ANTIMICROBIALS AND ANTIMICROBIAL RESISTANCE: TWO STUDIES INVESTIGATING DIFFERENT WAYS TO TACKLE THE ISSUE

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LIST OF ABBREVIATIONS

AMP	Adenosine Monophosphate
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
CFU	Colony Forming Units
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide
ESBL	Extended Spectrum Beta-lactamases
FA	Fatty Acid
FBS	Fetal Bovine Serum
FFA	Free Fatty Acid
iNTS	Invasive Non-Typhoidal Salmonella
L-PG	Lysyl-phosphotidylglycerol
LA	LB Agar
LB	Lysogeny Broth
LDH	Lactate Dehydrogenase
LLA	Linoleic Acid
LPS	Lipopolysaccharide
MBC	Minimum Bactericidal Concentration
MDR	Multidrug Resistant
MG	Monoglyceride
MRSA	Methicillin-resistant Staphylococcus aureus
NP	Nanoparticle

OD	Optical Density
OMV	Outer Membrane Vesicle
PBP	Penicillin Binding Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGA	Poly(glycerol-adipate)
RND	Resistance Nodulation Division
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SOC	Super Optimal Broth with Catabolite Repression
SS1	Sydney Strain 1
TLR4	Toll-like Receptor 4
WHO	World Health Organisation
WT	Wild-type

Chapter 1

An Introduction to Antimicrobials and Antimicrobial Resistance

1.1 The History of Antimicrobials

The discovery of antimicrobials has been revolutionary for modern medicine, with the earliest antimicrobial discovery credited to Paul Ehrlich in 1909. He discovered a cure for syphilis that was marketed under the name Salvarsan. This drug was widely popular and for decades was the most frequently prescribed drug until the introduction of penicillin (1). Alexander Fleming's discovery of penicillin in 1928 is a famous story (2), however the antimicrobial effects of the *Penicillium* mould had also been recorded in the 19th century (3). A purification protocol that would be feasible for mass-production of penicillin wasn't elucidated until 1940 which allowed for large clinical trials and the eventual availability of penicillin to the open market (3-5). Penicillin was deemed a 'miracle drug' and marked the beginning of the antibiotic 'Golden-era' that lasted until the 1960s. Most of the drugs discovered during this era were found by screening soil derived actinomycetes (6).

Crystallisation of penicillin led to the discovery of the beta-lactam ring it contains, giving rise to the name of this class of antibiotic as the beta-lactams (Figure 1a) (3). The next class of antibiotic to be discovered was the aminoglycosides, such as streptomycin, in 1943, quickly followed by the tetracyclines in 1944. A new class of antibiotic was discovered every few years until the 60s when the last broad-spectrum antibiotic compound was discovered. Lipopeptides, such as daptomycin, were discovered in the 1980s however they only show efficacy towards gram positive bacteria (6). A summary of the discovery of some key antibiotic classes is listed in Table 1. With the introduction of each new antibiotic class, resistance was normally reported within a few years. Increasing emergence of resistance mechanisms and the stagnation of antimicrobial drug discovery has led to the World Health Organisation (WHO) categorising antimicrobial resistance as a global threat (7).

Table 1. A Summary of the discovery and mechanism of action of the key antibiotic classes. Adapted from Lewis K. (2013) (6).

Class (example)	Year of discovery	Year of Introduction	Year Resistance first reported	Mechanism of action
Beta-lactams (Penicillin)	1928	1938	1945	Inhibits cell wall synthesis
Aminoglycosides (Streptomycin)	1943	1946	1946	Binds 30S ribosomal subunit
Tetracycline (Chlortetracycline)	1944	1952	1950	Binds 30S ribosomal subunit
Chloramphenicols (Chloramphenicol)	1946	1948	1950	Binds 50S ribosomal subunit
Macrolides (Erythromycin)	1948	1951	1955	Binds 50S ribosomal subunit
Glycopeptides (Vancomycin)	1953	1958	1960	Inhibits cell wall synthesis
Rifamycins (Rifampicin)	1957	1958	1962	Binds RNA pol Beta subunit
Quinolones (Ciproflaxin)	1961	1968	1968	Inhibits DNA synthesis
Lipopeptides (Daptomycin)	1986	2003	1987	Depolarises cell membrane

1.2 Mechanisms of Action: Beta-lactams, Aminoglycosides and Lipopeptides

The beta-lactam class of antibiotics, including notably penicillin, amoxicillin and carbenicillin, target Penicillin Binding Proteins (PBPs) which are enzymes involved in synthesising the bacteria cell wall. PBPs functions include glycosyltransferase activity which polymerises glycan strands or cross-linking these strands via transpeptidase activity. Some PBPs perform both functions while others are only involved in cross-linking. The transpeptidase active site on the PBPs is the primary target of beta-lactam antibiotics and undergoes covalent modifications (8). These modifications lead to a reduction in cell wall stability due to an imbalance between cell wall synthases and hydrolases. This results in cell lysis (9, 10).

Aminoglycosides are broad spectrum antibiotics that are characterised by their aminocyclitol ring backbone that is saturated with hydroxyl and amine substitutions. The aminocyclitol ring in most clinically relevant aminoglycosides is streptamine or 2-deoxystreptamine. The one exception is streptomycin which contains a streptadine molecule (Figure 1b). Streptomycin was the first aminoglycoside discovered from *Streptomycese griseus* in the 1940s by Schatz and Bugle (11). Aminoglycosides are strongly cationic and can therefore bind to a number of negatively charged bacterial molecules including DNA, RNA and the lipopolysaccharide (LPS) of bacterial cell walls (12). Aminoglycosides passively bind to the outer membrane of gram negative bacteria and diffuse into the periplasmic space via porins. Transport into the cytoplasm of the bacterial cell requires metabolic energy from an oxygen dependent process. This limits the antibiotics efficacy in anaerobic conditions (12, 13). Once in the cytosol of the bacteria aminoglycosides bind to the 30S ribosomal subunit in another energy dependent process. The proof reading process is compromised due to the binding of the antibiotic and translational accuracy is reduced. The inability to produce correct proteins leads to cell death (14, 15).

Daptomycin, from the lipopeptide class of antibiotics, was discovered in the 1980s and is a natural fermentation product of the soil actinomycete *Streptomyces roseosporus* (16, 17). Eli Lily, who discovered daptomycin, shelved the antibiotic due to safety issues that arose during clinical trials. The licence was then sold to Cubist who developed the drug which got FDA approval for the treatment of serious skin infections in 2003. In 2006 the drug was also approved for treatment of *Staphylococcus aureus* bacteraemia (17). Daptomycin is a cyclic lipopeptide that contains 13 amino acids and a decanoyl side chain (Figure 1c) (18). Daptomycin contains a number of non-standard amino acids. These include three D-amino acids, ornithine, kynurenine and 3-methyl-glutamic acid (19, 20). The lipophilic tail is important in the

function of daptomycin as it can insert into the cell membrane of gram positive bacteria (21).

Early studies to determine the mechanism of action of daptomycin showed that it caused K^+ leakage. This prompted the hypothesis that daptomycin's site of action is at the cell membrane or cell wall (22, 23). Later studies in the 1990s then showed that daptomycin could be found bound to bacterial cell walls and membranes and it was not found in the cytosol, furthering this idea that the site of action is at the cell wall or membrane (24). However, it is still not clear what the initial binding event is between daptomycin and the bacterial cell membrane (19). Daptomycin's action is calcium-dependent. In the absence of calcium, daptomycin forms a defined structure that is not particularly amphiphilic, however in the presence of a 1:1 calcium ratio daptomycin is thought to form micelles (25). It is speculated that the micelles may act as a way of transporting daptomycin in high localised concentrations to the bacterial cell membrane (26). The multimer micelle disassociates once it becomes in contact with the bacterial cell membrane which enables the monomers of daptomycin to insert into the phospholipid bilayer (20, 25). It is then thought that this insertion causes membrane curvature and an oligomerisation step causes a pore to be formed in the membrane leading to K^+ leakage and eventually cell death (25). Early studies suggested that the main action of daptomycin was the inhibition of cell wall macromolecule synthesis (24), however it is argued that this is just a downstream effect of the membrane permeabilisation (20).

Unfortunately, bacteria have developed a wide range of strategies to overcome the actions of these antibiotics. The pharmaceutical industry has reduced their antimicrobial research as antimicrobial drugs are no longer deemed a good investment (27). This stagnation of drug discovery combined with ever increasing resistance rates has led to an uphill battle against infectious diseases.

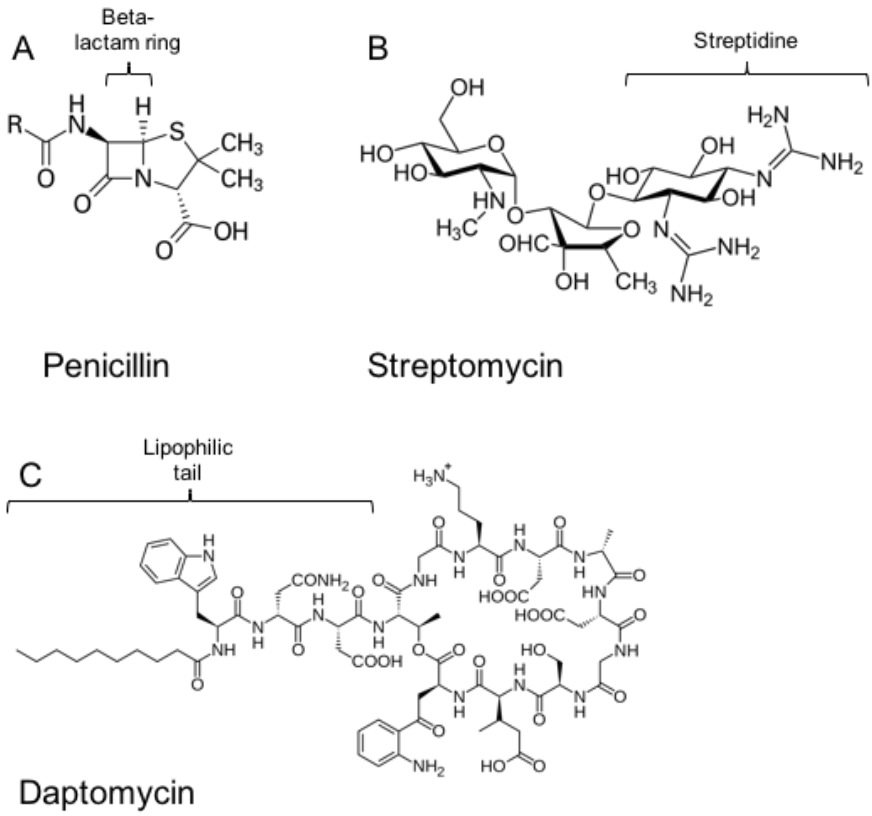


Figure 1. Chemical structures of penicillin (A), streptomycin (B) and daptomycin (C).

1.3 Mechanisms of Antimicrobial Resistance

Antimicrobial resistance is an increasing global threat. Over 63,000 patients die from hospital acquired bacterial infections in the US every year (1). In 2016 a patient in the US died from an Enterobacteriaceae infection that was resistant to all available antimicrobial drugs (28). As well as detriments to health antimicrobial resistance poses a huge economic burden with multidrug resistant bacterial infections estimated to cost over €1.5 billion each year in the EU. This includes healthcare costs as well as loss of productivity (1).

Antimicrobial resistance is not a modern phenomenon as resistance occurs inherently in nature. Evidence of genes encoding various resistance mechanisms against beta-lactams and tetracyclines was

discovered in DNA recovered from 30,000-year-old permafrost sediments (29). This obviously pre-dates the selective pressure that has arisen from the modern clinical use of antibiotics.

Bacteria can gain resistance to antimicrobials through mutations in their chromosomes or by gaining resistance genes via horizontal gene transfer. However, some bacteria can be inherently resistant to certain antimicrobials (30). Inherent resistance can occur when a bacterial species doesn't have the specific target of the antimicrobial. Gram-negative bacteria, for example, are inherently resistant to daptomycin. Upon combining with Ca^{2+} ions, daptomycin becomes positively charged and, compared to Gram-positive bacteria, Gram-negative bacteria have a reduced amount of anionic phospholipid in the cytoplasmic membrane. Thus, the efficiency with which daptomycin inserts into the cytoplasmic membrane is reduced (31).

Lack of intrinsic resistance can be overcome rapidly in a number of ways. There are 3 main strategies that bacteria have developed to become resistant to antimicrobials. They are; reduction of access to the antimicrobial target, modification of the target, and modification to the antimicrobial (32).

1.3.1 Reducing antimicrobial access to their target

Bacteria can reduce their permeability to prevent the drug from entering into the cell. This is seen inherently in Gram-negative bacteria as their outer membrane serves as a permeability barrier to a number of antibiotics. However, some drugs enter the bacterial cell via porins and therefore reduction in the expression of porins can lead to increased resistance (30). Resistance to carbapenem has been reported in Enterobacteriaceae that doesn't produce carbapenemase. These species have mutations that causes a decrease in the production of porins (33). Mutations can also lead to increased specificity of porins, therefore less types of molecules can pass through (30).

Pumping out the antimicrobial before it reaches its target is another key mechanism of resistance. This occurs via efflux pumps. Efflux pumps pump out the antimicrobial molecules at a rate that prevents the intracellular concentration getting too high therefore preventing the drug from working (32). Efflux pumps are found in all bacteria but not all pumps are clinically relevant. Pumps which have the ability to transport a large range of substrates are known as multidrug resistant (MDR) efflux pumps (30). The best characterised MDR pumps are the Resistance Nodulation Division (RND) family which are found in Gram-negative bacteria. For example, AcrB confers multidrug resistance to *Escherichia coli* (34). Efflux pumps can also confer resistance to tetracycline (35). The increase in expression of the MDR pumps is often caused by mutations in genes involved in regulating the expression of the pumps. For example, the mutation could be in a transcription factor or a local repressor (36-38). Unfortunately many MDR pump genes have been mobilised on plasmids meaning the resistance can rapidly spread (30).

1.3.2 Changing the antimicrobial target

By editing the specific target of a particular antimicrobial, you can reduce the affinity with that it reacts. The modification must retain the function whilst diminishing the activity of the drug to be successfully resistant (30). An example of target modification can be seen in *Streptococcus pneumoniae*. The bacteria take up DNA and undergo transformation to form 'mosaic' genes. In *S. pneumoniae* mosaic PBP genes have been reported from recombination from *Streptococcus mitis* DNA. These encode PBPs that have a reduced affinity for penicillin therefore conferring resistance (39).

Another example of target modification occurs in methicillin-resistant *Staphylococcus aureus* (MRSA). The staphylococcal cassette chromosome *mec* (SCCmec) element contains the gene *mecA*. This gene encodes PBPs that are resistant to beta-lactams. In the presence of beta-lactams, bacteria that have acquired the element can still

undertake cell wall synthesis using these insensitive PBPs despite the inhibition of the native PBPs (40, 41).

Daptomycin resistance can occur in *S. aureus* due to a mutation in the *mprF* gene. mprF encodes a multi-peptide resistance factor that is involved in synthesising and transporting lysyl-phosphotidylglycerol (L-PG) to daptomycin's target: the cell membrane (42). L-PG reduces the overall negative charge of the bacterial cell envelope (43). This slight increase in positive charge of the membrane decreases the binding of the positively charged daptomycin-calcium complex (30). This change in membrane charge also repels other cationic peptides such as human defensins (44).

Targets can also be modified via methylation. This has been reported in aminoglycoside resistant isolates of Enterobacteriacae (45). The *armA* gene encodes a methyltransferase that adds a methyl group to the aminoglycoside binding side of the 30S ribosomal subunit. This confers resistance by reducing the affinity between the aminoglycoside and its target site (46).

1.3.3 Modifying the antimicrobial directly

As well as modifying the drug target, bacteria can modify the drug itself rendering it inactive. A key example of this is the hydrolysis of beta-lactam antibiotics via beta-lactamases. Beta-lactamases hydrolyse the beta-lactam ring forming a penicilloic acid product. This product is inactive against PBPs and defunct as an antimicrobial (32). Extended spectrum beta-lactamases (ESBL) can hydrolyse a wide range of substrates posing a real issue in the clinic. Beta-lactamases are secreted into the periplasm of Gram-negative bacteria to inactive the antimicrobial before it comes in contact with its target PBP on the cytoplasmic membrane. One beta-lactamase has the ability to inactivate 1000 penicillin molecules per second (32). ESBL producing bacteria is resistant to all beta-lactams except carbapenems, however

carbapenemases, such as metallo-beta-lactamases, exist. Worryingly a number of bacterial isolates, such as *Klebsiella pneumoniae,* have been reported to contain both ESBL and carbapenemases (47).

In the same way bacteria can add chemical groups, such as methyl groups, to modify the antimicrobial target, bacteria can add certain groups to the antimicrobial directly in order to deactivate it. The deactivation occurs by preventing the antimicrobial from binding to its target (30). This is often seen in resistance to aminoglycosides due to the exposed amide and hydroxyl groups on these antibiotics. The three main enzyme classes involved in this method of resistance to aminoglycosides are; acetyltransferases, which acetylate the antibiotic, adenylyltransferases and phosphoryl transferases which add adenosine monophosphate (AMP) and PO3 groups respectively. These modified aminoglycosides have a reduced affinity for the 30S ribosomal subunit (32). In China, a genomic island has been discovered in isolates of *Campylobacter coli* from chickens that encodes aminoglycoside modifying enzymes from all main classes. This island confers resistance to many aminoglycoside antibiotics including those used clinically to treat *Campylobacter* infections such as gentamycin (48).

1.4 Combating Antimicrobial Resistance

A major step towards combating resistance is encouraging sensible prescribing practices, as well as educating the public on the correct use of antibiotics. For example, not using antibiotics for the common cold and completing the full course of antibiotics prescribed. However, this only slows the progression of resistance, not halt it completely. Attempts at reducing the use of antibiotics in farming has been made with an EU wide ban on the use of all antibiotics as growth promoters in animals (49). It has proven difficult to implement a global strategy at reducing antibiotic use, however, with the US and China still using antimicrobials as growth promoters. Antibiotics are also unregulated in many developing countries and sold as over the counter medications adding to the issue (50).

Another short-term solution is the cycling of antibiotics. This reduces the selection pressure and can give antibiotics a longer life-span before resistance is widespread but this is not a permanent solution (51). Treating patients with a combination of antibiotics is another strategy to reduce the prevalence of resistance. The compounds must have different modes of actions and extensive pharmacodynamics details is needed. This is to ensure that the correct concentration is used and that the two different drugs arrive at their target site enabling the maximum result (50). Clavulonic acid can be used in combination with beta-lactam antibiotics as it inhibits beta-lactamase enzymes. However, resistance to clavulanate has now been reported (52).

There is a need for a long-term solution to the rise of antimicrobial resistance. More people in the US die of MRSA infections than of HIV/AIDS and TB combined (53). At the current rate, we are looking at a return to the pre-antibiotic era which would have major implications to modern medicine.

Two different ways to combat antimicrobial resistance, in two different bacterial species, have been investigated in this thesis.

The first research project aimed to improve antibiotic delivery for more efficient eradication of *H. pylori*. If antibiotic delivery systems can deliver drugs directly to the gastric mucosal niche of the bacteria, rather than working systemically, then lower concentrations of drug would be needed. Additionally, fewer side effects could result from decreased exposure of gut commensal bacteria to the drug. The work entailed developing a novel drug delivery system and incorporating fatty acids with potent antimicrobial activity. Polymer-based nanoparticles were loaded with linoleic acid and assessed for their ability to kill *Helicobacter pylori in vitro*.

The second project was focused on prevention of disease. If the human immune system can efficiently eradicate an infection, then there is no

need for antibiotic treatment. Vaccines have resulted in the complete eradication of diseases such as smallpox, and, in most of the world, polio. In this study, outer membrane vesicles (OMVs) were investigated as a potential vaccine against *Salmonella enteritica* serovar Typhimurium. OMV vaccines have shown promise for bacteria such as *Neisseria meningitides*, with currently ongoing clinical trials. OMVs contain the native bacterial LPS which at small concentrations can be severely toxic in humans. The aim of this study was to reduce the toxicity of *S*. Typhimurium LPS and purify and study the corresponding OMVs.

The fight against antimicrobial resistance is an uphill struggle however it is important that the issue is not swept under the rug. A return to the pre-antibiotic era would be disastrous for modern medicine, therefore all efforts made to combat the issue are important.

Chapter 2

Project 1: Antimicrobial properties of linoleic acid loaded nanoparticles on *Helicobacter pylori*

Antimicrobial properties of linoleic acid loaded nanoparticles on *Helicobacter pylori*

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Abstract

Helicobacter pylori is a leading cause of gastric cancer worldwide. Due to increasing resistance rates, it is becoming more challenging to eradicate *H. pylori* infections. Fatty acids have been extensively studied as potential novel antimicrobial therapeutics, however their insolubility in aqueous buffers leads to poor *in vivo* efficacy. In this study, we have developed a novel nanoparticle drug delivery system with the ability to administer linoleic acid (LLA) to *H. pylori* Sydney Strain 1 (SS1). Our nanoparticles consist of a poly(glycerol-adipate) backbone bearing either butyryl, octanoyl or stearoyl side chains. LLA loaded and unloaded nanoparticles were tested for their antimicrobial activity. Nanoparticles bearing butyryl side chains showed the most promise as unloaded they were capable of eradicating *H. pylori*. There was minimal cytotoxicity to human gastric AGS cells and human intestinal Caco-2 cells at the MBC of the nanoparticles. We have demonstrated a successful system that can deliver insoluble fatty acids to bacteria and improves efficacy when compared to the free fatty acid form.

Introduction

Helicobacter pylori (*H. pylori*) is thought to infect over 50% of the world's population and is a major cause of gastric cancer and peptic ulceration (54). The current treatment regimen includes one proton pump inhibitor drug and two antibiotics, usually amoxicillin or metronidazole and clarithromycin (54, 55). However, in recent years eradication rates have significantly decreased and in most parts of the world have already

reduced to less than 80% (56). Failure of this treatment is mainly associated to a rapid increase of bacterial resistance to standard antibiotics, reaching rates as high as 60-70% to metronidazole (56). Moreover, high localized concentrations of these drugs at the site of infection are difficult to achieve due to degradation in the acidic gastric environment and reduced gastric residence time (57, 58).

In an effort to find novel therapeutics, various fatty acids (FAs) have been investigated for their antimicrobial activity and their safety. Unsaturated FAs, such as Linolenic acid (C18:3) and Linoleic acid (LLA) (C18:2), have been identified as the most potent for a variety of bacteria, including *H. pylori* (59-64). The minimum bactericidal concentration (MBC) required to kill 50% of *H. pylori* was 0.2 and 0.5 mM for Linolenic acid and LLA respectively (63). FAs showed further promise as they created a lower frequency of spontaneous resistance when compared to antibiotics such as metronidazole (62). This is likely to be related to FA's site of action as they act mechanically, increasing permeability of the bacterial cell membrane, rather than at the metabolic stage (65).

The promise of FAs as a potential therapeutic was dampened when they were taken to clinical trials. Duggan et al. reported that there was no significant difference in infection severity between *H. pylori* infected patients who were given either fatty acids or a placebo (66). This is likely due to poor solubility and absorption in the stomach, resulting in a reduced local effect (67). Encapsulation of FAs into nanoparticles, such as liposomes, has emerged as an effective strategy to improve the stability of FAs in the gastric environment and provide controlled localized delivery of the drug in the stomach. Thamphiwatana *et al.* recently reported an increased *in vivo* therapeutic efficacy of linolenic acid when encapsulated in liposomal particles compared to free linolenic acid and standard triple-therapy against *H. pylori* (56, 68, 69). However complete eradication could not be achieved.

This study describes the synthesis of biodegradable nanoparticles (NPs) based of poly(glycerol adipate) (PGA) polymer as potential drug carrier for the localized delivery of LLA against *H. pylori*. Taresco *et al.* demonstrated the enzymatic degradability of PGA and a series of acyl derivatives, bearing butyryl, octanoyl or stearoyl side chains and their respective nanoparticles (C4, C8 and C18 respectively) (Figure 1) (70, 71). In this work we evaluated the potential of these nanoparticles to incorporate LLA and to eradicate *H. pylori in vitro*. The acyl derivatives, due to the presence of the fatty acid side chains, were also evaluated for their potential antimicrobial activity in the absence of LLA.

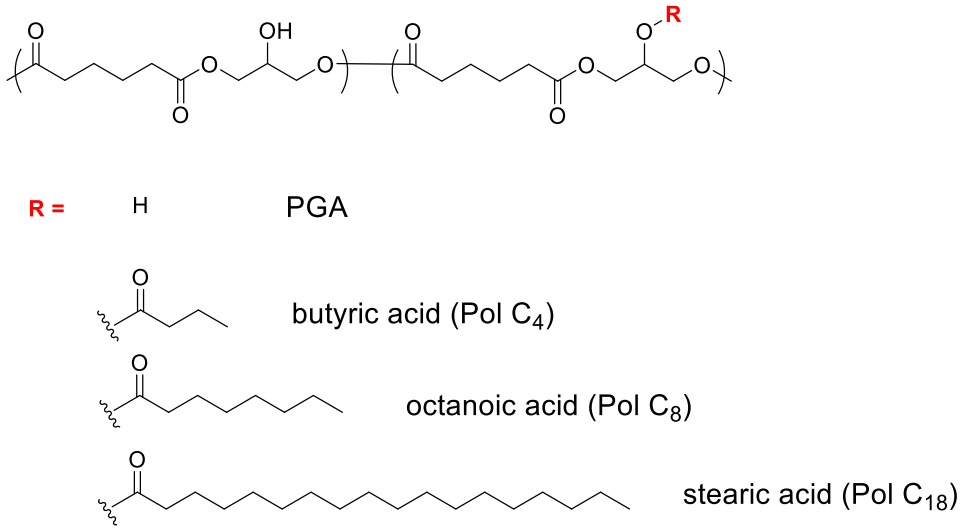


Figure 1. PGA polymer and acyl modifications

Methods

Bacterial culture

H. pylori Sydney strain 1 (SS1) was used throughout this study. *H. pylori* was grown routinely on blood agar base 2 plates supplemented with 5% horse blood (Oxoid) at 37˚C under microaerobic conditions (86% N_2, 6% O_2, 5% CO_2, 3% H_2). For experiments, overnight cultures were produced by inoculating Brain Heart Infusion (BHI) media (Oxoid) supplemented with 5% Foetal Bovine Serum (FBS) (Sigma) (BHI/5% FBS) with fresh

colonies on agar plates. Cultures were incubated at 37˚C with moderate shaking under microaerobic conditions as previous mentioned.

General procedure for nanoparticle synthesis

Nanoparticles were produced using method adapted from Taresco *et al.* (71). PGA, C4, C8 and C18 polymers (25 mg) (kind gift from Dr V Taresco, University Of Nottingham) were dissolved in 1 ml of acetone with and without 15 mg/ml of LLA (Sigma-Aldrich). These mixtures were vortexed and briefly sonicated for 30-45 seconds to aid dissolution. Each solution was added dropwise to 2.5 ml of deionised water (dH_2O) under constant stirring. The solutions were left under constant stirring for 3-4 hours at room temperature to allow the acetone to evaporate. Triton-X 100 (10 µL of 0.1% v/v) (Sigma) was added and allowed to react for 15 minutes at room temperature. The samples were run through a PD-10 column (GE Healthcare) according to manufacturer's instructions using dH_2O as the eluent. The concentration of nanoparticles in each sample was determined by weighting a freeze-dried aliquot of known volume. Hydrodynamic volume and zeta potentials were determined using dynamic light scattering (DLS) and zeta potential on a Zetasizer ZS (Malvern Instruments) in water. All measurements were repeated three times at 25 ˚C.

LLA Quantification

200 µL of NPs were freeze dried and dissolved in 500 µL deuterated methanol (MeOD) (Acros Organics). Benzoic acid (100 µL, 5 mg/mL) (Sigma-Aldrich) was added as the internal standard. Samples were sonicated for 45 seconds to aid dissolution and then analysed via ^1H NMR.

In vitro bactericidal activity of LLA NPs against H. pylori SS1

H. pylori overnight cultures were centrifuged at 3000 rpm for 5 minutes. The pellet was resuspended in 2 mL BHI/5% FBS media and adjusted to give an optical density at 600nm (OD_{600}) of 1.0 which equates to

approximately $1x10^8$ colony forming units (CFU)/ml. 10 µL of bacterial suspension was added to flat bottomed wells of a 96 well plate in triplicate. 20 µL of NP and 30 µL of NP_LLA were added to individual wells in triplicate. Final concentrations of 500 and 75µg/mL for NP and NP_LLA were used respectively. BHI/5% FBS media was added to give a total well volume of 200 µL. A triplicate of wells contained only bacteria and media was added as the negative control and LLA at 150 µg/mL was the positive control. Plates were incubated and shaken at 37˚C under microaerobic conditions for 4 hours and 48 hours. After incubation, 10 µL of 10-fold serial dilutions from 10^{-1} to 10^{-5} of the bacterial suspension were plated onto blood agar phase 2 plates. After 3 days incubation under microaerobic conditions at 37 ˚C colonies were counted to determine the CFU/ml.

Minimum Inhibitory Concentration of NP against H. pylori SS1

C4, C4_LLA and LLA were diluted in BHI/5% FBS media to give concentrations ranging from 0-0.36 mM for C4_LLA and LLA, and 0-3.0 mM for C4. The samples were added in triplicate to wells of 96 well plate containing 10 µL of H. pylori and BHI/5% FBS was added giving a total well volume of 200 µL. Plates were then incubated and shaken for 24 hours at 37˚C under microaerobic conditions. CFU was determined as previously described.

LDH cytotoxicity assays for NPs

To determine the potential *in vivo* toxic effects AGS and Caco-2 cell lines were used, representing gastric and intestinal cells respectively (European Centre for Authenticated Cell Cultures, AGS: 89090402, Caco-2: 86010202 (72)). AGS cells were maintained in F-12 Ham media (Sigma-Aldrich) while Caco-2 cells were maintained in Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich). Each cell line was seeded at $1x10^4$ cells/100µL in a 96 well plate and incubated overnight at 37˚C in 5% CO_2. 10 or 20 µL of NP or NP_LLA was added in triplicate cells giving a range of final concentrations from 0-0.72 mM. 10 µL of sterile water was added in triplicate to cells as the spontaneous LDH

activity control. Plates were incubated for 24 at 37˚C in 5% CO_2. The LDH assay was run according to the manufacturer's instructions (Pierce). Absorbance was read at 490nm using 680nm as a reference. The % cytotoxicity was determined by the following equation:

$$\%\ cytotoxicity = \frac{NP\ treated\ LDH\ activity - background\ LDH\ activity}{maximum\ LDH\ activity - background\ LDH\ activity} \times 100\ .$$

Results

Synthesis and characterisation of NPs

NPs were produced adapting a protocol developed by Taresco *et al.*, via a nanoprecipitation method. DLS analysis showed a narrow particle size distribution with diameter ranging between 175 to 220 nm for the unloaded NPs. Addition of LLA caused an increase in the particle size of the nanoparticles, between 220 and 313 nm, indicating successful incorporation of the drug, with the exception of C18. It is worth noticing that C18_LLA formed a large precipitate upon addition of LLA and was therefore excluded from subsequent experiments (Table 1). Zeta potential analysis of unloaded NPs indicated a negative surface charge for all the NPs, due to the presence of the hydroxyl group in the polymer chains. Incorporation of LLA induced a reduction in the zeta potential for PGA_LLA (-38 to -58 mV) while it remained substantially unaffected in C4, C8 and C18_LLA, thus suggesting the effect of the polymer fatty acid side chains to incorporate LLA in the inner core of the NPs.

Table 1. Hydrodynamic volume and surface charge of NPs

Sample	Size (nm)	Polydispersity Index (PDI)	Zeta potential
PGA	175.5	0.07	-38.0
PGA_LLA	220.3	0.12	-58.3
C4	122.8	0.11	-41.8
C4_LLA	224.8	0.17	-44.6
C8	229.7	0.12	-44.8
C8_LLA	313.2	0.19	-44.1
C18	188.5	0.07	-42.6
C18_LLA*	179.9	0.14	-39.2

PGA_LLA, C4_LLA, C8_LLA, C18_LLA: NP loaded with LLA
*precipitated during synthesis

Quantification of LLA and polymer content

LLA concentration was determined by 1H NMR. The internal standard (Benzoic acid) produced a peak at 7.98 ppm (2CH_{aromatic}) and this was integrated and used as a reference for the diagnostic peak of LLA at 2.78 ppm (CHCH_2CH) (Figure 2). PGA_LLA, C4_LLA and C8_LLA contained similar concentrations of LLA, suggesting similar encapsulation rates (Table 2). Polymer content of unloaded NPs was determined to be 5 mg/mL with the exception of C4 which was 7.5 mg/mL. For experiments C4 was diluted in dH_2O to 5 mg/mL giving all NPs the same starting concentration.

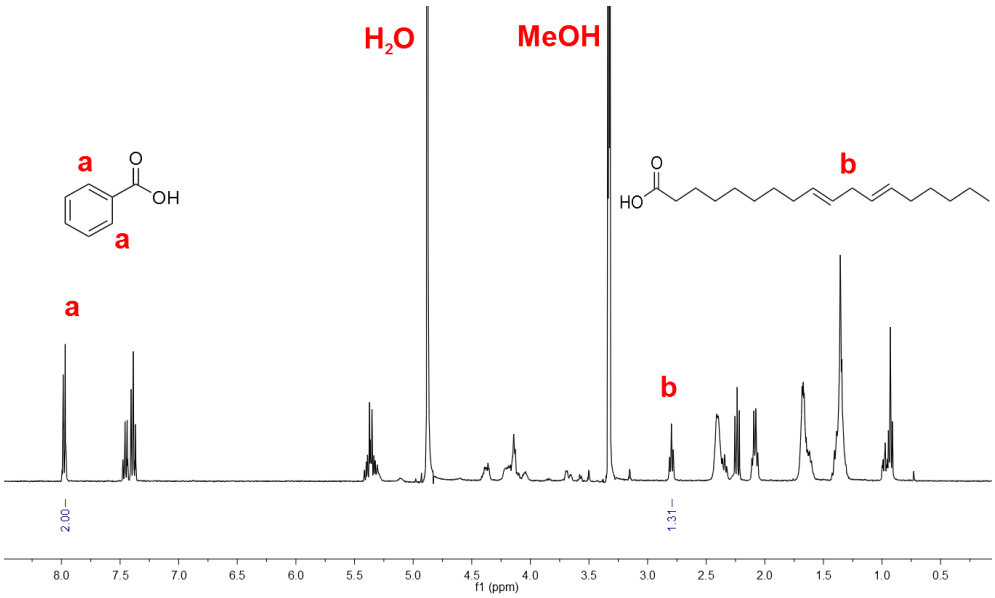


Figure 2. A representative spectrum of a ^{1}H NMR of C4_LLA with assignments of the peaks relative to the internal standard (a) and LLA (b), used for LLA loading quantification.

Table 2. Concentration of NP in solution and LLA loading

	NPs		LLA	
Sample	mg/mL	mM	mg/mL	mM
PGA	5	21.4	n/a	n/a
C4	7.5	22.5	n/a	n/a
C8	5	16.1	n/a	n/a
C18	5	16.1	n/a	n/a
PGA_LLA	n/a	n/a	2.0*	7.2
C4_LLA	n/a	n/a	2.2	7.9
C8_LLA	n/a	n/a	2.0	7.2

*sample had to be further concentrated. All preceding NPs showed similar concentrations

Antimicrobial activity of loaded and unloaded nanoparticles

Antimicrobial efficacy of NPs against *H. pylori* was determined by viable count assay. All LLA NPs induced a reduction in CFUs indicative of antimicrobial activity, however C4_LLA and C8_LLA had more potent effects compared to PGA_LLA. By 4 hours C8_LLA had completely

eradicated the bacteria and C4_LLA had also resulted in this by 48 hours (Figure 3a). This suggested that the acyl modified polymers may have an extra antimicrobial property and further studies showed that this was true. C4 exhibited the most potent antibacterial response by completely eradicating bacteria by 48 hours. C8 and C18 showed a slight reduction in CFU at 48 hours that was significantly less than non-modified PGA NP (Figure 3b).

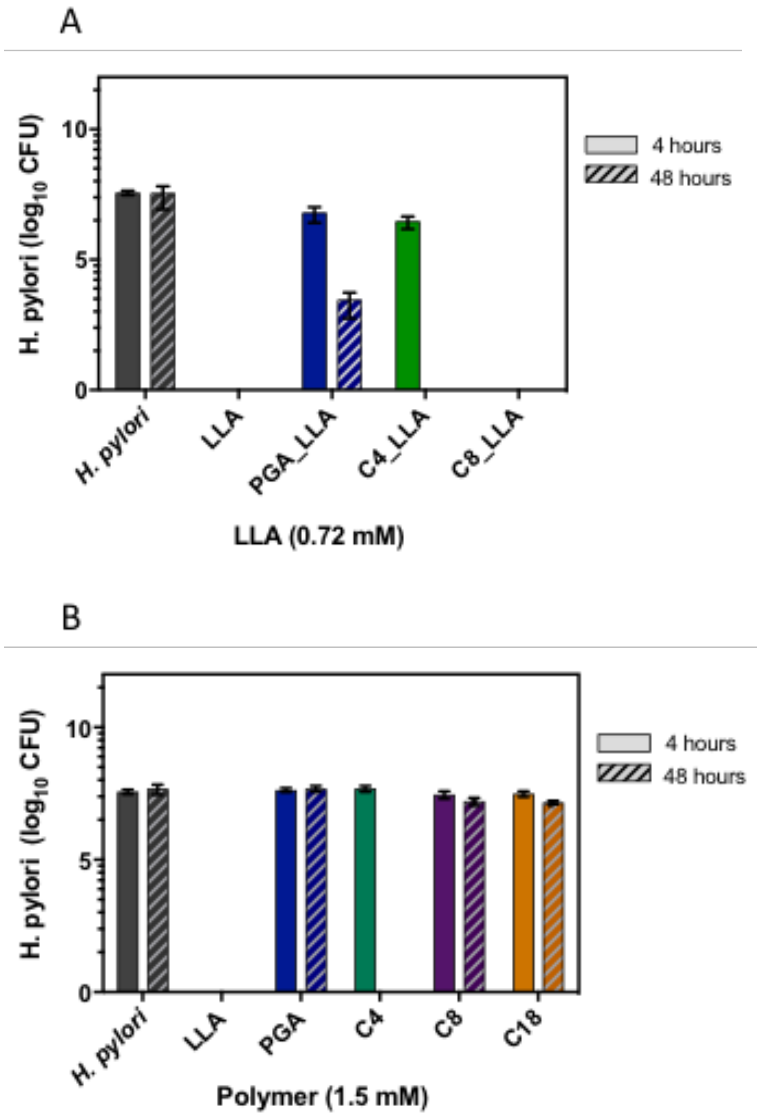


Figure 3. Bactericidal activity of NPs. CFU of *H. pylori* incubated for 4 and 48 hours with LLA loaded NPs (A) or unloaded NPs (B). N=3

Determination of the MBC of C4, C4_LLA and LLA

C4 was selected from previous experiments as the most promising NP, and was used for further evaluation. The MBC was defined as the minimum concentration required to kill 99.9% of the bacteria. This was achieved at a concentration < 0.09 mM for C4_LLA. No colonies were observed at 0.12 mM of C4_LLA (35 µg/mL). The MBC of LLA alone was > 0.18 mM with complete eradication at 0.27 mM (75 µg/mL) (Figure 4a). No MBC could be determined for newly synthesized C4 NPs, with minimal antibacterial activity exhibited at the highest concentration tested. Due to the disparity between this result and our previous results we tested the MBC of older NPs and complete eradication could be achieved at concentrations of <1.9 mM (Figure 4b). This suggests the NPs showed signs of slight degradation as time from synthesis increases (Supplementary S1).

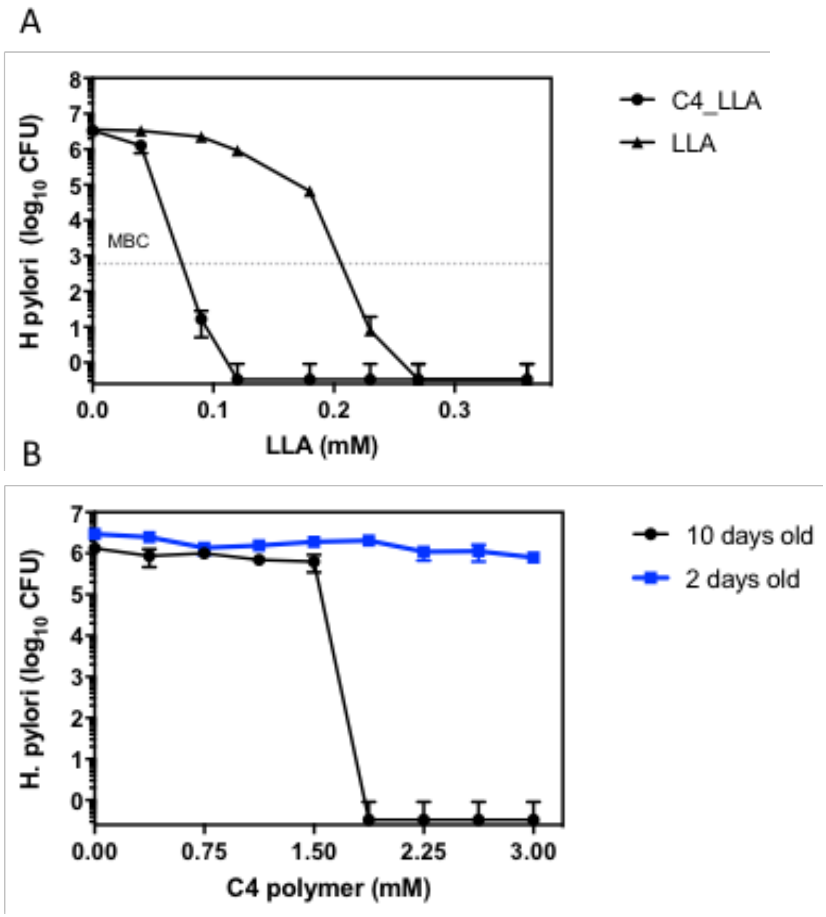


Figure 4. Minimum bactericidal concentration (MBC) of LLA and C4_LLA for H. pylori (A). MBC of 2 day and 10 day old C4 NP for H. pylori (B). MBC was defined as the concentration required to kill 99.9% of H. pylori CFU (4-log decrease). N=3

Cytotoxicity of C4, C4_LLA and LLA on AGS cell line

Cytotoxicity assays were carried out on a human gastric cell line (AGS) to obtain a preliminary determination of the likely effects *in vivo*. C4_LLA was slightly more toxic compared to free LLA. At the highest concentration tested there was 75% cell viability and 83% at the lowest concentration tested (Figure 5a). C4 alone exhibited no cytotoxicity (Figure 5b) which suggested that the action of delivery of LLA that occurred with loaded NPs was causing the slight cytotoxic effects. We also investigated cytotoxicity to an intestinal epithelial cell line (Caco-2) and achieved similar results as with the AGS cells (Supplementary S2).

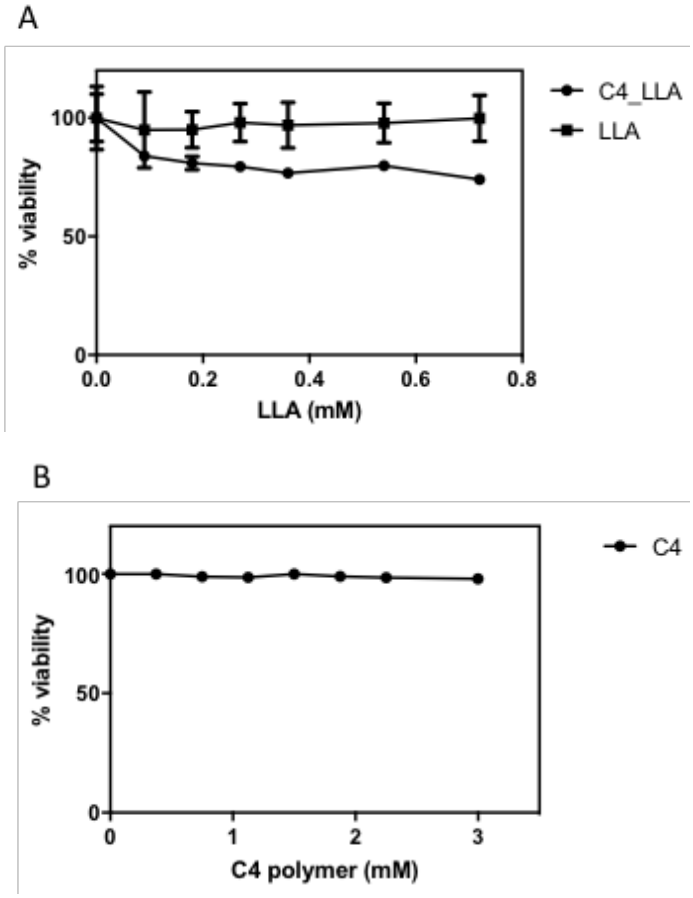


Figure 5. LDH cytotoxicity assay of NPs on AGS cells. The effect of LLA and C4_LLA (A) and C4 NP (B) on percentage AGS cell viability. N=3

Discussion

The therapeutic potential of antimicrobial lipids, namely free fatty acids (FFA) and their monoglyceride forms (MG), has been recognized for decades (59, 60, 62). Many studies have established that the MG form is more effective than the free fatty acid form of the saturated FAs (62). By acting as mild surfactants, these molecules exhibited broad bacteriostatic and bactericidal activity spectrum against a variety of microorganism. This unique mechanism of action allows to maintain high barrier to the development of bacterial resistance, as compared to traditional antibiotics. However, although *in vitro* antimicrobial lipids showed great promise in eradicating *H. pylori*, their effectiveness *in vivo* was limited (66). A major hurdle in the oral delivery of FAs and MGs is their lack of solubility in aqueous buffers which leads to aggregation and poor absorption. To overcome this problem, we developed a drug delivery system which can entrap high amounts of our chosen FA (LLA) and release it in the presence of *H. pylori* by degradation of the polymer, thus increasing the antimicrobial effect.

A series of polymers based on the enzymatically degradable PGA polymer backbone were selected to produce nanoparticles able to incorporate LLA. These polymers were designed to bear acyl pendant chains, mimicking short-, medium- and long- fatty acid chains. It was thought that these chains may enhance LLA entrapment while also exhibiting a synergistic antimicrobial effect.

We successfully synthesised homogenous NPs that ranged in size from 122-229 nm for unloaded NPs and 179-313 nm for LLA loaded NPs. This range is suitable for oral delivery with the aim of penetrating the gastric mucus (73). High loading of up to 2.2 mg/mL was achieved for all polymers, however acyl modifications did not cause an increase in loading. The acyl modifications did however affect the way the drug is entrapped. Further investigation of the effect acyl modifications has on drug loading could use polymers with a range of percentage

modification. This would show whether increasing amounts of acyl modification yields higher drug concentrations. PGA's decrease in zeta potential showed a superficial loading of LLA while the other polymer's entrapped LLA into the core of the NP.

The NPs were then investigated for any antimicrobial activity against H. pylori. C4_LLA and C8_LLA displayed a higher activity than PGA_LLA with C8_LLA eliciting a more rapid response that was equal to free LLA dissolved in 5% DMSO. We have also shown in this study that the non-loaded NPs cause a decrease in CFU after 48 hours with C4 completely eradicating the bacteria. As C8_LLA had the fastest effect on CFU it would have been expected that C8 alone would have the most potent effect out of the non-loaded NPs. It is hypothesized that this could be related to differences in the release profile of LLA between C4_LLA and C8_LLA. These results suggest that C8_LLA may undergo a burst release therefore eliciting a more rapid killing of the bacteria, while C4_LLA undergoes a more controlled release.

The results from our bactericidal assays show minimal change in *H. pylori* CFU between 4 and 48 hours in the negative control. A larger increase in CFU may have been expected. Doing a growth curve of the *H. pylori* SS1 would have clarified this potentially abnormal result. A likely reason for the minimal change is that by 48 hours the bacteria will start to die due to lack of nutrients in the media. If so, then future bactericidal experiments may benefit from determining the CFU at a lower time point, for example, 24 hours.

Due to the results from our bactericidal assays, C4 and C4_LLA were chosen as the most promising NP and were used for further investigation. To get a better understanding of the NP potency an MBC assay was carried out. C4_LLA was shown to be more effective than free LLA. Our findings are lower than reported literature (63, 68), this is likely to be caused by the increased incubation time used in this study.

During initial MBC studies of C4 the MBC was unable to be determined. This was inconsistent with our previous findings which showed complete bacterial eradication at 1.7 mM of C4. The MBC assay was initially carried out on freshly synthesised NPs while the bactericidal assay used 10 day old NPs. The DLS data showed that the NPs remained monodispersed (data not shown), however when the bactericidal activity of NPs at different times since synthesis was compared, there was a clear increase in activity as time from synthesis increases (Supplementary S1). The NPs have previously been shown to be stable for 3-4 weeks and are biodegradable via several enzymes (70, 71). Enzymatic cleavage sites are present at the side chain, inducing release of free fatty acid (butyric acid) or at the polymer backbone, releasing MG-like derivatives of butyric acid. Therefore, as the C4 begins to degrade there is already small amounts of FFA or MG-like butyric acid in solution thus causing a more rapid effect. Hence, the degradation profile must be investigated and considered when carrying out future experiments to prevent further inconsistencies.

LLA is a promising therapeutic as it is the most prevalent polyunsaturated fatty acid in the human diet and is an approved food preservative (74). There have been a number of clinical trials that have evidenced the safety of LLA in humans (75-77). Our cytotoxicity assays displayed low toxicity at the MBC of LLA, C4_LLA and C4. There was a slight increase in cytotoxicity at the higher concentrations tested. Cytotoxicity may be expected however if the LLA is able to interact with the cell membrane, and this was seen at high LLA concentrations in C4_LLA. Free LLA forms macroscopic aggregates when added to the media and therefore may not interact with the cell membrane. C4_LLA releases the LLA in close proximity to the cells and at concentrations below the critical micellar concentration thus increasing the absorption of LLA into the cell membrane and its ability to act as a mild surfactant.

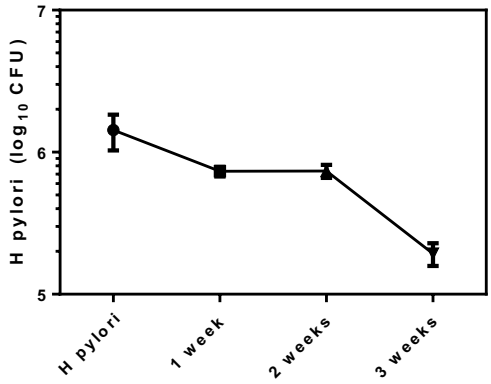
We have produced a novel drug delivery system that enables effective administration of insoluble antimicrobial fatty acids. The delivery system

alone elicits an antimicrobial effect providing a synergistic response. Our novel system improves the efficacy of LLA when compared to the free form and in the future could provide a way to administer other insoluble drugs, such as the antibiotic: clarithromycin.

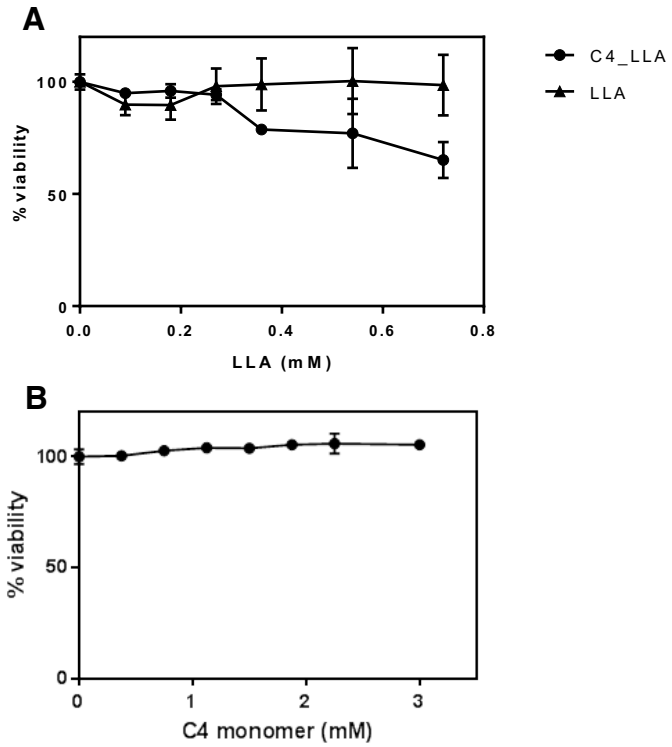
Acknowledgements

The work was financially supported by the Wellcome Trust. I would like to thank Dr K. Robinson and Dr N. Francini for all their help and support. Also, thanks to Dr V. Taresco for supplying the polymers used in this study.

Supplementary Information



Supplementary S1. The CFU of *H. pylori* after 24 hours incubation with C4 at different times since synthesis.



Supplementary S2. LDH cytotoxicity assays on intestinal CaCo-2 cells. The effect of LLA and C4_LLA (A) and C4 (B) on % cell viability.

Chapter 3

Project 2: Production and investigation of outer membrane vesicles as a potential vaccine against invasive non-Typhoidal *Salmonella*

Production and investigation of outer membrane vesicles as a potential vaccine against invasive non-Typhoidal Salmonella

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Abstract

Invasive non-Typhoidal Salmonella disease is caused by *Salmonella enterica* bloodstream infections, predominantly serovars Enteritidis and Typhimurium. It causes the greatest disease burden in low income countries such as sub-Saharan Africa. Outer membrane vesicle (OMV) vaccines are self-adjuvanting with a low-cost production process making them an ideal candidate for this disease. The aim of this study was to produce purified Salmonella Typhimurium OMVs containing detoxified LPS. We successfully produce *S*. Typhimurium OMVs by deletion of the *tolR* gene. With the goal of detoxifying lipid A we attempted to also delete *pagP* and *msbB* genes to yield a fully penta-acetylated lipid A profile. In this study, we created and investigated Δ*tolR*Δ*pagP* strains and OMVs against wild type S. Typhimurium and Δ*tolR* strains and OMVs. We have shown that deletion of Δ*tolR* leads to a loss in long LPS O-chain and that modifying lipid A via *pagP* may change O-chain phenotype. This provides an early step into understanding OMVs as vaccines and putting them into clinical use.

Introduction

Salmonella enterica is responsible for a considerable disease burden worldwide with varying severity between countries. In high-income countries infection normally leads to self-debilitating diarrhoeal disease whilst in low income countries infection can lead to more serious bloodstream infections. There are four main serovars of *S. enterica*;

Typhi, Paratyphi, Typhimurium and Enteritidis. Serovars Typhimurium and Enteritidis cause invasive non-typhoidal Salmonella (iNTS) which has emerged as a predominant bloodstream infection in sub-Saharan Africa (78-81). The estimated incidence of iNTS cases is thought to be approximately 1.4 million globally with a 20-25% mortality rate (78, 82). There is currently no vaccine against iNTS disease therefore there is a push to find a suitable vaccine that will be effective against adults as well as children, as children bear the brunt of the disease burden (79, 83).

A novel vaccine mechanism is the use of outer membrane vesicles (OMVs). OMVs are outer membrane spheres that contain periplasmic content and are found naturally in all gram-negative bacteria studies to date (84). OMVs allow the conformation of proteins and other membrane components to remain intact (79, 85). Protection from *Salmonella* infections requires the innate and the adaptive immune responses (86-88), and previous studies have shown OMV vaccines to activate both these arms of the immune system via activation of toll-like receptor 4 (TLR4) on macrophages and dendritic cells (85, 89). Mice models injected with Salmonella OMVs have been shown to be protected against future infection (85).

OMVs are produced naturally during bacterial cell growth (85, 89). Reduced cross-linking between the outer membrane and the peptidoglycan layer is thought to cause bulging that eventually leads to fission and detachment of the bacterial membrane. What exactly causes this reduction in crosslinking remains unclear (90). Rate of OMV production naturally however is relatively low. A mechanism for increasing the OMV yield has been discovered via deletion of the *tolR* gene (91, 92). This increase in yield allows for a low-cost production process making it an ideal vaccine candidate for lower income countries (92, 93). The *tolR* gene encodes a colicin uptake protein and the mechanism of why it causes ‘hyper-blebbing’ when deleted is unclear (89).

One key issue with *Salmonella* OMVs is the presence of lipopolysaccharide (LPS), which in its wild-type form can be extremely toxic to humans (94). LPS consists of 3 components; lipid A, core polysaccharide and O-linked polysaccharide. Lipid A is a key endotoxin and the leading cause of septic shock in gram negative bacterial infections (94). There has been a focus therefore on reducing the toxicity of lipid A in order to increase safety while maintaining the immunogenicity of OMV vaccines. It has previously been shown that penta-acetylated lipid A, as opposed to the classical hexa-acetylated lipid A, has decreased reactogenicity (93, 95). One study has shown that deletion of *pagP* and *msbB* genes leads to a fully penta-acetylated lipid A profile (93).

The PhoPQ system is a key regulator of lipid A modification and can increase bacterial resistance. PagP, a member of the PhoPQ pathway is a well conserved outer membrane (OM) protein that transfers a palmitate chain from the phospholipid layer to lipid A. This produces hepta-acetylated lipid A which confers greater resistance to stresses (96, 97). Therefore, we would predict that strains with no functioning PagP would remain hexa-acetylated.

MsbB is an enzyme that catalyses the addition of a myristoyl chain to lipid A that forms part of its anchor (94). Deletion of the *msbB* gene leads to growth defects and reduced virulence (94, 98). Studies have also shown that salmonella lacking this myristoyl transferase activity have reduced TLR4 activation (98). Deletion of both *msbB* and *pagP* has been shown to produce penta-acetylated lipid A that has a reduced reactogenicity profile compared to the WT (93, 99).

We describe in this study the attempt to produce a *S. enterica* serovar Typhimurium (from here denoted *S.* Typhimurium) SL1344 mutant with *pagP*, *msbB* and *tolR* genes knocked out. This was with the aim to produce OMVs that contain a detoxified lipid A when compared to single *tolR* knock out which represented the wild-type (WT) OMVs.

Unfortunately, the *msbB* gene was unable to be knocked out after several attempts, therefore further OMV analysis was carried out on WT OMVs and double *tolR pagP* mutant OMVs, as well as their corresponding strains. We predict the double mutant will be more reactogenic than the WT OMVs as the lipid A should be uniformly hexa-acetylated.

Materials and Methods

Culture media and growth conditions.

Bacterial strains were inoculated in lysogeny broth (LB) consisting of 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl. Overnight cultures were grown in 5 mL LB in 50 mL centrifuge tubes. Other liquid cultures were grown in 50 mL LB in 250 mL conical flasks unless otherwise stated. All liquid cultures were shaken at 180 rpm. LB agar (LA) consisted of 1.5% w/v nutrient agar and LB. If needed, 100 μg/mL kanamycin, 100 μg/mL carbenicillin and 100 μg/mL chloramphenicol was added to the media.

Strains and mutant generation.

Salmonella enterica serovar Typhimurium SL1344 was used as the parent strain in this study (Wray et al., 1978). *PagP* and *tolR* genes were knocked out following the lambda red recombinase method previous described (100) (Figure 1). Briefly, kanamycin resistance cassettes were produced via polymerase chain reaction (PCR) using primers with homology regions to flanking regions of *pagP* and *msbB* respectively (Table 1), and using the pkD4 plasmid as a template. The chloramphenicol resistance cassette with flanking homology regions to tolR were amplified from the Salmonella library (101) using primers listed in Table 1. Electrocompetent SL1344 cells were transformed with pKD46, which contains the λ red recombinase genes under the control of an arabinose inducible promoter. This plasmid is also heat sensitive and encodes ampicillin resistance. These strains were transformed with the resistance fragments previously made. *PagP* and *msbB* was

replaced with the kanamycin resistance gene, *aph* (aminoglycoside phosphotransferase), and *tolR* was replaced with the chloramphenicol resistance gene, *cat* (chloramphenicol acetyltransferase). The kanamycin cassette was removed from the *pagP::aph* strain before *msbB* transformation was attempted. The *pagP::aph* strain was transformed with temperature sensitive pCP20 which expresses ampicillin resistance and thermal inducible FLP recombinase which removed the resistance cassette. Transformed cells were recovered in SOC media (Invitrogen). All strains, mutants and corresponding abbreviations are listed in Table 2. To ensure the gene of interest has been replaced, primers of approximately 20 base pair homology flanking the gene were used to amplify the coding sequence, using single transformed colonies plated on the appropriate antibiotic LA as a template. All primers used in this study were provided by Alta Biosciences.

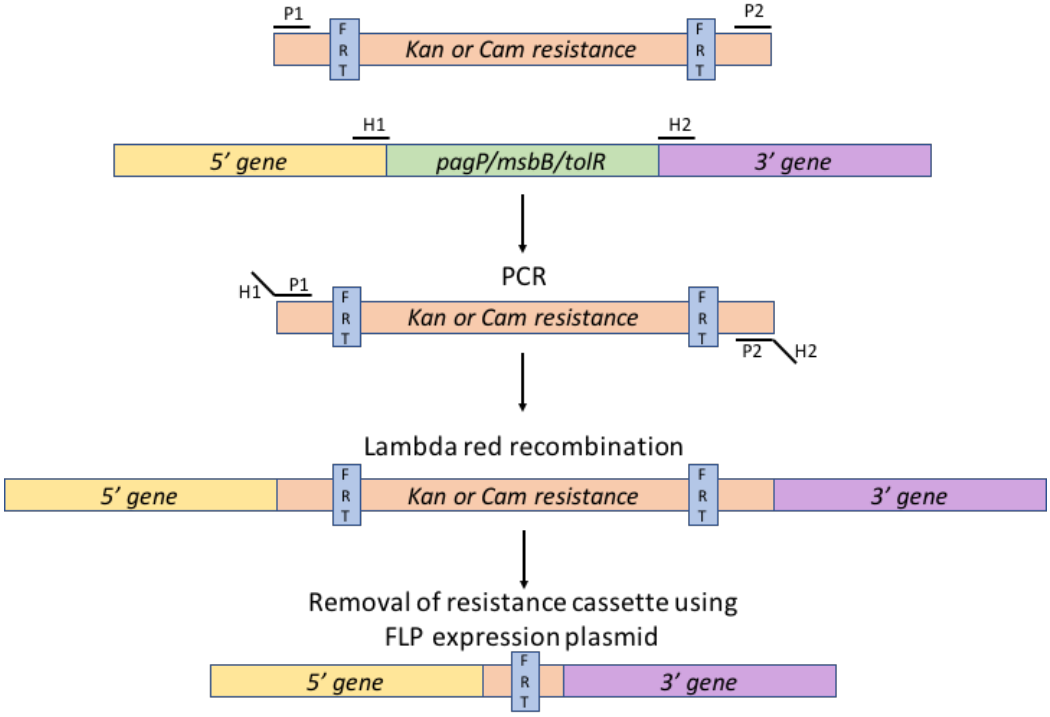


Figure 1. Sequence of steps for λ red recombination. A linear fragment containing the chosen resistance cassette with homology regions upstream (H1) and downstream (H2) of the gene of interest is generated. P1 and P2 represent the priming sites. The competent strain is transformed with the linear fragment and recombines to replace the gene of interest. A FLP machinery flips out the resistance cassette leaving a small scar on the genome. FRT= FLP recognition target, Kan= kanamycin, Cam= chloramphenicol. Adapted from Datsenko and Wanner (2000).

Table 1. Primers used to produce linear resistance fragments

Primer Name	Primer Sequence
tolR forward	ATATCGGTCTGTTTGGCACC
tolR reverse	CAAACGAACTCCAGATCAGC
msbB forward	AGGTAGTACAGGGTTTGTCAGCATAAAGCCTCTCTTACGAGAGGCT TTATTTAGTGTAGGCTGGAGCTGCTTCG
msbB reverse	AGACGTCGCTACACTATTCACAATTCCTTTTCGCGTCAGCAGACCC TCATATGAATATCCTCCTTGA
pagP forward	GGAGCGCGTGACGGTTCTGAGTGCTAAATCAAACGCCGTTAACCC GATGTGTAGGCTGGAGCTGCTTC
pagP reverse	GTACAACAATTGTGATGCATTTTGTCCAGTCGAACTTTGCGAAAAA GTGATAAATATGTAATGAATATCCTCCTTA

Table 2. Strains used and produced and their corresponding abbreviations used in this study

Strain	**Genotype**	**Abbreviations used in this study**
Salmonella SL1344	*Salmonella enterica* subsp. *enterica* serovar Typhimurium	SL1344
SL1344 *pagP::aph*	*Salmonella* Typhimurium SL1344 with pagP disrupted with a kanamycin cassette	*pagP::aph*
SL1344 Δ*pagP*	*Salmonella* Typhimurium SL1344 with a deletion in *pagP*	Δ*pagP*
SL1344 *tolR::cat*	*Salmonella* Typhimurium SL1344 with *tolR* disrupted with a chloramphenicol cassette	Δ*tolR*
SL1344 Δ*pagP tolR::cat*	*Salmonella* Typhimurium SL1344 with a deletion in *pagP* and *tolR* disrupted with a chloramphenicol cassette	Δ*tolR*Δ*pagP*

OMV production and purification

Overnight cultures of Δ*tolR* and Δ*tolR*Δ*pagP* were used to inoculate 300 mL of LB to an OD_{600} of 0.05. Once an OD_{600} of 1 is achieved cultures were centrifuged at 4000 g for 10 minutes at 4°C. The supernatants were passed through a 0.22 μm filter. Filtrates were ultracentrifuged at 45,000 rpm using a 70 TI rotor (Beckman Coulter) at 4°C for 2 hours. The

resulting pellets were resuspended in cold phosphate buffered saline (PBS) and centrifuged for 45 minutes under the same conditions and speed. Pellets were resuspended in approximately 1 mL of PBS.

Quantifying OMVs

OMV amounts are expressed as total protein concentration. To quantify the OMVs a Bradford assay was used. Bovine serum albumin (BSA) was used at known concentrations to provide a standard curve with which to compare to the OMV solutions. Δ*tolR* OMVs gave a yield of 115 μg/mL and Δ*tolR*Δ*pagP* OMVs gave a yield of 135 μg/mL.

Visualising the LPS

500 μL of liquid cultures of SL1344, Δ*tolR*, Δ*pagP* and Δ*tolR*Δ*pagP* at OD_{600} of 2 were centrifuged at 14,000 g for 5 minutes. Pellets were resuspended in 100 μL of 2x laemmli sample buffer (Qiagen) and incubated at 100°C for 4 minutes, followed by 5 minutes at -80°C, then a third incubation step at 100°C for 4 minutes. The samples were centrifuged at 14,000 g for 2 minutes and 80 μL of the supernatant was combined with 5 μL of 5 mg/mL proteinase K (Qiagen) and incubated at 60°C for 1 hour. Proteinase K was deactivated by incubating at 95°C for 5 minutes. SDS-PAGE was used to separate the LPS extract on a Tris-Glycine gel (Invitrogen) and it was visualised by following manufacturer's instructions for silver staining (Invitrogen).

Light transmission platelet aggregometry

Plasma containing platelets from two separate donors were used. 225 μL of platelet rich plasma was mixed with 25 μL of each sample at varying concentrations. The samples tested were Δ*tolR* OMVs and Δ*tolR*Δ*pagP* OMVs at final concentrations of 0.1 ng/mL, 1 ng/mL and 10 ng/mL. The PBS used to dilute the OMVs to the correct concentration was used as the negative control while thrombin activating receptor peptide at 25 mM was the positive control. Platelet free plasma was provided as a blank. Samples are placed in a PAP-8E aggregometer

(Bio/Data) which mixes the samples and measures the change in light transmission through the sample over time. Data is read out as percentage aggregation.

Results

Construction of SL1344 ΔtolRΔpagP strain

The *pagP* gene was successfully replaced by the larger *aph* gene which encodes for the kanamycin cassette. The *tolR* gene was replaced by the larger *cat* gene encoding chloramphenicol resistance. This was confirmed by visualisation of the PCR product on a 1% agarose gel (Figure 2A). Multiple efforts were unable to yield the *msbB* mutant. Insertion fragments containing 50, 70 and 90 base pair flanking homology regions were unable to replace the *msbB* gene with a kanamycin cassette (Figure 2B).

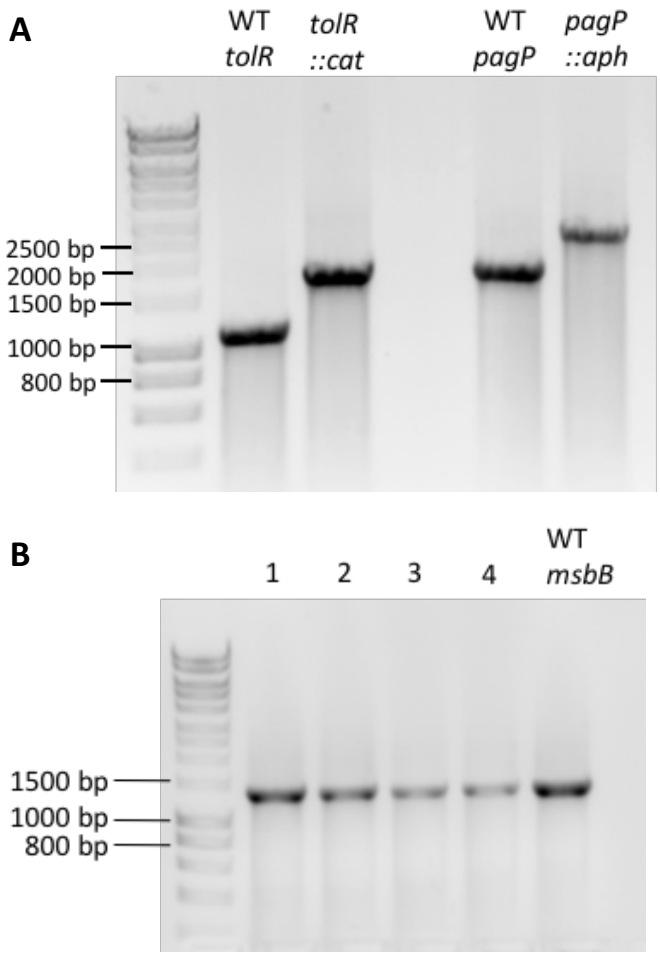


Figure 2. Visual confirmation that the resistance cassettes have inserted correctly. (A) *tolR* and *pagP* successful transformations showing larger chloramphenicol (*cat*) and kanamycin (*aph*) gene insertions respectively. (B) Unsuccessful *msbB* transformations. 1-4 represents different individual kanamycin resistant colonies which do not differ in size from the wild type (WT).

Visualisation of LPS

The LPS of SL1344 (WT), Δ*tolR*, Δ*pagP* and Δ*tolR*Δ*pagP* was successfully visualised via SDS-PAGE and silver staining (Figure 3). Strains lacking the *tolR* gene contain no extra-long chain O-antigen. *PagP* mutants appear to contain less of this extra-long chain O-antigen compared to SL1344 wildtype (Figure 2). The silver stained LPS profile also seems to show larger lipid A bands in mutants that contain PagP. This may be due to the mixed population of acylation states of lipid A, however it may also be due to different loading amounts, therefore no definitive conclusion can be drawn from this observation.

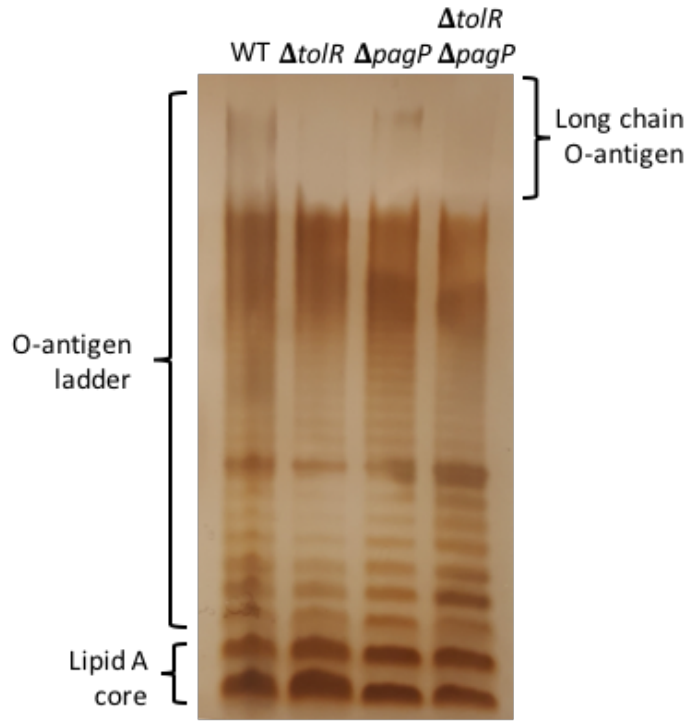


Figure 3. Visualisation of LPS via SDS-PAGE and silver staining on a Tris-Glycine gel. LPS extracted from wild type, *ΔtolR, ΔpagP* and *ΔtolRΔpagP S.* Typhimurium strains.

Platelet Aggregation after exposure to OMVs

The results of the pilot platelet aggregation study did not yield informative results due to the apparent contamination of samples or

tubes in donor 1 (Figure 4). PBS caused approximately 70% platelet aggregation, which is much higher than anticipated. The results are also conflicting as samples which are diluted in the same PBS show lower aggregation amounts than the PBS alone. There also unexpectedly appears to be no correlation between decreasing concentrations of OMV and percentage aggregation. Platelets from donor 2 were mostly unresponsive to OMVs, with only the positive control eliciting substantial aggregation (Supplementary S1). It is not unusual for there to be discrepancies between donors however the results give completely contradictory results. These results also do not allow us to determine whether the OMVs with modified lipid A are more aggregative than the non-modified version or not.

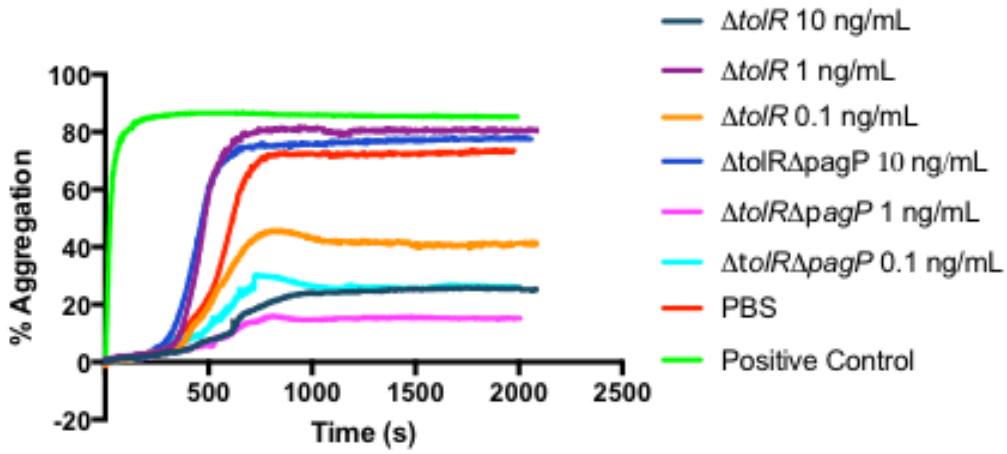


Figure 4. Light transmission platelet aggregometry assay. Platelets from donor 1 exposed to *ΔtolR* wild type OMVs and *ΔtolRΔpagP* OMVs and percentage aggregation recorded over time. PBS was used as a negative control and 25 mM thrombin receptor activating peptide is the positive control.

Discussion

Salmonella diseases cause the greatest burden to countries of a low income. There is therefore a push to create a vaccine that has a simple and low cost production process (83). iNTS is a disease that mainly affects sub-Saharan Africa with children, HIV patients, the malnourished

and other immunocompromised individuals being the worst affected (102). Symptoms of iNTS are non-specific and similar to those of malaria therefore diagnosis can present an economic burden that might not be feasible to these countries (79, 102). OMVs have emerged as a potential solution to low cost vaccines for low income countries. A range of bacterial OMVs have been investigated with a meningococcal OMV vaccine currently in clinical trials (91, 93, 103).

Lipid A at very small doses can be fatal in humans therefore it is of utmost importance to reduce its reactogenicity before administration in humans. This study was based around previous work that demonstrated that lipid A could be detoxified by deletion of *msbB* and *pagP* (93). Despite following that method and using the same primers for fragment production we were unable to knock out *msbB*. Our fragment containing homology regions flanking the *msbB* gene appeared to insert into another area of the genome. Future work may involve sequencing these strains to understand where the fragment is inserting. In this work, a SL1344 strain was used compared to SL1418 used in the methodology we were following. This may explain the discrepancy between our work and the previous study.

As mentioned previously, *msbB* mutants have growth defects in LB and this could be another reason for the inability in this study to successfully delete the gene (104). Transformed cells were recovered in nutrient rich SOC media however they were then plated on LA. Future work could use different media to grow recovered *msbB* mutant strains. It is also worth noting that the *msbB* mutant is not present in the *Salmonella* library (101) suggesting a difficulty in creating a viable mutation in this gene.

Our results have shown that the 'hyper-blebbing' tolR mutants show no long chain O-antigen. This has not previously been described in the literature and shows OMVs may have altered O-chain compared to the WT strain. Due to the blebbing nature of the Δ*tolR* strain, the O-chain

may not have enough time to build to longer lengths. To confirm this hypothesis the experiment could be carried out again but on the OMVs themselves rather than the strains. The Δ*pagP* strain had reduced long chain O-antigen suggesting that modifying lipid A may modify the O-chain phenotype. There appears to be larger lipid A bands in strains that contain a functional PagP which could represent the mixed hepta- and hexa-acetylated lipid A. Although the samples were normalised to the same OD_{600} and therefore number of cells, the LPS loading cannot be guaranteed to be the same after processing and no conclusions can confidently be made about the different acylation steps using this method.

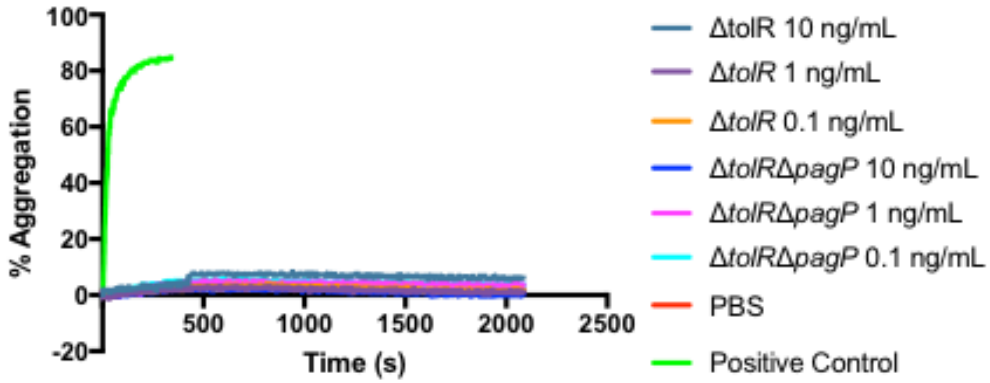
Previous work on OMVs from *Escherichia coli*, *Porphyromonas gingivalis* and *Neisseria meningitidis* strains has shown that they contribute to platelet aggregation (105-107). Purified S. Typhimurium LPS has been shown to be a potent inducer of platelet aggregation (108). We wanted to investigate whether altering lipid A would alter the aggregative effect. Platelet aggregation *in vivo* can cause issues such as microthrombosis and organ dysfunction. This is seen in fulminant meningococcal septicaemia. One study was able to show that meningococcal-derived OMVs can cause the platelet aggregation and activation that can lead to this increased disease severity (106). OMVs could not be administered to humans if the aggregative effect was large enough to cause adverse effects. Our aim at elucidating the aggregative effect of our *S.* Typhimurium OMVs wasn't successful in this initial pilot study. The experiment would need to be repeated with freshly filtered PBS and diluted OMVs. It would also be beneficial to extend the range of concentrations tested. We would predict that the Δ*tolR*Δ*pagP* OMVs would be more aggregative than the Δ*tolR* OMVs, whereas we would expect the Δ*tolR*Δ*pagP*Δ*msbB* OMVs to be less aggregative due to the expected increased and reduced reactogenicity of the lipid A in this mutant respectively.

In summary, we have successfully produced *S*. Typhimurium SL1344 OMVs in both wild type and modified lipid A orientations. We have shown that the method of mass producing OMVs via deletion of the *tolR* gene causes LPS O-chain deviations. Initial future work should focus on deleting the *msbB* gene to get OMVs with reduced endotoxicity as opposed to the currently predicted increased toxicity of the Δ*tolR*Δ*pagP* strain. The work would also benefit from ELISA studies, to confirm our prediction that the Δ*tolR*Δ*pagP* is more toxic, via measuring IL-6 release from cells treated with OMVs. A Limulus Amebocyte assay would also give quantitative results relating to LPS toxicity. More work needs to be carried out in order to elucidate the true potential of OMVs as a vaccine against iNTS.

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Supplementary Data



Supplementary S1. Light transmission platelet aggregometry assay. Platelets from donor 2 exposed to *ΔtolR* wild type OMVs and *ΔtolRΔpagP* OMVs and percentage aggregation recorded over time. PBS was used as a negative control and 25 mM thrombin receptor activating peptide is the positive control.

Chapter 4

Perspectives for the next 3 years

4.1 Overview and Aims of the PhD project

The first project is the study that will be continued for the rest of the PhD programme. The preliminary results were promising and there are lots of viable directions to move the project in.

The 3 main aims of the project will be:

1. To formulate a polymer nanoparticle drug delivery system against *H. pylori*

2. To formulate a liposomal nanoparticle drug delivery system against *H. pylori*

3. To use *in vivo* models to investigate the most promising formulation

4.2 Formulating new drug delivery systems

Formulation of the nanoparticle drug delivery systems will follow two experimental strands. The first will continue investigating the polymer nanoparticles introduced in chapter 2, the second will study liposomes. Liposomes are spherical membranes encasing a hydrophilic core. Previous literature has shown the ability to incorporate the fatty acid, linolenic acid, into the liposomal membrane. These studies demonstrated antimicrobial effects of the liposomes against H. pylori both *in vitro* and *in vivo* (68, 69).

The aim of our nanoparticles is to have a dual functionality via antimicrobial fatty acids and antibiotics. We will incorporate a range of antimicrobials into our polymer and liposomal nanoparticles, such as LLA, linolenic acid, metronidazole, clarithromycin and amoxicillin. In our polymer nanoparticles, the antibiotics will be entrapped similarly to the fatty acids. In the liposomes, however, there is a different mechanism of capture. The fatty acids insert and become part of the liposomal membrane while hydrophilic antibiotics are entrapped into the core of the liposome. The liposomes fuse with the bacterial membrane releasing

the antibiotic core directly onto the bacterium while increasing concentrations of fatty acid leads to membrane instability and cell lysis. As well as changing the drug formulation, using a range of different polymers to form nanoparticles is a viable option, for example, polymers which bind to *H. pylori*, as well as our PGA-backbone polymers.

We will determine the most promising formulation via a number of *in vitro* assays, such as our bactericidal and cytotoxicity assays utilised in chapter 2. The formulation which has a combination of both high antibacterial and low cytotoxic effects will be chosen as the most promising nanoparticle. This nanoparticle will then be coated in the adhesion, LabA. LabA is expressed by H. pylori and binds to MUC5AC gastric mucins (109). If our nanoparticles express this adhesion, then they can bind to the gastric epithelia and therefore be in close proximity to *H. pylori* in their environmental niche. To determine binding efficiency *in vitro*, nanoparticles can be FITC-labelled and incubated with MUC5AC overexpressing AGS cells. The cells are then washed and fluorescence can be measured via a plate reader. The greater the fluorescence, the greater the binding.

4.3 *In vivo* testing of selected nanoparticle formulation

The formulation that performs best in the *in vitro* assays and once coated in LabA adhesions, will be investigated using mice models. Mice will be infected with *H. pylori* and treated with either the standard triple therapy or with the nanoparticle formulation. The triple therapy (as used in humans) is a combination of omeprazole, metronidazole and clarithromycin given orally twice a day (110). Antibacterial efficacy will be quantified by euthanizing the mice after 7 days of treatment and dissecting their stomachs. The stomach tissue will be disrupted and then serially diluted. These dilutions will be plated and CFU counts per gram of stomach tissue will be determined. This allows a comparison between standard therapy and the nanoparticle formulation

We also aim to assess the gastric retention of the nanoparticle formulations. Nanoparticles will be radiolabelled with ^{99m}Tc-HMPAO. This allows for non-invasive tracking via nanoSPECT-CT imaging, as previously demonstrated by Hwang *et al.* (111). This is important to ensure maximal retention at the site of infection and also minimise exposure of intestinal bacteria to the antibiotics. Reduced exposure of intestinal microflora to the antimicrobials should help to reduce the severity of adverse effects.

4.4 Closing Remarks

Antimicrobial resistance is an ever-increasing issue. Serious effort must be made to prevent a return to the pre-antibiotic era. This future project has the potential to yield a novel therapeutic for an infection that treatment for is failing due to resistance. As well as tackling resistance, novel and more effective treatment of *H. pylori* can have economic benefits by reducing the burden of *H. pylori* diseases, such as gastric cancer and peptic ulcers, on the NHS. The issue of resistance is not one that will disappear without a global effort to combat it.

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