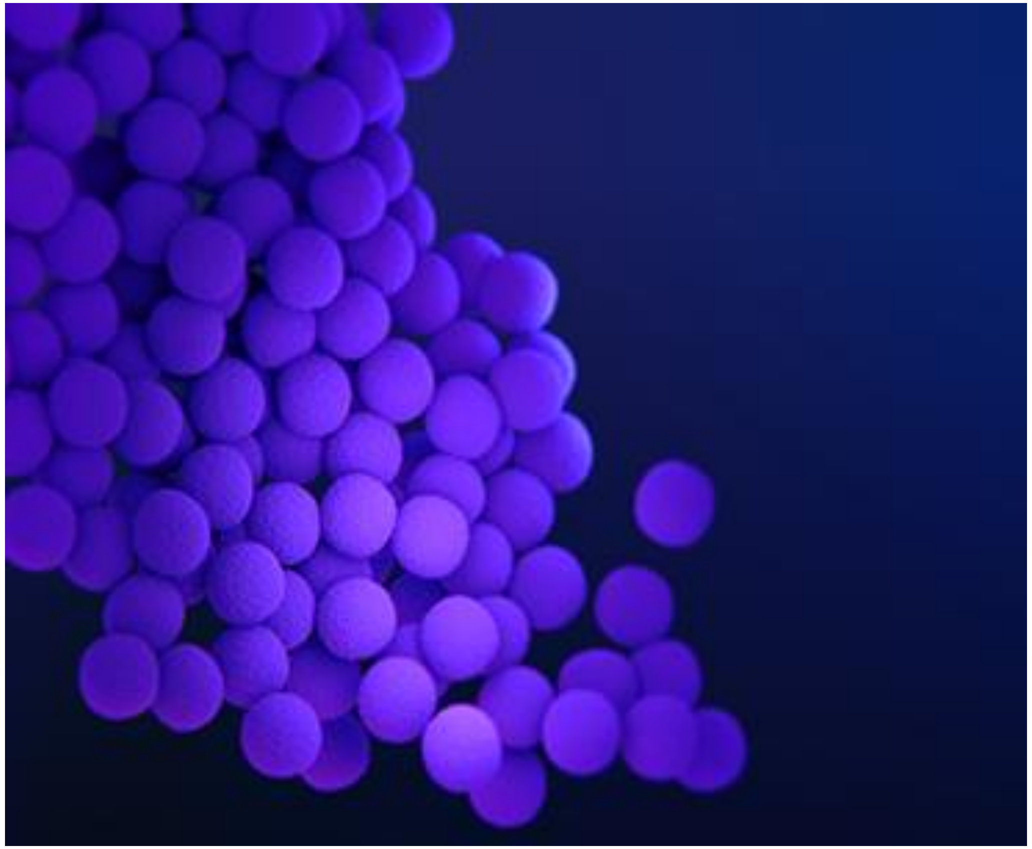
Synergies and Bactericidal Properties of Copper (Cu) and Zinc (Zn) Nanoparticles in Wound Healing and Other Potential Antimicrobial Treatment for the Healthcare Industry



Arjun Ghosh
Los Alamitos High School

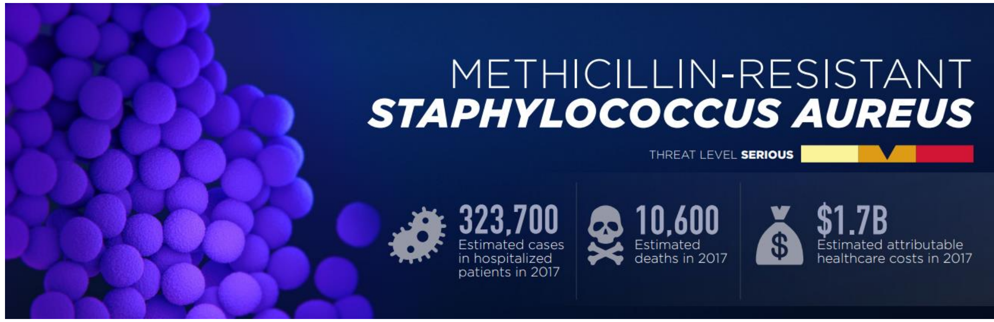
Methicillin-Resistant Staphylococcus Aureus (MRSA), Image credits: Centers for Disease Control and Prevention and Adobe Stock Photo

Abstract

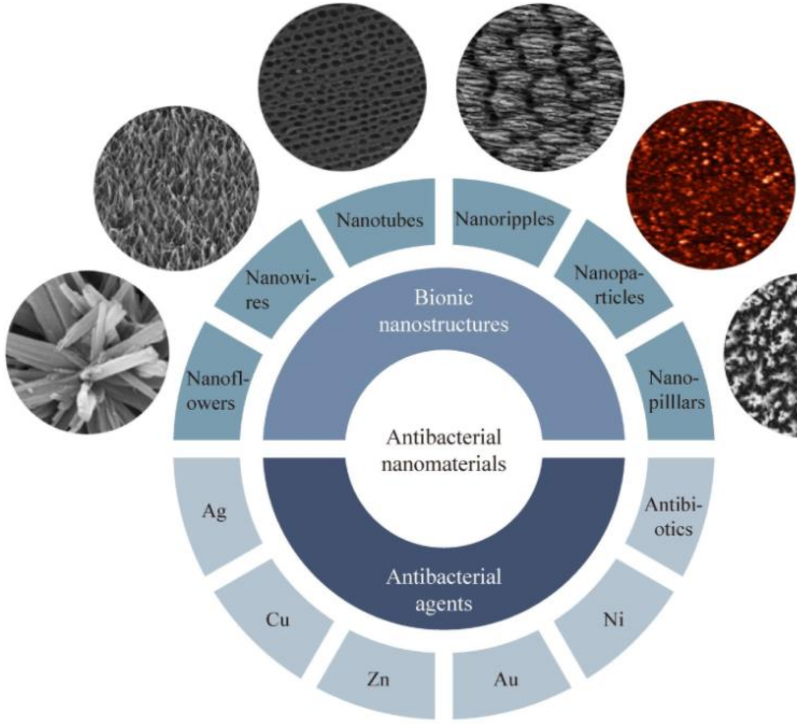
My interest in nanomaterial-based wound treatment was inspired by a personal event. My grandfather developed a deep pressure sore on his lower back during his treatment at a hospital. While researching the silver nanoparticle-based treatment prescribed to him, I learnt about the cytotoxic effects of silver and the major global problem involving antibiotic-resistant bacteria such as Methicillin-Resistant Staphylococcus Aureus (MRSA) in hospitals. Metal nanoparticles are being used increasingly as an alternative to antibiotics for wound treatment and infection prevention due to their bactericidal properties. For my experiment, I wanted to develop safe and inexpensive wound healing products using Copper and Zinc Nanoparticles as alternatives. In my project, I use Copper (CuNP) and Zinc (ZnNP) Nanoparticles with agar powder, glycerin, and water and establish the effectiveness of the compounds. I take two approaches i. as a topical gel using CuNp and ZnNp; ii. a Np-infused bandage using CuNP and ZnNP. I test on E.coli (gram-negative) and B. subtilis (gram-positive). A crucial step was determining the Minimum Bactericidal Concentration (MBC) to establish the safe quantity of nanomaterial use. My results prove CuNPs to be significantly (~2–30 times) more effective than ZnNPs at the same concentration. I also demonstrate the synergistic properties of CuNP and ZnNP as the combination of CuNP and ZnNP is more effective in killing both E.coli and B.subtilis than CuNP and ZnNP applied alone. This also implicates that a simple strategy of combining the two Nps has the potential to inhibit gram-positive antibiotic resistant bacteria such as MRSA.

Problem

- Antibiotic resistant bacteria are a major problem in hospitals in the United States and globally
  - Cases of antibiotic resistant bacteria/super bugs is increasing at an alarming pace. This is a major problem in hospitals in patients with open wounds, burns as they have the potential for infection.
  - 2.8 million antibiotic-resistant bacteria infections occur in the United States each year, and more than 35,000 people die as a result (2019 report)
  - For MRSA alone, there were 323,700 estimated hospitalized cases, 10,600 deaths, and $1.7B estimated healthcare costs (2017 CDC Report)
- Metal Nanoparticles such as silver (Ag), gold (Au), copper (Cu), and Zinc (Zn) offer an alternative to antibiotics and are being used increasingly for wound treatment and infection prevention due to their bactericidal properties
- However, silver-based treatments have cytotoxic effects on tissues, can cause delayed healing, and have growing concerns of development of antimicrobial resistance (FDA study, 2016).
- Gold treatments at inhibitory/bactericidal concentrations is expensive



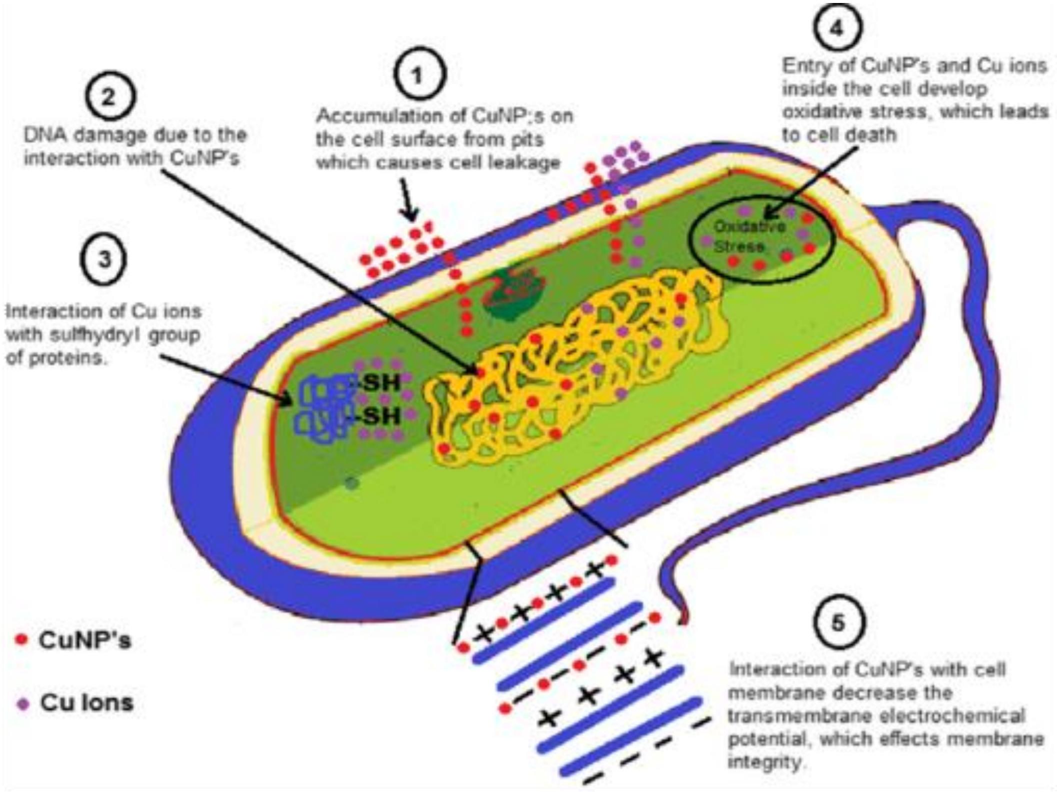
https://www.cdc.gov/



Nanometals with Antibacterial Properties
https://www.frontiersin.org/

Introduction (Background Research)

- Nanoparticles are effective due to their large surface area, small size allowing to penetrate cell wall, and release of Np (+) ions at a large rate to neutralize negatively (-) charged bacteria
- Mechanism of anti-bacterial activity of nanoparticles against bacterial cell: Production of reactive oxygen species (ROS) elevates membrane lipid peroxidation and causes membrane leakage of reducing sugars, DNA, proteins, and reduces cell viability
- Cu and Zn exhibit strong antibacterial activity; Cu and Zn are part of micronutrient in humans (Prado et al., 2012, Djoko et al., 2015)
- Cu and Zn hybrid nanoparticles exhibit synergistic properties and significantly reduced bacterial growth in tomato plants (Carvalho et al., 2019)
- Cytotoxicity of Cu is less than Ag (Ostaszewska et al., 2018); Cytotoxicity of NPs depends on size and form
- Both Cu and Zn are less expensive than Ag or Au Nanoparticles



https://www.researchgate.net/figure/Mechanism-for-antibacterial-activity-of-copper-nanoparticles_fig6_322097891

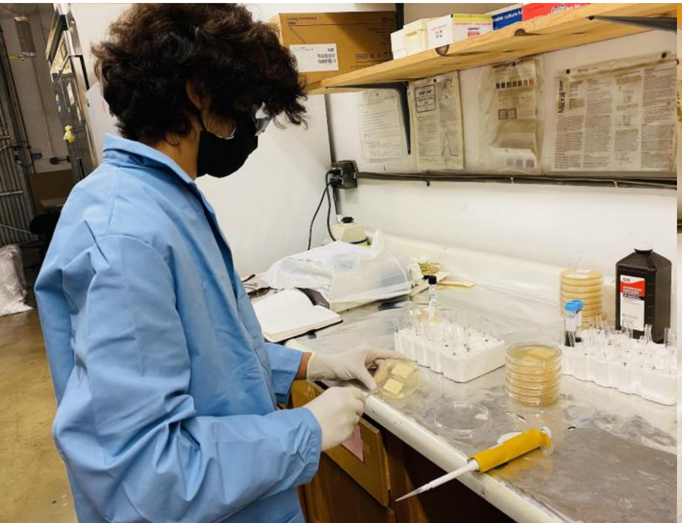
Hypothesis

My hypothesis is that Copper Nanoparticles (CuNPs) and Zinc Nanoparticles (ZnNPs) have synergistic properties and can be used in combination to improve efficiency of individual Nanoparticles (NPs) in combating bacteria that forms in wounds, burns, which are commonly found to cause infections in hospitals.

- My primary objective of this research is to develop an effective and safe antibacterial treatment gel and bandage using CuNPs and ZnNPs
- My secondary objective is to combine ZnNPs and CuNPs to demonstrate their synergy and increased effectiveness when put together
- I would like to establish a Minimum Bactericidal Concentration (MBC) of the individual NPs and the combined product for use in antimicrobial treatment in the healthcare industry.

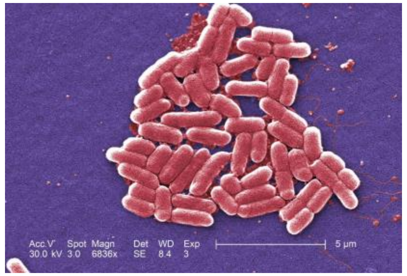
Materials

- Copper Nanoparticles 25nm diameter (Sigma-Aldrich 774081-5G)
- Copper Nanopowder ~425µm diameter (Sigma-Aldrich 266086-25G)
- Zinc Nanoparticles 40-60nm diameter (Sigma-Aldrich 578002-5G)
- Escherichia coli (E. coli) (ATCC 8739 LOT 483-1051-82022-05-310483U)
- Bacillus subtilis (B. subtilis) (ATCC 19659 LOT 540-21-22021-10-310540U)
- Tryptic Soy Agar Plates (Hardy Diagnostics G60 LOT 474621)
- Tryptic Soy Broth Tubes (Hardy Diagnostics K82 LOT 475069)
- Deionized water, Agar Powder, NaCl (salt), Coffee Filter, Cloth (bandage material) Metal loop, Glass Tubes
- Incubator, Autoclave

Procedure

Monitoring Bacteria



E.Coli gram-negative

B.Subtilis gram-positive

Test Variables

Copper Nanoparticles 25nm (CuNPs)

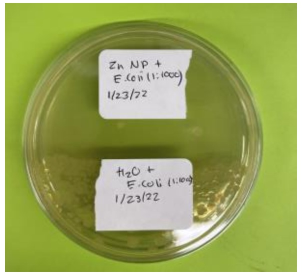
Copper Nanopowder ~425µm

Zinc Nanoparticles 40nm (ZnNPs)

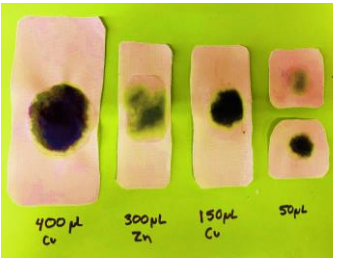
25nm CuNP & 25nm ZnNP combination

Methods to demonstrate Bactericidal Properties and Synergies between CuNP and ZnNP

Direct Application of NP samples to bacteria



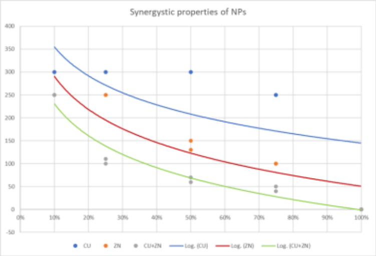
Wound dressing/ Bandage Simulation Experiment with NP



48 hr. incubation at 37°C

Observation Parameters

Minimum Bactericidal Concentration



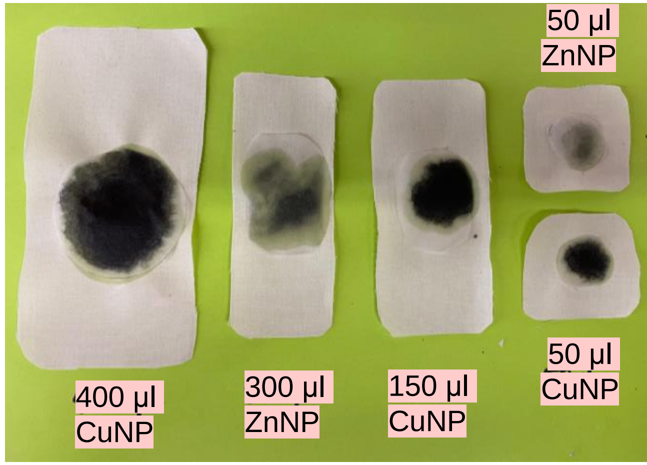
Average of Bacterial Colony count to establish individual and combined MBC of CuNP and ZnNP

- Bactericidal efficiency/ effectiveness in destroying bacteria

Procedure (Continued)

Multiple methods used to test bactericidal activities of Copper and Zinc NPs

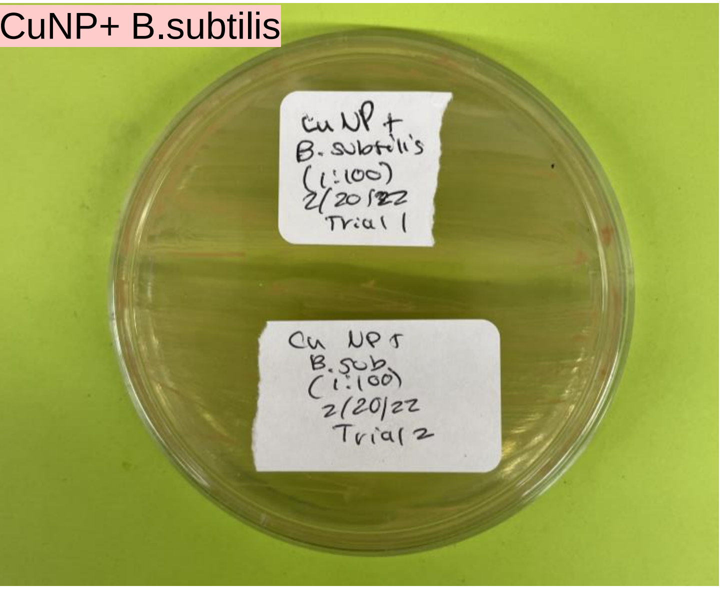
a) Direct Application of CuNp samples to bacteria
b) Direct Application of ZnNp samples to bacteria
c) Wound dressing/Bandage Simulation – NP samples applied to coffee filter



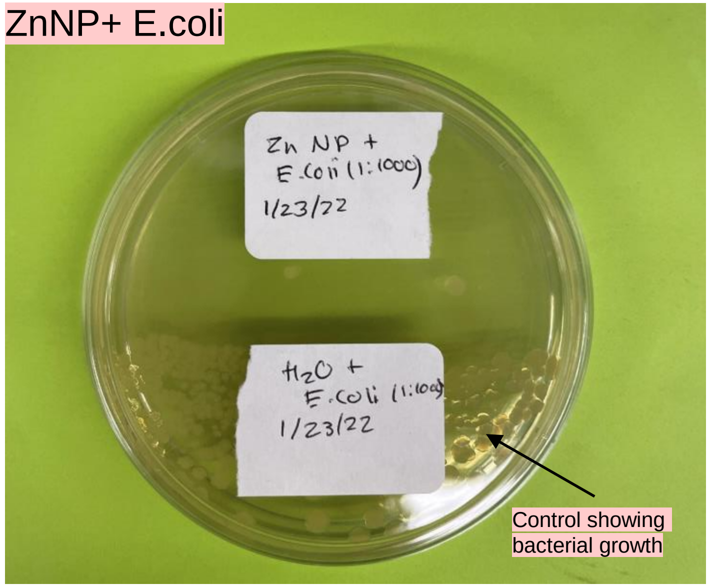
Sample bandages using coffee filters- infused with CuNP, ZnNP and combination of CuNP + ZnNP

Design of Experiment carefully prepared using a systematic set of steps to establish Minimum Bactericidal Concentration (MBC)

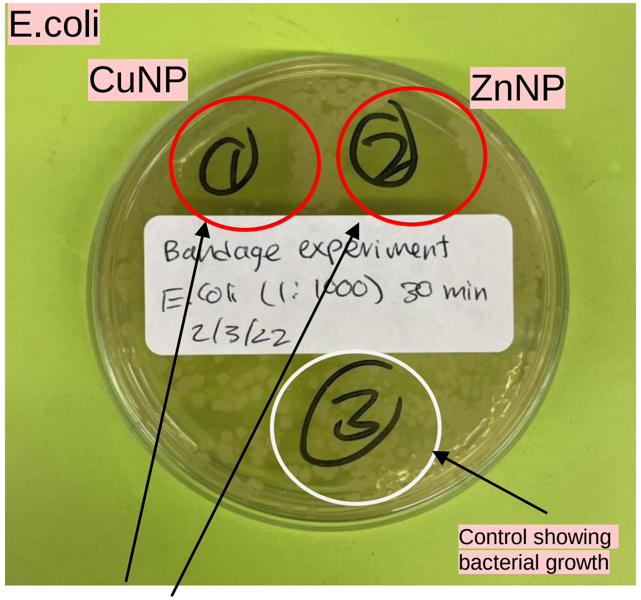
a) Single Nanometal MBC calculated for CUNP and ZnNP individually
b) MBC for CuNP and ZnNP combination established using individual MBCs at various dilution levels/concentration (100% or base MBC, 75%, 50%, 25%, 10%)



Direct CuNP application showing no bacterial growth in B.subtilis



Direct ZnNP application showing no bacterial growth in E.coli



Bandage simulation- shows areas with no bacterial growth in E.coli

Results

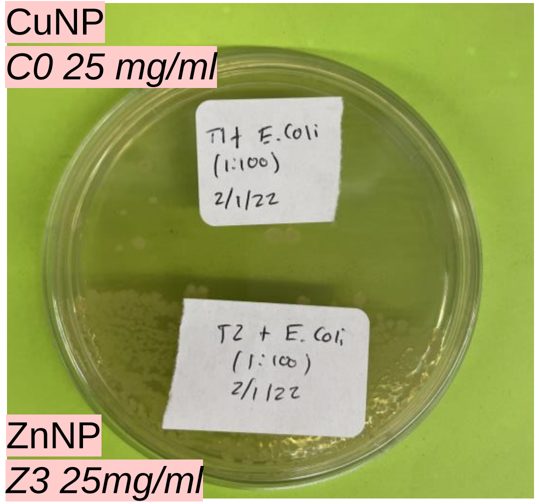
		Average No. of Colonies			
		E.Coli (1:100)		***B.subtilis (1:100)***	
Cu NP dilutions:	*mg/ml*	*Trial 1*	*Trial 2*	*Trial 1*	*Trial 2*
C0	***25.00***	*0*	*0*	***0***	***0***
C1	*12.50*	*0*	*0*	*100*	*80*
C2	*6.25*	*0*	*0*	*120*	*120*
C3	*3.13*	*0*	*0*	*120*	*130*
C4	***1.56***	***0***	***0***	*150*	*150*
C5	*0.78*	*5*	*15*	*170*	*170*
C6	*0.39*	*30*	*40*	**	**
Zn NP dilutions	*mg/ml*	*Trial 1*	*Trial 2*	*Trial 1*	*Trial 2*
Z1	***50.00***	***0***	***0***	***0***	***0***
Z2	*37.50*	*100*	*100*	*20*	*20*
Z3	*25.00*	*>300*	*>300*	*30*	*30*

Establishing Minimum Bactericidal Concentration (MBC) for CuNP and ZnNP

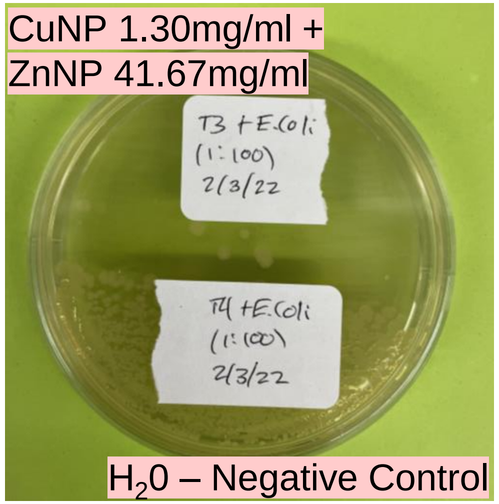
- *6 concentrations, 2 trials performed for CuNP*
- *3 concentrations, 2 trials performed for ZnNP*

** *Was not tested*

- Minimum Bactericidal Concentration (MBC) for CuNP was between C4 and C5 (0.78-1.56 mg/ml) for E.coli and between C0 and C1 (12.5-25.0 mg/ml) for B.subtilis
- MBC for ZnNP was between Z and Z2 (37.5-50.00 mg/ml) for both E.coli and B.subtilis.
- CuNP demonstrated MBC at very low concentration when compared with ZuNP - **~30x** less for E.coli and **2x** less for B.subtillis



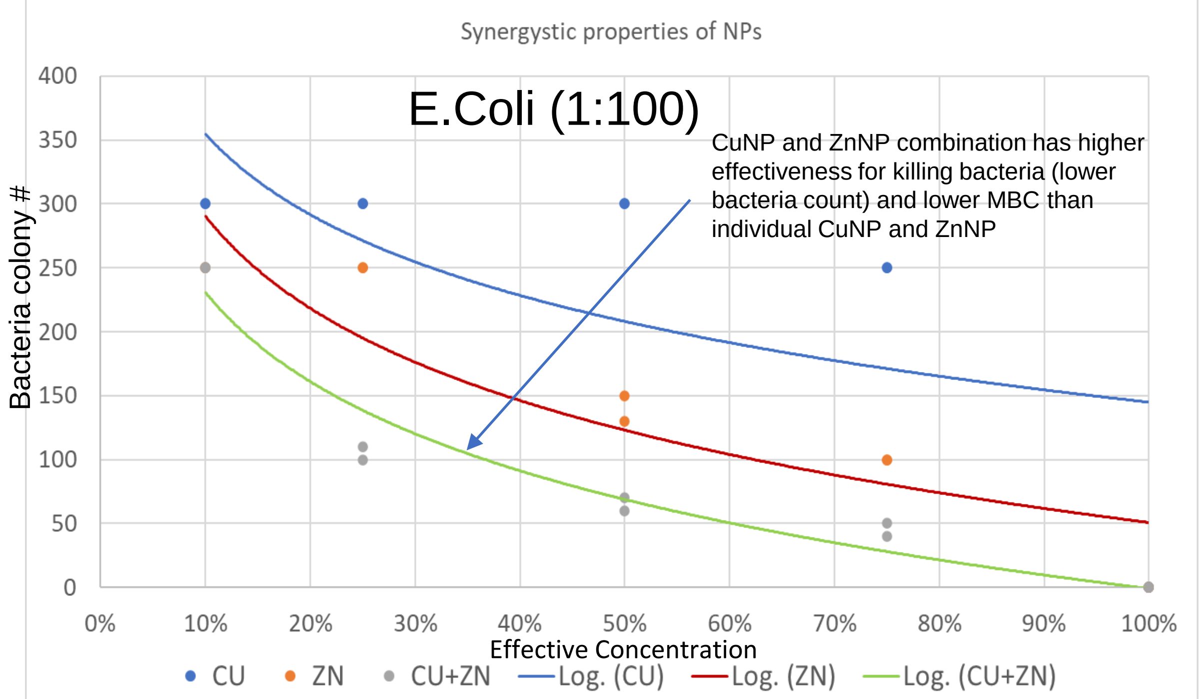
Top - CuNP conc. C0 with no bacterial growth
Bottom- ZnNP conc. Z3 shows bacterial growth



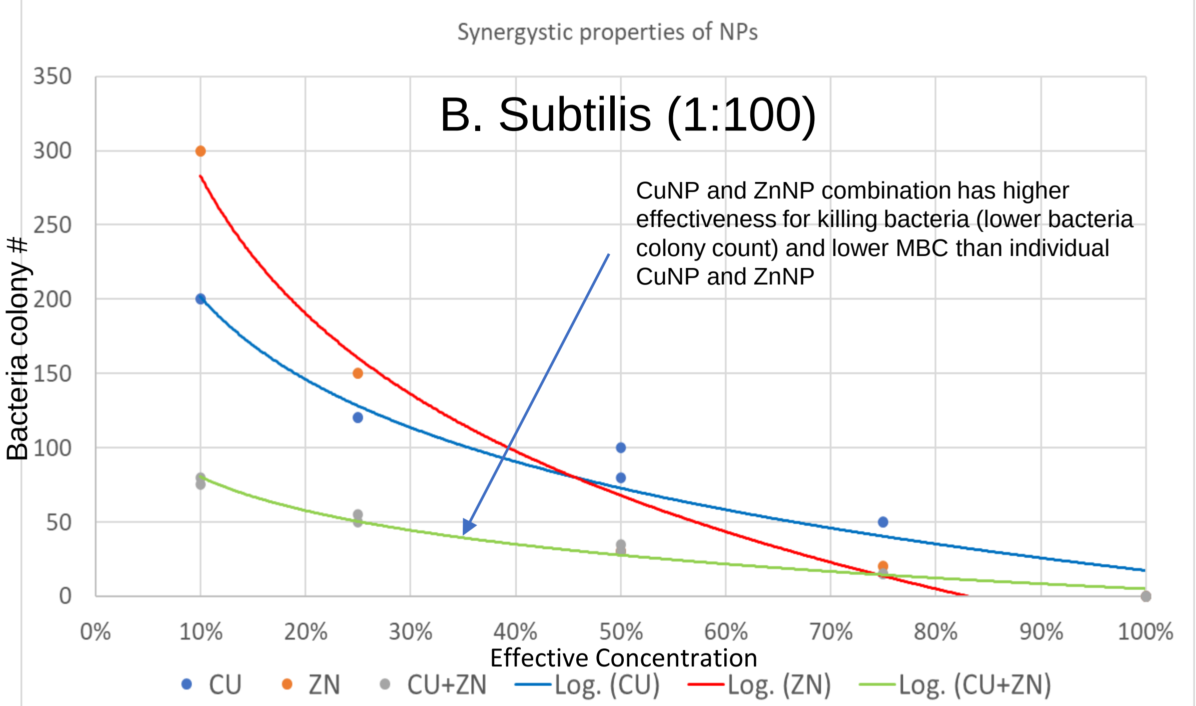
Top – Combined CuNP conc. 1.3mg/ml and ZnNP 41.67mg/ml with no bacterial growth
Bottom- Negative control shows bacterial growth

Results (Cont.)

Establishing Minimum Bactericidal Concentration (MBC) for CuNP and ZnNP combined



Graph showing E.coli colony count vs. Effective concentration for CuNP, ZnNP, [CuNP+ZnNP]



Graph showing B. subtilis colony count vs. Effective concentration for CuNP, ZnNP, [CuNP+ZnNP]

E.Coli (1:100)

		CuNP		**ZnNP**		**CuNP + ZnNP**
	Effective Concentration %	Concentration mg/ml	Bacteria Colony#	Concentration mg/ml	Bacteria Colony#	Bacteria Colony#
T1	100%	1.30	0	41.67	0	0
T2	100%	1.30	0	41.67	0	0
T1	75%	0.98	250	31.25	100	40
T2	75%	0.98	250	31.25	100	50
T1	50%	0.49	300	15.63	130	60
T2	50%	0.49	300	15.63	150	70
T1	25%	0.12	300	3.91	250	100
T2	25%	0.12	300	3.91	250	110
T1	10%	0.01	300	0.39	250	250
T2	10%	0.01	300	0.39	250	250

B.Subtilis (1:100)

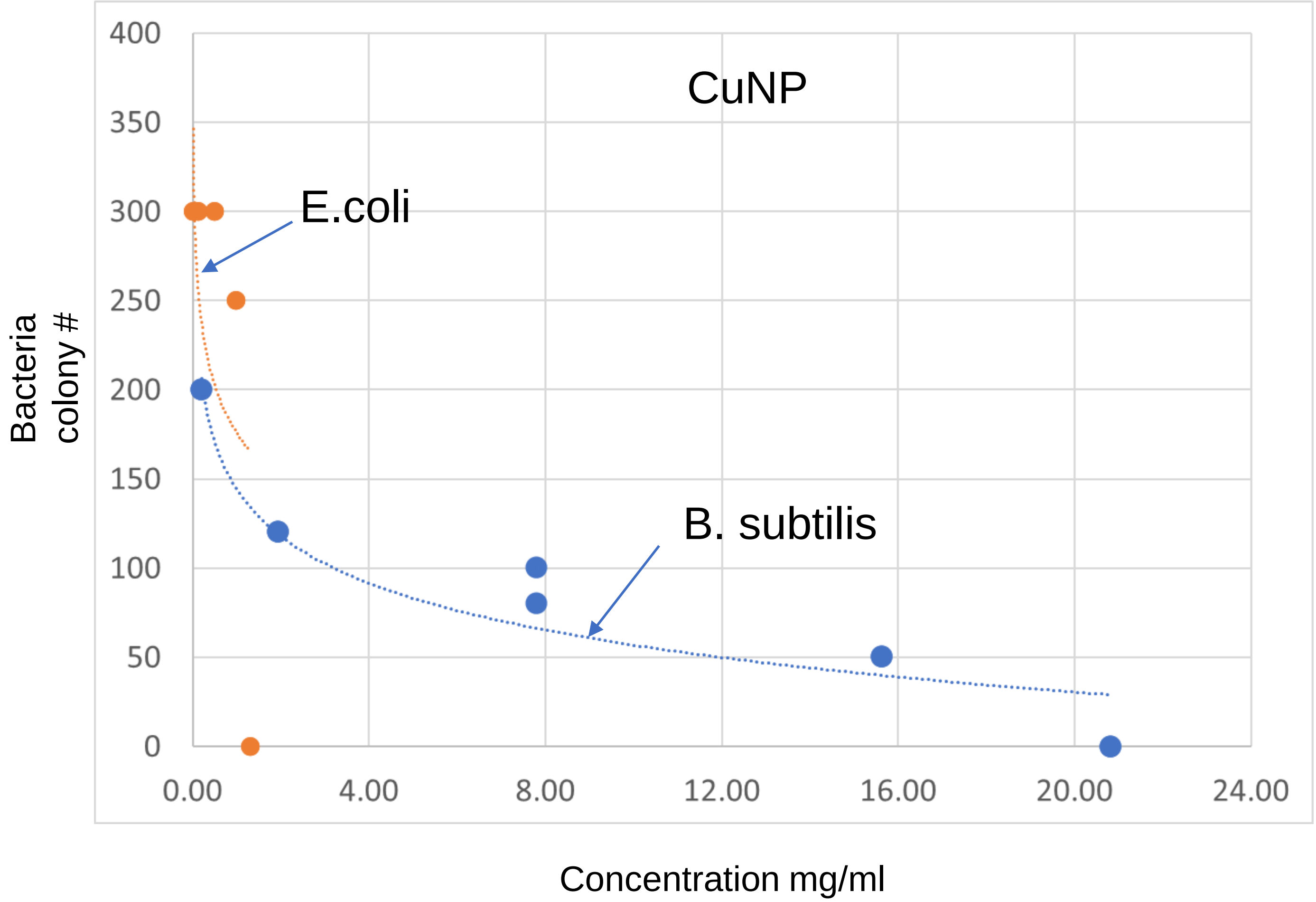
		CuNP		**ZnNP**		**CuNP + ZnNP**
	Effective Concentration %	Concentration mg/ml	Bacteria Colony#	Concentration mg/ml	Bacteria Colony#	Bacteria Colony#
T1	100%	20.83	0	41.67	0	0
T2	100%	20.83	0	41.67	0	0
T1	75%	15.63	50	31.25	20	15
T2	75%	15.63	50	31.25	20	15
T1	50%	7.81	100	15.63	30	30
T2	50%	7.81	80	15.63	30	35
T1	25%	1.95	120	3.91	150	50
T2	25%	1.95	120	3.91	150	55
T1	10%	0.20	200	0.39	300	80
T2	10%	0.20	200	0.39	300	75

Discussion

1) Nanoparticles are effective due to their small size (25 nm CuNP and ZnNP) allowing to penetrate cell wall. Use of 425µm Copper Nanopowder on E.coli and B.subtilis did not have any bactericidal effect

2) CuNP was more effective than ZnNP at same concentrations:

- Minimum Bactericidal Concentration for CuNP was between **0.78-1.56 mg/ml** for E.coli and between **12.5-25.0 mg/ml** for B.subtilis

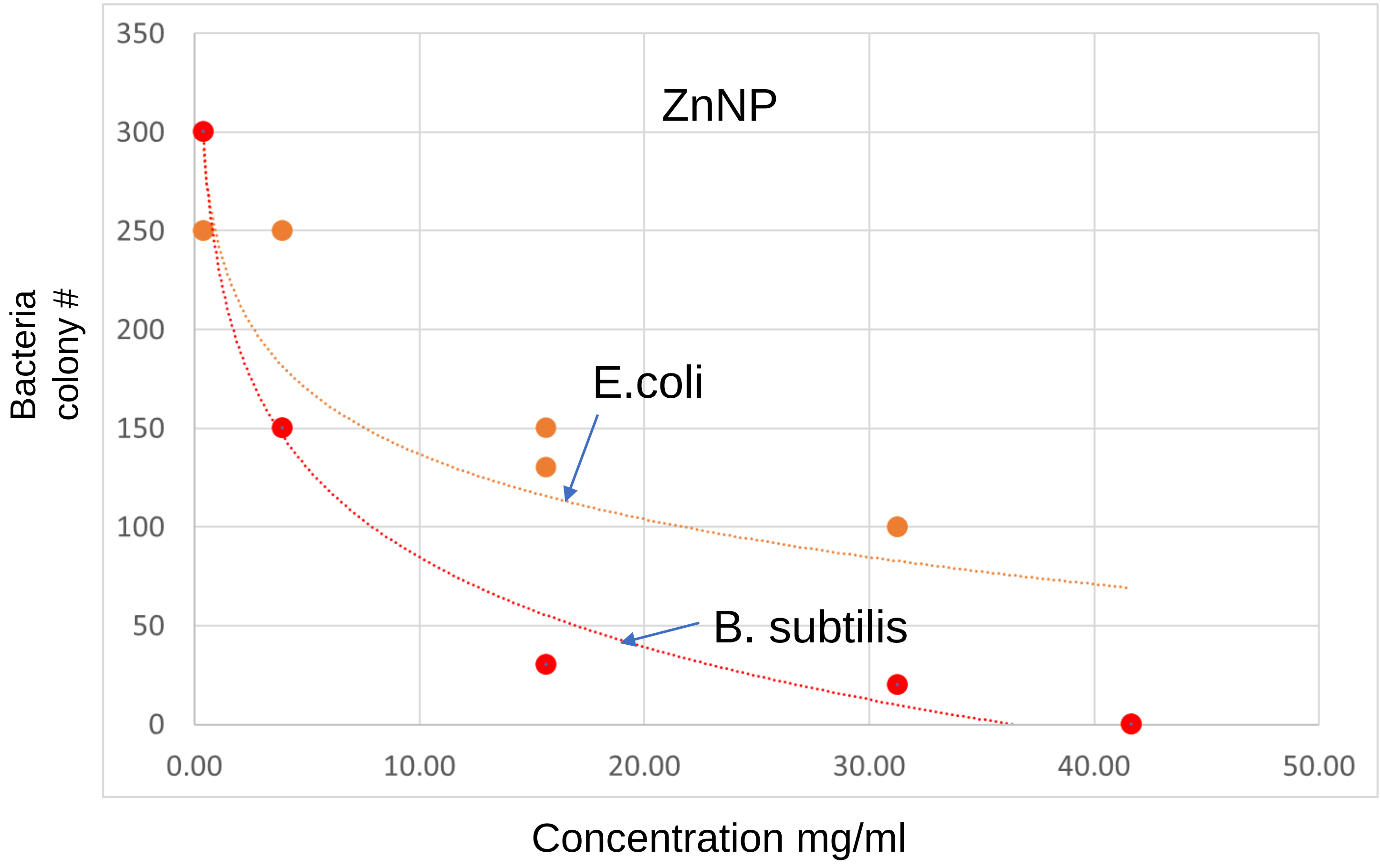


Discussion (Cont.)

1) ZnNP was also effective but at a higher concentration than CuNP
- Minimum Bactericidal Concentration for ZnNP was **between 37.5- 50.00 mg/ml** for both E.coli and B.subtilis

2) CuNP and ZnNP combination demonstrated higher effectiveness for killing bacteria and lower Minimum Bactericidal Concentration than individual CuNP and ZnNP:
- For E.coli MBC was **1.30 mg/ml** CuNP, **41.67mg/ml** ZnNP
- For B.subtilis MBC was **20.83 mg/ml** CuNP, **41.67mg/ml** ZnNP

3) Overall, higher concentration of NPs needed for B.subtilis than E.coli to have bactericidal effects



Conclusion

Through my experiment I concluded:

Copper Nanoparticles (CuNPs) and Zinc Nanoparticles (ZnNPs) have synergistic properties and can be used in combination to improve efficiency of individual Nanoparticles (NPs) in combating bacteria that forms in wounds, burns, which are commonly found to cause infections in hospitals.

- Copper Nanoparticles (CuNPs) are effective on both E.coli and B.subtilis at lower concentrations
- Zinc Nanoparticles (ZnNP) are effective in killing bacteria at higher concentrations
- Nanoparticle size has a large effect on the killing power of the nanometals that were tested
- Minimum Bactericidal Concentration (MBC) for CuNP in E.Coli is relatively low
- Minimum Bactericidal Concentration (MBC) for ZnNP is relatively high for both E.coli and B.subtilis
- CuNP and ZnNP infused based wound dressing has potential as a method of infection control

Reflection/Application

Through my experiment I established quantitative Minimum Bactericidal Concentration (MBC) for combined nanoparticles CuNP and ZnNP.

My project demonstrated the synergistic properties of CuNP and ZnNP as the combination of CuNP and ZnNP is more effective in killing both E.coli and B.subtilis than CuNP and ZnNP applied alone.

- My project indicate that a simple strategy of combining the two NPs has the potential to inhibit gram-positive antibiotic resistant bacteria such as MRSA.

- As a next stage of this project, I will extend these findings to design experiments and explore opportunities of research at university research labs involved in antibiotic resistant bacteria MRSA such as UC Irvine Medical Center (Project Clear), Los Angeles Biomedical Research Institute (LABioMed) at Harbor-UCLA Medical Center.

- I will also explore effective wound healing and bactericidal product strategies for use in the healthcare industry such as CuNP/ ZnNP infused wound dressings, surface treatment for medical equipment and topical gel-based applications

References Cited

1. Carvalho, R., Duman, K., Jones, J.B. et al. Bactericidal Activity of Copper-Zinc Hybrid Nanoparticles on Copper-Tolerant Xanthomonas perforans. Sci Rep 9, 20124 (2019). https://doi.org/10.1038/s41598-019-56419-6
2. CDC. Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2019.
3. CDC. Factsheet on Methicillin-Resistant Staphylococcus Aureus (MRSA). 2017. https://www.cdc.gov/drugresistance/pdf/threats-report/mrsa-508.pdf
4. Djoko, K. Y., Ong, C. L., Walker, M. J., & McEwan, A. G. (2015). The Role of Copper and Zinc Toxicity in Innate Immune Defense against Bacterial Pathogens. The Journal of biological chemistry, 290(31), 18954–18961. https://doi.org/10.1074/jbc.R115.647099
5. Food and Drug Administration (FDA) Executive Summary, Classification of Wound Dressings Combined with Drugs, Prepared for the Meeting of the General and Plastic Surgery Devices Advisory Panel, September 20-21, 2016,
6. Ostaszewska, T., Śliwiński, J., Kamaszewski, M., Sysa, P., & Chojnacki, M. (2018). Cytotoxicity of silver and copper nanoparticles on rainbow trout (Oncorhynchus mykiss) hepatocytes. Environmental science and pollution research international, 25(1), 908–915. https://doi.org/10.1007/s11356-017-0494-0
7. Prado J V, Vidal A R, Durán T C. [Application of copper bactericidal properties in medical practice]. Rev Med Chil. 2012 Oct;140(10) 1325-1332. doi:10.4067/s0034-98872012001000014. PMID: 23559292.