Supplementary Information (SI) for Nanoscale.

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

Craig J. Neal[1], Abinaya Sindu Pugazhendhi[2], Elisabeth M. Shiffer[3], Candace R. Fox[3], Muhammad Hassan Mehmood Kalyar[3], Elayaraja Kolanthai[1], Katarzyna Stan-Glowinska[4], Daniel Brown[5], Khao Minh Ta[5], Joanna Wojewoda-Budka[4], Natalia Sobczak[4], Marco Molinari[5], Griffith D. Parks[3], Melanie J. Coathup[2], Sudipta Seal[1,3]

[1] Materials Science & Engineering Department, Advanced Materials Processing and Analysis Center (AMPAC), University of Central Florida, Orlando, FL, 32816, United States

[2] Biionix Faculty Cluster, College of Medicine, University of Central Florida, Orlando, Florida 32827, United States

[3] Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, Florida 32827, United States

[4] Institute of Metallurgy and Materials Science, Polish Academy of Sciences, Krakow, 30-418, Poland

[5] Department of Physical and Life Sciences, School of Applied Sciences, University of Huddersfield, Huddersfield, HD1 3DH, UK

Effect of temperature on Ag/Zn-CNP activity. At 4°C, the potency of Ag/Zn-CNP was studied in the Tris-NaCl medium against all three strains. After 1 h of incubation about 1 log reduction was found and around 3-log reduction was observed after 24 h in P. aeruginosa. S. aureus showed a 2-log reduction after 3 hours and after 24 hours a 4-log reduction was detected. In the case of MRSA, no significant reduction was found after 3 h but at 24 h incubation a 2-log reduction in CFU was noticed (fig. S1 , C). However, Ag/Zn-CNP was able to significantly reduce the tested organism, indicating the Ag/Zn-CNP was able to hold antibacterial efficacy even at low temperature.

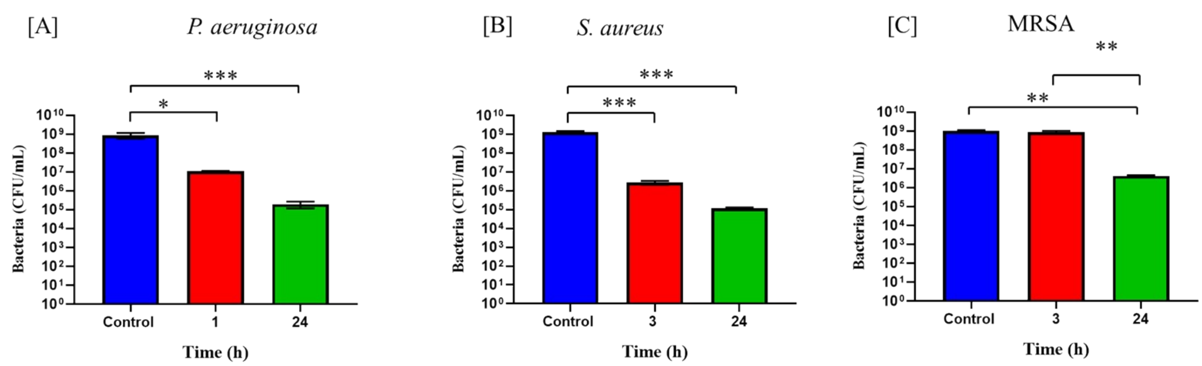

Figure S1 Effect of temperature on Ag/Zn-CNP activity. (A) A 1-log reduction in P. aeruginosa growth was observed at 1 h following supplementation with Ag/Zn-CNP and about ~3-log reduction was found after 24 h of incubation. (B) When S. aureus was treated with Ag/Zn-CNP a 2-log reduction was observed after 3 h and around 4-log reduction was noted after 24 h of incubation. (C) In MRSA group no significant reduction in growth was observed after 3 h treatment with Ag/Zn-CNP whereas after 24 h, a ~2-log reduction in growth was observed. (*p<0.05, **p<0.01, ***p<0.001).

Cytotoxicity study on Ag/Zn-CNP. The Ag/Zn-CNP cytotoxicity effect on macrophage cells was studied at an increasing concentration from 0-400 µg/mL. On day-1 no significant differences were observed at 4 and 6 µg/mL showing that no detectable cytotoxicity to cells, indicating preserved cell viability (Fig. S2 A). A significant decrease in viability was noted at 30 µg/mL and at higher concentration (100 and 400 µg/mL) complete loss of viability was observed (Fig. S2 C). Hence. The cells were able to tolerate low concentration but Ag/Zn-CNP at higher doses are strongly cytotoxic to the cells. At 6 µg/mL on day 3 showed a significant ($p>0.01$) (Fig. S2 B) decrease in metabolic activity of cells but no death cells were noted (Fig. S2 C) . But at 30 µg/mL significant loss of viable (green) signal was observed and complete loss of cells were noted at 100 and 400 µg/mL.

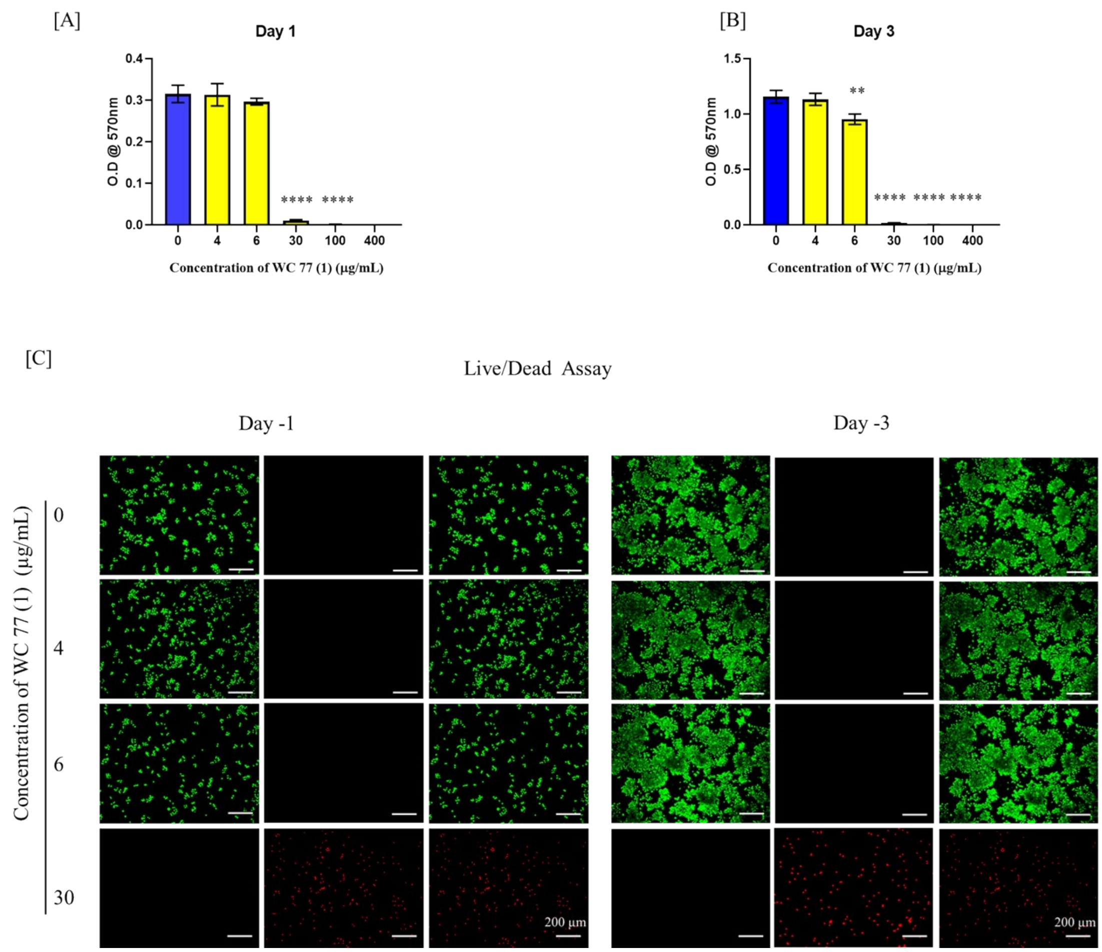

Figure S2 Cytotoxicity study on macrophage (RAW 264.7) cells. [A] Cell metabolic activity was assessed and the response to Ag/Zn-CNP was quantified using MTT assay on day 1 no significant change in cell metabolic activity was observed when the macrophage cells are treated up to 6 µg/mL. A complete reduction was observed when the concentration was increased more than 30 µg/mL. (B) On day a significant reduction ($p<0.01$) in cell metabolic activity was observed in macrophage cells when treated with 6 μg/mL of Ag/Zn-CNP and a complete reduction ($p<0.0001$) was observed when treated with more than 30 μg/mL. [C] Live/Dead assay on day 1 and day 3. (*$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$).

Table S1. Tabulated adsorption energies (eV) of all the configurations simulated using DFT calculations. Hydrogen peroxide (H_2O_2) and its dissociated forms (H/OO/H, H/OOH, HO/OH) are adsorbed on the {111}, {110} and {100} surfaces of cerium dioxide at different surface compositions. The label Ag represents the presence of a surface Ag3 cluster. The label Zn represents that a surface Ce atom has been replaced with a Zn atom.

			Surface Composition	
Surface Plane	**Surface Stoichiometry**	**Adsorbate**	**Zn**	**Ag/Zn**
{111}	CeO_2	H_2O_2	-	-
		H/OO/H	-3.84	-2.19
		H/OOH	-1.42	-0.56
		HO/OH	0.50	-0.38
	$CeO_{2-x(SL)}$	H_2O_2	-0.62	-
		H/OO/H	-2.69	-1.54
		H/OOH	-1.93	-1.25
		HO/OH	-0.83	-1.73
	$CeO_{2-x(SLL)}$	H_2O_2	-0.59	-0.61
		H/OO/H	-1.39	-0.82
		H/OOH	-0.72	-1.74
		HO/OH	0.79	-2.15
{110}	CeO_2	H_2O_2	-1.08	-1.05
		H/OO/H	-3.00	-2.42
		H/OOH	-1.09	-1.22
		HO/OH	-2.18	-2.77
	$CeO_{2-x(SL)}$	H_2O_2	-0.79	-1.26
		H/OO/H	-2.07	-2.45
		H/OOH	-1.68	-1.78
		HO/OH	-0.68	-2.92
	$CeO_{2-x(SLL)}$	H_2O_2	-1.90	-0.99
		H/OO/H	-2.60	-1.68
		H/OOH	0.20	-1.19
		HO/OH	-0.52	-3.69
{100}	CeO_2	H_2O_2	-	-
		H/OO/H	-3.66	-2.47
		H/OOH	-2.03	-3.33
		HO/OH	-3.34	-1.03
	$CeO_{2-x(SL)}$	H_2O_2	-	-
		H/OO/H	-2.53	-2.73
		H/OOH	-2.19	-2.23
		HO/OH	-1.53	-2.78
	$CeO_{2-x(SLL)}$	H_2O_2	-	-
		H/OO/H	-3.14	-3.29
		H/OOH	-2.94	-

		HO/OH	-2.32	-

Figure S3. Density Functional Theory calculations and Configurations. Adsorption energies of molecular H2O2 and its dissociation products (HO/OH, H/OOH, H/OO/H) onto the {111}, {110} and {100} surfaces of stoichiometric Zn@CeO2 and Zn/Ag@CeO2, oxygen deficient Zn@CeO2-x(SL) and Zn/Ag@CeO2-x(SL) where the oxygen vacancy is positioned in the top layer, and oxygen deficient Zn@CeO2-x(SSL) and Zn/Ag@CeO2-x(SSL) where the oxygen vacancy is positioned in the subsurface layer. All values are calculated using DFT simulations. Ce, O, Ag and H as green, red, grey, and white spheres, and oxygen vacancies as blue squares.

TableS2. List of configurations with details of geometric and magnetic symmetry of each configuration alongside the number of Ce^{3+} and the nature of the adsorbed species after minimization using DFT calculations. Where attempts to stabilize certain configurations have been unsuccessful, the label n/a is used. All the configurations are symmetric in terms of their geometry and distribution of Ce^{3+}

Surface Configurations	**Geometric Symmetry**	**Symmetrical Distribution of Ce^{3+}**	**Number of Ce^{3+}**	**Adsorbed Oxygen Species**
H_2O_2_{111}_Zn@CeO_2	n/a	n/a	n/a	n/a
H/OO/H_{111}_Zn@CeO_2	Yes	Yes	0	Oxygen Triplet
H/OOH_{111}_Zn@CeO_2	Yes	Yes	0	Hydroperoxyl Radical
HO/OH_{111}_Zn@CeO_2	Yes	Yes	0	Hydroxyl Radical
H_2O_2_{111}_Zn@$CeO_{2-x(SL)}$	Yes	Yes	0	H_2O_2
H/OO/H_{111}_Zn@$CeO_{2-x(SL)}$	Yes	Yes	2	Superoxide
H/OOH_{111}_Zn@$CeO_{2-x(SL)}$	Yes	Yes	0	Hydroperoxide Anion
HO/OH_{111}_Zn@$CeO_{2-x(SL)}$	Yes	Yes	0	2 Hydroxyl Radical, 2 Hydroxide Ion
H_2O_2_{111}_Zn@$CeO_{2-x(SSL)}$	Yes	Yes	0	H_2O_2
H/OO/H_{111}_Zn@$CeO_{2-x(SSL)}$	Yes	Yes	4	Oxygen Triplet
H/OOH_{111}_Zn@$CeO_{2-x(SSL)}$	Yes	Yes	0	Hydroperoxide Anion
HO/OH_{111}_Zn@$CeO_{2-x(SSL)}$	Yes	Yes	0	Hydroxyl Radical
H_2O_2_{111}_Ag/Zn@CeO_2	n/a	n/a	n/a	n/a
H/OO/H_{111}_Ag/Zn@CeO_2	Yes	Yes	2	Oxygen Triplet
H/OOH_{111}_Ag/Zn@CeO_2	Yes	Yes	2	Hydroperoxide Anion
HO/OH_{111}_Ag/Zn@CeO_2	Yes	Yes	0	2 Hydroxyl Radical, 2 Hydroxide Ion
H_2O_2_{111}_Ag/Zn@$CeO_{2-x(SL)}$	n/a	n/a	n/a	n/a
H/OO/H_{111}_Ag/Zn@$CeO_{2-x(SL)}$	Yes	Yes	4	Superoxide
H/OOH_{111}_Ag/Zn@$CeO_{2-x(SL)}$	Yes	Yes	2	Hydroperoxide Anion
HO/OH_{111}_Ag/Zn@$CeO_{2-x(SL)}$	Yes	Yes	0	Hydroxide Ion
H_2O_2_{111}_Ag/Zn@$CeO_{2-x(SSL)}$	Yes	Yes	2	H_2O_2
H/OO/H_{111}_Ag/Zn@$CeO_{2-x(SSL)}$	Yes	Yes	6	Oxygen Triplet
H/OOH_{111}_Ag/Zn@$CeO_{2-x(SSL)}$	Yes	Yes	2	Hydroperoxide Anion
HO/OH_{111}_Ag/Zn@$CeO_{2-x(SSL)}$	Yes	Yes	0	Hydroxide Ion
H_2O_2_{110}_Zn@CeO_2	Yes	Yes	0	H_2O_2
H/OO/H_{110}_Zn@CeO_2	Yes	Yes	0	Oxygen Triplet
H/OOH_{110}_Zn@CeO_2	Yes	Yes	0	Hydroperoxyl Radical
HO/OH_{110}_Zn@CeO_2	Yes	Yes	0	Hydroxide Ion
H_2O_2_{110}_Zn@$CeO_{2-x(SL)}$	Yes	Yes	0	H_2O_2
H/OO/H_{110}_Zn@$CeO_{2-x(SL)}$	Yes	Yes	2	Superoxide
H/OOH_{110}_Zn@$CeO_{2-x(SL)}$	Yes	Yes	0	Hydroperoxide Anion
HO/OH_{110}_Zn@$CeO_{2-x(SL)}$	Yes	Yes	0	2 Hydroxyl Radical, 2 Hydroxide Ion

H_2O_2_{110}_Zn@$CeO_{2-x(SSL)}$	Yes	Yes	0	H_2O_2
H/OO/H_{110}_Zn@$CeO_{2-x(SSL)}$	Yes	Yes	4	Oxygen Triplet
H/OOH_{110}_Zn@$CeO_{2-x(SSL)}$	Yes	Yes	2	Hydroperoxyl Radical
HO/OH_{110}_Zn@$CeO_{2-x(SSL)}$	Yes	Yes	0	2 Hydroxyl Radical, 2 Hydroxide Ion
H_2O_2_{110}_Ag/Zn@CeO_2	Yes	Yes	0	H_2O_2
H/OO/H_{110}_Ag/Zn@CeO_2	Yes	Yes	2	Oxygen Triplet
H/OOH_{110}_Ag/Zn@CeO_2	Yes	Yes	0	Hydroperoxide Anion
HO/OH_{110}_Ag/Zn@CeO_2	Yes	Yes	0	Hydroxide Ion
H_2O_2_{110}_Ag/Zn@$CeO_{2-x(SL)}$	Yes	Yes	2	H_2O_2
H/OO/H_{110}_Ag/Zn@$CeO_{2-x(SL)}$	Yes	Yes	2	Peroxide Ion
H/OOH_{110}_Ag/Zn@$CeO_{2-x(SL)}$	Yes	Yes	2	Hydroperoxide Anion
HO/OH_{110}_Ag/Zn@$CeO_{2-x(SL)}$	Yes	Yes	2	Hydroxide Ion
H_2O_2_{110}_Ag/Zn@$CeO_{2-x(SSL)}$	Yes	No	2	H_2O_2
H/OO/H_{110}_Ag/Zn@$CeO_{2-x(SSL)}$	Yes	Yes	6	Oxygen Triplet
H/OOH_{110}_Ag/Zn@$CeO_{2-x(SSL)}$	Yes	Yes	2	Hydroperoxide Anion
HO/OH_{110}_Ag/Zn@$CeO_{2-x(SSL)}$	Yes	Yes	2	Hydroxide Ion
H_2O_2_{100}_Zn@CeO_2	n/a	n/a	n/a	n/a
H/OO/H_{100}_Zn@CeO_2	Yes	Yes	0	Superoxide
H/OOH_{100}_Zn@CeO_2	Yes	Yes	0	Hydroperoxide Anion
HO/OH_{100}_Zn@CeO_2	Yes	Yes	0	Hydroxide Ion
H_2O_2_{100}_Zn@$CeO_{2-x(SL)}$	n/a	n/a	n/a	n/a
H/OO/H_{100}_Zn@$CeO_{2-x(SL)}$	Yes	Yes	4	Strained Oxygen Triplet
H/OOH_{100}_Zn@$CeO_{2-x(SL)}$	Yes	Yes	0	Hydroperoxide Anion
HO/OH_{100}_Zn@$CeO_{2-x(SL)}$	Yes	Yes	0	Hydroxide Ion
H_2O_2_{100}_Zn@$CeO_{2-x(SSL)}$	n/a	n/a	n/a	n/a
H/OO/H_{100}_Zn@$CeO_{2-x(SSL)}$	Yes	Yes	4	Oxygen Triplet
H/OOH_{100}_Zn@$CeO_{2-x(SSL)}$	Yes	Yes	0	Hydroperoxide Anion
HO/OH_{100}_Zn@$CeO_{2-x(SSL)}$	Yes	Yes	0	Hydroxide Ion
H_2O_2_{100}_Ag/Zn@CeO_2	n/a	n/a	n/a	n/a
H/OO/H_{100}_Ag/Zn@CeO_2	Yes	Yes	2	Oxygen Triplet
H/OOH_{100}_Ag/Zn@CeO_2	Yes	Yes	2	Hydroperoxide Anion
HO/OH_{100}_Ag/Zn@CeO_2	Yes	Yes	0	Hydroxide Ion
H_2O_2_{100}_Ag/Zn@$CeO_{2-x(SL)}$	n/a	n/a	n/a	n/a
H/OO/H_{100}_Ag/Zn@$CeO_{2-x(SL)}$	Yes	Yes	2	Peroxide Ion
H/OOH_{100}_Ag/Zn@$CeO_{2-x(SL)}$	Yes	Yes	2	Hydroperoxide Anion
HO/OH_{100}_Ag/Zn@$CeO_{2-x(SL)}$	Yes	Yes	0	Hydroxide Ion
H_2O_2_{100}_Ag/Zn@$CeO_{2-x(SSL)}$	n/a	n/a	n/a	n/a
H/OO/H_{100}_Ag/Zn@$CeO_{2-x(SSL)}$	Yes	Yes	6	Strained Oxygen Triplet
H/OOH_{100}_Ag/Zn@$CeO_{2-x(SSL)}$	n/a	n/a	n/a	n/a
HO/OH_{100}_Ag/Zn@$CeO_{2-x(SSL)}$	n/a	n/a	n/a	n/a

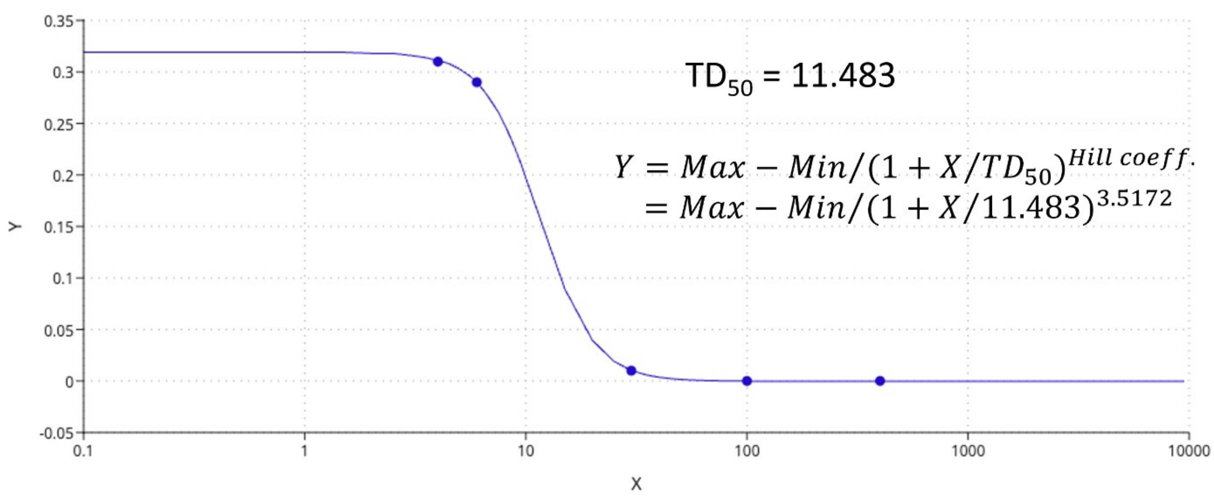

Figure S4. Regression analysis of the macrophage response to Ag/Zn-CNP on day 1 of culture and Calculation of Therapeutic Index. The therapeutic index (TI) measures the safety margin of a novel product by comparing the dose that produces a desired effect to the dose that causes toxicity. The TD_{50} of Ag/Zn-CNP was determined by regression analysis of macrophage culture response to Ag/Zn-CNP (culture time: 1 day; Fig. S2) and calculated as 11.483 µg/mL. In the absence of surface fouling biomolecules, the MIC (or ED_{50}) of *P. aeruginosa* was 4 µg/mL, and 6 µg/mL for both *S. aureus* and MRSA. Therefore, a TI (TD_{50}/EC_{50}) of 2.87 was calculated for *P. aeruginosa* and 1.91 for both *S. aureus* and MRSA. Similarly, using an EC_{50} for RV14 and VSV of 20 ug/ml and 150 ug/ml (derived from results presented in Fig. 2), yielded TIs of 0.574 and 0.077, respectively. However, for bacterial studies performed in the presence of biomolecules, it was noted that the MIC of *P. aeruginosa* increased to 30 µg/mL and the MIC of *S. aureus* and MRSA both increased to 400 µg/mL. Thus, it is indicated that when in the presence of biomolecules, the effective therapeutic dose is higher than the concentration which causes toxicity to host cells. This highlights a further need for research developing measures that minimize biofouling or that otherwise limit physicochemical processes inducing host toxic responses prior to the material's use as an antimicrobial, *in vivo*.