Wound Healing Promoted by Broad-Spectrum, Phage Structure Mimicking, Synthetic Antibacterial Nanoparticles as a Topical or an Intravenous Formulation, which Reduced MDR ESKAPE Pathogens Induced Infections in Wound Models

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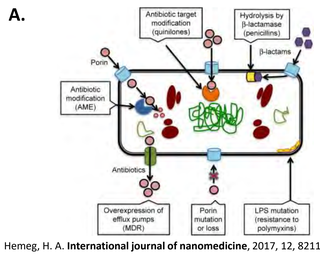
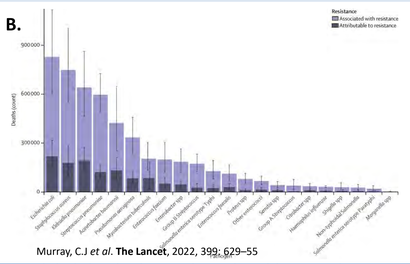
Introduction: The increasing frequency of nosocomial infections caused by antibiotic-resistant microorganisms concurrent with the stagnant discovery of new classes of antibiotics has made the development of new antibacterial agents a critical priority. Our approach is an antibiotic-free strategy drawing inspiration from bacteriophages to combat antibiotic-resistant bacteria. We developed a nanoparticle-based antibacterial system that structurally mimics the protein-turret distribution on the head structure of certain bacteriophages and explored a combination of different materials arranged hierarchically to inhibit bacterial growth and ultimately kill pathogenic bacteria. Here, we describe the synthesis of phage-mimicking antibacterial nanoparticles (PhANPs) consisting of silver-coated gold nanospheres distributed randomly on a silica core. The Gold-silver nanoalloys were further modified with antimicrobial peptides or polymers. Structurally, our nanoparticles mimicked the bacteriophages of the family Microviridae by up to 88%. These phage-mimicking ANPs were tested for bactericidal efficacy against seven clinically relevant nosocomial pathogens (*Staphylococcus aureus USA300, Pseudomonas aeruginosa FRD1, Enterococcus faecalis, Corynebacterium striatum, Streptococcus Pyogenes, Klebsiella pneumoniae, and Acinetobacter baumannii*) and for biocompatibility with skin cells. The antibacterial efficacy was > 99.999% against all bacteria in the liquid phase (topical) and on an immobilized phase (implants, bandages). Importantly, the phage-mimicking ANPs did not show any cytotoxic effects against human skin keratinocytes. Our mouse wound healing results also confirmed *in vivo* biocompatibility with enhanced wound healing effect. Our results indicate that phage-mimicking antimicrobial nanoparticles are a highly effective, alternative antibacterial agent, which may be suitable for standalone treatments or for co-administration with existing available formulations. TRL ≥4

I. Antibiotic Resistance and its Effect on Infection Induced Mortality



Hemeg, H. A. **International journal of nanomedicine**, 2017, 12, 8211.



Murray, C.J *et al.* **The Lancet**, 2022, 399: 629–55

A. Drug resistance mechanisms evolved by bacteria comprise hydrolysis by β-lactamases, modification of drug targets or antibiotics, loss or mutation of porins and overexpression of efflux pumps.

B. Global deaths (counts) attributable to and associated with bacterial antimicrobial resistance by pathogen, 2019. Estimates accounted for the co-occurrence of resistance to multiple drugs. P-value ≤ 0.05

At the current low rate of antibiotic discovery and development, we may lose the race to contain antibiotic-resistant bacterial strains. Without urgent action, antibiotic-resistant infections will kill more patients per year by 2050 than all cancers combined. There is a pressing need for a new class of antibacterial.

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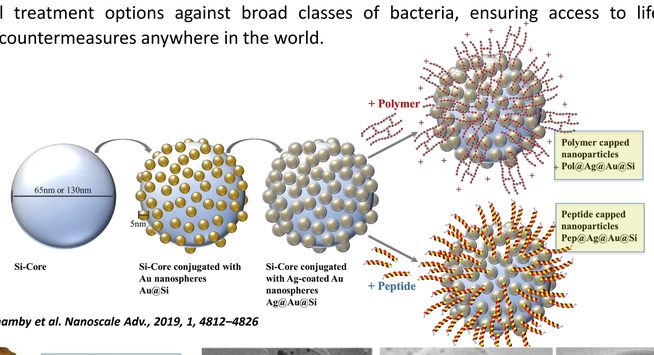
II. Modularly Assembled Phage-Mimicking Antibacterial Nanoparticles (PhANPs)

Evolutionarily phages have been successful in targeting and killing bacteria for millions of years. We are tapping into that evolutionary advantage with our phage-mimicking nanoparticles to contain the emergence of new antibiotic resistances. This is a **tunable** platform technology that creates universal treatment options against broad classes of bacteria, ensuring access to life-saving medical countermeasures anywhere in the world.



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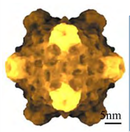
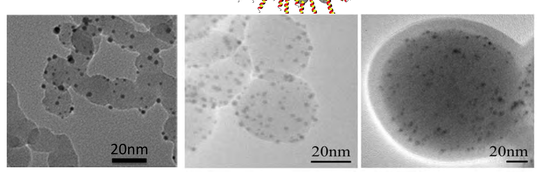


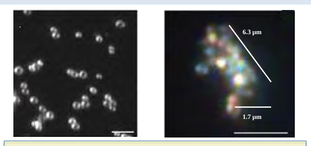
Bacteriophage **ΦX174** is 50% similar to PhANPs based on turret interspacing



Silica diameter = 24.9 ± 3.5 nm 45.1 ± 8.3 nm 128.3 ± 10.4 nm

Gold-Silver nanoalloy (GSAN) diameter = 5 ± 1.6 nm

III. PhANPs mode of Antibacterial Action



Control *S. aureus* USA300. Diameter: 1.3 μm

With PhANPs, *S. aureus* USA300. die by non-division

Phage-mimicking structure of our PhANPs allows them to structurally and chemically prolong their interaction with the bacterial membrane, thus interrupting bacterial cell division, leading to death of the bacteria

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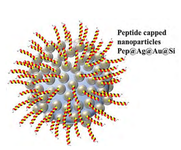
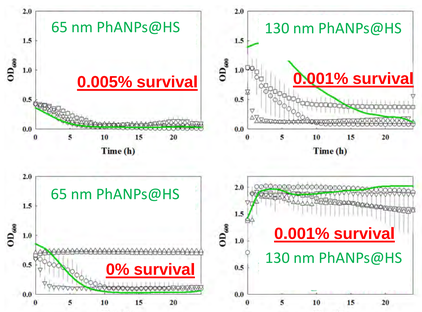
IV. MIC and Evolution of Resistance

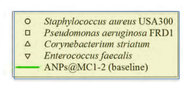
S.No.	Bacteria Strain	MIC (μM)	MIC after 28 days of serial culture in 2x MIC (μM)
1.	S. aureus USA300	7.29 - 9.51	No change
2.	A. baumannii BA1605	8.50 - 11.11	No change
3.	K. pneumoniae	4.65- 10.58	No change
4.	S. pyogenes	6.71 - 8.62	No change
5.	P. aeruginosa FRD1	2.81 - 17.06	No change
6.	C. striatum	4.64 - 10.77	Not tested
7.	E. faecalis	5.19 - 6.98	Not tested

After >1000 generations of *bacteria*(28 days of culture) were exposed to three different concentrations of PhaNP@Syn71 (MIC50, MIC90, 2xMIC) the population of bacteria exposed to 2xMIC concentration of PhaNPs@Syn71 **showed no signs of evolved resistance thus highlighting the need for proper dosage being used consistently when treating an infection site.** Our antibacterial dosages *in vivo* are significantly higher than the MIC because of the high biocompatibility of our formulation, which further negates the emergence of resistance.

V. Broad Spectrum, High Bactericidal Activity, of Antimicrobial Peptide Capped PhANPs

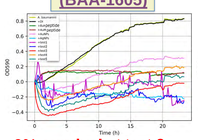






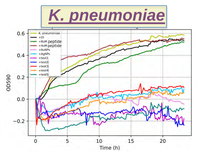
References:
1. J Antibiot (Tokyo). 2018 Jun;71(6):592-600
2. PhANP 10.1039/C3NR02639F
3. S. pyogenes in vivo wound infection model 10.26434/ chemrxiv 2023 hcs0b

A. baumanii (BAA-1605)



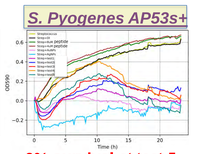
0% survival at test 2 Concentration.

K. pneumoniae



0% survival at test 2 Concentration.

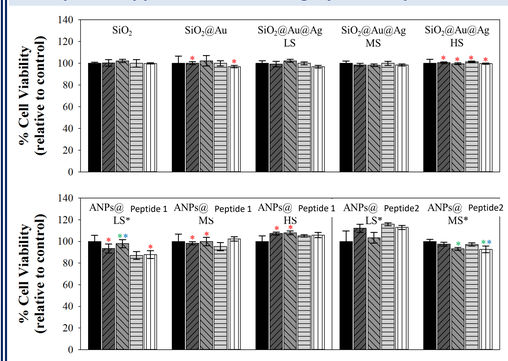
S. Pyogenes AP53s+

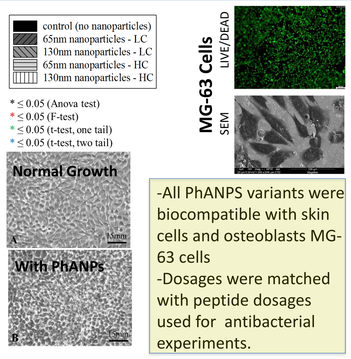


0% survival at test 5 Concentration.

- The peptides assume a helical structure inside hydrophobic membranes.
- Near 100% bacterial death.
- Broad spectrum activity against 7 clinically derived strains. 4 gram (+) and 3 gram (-) bacterial strains
- We have highlighted consistently, high efficacy bactericidal activity of peptide modified PhANPs while using 2 order of magnitude lower peptide concentration.
- The free peptides at the same dosage had insignificant bactericidal effect.

VI. Peptide Capped PhANPs are Highly Biocompatible with Human Keratinocytes and Osteoblasts



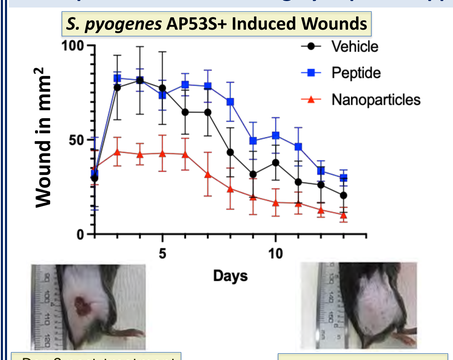


-All PhANPS variants were biocompatible with skin cells and osteoblasts MG-63 cells

-Dosages were matched with peptide dosages used for antibacterial experiments.

In general, the use of silica-based or gold-based materials are not considered harmful to humans. The phage-mimicking structure of our PhANPs increased the biocompatibility towards eukaryotic cells as was evident from our tests on model skin cell lines (HaCaT) and Osteoblasts (MG-63). Additionally, the Environmental Protection Agency (EPA) has established a chronic oral Reference Dose (RfD) of 5 μg per kg per day for silver which broadly translates to 250 μg to 750 μg per person per day. Our phage-mimicking ANPs exhibit a maximum silver content of 189.22 μg ml^{-1} and a predicted amount of <1 μg ml^{-1} silver ions leaching into solution, which are well below the EPA prescribed safety limits.

VII. Improved Wound Healing by Peptide Capped PhANPs in a Mouse Wound Model



Day 0 post-treatment

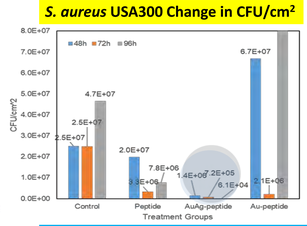
Day 20 post-treatment

- N = 4 mice per cohort. **One intravenous (IV) dose + daily topical doses** of PhANPs peptide showed **immediate stabilization of wound size** followed by inflammation free healing due to rapid lysis of bacteria in wounds . In comparison peptide only controls or vehicle only controls showed increase in wound size before the healing trend kicked in.
- The wounds healed within a 20-day period
- Antibiotic free, wound stabilization followed by wound healing, and thereby can be used in any stages of infection without concern for the emergence and the spread of new antibiotic resistance. ***S. pyogenes in vivo wound infection model 10.26434/ chemrxiv 2023 hcs0b***

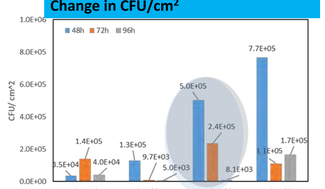
VIII. Treating and Monitoring MDR Bacterial Load in Rat Wound Model 24h Post-Infection

Topical Wound Infection Model in Sprague Dawley Rats

S. aureus USA300 Change in CFU/cm²



Carbapenem resistant A. baumannii Change in CFU/cm²

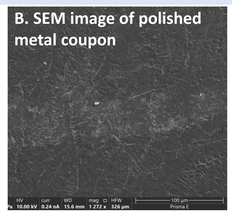


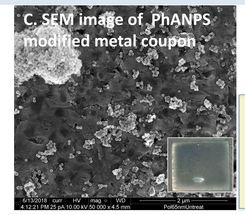
- *MRSA, or MDR A. baumannii* were applied to biopsy punched induced skin lesions, to create topological wound infections.
- Three cohorts. Cohort1 was sacked 48h post-bacteria infection, Cohort2 was sacked 72h post-bacteria infection, and Cohort3 was sacked 96h post-bacteria infection.
- Cohort 1= 1 dose; Cohort 2 = 2 doses, 24h apart; Cohort3 = 3 doses and each 24h apart.
- The PhANPs-peptide (AuAg-peptide) reduced bacterial load by 100-fold to 1000-fold in S. aureus USA300 in comparison to the no treatment groups.
- The PhANPs-peptide (AuAg-peptide) reduced bacterial of *A. baumannii BA1605* load by 100-fold after 3-doses in comparison to 1-dose or to the no treatment groups.

IX. Immobilizing Polycationic Polymer Capped PhANPs on Implants to Create Cytocompatible Antibacterial Surfaces

A.

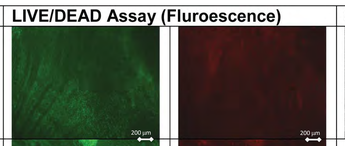
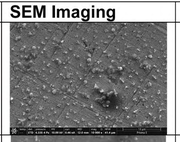
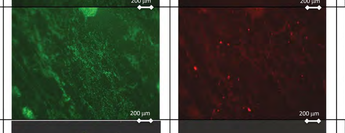
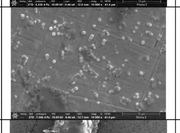
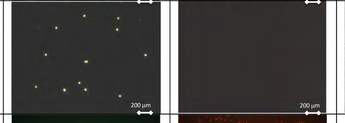
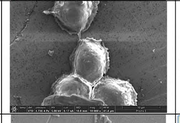
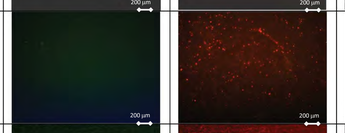
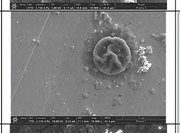
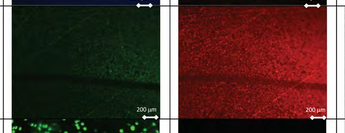
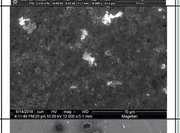
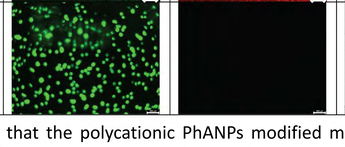
NP&polymer coated metal coupons





1-inch , PhANPs modified, implant grade metal (inset)

Implant grade metal coupons were sourced from Zimmer Biomet. Polycationic polymers of different molecular weights and structures, were capped on PhANPs and immobilized on the metal coupons. PhANPs modification on metal coupons was confirmed using SEM, EDX spectroscopy and IR-spectroscopy. *S.aureus USA300* or *P.aeruginosa FRD1* or HaCaT human cells were exposed to the PhANPs modified metal coupons surfaces for 24 hours. The presence and viability of the bacteria or cells on the PhANPs modified metal coupons was assessed using fluorescent LIVE/DEAD assay, as well as SEM imaging.

	Sample id.	LIVE/DEAD Assay (Fluroescence)	SEM Imaging	Viability
Implant Grade Metal Coupon as is, NO MODIFICATIONS	*P.aeruginosa* FRD1 (Gram negative)			Viable Bacteria
	S.aureus USA300 (Gram Positive)			Viable Bacteria
	HaCaT Cells. Model human skin cell lines			Viable HaCaT cells but no spreading
Implant Grade Metal Coupon coated with polycationic polymer presenting PhANPs	*P.aeruginosa* FRD1 (Gram negative)			Zero viable bacteria and insginifcant surface adhesion
	S.aureus USA300 (Gram Positive)			Zero viable bacteria on surface
	HaCaT Cells. Model human skin cell lines			Viable HaCaT cells with normal spreading

SEM imaging confirmed that the polycationic PhANPs modified metal coupons promoted better cell adhesion(HaCaT, MG-63). LIVE/DEAD assay utilizing calcein-AM esters and propidium iodide staining showed 100% viable HaCaT cells, 24h post seeding, thus confirming cytocompatibility. Additionally, on the polycationic PhANPs modified metal coupons, there were zero viable *P.aeruginosa FRD1* or *S.aureus USA 300*. Interestingly, the modifications prevented *P.aeruginosa FRD1* adhesion as well.

X. Benefits of Proposed Technology

- **>99.999% kill-rate** against antibiotic-resistant *S. aureus* USA300, *P. aeruginosa* FRD1, *C. striatum*, *E. faecalis*, *A .baumanii*, *K. pneumoniae*, and *S.pyogenes AP53s+*
- Utilized a **10^{1}- 10^{2} lower concentration** of antibacterial peptides thus saving material cost.
- **100% biocompatibility** to human skin cells (HaCaT) and osteoblasts (MG-63)
- Retains antibacterial efficacy in liquid-phase and on solid-phase.
- Assembled using well characterized, generally regarded as safe components.
- Can be stored frozen, refrigerated, or at room-temperature. Retained activity after multiple freeze-thaw cycles over the course of a year
- **Maturity of Technology: TRL 4 Tested in mouse and rat wound infection models.**

XI. Future Plan of Action and Potential Collaborations

In summary, Our rational modular approach provides a flexible platform for efficient customization of Phage-mimicking antibacterial nanoparticles, for multiple application modes, with tailored surface functionality as summarized in the table below.

Product	Therapeutic Class	Applications	Novelty
Phage-mimicking, tunable spectrum, antibacterial nanoparticles	Antibiotic free, modularly assembled, synthetic phage mimic.	- Topical applications to prevent infections from taking root - Antibacterial coatings on medical devices, surgical instruments, implants, perishables, etc. - Promotes wound healing *in vivo*	- Hard for bacteria to develop resistance - Low-immunogenicity in comparison to phage therapy - Tunable spectrum to make antibacterial action broad-spectrum or specific to one bacterial species.

- **We are looking for p**artnerships and collaborations to validate our platform technology in DoD relevant wound-infection models, DoD relevant combat scenarios, and implant models *in vivo*.
- Partnerships to explore complementarity of PhANPs to existing or new broad-spectrum antibacterial, by utilizing PhANPs as a carrier or as a companion therapeutic. Target other DoD relevant pathogenic bacteria.
- Partnerships that can accelerate our technology to TRL≥ 5, to aid translation into the field.

Acknowledgements:

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