) after treating them with 4 µg/mL in P. aeruginosa and 6 µg/mL in S. aureus. (B , D) AgCNP resistant *P. aeruginosa* and *S. aureus* treated with Ag/Zn-CNP (10, 20, 30, 40, 50 and, 60 µg/mL. Cells are undetected (Un) at 50 µg/mL in *P. aeruginosa* and 60 µg/mL in *S. aureus*. The results are shown as mean ± standard error, and the experiments were conducted in triplicate. *p<0.05, **p<0.01, ***p*<0.001, and ****p*<0.0001. (data for the pure and Ag@CeO_2 systems is taken from Pugazhendhi et al. (1) and Wei et al. (2-4)).*

measured for corresponding AgCNP-resistant strains (Figs. 5F and H, ~50 and 60 µg/mL, for *P. aeruginosa* and *S. aureus*, respectively) suggesting some increase in bacterial robustness towards Ag/Zn-CNP exposure as well as demonstrating a persistent antimicrobial efficacy for the Ag/Zn-CNP formulation. The overall susceptibility of Gram-positive *Pseudomonas* was slightly lower than that of Gram-negative *Staphylococcus*.

3.5 Antimicrobial Function Discussion

In our previous studies of AgCNP formulations towards viruses and bacteria, an initial probe into respective mechanisms of antimicrobial action was performed. Treatment of an enveloped parainfluenza virus (PIV5; negative-sensed, single-stranded RNA virus) with AgCNP (AgCNP$_{ctrl}$ in the present study) induced an aggregation of virions, suggested by a dose-dependent decrease in hemagglutination of red blood cells by NP-treated PIV5 inocula, and by gradient centrifugation studies. Importantly, this observation of NP-induced virus aggregation in centrifugation occurred without appreciable segregation of virions to lower density gradient bands. This observation allows an important insight: specifically, that the dominant mechanism of virus inactivation is likely not binding protein, or general surface, degradation/modification due to disruption of the integrity of the virus particle, but rather that the NPs bind virus surface sites and inhibit an early stage in infection. In the same study, a lab strain of non-enveloped rhinovirus (RV14; single-stranded, positive-sensed RNA virus) was produced which was found to be substantially less sensitive to AgCNPctrl than the parental strain. Genomic analysis identified five point mutations, two of which induced changes in amino acid identity (the other three being silent mutations in VP3 protein encoding region) and both in a superficial/exterior region of a surface binding protein subunit (VP1). While these findings are not direct evidence, the loss of virus inactivation and the presence of amino acid mutations which converted hydrophilic to hydrophobic amino acid expressions implicate these proteins in the virus inhibition mechanism. Our prior work (3) showed that AgCNP-treated viruses had lost receptor binding. It is possible that the ability of Ag/Zn-CNPs to bypass AgCNP-resistance reflects i) prolonged interactions of Ag/Zn-CNPs with virions, ii) improved access of Ag/Zn-CNPs to the virus receptor binding domain, or iii) higher denaturation of viral capsid proteins leading to increased virus aggregation and/or blocks in uncoating of virus during infection.

Corresponding studies of AgCNP$_{ctrl}$ treatments on bacterial species (*P. aeruginosa*, *S. aureus*, MRSA) evidence effects from reactive oxygen species (ROS) generation, DNA damage, modulation of cellular oxygen metabolism, and particle adsorption on cell viability(1). For all tested bacteria, cell lysis was observed. Interestingly, despite the apparent multitude of contributing antibacterial mechanisms, results from the present study further demonstrate the potential to produce AgCNP-resistant strains of *P. aeruginosa* and of *S. aureus*. Ag/Zn-CNPs retain antibacterial activity towards these strains: suggesting an incomplete overlap in the dominant mechanisms producing the antibacterial outcome. Measurements of antibacterial

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performance at 4°C (Fig. S1) reflect a retention in formulating antibacterial activity and suggest that the dominant antibacterial processes are not appreciably rate-controlled or limited by thermal activation, including metabolic processes. Follow-on studies which further probe the observed resistance are underway, though results from the current study note a more complex phase distribution of formulation components (Fig. 1) which may modify surface interactions between bacteria surface structures and the Ag/Zn-CNPs and density functional theory (DFT) studies suggest a more favorable generation of ROS relative to AgCNPs.

In the presented DFT investigation (Fig. 6, Table S1), we studied hydrogen peroxide (H_2O_2) and its dissociated products (H/OO/H, H/OOH, HO/OH) adsorbed on the {111}, {110} and {100} surfaces of cerium dioxide at different surface compositions including stoichiometric pure, Ag@CeO_2, Zn@CeO_2 and Zn/Ag@CeO_2, and oxygen deficient pure, Ag@$CeO_{2-x(SL)}$, Zn@$CeO_{2-x(SL)}$ and Zn/Ag@$CeO_{2-x(SL)}$ where the oxygen vacancy is positioned in the top layer, and oxygen deficient pure, Ag@$CeO_{2-x(SSL)}$, Zn@$CeO_{2-x(SSL)}$ and Zn/Ag@$CeO_{2-x(SSL)}$ where the oxygen vacancy is positioned in the subsurface layer (data for the pure and Ag@CeO_2 systems is taken from Pugazhendhi et al. (1) and Wei et al (2)). Collectively, the computational results suggest that Ag/Zn-CNPs are more efficient in their generation of antimicrobial ROS and, therefore, potentially better able to induce virus/bacteria inactivation by an ROS-mediated pathway, relative to AgCNP or CNP alone. Results of the produced calculations, specifically, identify a less negative interaction energy for ROS with Ag/Zn-CNPs; hence, the radicals kill virus/bacteria more effectively. This relationship can be interpreted as a greater ease in ROS becoming exchanged with the surrounding environment.

Considering all the models studied for all the three surfaces and the three compositions, in 55% and 69% of the models, the adsorption of ROS on Ag/Zn@CeO_2 is less favorable than on Ag@CeO_2 and CeO_2, respectively (Fig. S3, Table S2). Considering these relationships with respect to specific surface morphologies: (1) In 60%, 50% and 57% of the {111}, {110} and {100} models, respectively, the adsorption of ROS on Ag/Zn@CeO_2 is less favorable than on Ag@CeO_2 and (2) in 60%, 67% and 86% of the {111}, {110} and {100} models, respectively, the adsorption of ROS on Ag/Zn@CeO_2 is less favorable than on CeO_2. Alternatively, the data may be considered with respect to surface composition. From this representation, we find that (1) in 50%,70% and 44% of the CeO_2, $CeO_{2-x(SL)}$, $CeO_{2-x(SLL)}$ models, respectively, the adsorption of ROS on Ag/Zn surfaces is less favorable than on the Ag surfaces alone and (2) in 50%,90% and 67% of the CeO_2, $CeO_{2-x(SL)}$, $CeO_{2-x(SLL)}$ models, respectively, the adsorption of ROS on Ag/Zn surfaces is less favorable than on the pure surfaces alone. These results display that the presence of surface oxygen vacancies (in the $CeO_{2-x(SL)}$ models) drive the inefficiency of the Ag/Zn models compared to Ag and pure (without Ag) models.

Interestingly, despite the pronounced antimicrobial activity and favorable generation of ROS, exposure of a macrophage cell line to Ag/Zn-CNPs (Fig.S2) evidenced a non-toxic character at low concentrations: further supporting their potential use as broad-spectrum antimicrobial agents. While the current study was designed to evaluate efficacy in disinfection, calculation of therapeutic index (TI) values from bacterial studies mark a first step in assessing material efficacy as a nano-pharmaceutical

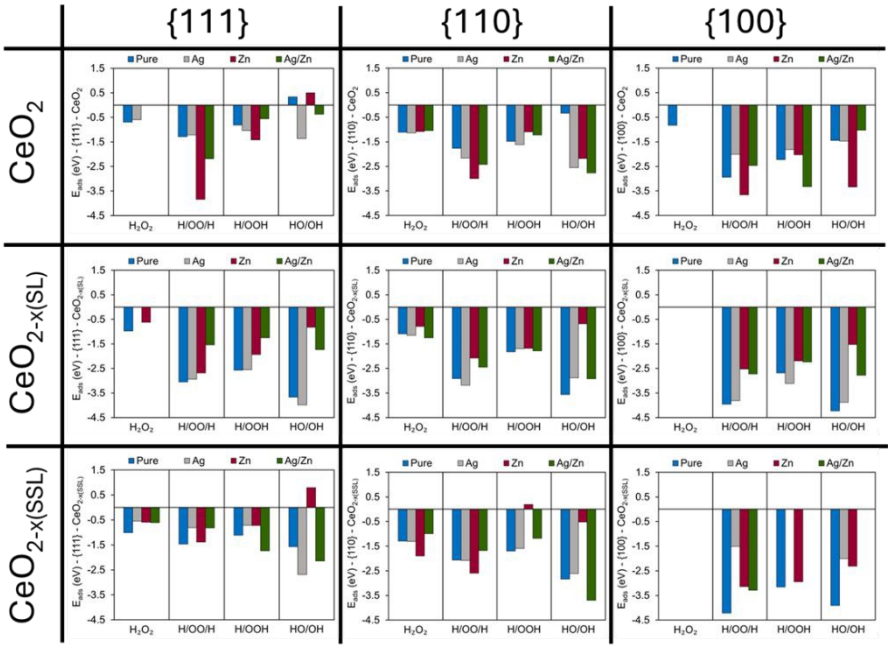

Figure 6. Density Functional Theory Schematic representation of the adsorption energies (eV). Density functional theory calculations of hydrogen peroxide (H_2O_2) and its dissociated forms (H/OO/H, H/OOH, HO/OH) adsorbed on the {111}, {110} and {100} surfaces of cerium dioxide at different surface compositions including stoichiometric pure, Ag@CeO_2, Zn@CeO_2 and Zn/Ag@CeO_2, and oxygen deficient pure, Ag@$CeO_{2-x(SL)}$, Zn@$CeO_{2-x(SL)}$ and Zn/Ag@$CeO_{2-x(SL)}$ where the oxygen vacancy is positioned in the top layer, and oxygen deficient pure, Ag@$CeO_{2-x(SSL)}$, Zn@$CeO_{2-x(SSL)}$ and Zn/Ag@$CeO_{2-x(SSL)}$ where the oxygen vacancy is positioned in the subsurface layer. Tabulated data can be found in Table *S1*.

(Fig. S4). Initial measurements in absence of biomolecules identified TI values of 2.87, 1.91, and 1.91 for *P. aeruginosa*, *S. aureus*, and MRSA, respectively. Similarly, TI values for RV14 and VSV were calculated as approximately 0.574 and 0.077, respectively. The data suggest a potential need for nanoparticle surface modification or engineering before use as a pharmaceutical. Follow-on studies of nanomaterial suitability and design for pharmaceutical applications will consider the antimicrobial efficacy of similar formulations in biological systems, with biological data and metrics such as TI, utilized for the design and optimization of nanomaterial formulations.

4. Conclusions

In the presented study, a multi-metal oxide nanoparticle formulation was produced containing zinc and silver-rich surface phases on cerium oxide nanoparticles (Ag/Zn-CNPs). Materials characterization and analyses noted a high relative proportion of cerium sites in a Ce^{3+} formal redox state, predominant metallic state for silver, and presence of zinc in both cationic Zn^{2+} and metallic Zn^0 states, as evident from XPS measurements. High-resolution transmission electron microscopy imaging and energy-dispersive spectroscopy evidence a complex phase distribution, with elemental mapping and fast Fourier transform crystal pattern analyses suggesting presence of CeO_2, Ag^0, and potentially $(Ag,Zn)_2$ phases. Treatment of bacterial (*P. aeruginosa*, *S. aureus*, MRSA) and virus (RV14 rhinovirus, VSV) species demonstrated a significant antimicrobial activity relative to a previously reported silver-modified cerium oxide nanoparticle formulation (AgCNP). Further, Ag/Zn-CNPs were found to retain antimicrobial activity towards *P. aeruginosa*, *S. aureus* strains, and RV14 lab strains which are insensitive to AgCNP treatment: further suggesting their improved utility for broad-spectrum antimicrobial technologies. Density functional theory calculations (considering presence/absence of Ce^{3+}-associated oxygen vacancies, assorted high atomic density surface facets) similarly suggest a greater ability to generate antimicrobial reactive oxygen species, relative to pure pristine or non-stoichiometric cerium oxide or silver-modified cerium oxide surfaces. When the antibacterial efficacy of Ag/Zn-CNP was compared with our previously published AgCNP activity (5), data show that in the absence of biomolecules, the MIC of Ag/Zn-CNP delivered superior activity towards Gram negative *P. aeruginosa* (4 µg/mL), as well as to Gram positive *S. aureus* and MRSA (both 6 µg/mL) when compared with AgCNPs (20 µg/mL (*P. aeruginosa*) and 50 µg/mL (*S. aureus* and MRSA, unpublished data)). Notably, and in the presence of media containing biomolecules (i.e., biofouling), the MIC of Ag/Zn-CNP was found to be lower and thus more potent (30 µg/mL) when compared to AgCNPs (100 µg/mL). However, Ag/Zn-CNPs were marginally less potent to *S. aureus* and MRSA (400 µg/mL) when compared to AgCNPs (300 µg/mL). Nevertheless, and critically, the present study reveals that Ag/Zn-CNPs effectively eradicated Ag-resistant *P. aeruginosa* and *S. aureus* strains. Further, and given that bacterial antimicrobial resistance is considered a severe and global public health crisis, data confirms that remarkably, resistant strains of *P. aeruginosa* and *S. aureus* could not be created after eight sequential sub-lethal exposure and re-culture cycles to Ag/Zn-CNP

Author contributions

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Griffith D. Parks- methodology, resources, supervision, writing- review & editing
Melanie J. Coathup- methodology, resources, supervision, writing- review & editing
Sudipta Seal- conceptualization, supervision, resources, writing- review & editing

Conflicts of interest

There are no conflicts of interest to declare

Data availability

The datasets supporting the findings of this study are available within the article and its supplementary information files.

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The Editor,
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Data Availability Statement

The datasets supporting the findings of this study are available within the article and its supplementary information files.

We hope that you find the manuscript suitable for publication in *Nanoscale*.

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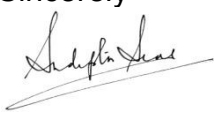

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