Development of antibiotic-encapsulated *Escherichia coli* outer membrane vesicles as novel antibiotic agents to overcome resistance mechanisms

by

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Introduction

Antibiotic resistance poses an immense global threat to human health, contributing to more than 1.27 million fatalities worldwide every year [1]. Antimicrobial resistance can affect people at any life stage and has impacts on healthcare, veterinary medicine, and agricultural industries [1]. The latest Antibiotic Resistance Threats Report in 2019 released by the Centers for Disease Control and Prevention reported more than 2.8 million antibiotic-resistant infections resulting in more than 35,000 deaths in the United States alone [1]. There is a growing number of untreatable infections due to intrinsic and developed methods of resistance to antimicrobial treatments. In 2017, the World Health Organization published a list of bacteria that pose a serious threat to humans and which new antibiotic treatments are needed. This list is largely composed of gram-negative bacterial strains [2]. Gram-negative bacteria are particularly refractory to antibiotics because of their double-lipid outer membrane structure which provides intrinsic resistance to antibiotics that act on intracellular targets [3]. The outer leaflet of the outer membrane is composed of lipopolysaccharides (LPS) which prevents penetration of hydrophobic molecules. The inner leaflet is composed of phospholipids which prevent the penetration of hydrophilic molecules [4]. Therefore, most antibiotics rely on protein channels called porins to enter bacterial cells. Bacteria have developed mechanisms of resistance by down-regulating or altering these porins or by affecting the barrier properties of the outer membrane lipid

bilayer itself [5,6]. Bacteria have also evolved to express enzymes that degrade antibiotics, making them ineffective [7].

Recent studies have focused on developing strategies to overcome these intrinsic and developed methods of resistance to improve antibiotic uptake by bacteria. One approach is to increase membrane permeability using membrane permeabilizers or adjuvants that affect membrane fluidity through interactions with LPS [6, 8]. Mechanisms of resistance can also be overcome by controlling the delivery of antibiotics into the bacteria using nano-based drug delivery to bypass the outer membrane by surface interactions [9]. Liposomes are a commonly used nano-based drug delivery vehicle due to their low toxicity, site-targeting, and controlled release of encapsulated contents [10]. However, liposomes have physical and chemical instability that shortens the shelf-life and influences the *in vivo* therapeutic index of the encapsulated drug [11]. Therefore, we propose the use of outer membrane vesicles (OMVs) to overcome the limitations of liposomes as a delivery platform.

OMVs are nanosized, spherical proteoliposomes secreted by gram-negative bacteria as part of the normal growth process [12]. The surface of OMVs is similar to that of gram-negative bacteria; a phospholipid bilayer with an outer layer of LPS and outer membrane proteins [12]. Due to the similar membrane structure, OMVs have been shown to fuse with the membrane of gram-negative bacteria and deliver encapsulated therapeutics to target cells [13]. However, it is unknown why OMVs are able to fuse with

target cells when bacterial cells do not readily fuse with each other [14]. OMVs have been characterized as biocompatible and suitable for encapsulating antibiotics for drug delivery [15]. Our goal is to use the intrinsic characteristics of OMVs to develop antimicrobial nanocarriers that can transport drugs across the cell membrane of target bacteria in a porin independent manner.

In the initial part of this project, we studied the encapsulation of antibiotics into the OMV lumen to create antibiotic-encapsulated OMVs (aOMVs). We want to explore the loading, encapsulation efficiency, optimal loading technique, and limitations of loading techniques. *In vivo* passive loading is the most commonly used method in which bacterial cells are incubated with sub-lethal concentrations of antibiotics to enable the loading of antibiotics during OMV production [16]. *In vitro* passive loading has also been used by incubating OMVs with high concentrations of antibiotics [17]. We investigated passive loading techniques as well as active loading techniques such as electroporation and sonication which have been shown to be effective at loading small molecules into membrane vesicles [18, 19]. The goal of the first aim is to systematically evaluate the effectiveness of these techniques to load antibiotics into OMVs for future experiments.

To analyze these techniques, we investigated the loading of fluoroquinolones, a class of broad-spectrum antibiotics that work by inhibiting type II DNA topoisomerases that are required for mRNA synthesis and DNA replication [20]. We tested norfloxacin (NOR), ciprofloxacin (CIP),

and moxifloxacin (MOX) to represent a diverse range of bacterial susceptibility, hydrophobicity, and molecular weight. Our results showed that different loading techniques are effective for different fluoroquinolones. Hydrophobic antibiotics showed more effective encapsulation by *in vivo* passive loading, while hydrophilic antibiotics showed more effective encapsulation by electroporation.

After demonstrating that OMVs can be readily loaded with small-molecule antibiotics, we investigated their effectiveness for antibiotic delivery. The second aim of the project is to determine the ability of aOMVs to cross bacterial membranes in the absence of key outer membrane channels. We also aim to determine the bactericidal capability of aOMVs in treating antibiotic-resistant bacteria. We will study OMVs' ability to promote the delivery of antibiotics to gram-negative bacteria and gram-positive to broaden the availability of drugs. Additionally, we delivered aOMVs to bacteria with a knockdown of the OmpF channel. OmpF is a crucial transport channel for antibiotics. Bacterial resistance against antibiotics includes downregulation of the OmpF porin channel. Seeking an alternative pathway to bypass the downregulation of the OmpF channel and maintain the antibacterial effectiveness of antibiotics is important. We hypothesize that aOMVs will be able to deliver more concentrated antibiotics to target bacteria in a porin-independent manner, therefore treating bacteria more effectively than free antibiotics.

The third aim of this project is to test the stability of aOMVs under

different storage conditions. Antibiotics can be degraded under certain storage conditions and by enzymatic degradation [7]. Resistant bacteria develop antibiotic degrading enzymes that break down antibiotics to make them ineffective. We aim to determine the stability of encapsulated antibiotics in OMVs compared to free antibiotics *in vivo*. We investigated the first part of this experiment where we determined aOMVs compatibility with certain enzyme inhibitors. Imipenem is commonly administered with relebactam which inhibits the beta-lactamase that gram-negative bacteria carry as a resistance mechanism [22]. We wanted to determine aOMVs' compatibility with relebactam for the delivery of imipenem to gram-negative bacteria to determine the efficacy of this treatment.

Methods

Aim 1: Loading of antibiotics into OMVs

1. Preparation of antibiotics

Norfloxacin (NOR), ciprofloxacin (CIP), and moxifloxacin (MOX) were our tested antibiotics for loading to represent a diverse range of bacterial susceptibility, hydrophobicity, and molecular weight. Additionally, these are broad-spectrum antibiotics used to treat gram-positive and gram-negative bacteria. A stock solution was prepared by dissolving 1mg antibiotic per 1mL PBS, with a final concentration of 1mg/mL.

2. Bacterial culture and OMV purification

A starter culture of Escherichia coli JC8031 strain was grown in LB-Lennox broth (Invitrogen Lennox L Broth Base) at 37ºC and 175 RPM for 8 to 12 hours until optical density (OD600) of 0.8 was reached. The culture was then diluted 1:100 with LB-Lennox media in a large flask and then cultured again at 37ºC and 175 RPM. Once the culture reached the late exponential phase of growth, the culture was centrifuged twice at 10,000 x g for 10 minutes at 4ºC. The supernatant was filtered through a 0.45-µm PES membrane filter from ThermoScientific. The filtered supernatant was concentrated using concentrator tubes (MilliporeSigma Amicon Ultra-15 centrifugal concentrator, Ultracel-50 regenerated cellulose membrane, 50 kDa MWCO) centrifuged at 5,000 x g and 4ºC for 10 minutes. The supernatant was then ultracentrifuged at 30,000 RPM using a Beckman Coulter Optima XPN 90 Ultracentrifuge, with a SW 32 Ti swinging bucket rotor at 4 °C for 2 hours. The resulting pellet was resuspended and washed twice with PBS. The resuspended OMVs were centrifuged at 5,000 x g at 4 °C for 5 minutes, or until no debris was observed. The OMVs were then filtered through a 0.45-µm syringe membrane filter (ThermoScientific, PES membrane). The purified OMVs were stored at -20 °C.

3. Characterization of OMVs

The purified OMVs' lipid content was determined by incubating 50 uL OMVs with 2 uL FMTM 4-64 dye (ThermoFisher Scientific) for 15 seconds in

a dark environment. The fluorescence was measured using an excitation wavelength of 515 nm and an emission wavelength of 640 nm on a PTI QuantaMaster Fluorometer. The protein concentration was determined by measuring the absorbance at a wavelength of 280 nm (A280) on a NanoDrop Spectrometer (ThermoFisher Scientific).

4. *In vivo* passive incubation

A 10 mL starter culture was prepared with LB Lennox broth and 200 µL glycerol stock of a resistant E. Coli strain previously developed in our lab. Once the starter culture reached the late exponential phase, it was diluted 1:100 with LB-Lennox media, and 0.05 % w/v antibiotics were added. The resistant E. coli grew to the late exponential phase, and then the OMVs were harvested as previously described. The resulting aOMVs were treated with 0.5 M EDTA for 3 h at 37 °C, then separated through an Amicon Ultra 3 kDa MWCO centrifugal filter at 14,000 x g for 30 m at 4 °C. The absorbance of the filtrate was measured.

5. In vitro passive incubation

Equal volumes of purified OMVs and 0.2 % w/v of one of the four antibiotics were incubated for 1 h and 24h at 37 °C.

6. Electroporation of OMVs with antibiotics

OMVs were mixed with electroporation buffer (10% glycerol and 500 mM sucrose, pH 7.0) and 0.2 % w/v of one of the three antibiotics in an 0.2

mm electroporation cuvette (Fisherbrand). A Bio-Rad Gene Pulser XCell was used to perform electroporation on OMVs, following a built-in exponential protocol. The capacitance was set at 50 μF, and the electrical resistance was set at infinite to indicate an open circuit. The antibiotic and OMV mixture were electroporated at 200, 400, or 700 V for 1 or 2 pulses, then incubated at room temperature for 1 h to allow OMV recovery.

7. Sonication of OMVs with antibiotics

Treatment of OMVs with sonication was developed from a published protocol [21]. The OMVs were mixed with the antibiotics and PBS in a 2:1:1 volume ratio. The mixture was sonicated in a bath sonicator (VWR) for 30 seconds at 35kHz, placed in ice for 60 seconds, then sonicated again for 30 seconds at the same setting. The mixture was then incubated at room temperature for 1 hour to allow for OMV recovery.

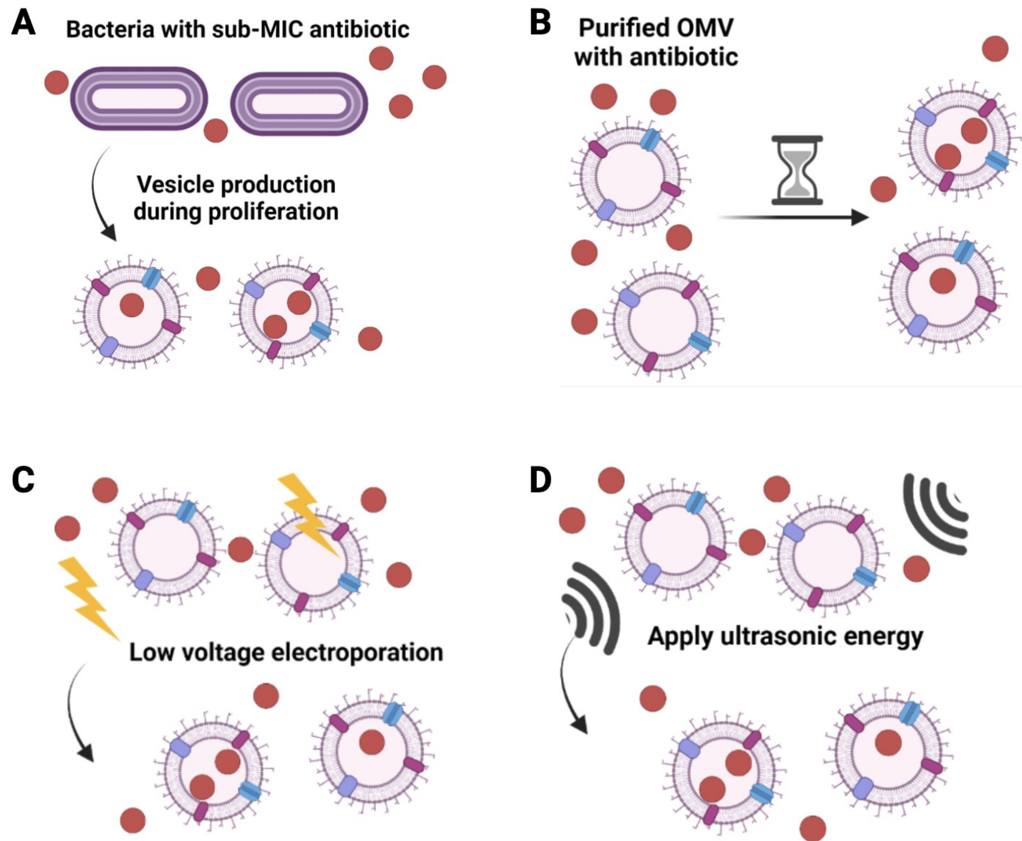


Figure 1: Methods for loading OMVs with antibiotics. A. Loading of antibiotics into aOMVs by *in vivo* passive loading. B. Loading of antibiotics into aOMVs by *in vitro* passive loading. C. Loading of antibiotics into aOMVs by electroporation. D. Loading of antibiotics into aOMVs by sonication.

8. Separation of encapsulated antibiotics

The aOMV mixtures were transferred to a 30-kDA molecular weight cutoff (MWCO) centrifugal filter (MilliporeSigma Amicon Ultra). The tubes were centrifuged at 14,000 x g for 15 minutes at 4ºC. The filtrate containing the encapsulated antibiotics was diluted (40x, 80x, and 160x), and the

absorbance was measured from 250 to 350 nm using a Tecan Infinite® 200 PRO plate reader.

The encapsulation efficiency (EE) was calculated using Eq 1, where aTot was the total mass of the antibiotic added to the OMV mixture, aFree was the mass of the encapsulated antibiotic determined by isolating the OMVs from the mixture and measuring the absorbance of the supernatant using UV-Vis spectrometry from 250 nm to 350 nm and comparing the calibration curves.

$$EE=\left(\frac{a_{Tot}-a_{Free}}{a_{Tot}}\right)*100\%\,(Eq.1)$$

The concentrate containing the aOMVs was filtered with 950 uL PBS through a 0.45-µm syringe filter (ThermoScientific, PES membrane). It was then analyzed by dynamic light scattering using an ALV/CGS-3 compact goniometer system at a wavelength of 632.8 nm and a 90º scattering angle for 180 seconds. The ALV software (ALV-7004) was used to calculate the size distribution.

Aim 2: Delivery of aOMVs to Bacteria

1. Incubation of Bacteria with Antibiotic Treatments

Laboratory strains and clinical isolate strains of both gram-negative and gram-positive bacteria were used. The gram-negative laboratory strain used was Escherichia coli W3110 and the clinical isolates were

Pseudomonas aeruginosa 01, 0238, and 0252. The gram-positive laboratory strain used was Streptococcus mutans NCTC 10449 and the clinical isolates were Staphylococcus aureus 0462 and 0484. Additionally, we used E. Coli BW25113 as a parent strain to analyze results when treating bacteria with the OMPF channel knocked down (E. Coli JW0912). The growing starter culture was diluted with media to reach an OD600 of ~0.2 before use. In a microcentrifuge tube, the diluted bacteria were treated with (1) free antibiotics, (2) aOMVs with the same antibiotic concentration as condition 1, (3) empty OMVs with the same OMV concentration as 2, or (4) empty OMVs and free antibiotics (Figure 1). The free antibiotics served as a control to show how regular antibiotics worked. The empty OMVs served as a control to show that the OMVs themselves did not kill the bacteria. The empty OMVs and free antibiotics served as a control to ensure the antibiotics did not act to multiply the slight inhibitory effect caused by empty OMVs. Antibiotic concentrations of 0.1 μg/mL, 0.5 μg/mL, 1.0 μg/mL, and 1.5 μg/mL were created for each condition, and three replicates were prepared for each condition. 150 uL were transferred from the microcentrifuge tube into a sterile 96-well plate, including negative and positive controls (media only and diluted bacteria) (Figure 2).

2. Measuring Absorbance with Plate Reader

The 96-well plate was placed in a Tecan Infinite® 200 PRO plate reader with a wavelength measurement of 600 nm, a total duration of 16

hours, a kinetic cycle interval of 1 hour, multiple reads per well in a circular 3x3 configuration with a 750 um border, 50 flashes, and no shaking.

3. Data Analysis

Data were analyzed at 12 hours for most gram-negative bacteria. The normalized absorbance was determined and plotted against the concentration of the treatment group.

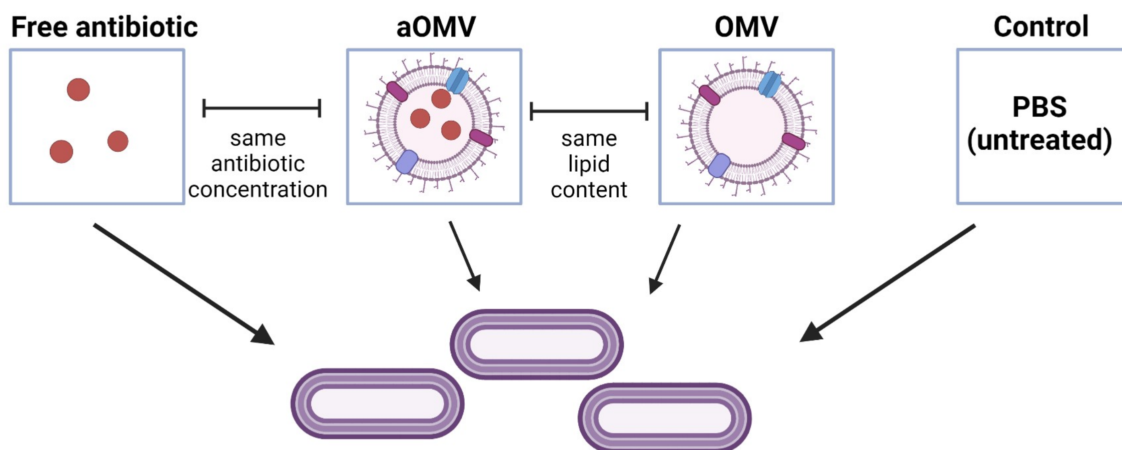


Figure 2: Schematic of experiments run for incubation of bacteria with antibiotic treatments.

Aim 3: Compatibility Assay

To test the compatibility of aOMVs loaded with imipenem with the administration of relebactam, the bacterial lysate of PA 0231 was obtained. This strain carried a metallo-beta-lactamase which was specific for carbapenems and would degrade imipenem. The lysate containing just the enzymes from this bacteria was isolated and combined with free imipenem

or OMV loaded imipenem and relebactam. The mixture was incubated at 37ºC for 3 hours under constant shaking conditions. They were then loaded onto an agar plate spread with E. coli W3110 and allowed to grow overnight. The zone of inhibition was then measured (Figure 3).

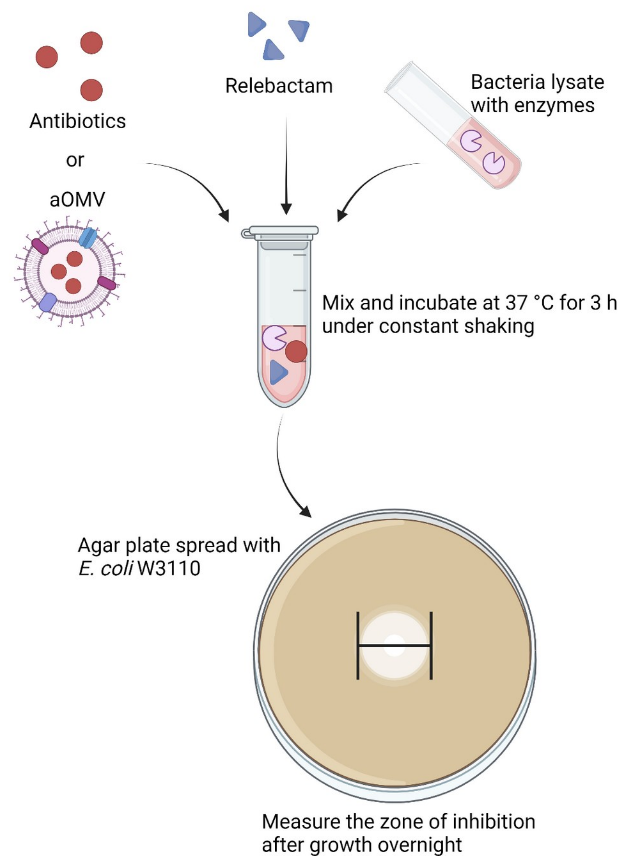


Figure 3: Compatibility assay methods.

Results

Aim 1: Loading of antibiotics into OMVs

The encapsulation of antibiotics into OMVs were separated into 2 methods, passive loading methods and active loading methods. Our passive loading methods proved to be less effective than active loading methods. *In vivo* passive loading showed little promise of loading OMVs, with less than 10% encapsulation efficiency for all antibiotics (Figure 4a). Similarly, *in vitro* passive loading was not effective at loading OMVs, but was more effective at loading than *in vivo* passive loading (Figure 4b). However, the encapsulation efficiency was below 30% for all antibiotics after 1 hour. The encapsulation efficiency of MOX increased after 24 hours, but the longer incubation time negatively impacts the loading of CIP and has no effect on the encapsulation of NOR. Encapsulation is the highest for the loading of MOX overall (Figure 4c). Electroporation was the most effective method of loading for all three antibiotics. The encapsulation was highest for all antibiotics at 400V with 2 pulses, and the loading of MOX was highest at this condition. Sonication loaded NOR and CIP more effectively than both passive incubation methods (Figure 4d). The loading of all antibiotics was below 36%. Overall, electroporation served as the most effective method for loading antibiotics into OMVs.

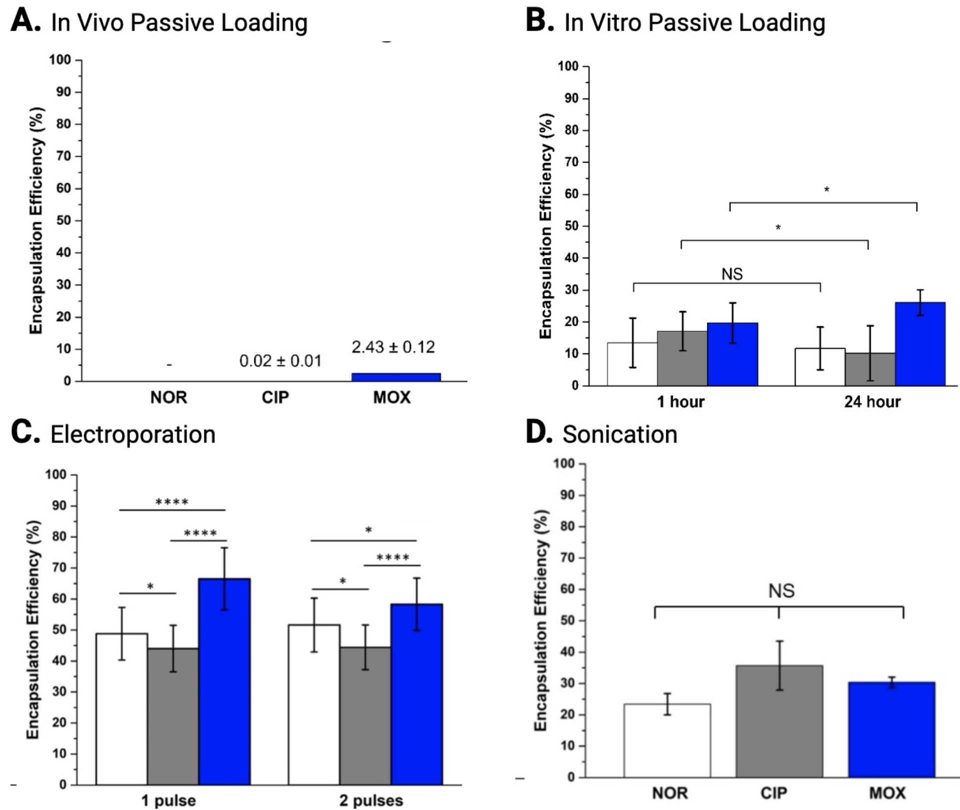


Figure 4: Loading of antibiotics into aOMVs.

Aim 2: Delivery of aOMVs to Bacteria

We tested the effectiveness of the aOMVs in treating laboratory strains and clinical isolate strains of both gram-negative and gram-positive bacteria. We found IMP-OMVs to be effective for both gram-negative and gram-positive bacteria. Starting with our gram-negative strains, for *E. coli* W3110, we found that aOMVs are as effective as free IMP (Figure 5a). For PA01, aOMVs are as effective as free IMP (Figure 5b). This is expected since PA01 is a laboratory strain and does not have a mechanism of resistance. For our clinical isolate strains, PA0252 and PA0238 have resistance mechanisms. For PA0252, at very low and very high

concentrations, IMP-OMVs are as effective as free IMP (Figure 5c). However, at the sub-inhibitory concentration (1.0 μg/mL), IMP-OMVs are significantly more effective than free IMP. For PA0238, IMP-OMVs are more effective than free IMP at all concentrations (Figure 5d).

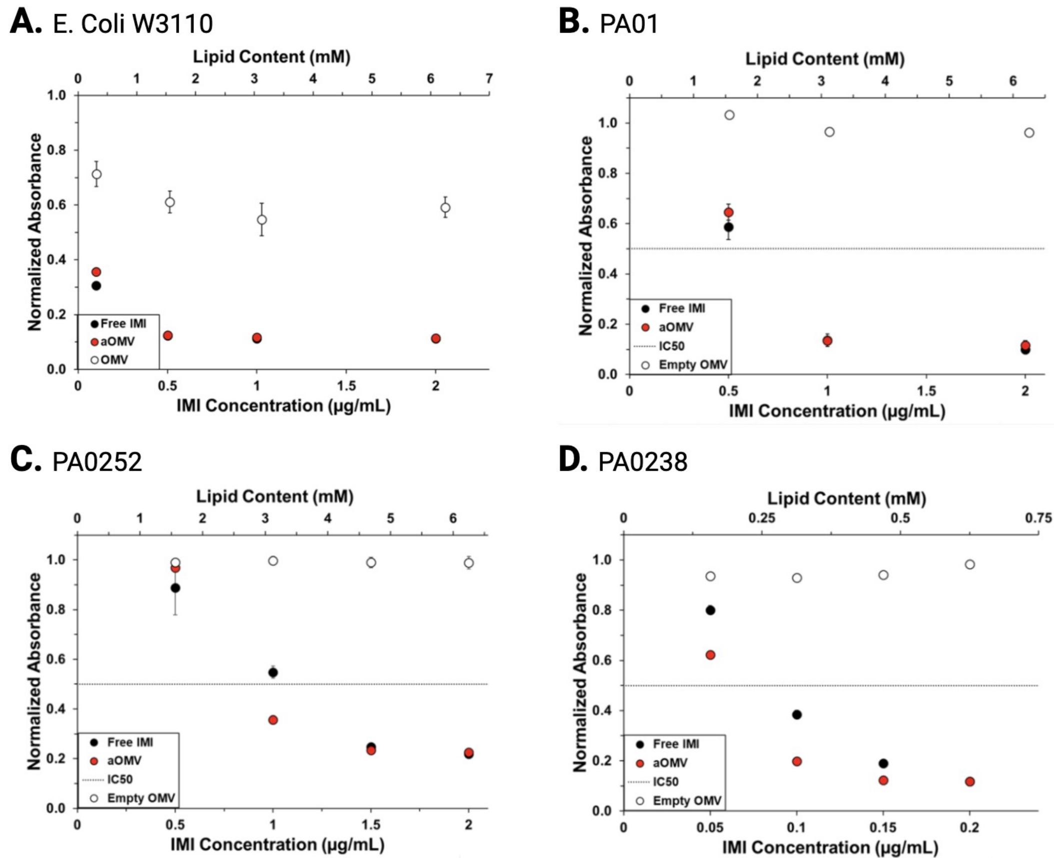


Figure 5: Delivery of aOMVs to gram-negative bacteria. A. *E. coli* W3110. B. *P. aeruginosa* 01. C. *P. aeruginosa* 0252. D. *P. aeruginosa* 0238

We also saw promising results for gram-positive bacteria. For the laboratory strain, *S. mutans*, aOMVs are more effective than free IMP (Figure 6a). For *S. aureus* 0462, aOMVs are more effective than free IMP (Figure 6b). For *S. aureus* 0484, aOMVs are as effective as free IMP (Figure

6c).

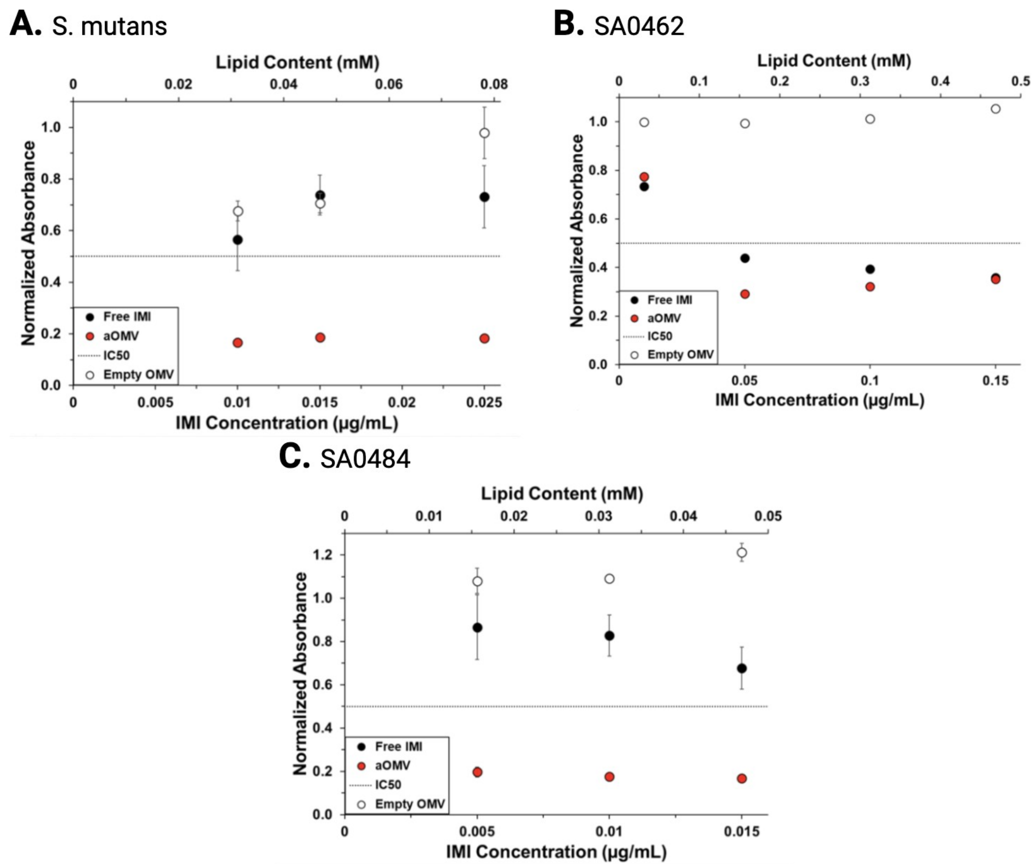
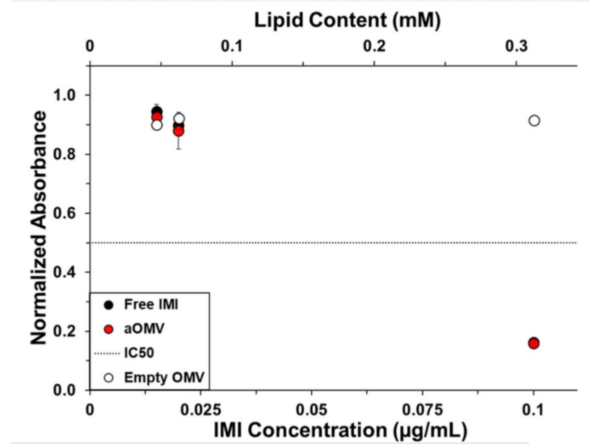


Figure 6: Delivery of aOMVs to gram-positive bacteria. A. *S. mutan*s. B. SA0462. C. SA0484.

In addition to the laboratory and clinical isolate strains of bacteria, we also tested bacteria with knockdowns of the OmpF porin channel. In the parent strain, *E. Coli* JW0912, aOMVs are as effective as free IMP (Figure 7a). In the knockout strain, *E. Coli* BW25113, aOMVs were more effective than free IMP at 0.1µg/mL (Figure 7b).



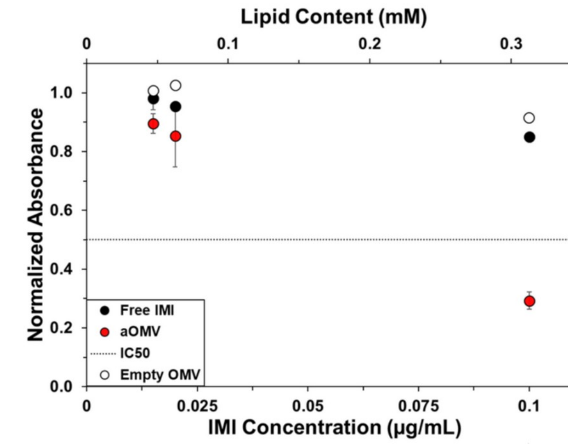


Figure 7: Delivery of aOMVs to porin channel knockouts. A. *E. Coli* JW0912. B. *E. Coli* BW25113.

Aim 3: Compatibility Assay

For the compatibility assay, we measured the zone of inhibition as an assessment for characterizing the efficacy of aOMVs with relebactam compared to free imipenem with relebactam. We found that the presence of inhibitors can potentiate the effect of aOMVs greater than free antibiotics. The zone of inhibition for aOMVs with relebactam was 20 mm where the zone of inhibition for the free antibiotics with relebactam was 17 mm (Figure 8). There was no inhibition for OMVs, bacterial lysate only, or adjuvant only.

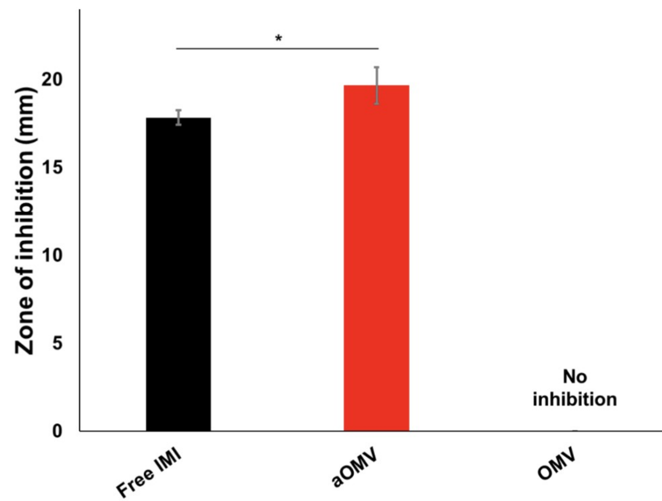


Figure 8: Compatibility Assay results.

Discussion

The findings underscore the potential of aOMVs as a promising strategy to combat antibiotic resistance. The study's multidimensional approach, encompassing loading techniques, bacterial delivery, and compatibility assessment, elucidates crucial facets in developing effective antimicrobial nanocarriers. The successful loading of small antibiotics into OMVs to create aOMVs, particularly through electroporation, highlights a pivotal breakthrough in overcoming resistance mechanisms. Moreover, the demonstrated efficacy of aOMVs in delivering antibiotics to both gram-negative and gram-positive bacteria, including those with OmpF channel knockdowns, signifies their versatility and potential in addressing diverse

resistance mechanisms. They also indicate that the delivery of aOMVs is porin-independent and have a higher bactericidal potency than free antibiotics. Additionally, they show that the inhibitory effect seen by aOMVs is not due to the presence of OMVs, as they do not have an inhibitory effect when administered independently. The compatibility assay further accentuates the superiority of aOMVs over free antibiotics, especially in the presence of resistance mechanisms. It proves that aOMVs can work effectively with adjuvants to enhance their inhibitory effect.

However, several challenges and avenues for future research remain. Further optimization of loading techniques and comprehensive assessment of aOMVs' safety and efficacy *in vivo* are imperative. Additionally, elucidating the mechanistic insights behind aOMVs' enhanced efficacy and exploring their potential in synergistic therapeutic approaches merit exploration. In conclusion, the study lays a solid foundation for harnessing OMVs as antimicrobial nanocarriers, offering a promising avenue in combating antibiotic resistance. Continued research in this domain holds the potential to revolutionize antimicrobial therapy and mitigate the looming threat of antibiotic resistance.

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