Application of Antibacterial Protein-Nanoparticle Conjugates Against a Multi-drug Resistant Strain Of Pseudomonas aeruginosa

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Introduction

- Antibiotics - traditionally used as chemotherapeutic agents to treat bacterial infections
- Recent statistics (CDC, 2006) indicate about 2 million cases of antibiotic-resistant infections each year; 90,000 patients die annually from such infections.
- $30 billion dollars spent on the cumulative effects of antimicrobial resistance each year (including multiple drug regimens, extra hospital day and additional medical care).

Antimicrobial proteins and peptides

Advantages

- Antibiotic-free approach
- Broad spectrum of antimicrobial action
- Peptides function as immunomodulators[1]
- Short treatment time

E.g – Lysozyme, Lactoferrin, Defensins, Lactoperoxidase, Cathelicidin

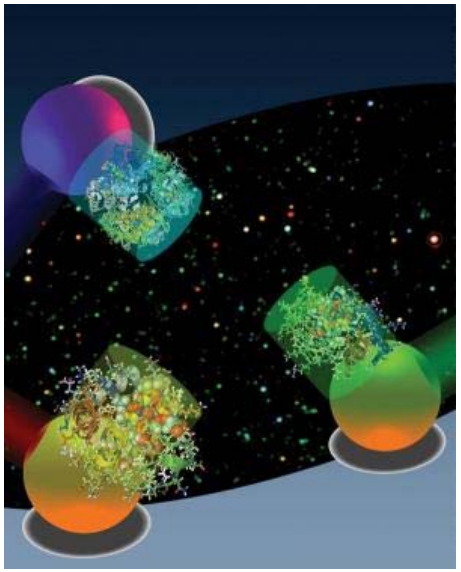
Disadvantages

- Systemic toxicity
- Low stability
- Delivery issues

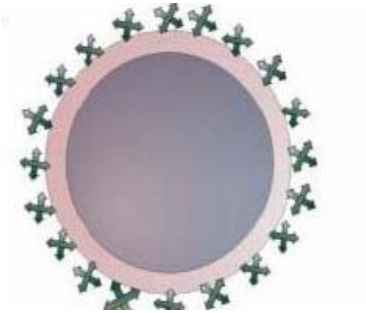
1. Jenssen, H., P. Hamill, and R.E. Hancock, Clin Microbiol Rev, 2006. 19 (3)

Nanoparticles – Targeting and delivery

- Bioavailability
- Minimum diffusional limitation
- High surface area to volume - Effective loading
- Specificity



Intrinsic properties of nanoparticles – Size, Charge etc



Study 1: To study the effect of nanoparticle charge in the targeting of antimicrobial proteins to gram-positive bacteria

Sample	Mean diameter of particles* (nm)	Area per charge group*	Surface charge density (groups/cm^2)	No. of functional groups per particle
Aliphatic amine particles **(+vely charged)**	20	65Å^2/NH_2	15.38*10^{12}	1930
R-CH_2Cl particles **(-vely charged)**	20	4848 Å^2/R-CH_2Cl 2194 Å^2/R-S0$_3^-$	2.07*10^{12} 4.5*10^{12}	26 56

* **Data supplied by manufacturer**

Overview

+

Polymeric nanoparticle Antimicrobial enzyme, e.g. lysozyme

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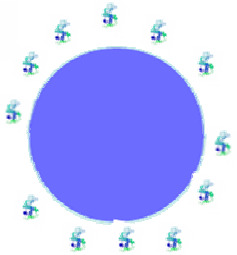
Covalent conjugation to the surface of nanoparticle



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Removal of adsorbed protein using non-ionic surfactants
(E.g Tween 20)

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Purification

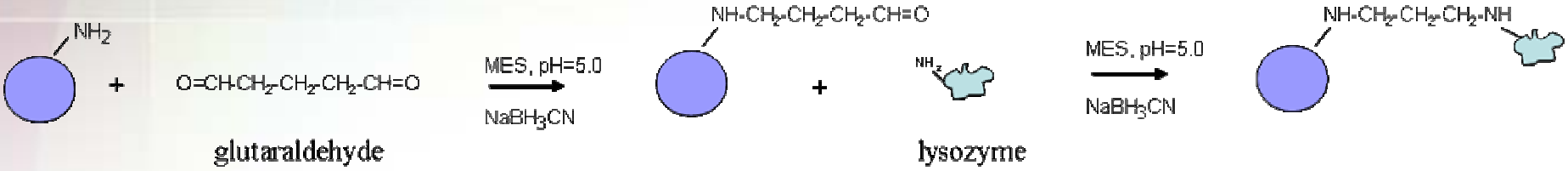
↙ ↓ ↘

Binding efficacy study Zeta potential analysis, AFM study, Live/dead cell imaging

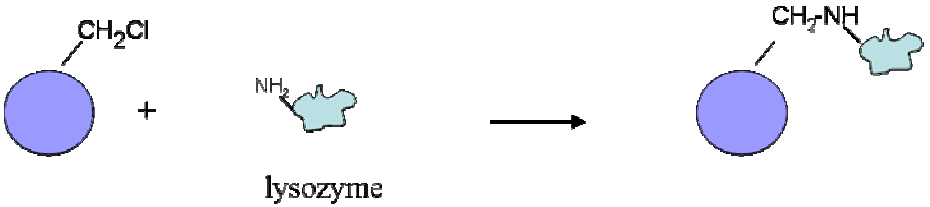
Antibacterial assay activity against free enzyme

Schematic – Protein conjugation to nanoparticles

Conjugation to aminated nanoparticles

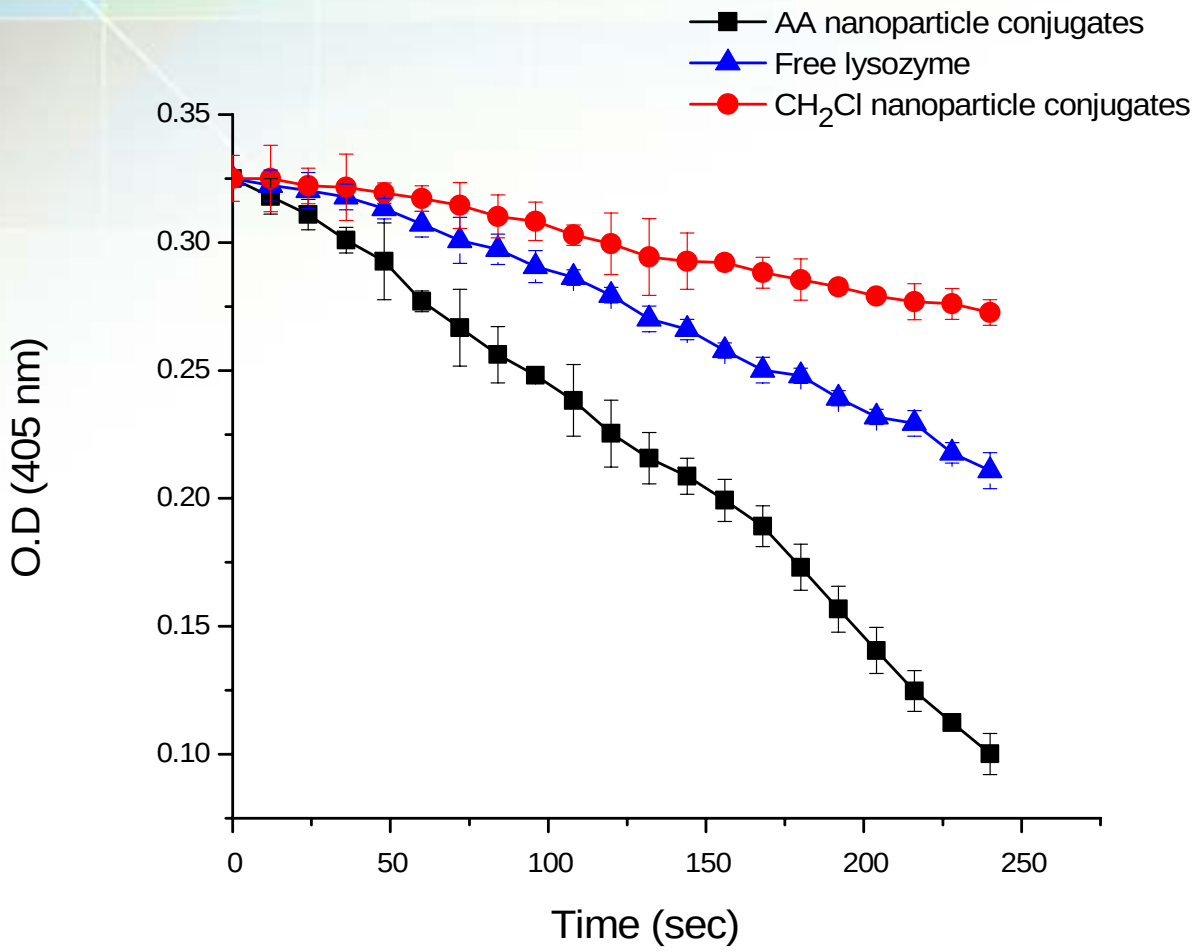


Conjugation to chloromethylated nanoparticles



Satishkumar R and Vertegel A – Biotechnology and Bioengineering, March 2008 (in press)

Rate of enzymatic activity - Bacterial lysis assay



- Bacterial cell substrate – Gram-positive; Micrococcus lysodeikticus

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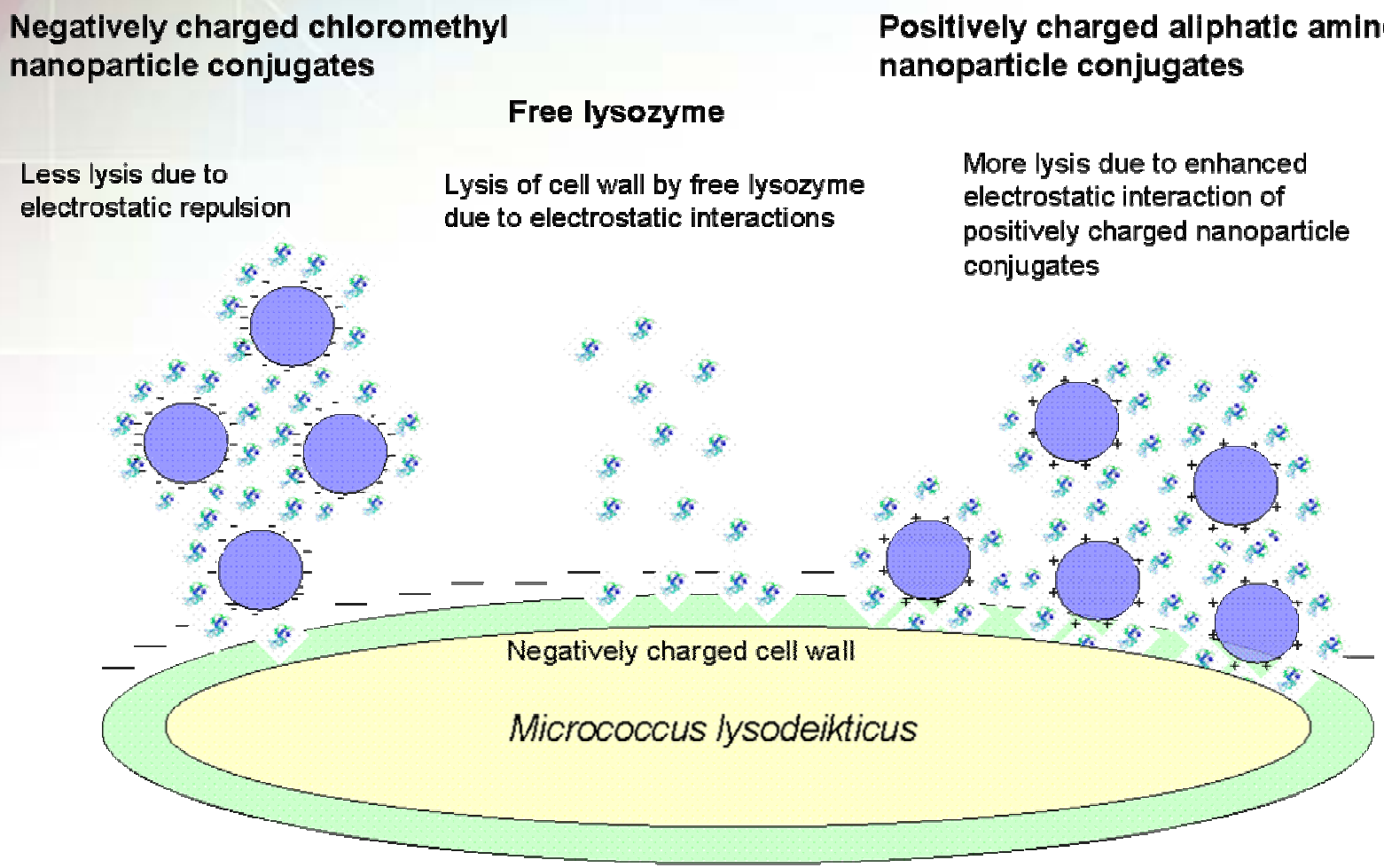
Effect of nanoparticle charge on bacteriolytic activity



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Zeta potential analysis

Sample	Zeta potential (mV)
Bacterial substrate	- 27.8 ± 1.1
Lysozyme conjugated to positively charged nanoparticles	+ 31.5 ± 1.7
Lysozyme conjugated to negatively charged nanoparticles	- 32.0 ± 1.6

- Correlation between charge and bacteriolytic activity
- Targeting better for positively charged nanoparticles

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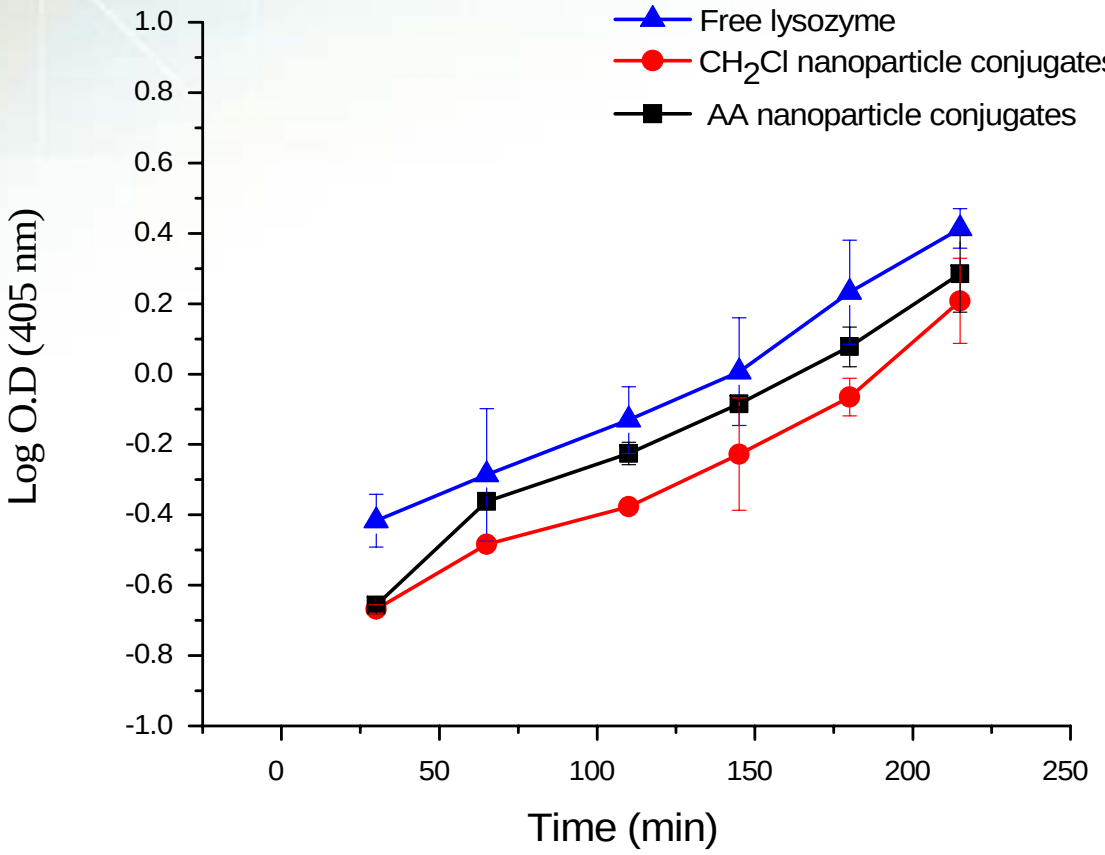
Activity assay with low molecular weight substrate



- PNP-$(GlcNAc)_5$ is a chromogenic pentachiteoside that serves as an alternative substrate for lysozyme

Satishkumar R and Vertegel A – Biotechnology and Bioengineering, March 2008 (in press)

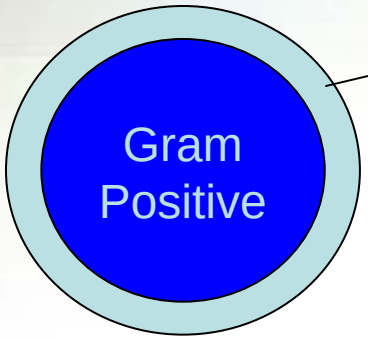
Conclusions

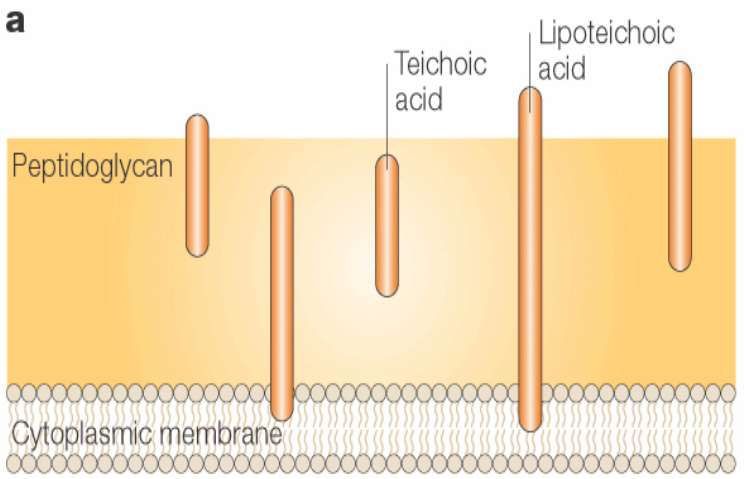
- Charge-directed targeting
- Higher antibacterial efficiency than free enzyme against a Gram-positive bacterium, Micrococcus lysodeikticus for positively charged protein-nanoparticle conjugates.

Study 2 : To test effectiveness of antibacterial activity of protein-nanoparticle conjugates against Gram-negative bacteria

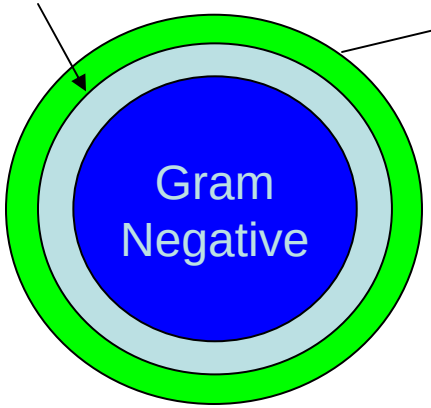
E.g. of Gram negative bacterium; *Escherichia coli, Salmonella, Enterobacteriaceae, Pseudomonas aeruginosa*

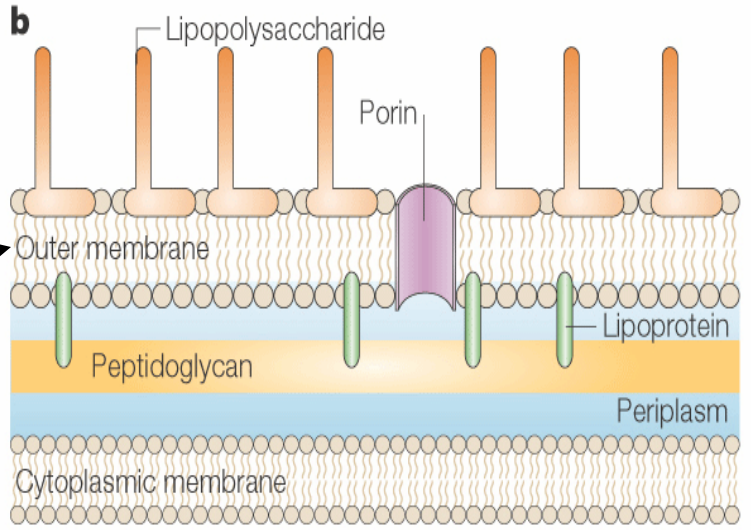
Bacterial cell wall





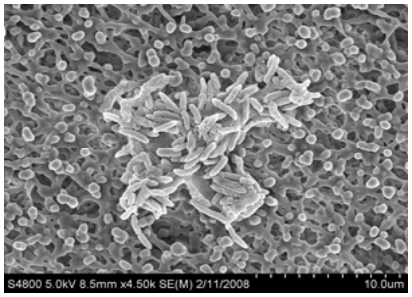
Outer
Membrane



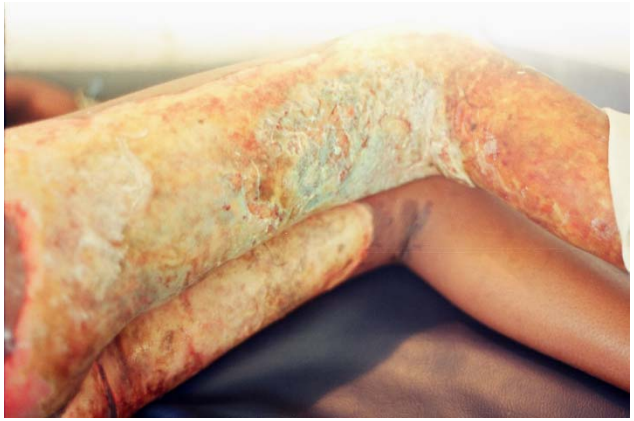


Pseudomonas aeruginosa

- Gram-negative bacterium
- Opportunistic pathogen
- Multi-drug resistant
- Low permeability of cell wall
- Biofilms



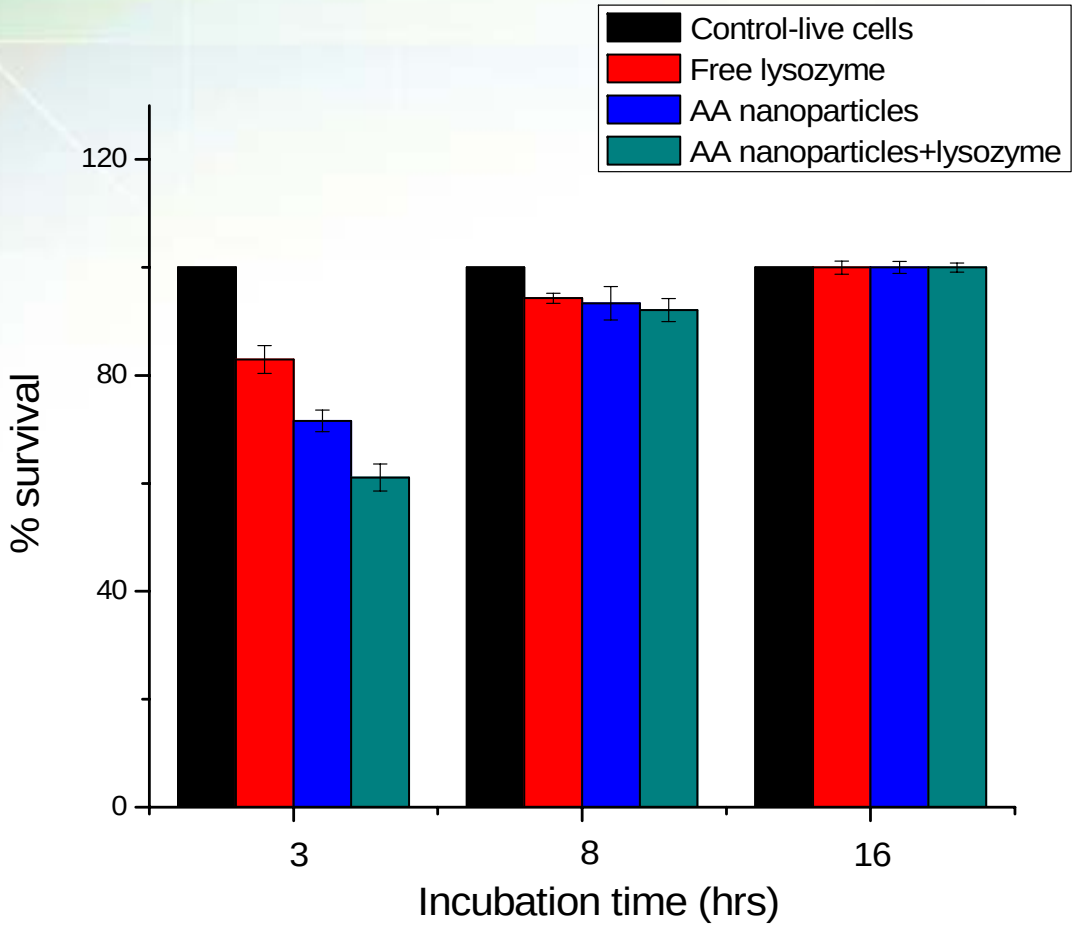
50 percent death in Immunocompromised patients



Materials and methods

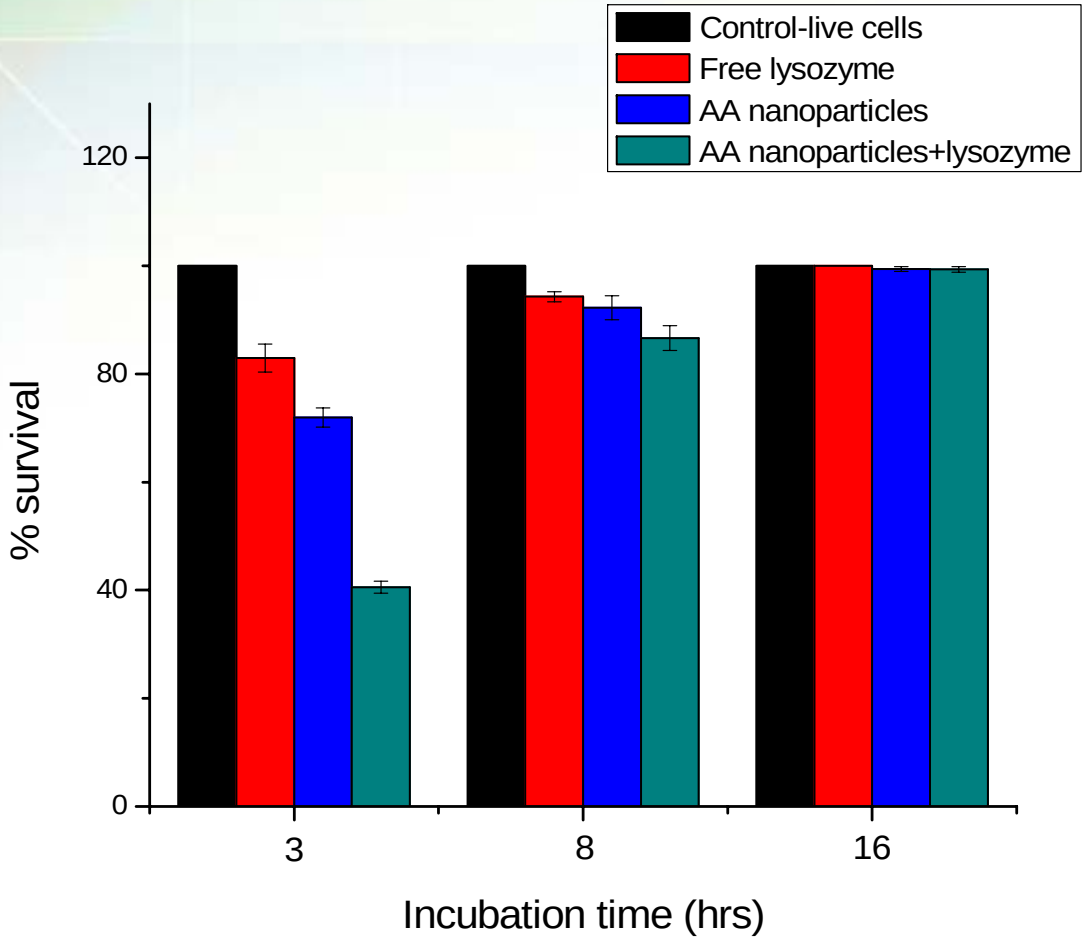
- Pseudomonas aeruginosa (ATCC® 10145) was prepared in nutrient broth and samples were grown to a mid-log phase
- Cell cultures were then centrifuged at 12000 x g and resuspended in 10mM potassium phosphate buffer.
- Cells were incubated with sample conjugates at 37 C with gentle shaking
- Aliquots of 100ul was taken at different time points (after 3, 8, 16 hrs) and then grown on agar in order to determine the number of colony forming units (CFU)

Antimicrobial assay – CFU method



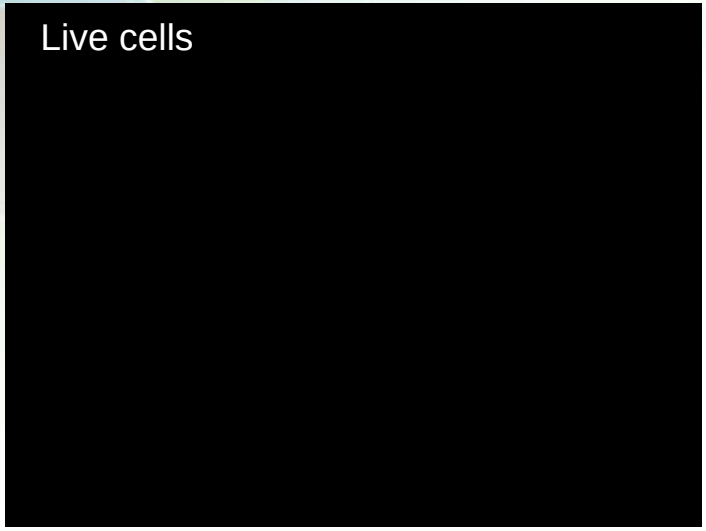
- Covalent coupling using Glutaraldehyde coupling
- Bacteriostatic
- Toxicity concern

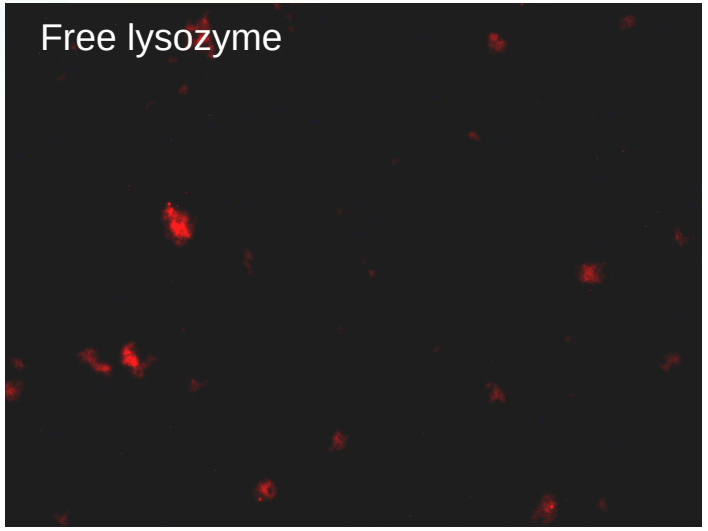
Antimicrobial assay – CFU method

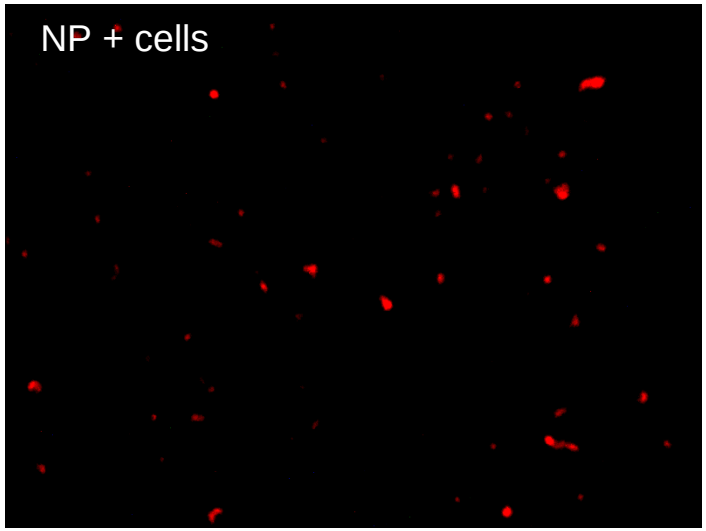


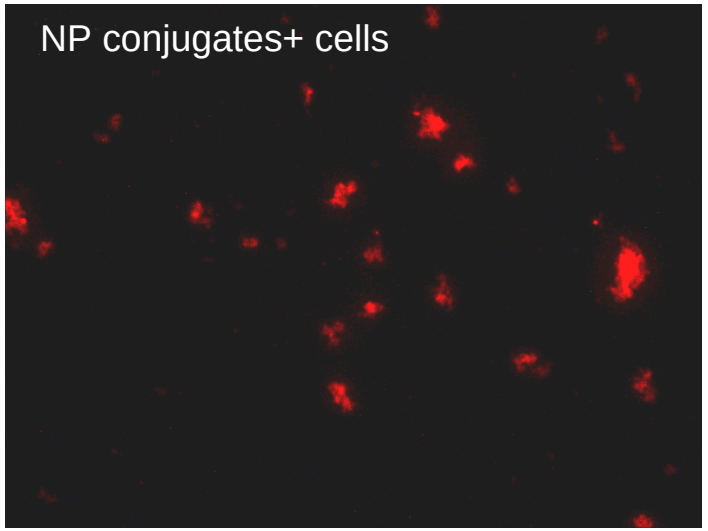
- Covalent coupling by EDC cross-linking
- Bacteriostatic
- Toxicity concern

Live/dead cell assay – 3 hrs post-treatment









Results and Discussion

- Synthesis of conjugates – purification and toxicity concerns
- Time dependent activity - Bacteriostatic
- Possible loss of activity (HEWL) against Gram-negative bacteria after covalent conjugation
- Delivery issues - Outer membrane (LPS)
- Increase dose - MIC

Conclusions

- Antibacterial activity of protein-nanoparticle conjugates was not significantly better than control nanoparticles over time
- Reduced charge-directed targeting against Gram-negative bacteria

Future work

- Reduce toxicity due to synthesis by improved methods of purification
- Alternative means of immobilization using different cross-linkers
- Different antimicrobial protein/peptides – more active against Gram-negative bacteria
- Antibody directed targeting

Acknowledgments

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