Bacterial Lipase Triggers the Release of Antibiotics from Digestible Liquid Crystal Nanoparticles

Chelsea R. Thorn\textsuperscript{1,2,4}, Andrew J. Clulow\textsuperscript{3}, Ben J. Boyd\textsuperscript{3,4}, Clive A. Prestidge\textsuperscript{1,4}\textsuperscript{*} and Nicky Thomas\textsuperscript{1,2,4}

\textsuperscript{1}School of Pharmacy and Medical Science, University of South Australia Cancer Research Institute, North Tce, Adelaide, SA 5000, Australia.

\textsuperscript{2}The Basil Hetzel Institute for Translational Health Research, Woodville, SA 5011

\textsuperscript{3}Drug Delivery Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Pde, Parkville, VIC 3052 Australia.

\textsuperscript{4}ARC Centre for Excellence in Bio-Nano Science and Technology, Australia

* Corresponding authors: Clive Prestidge (clive.prestidge@unisa.edu.au)
Abstract

In the advent of the post-antibiotic era, new strategies are urgently required to improve the efficacy of antimicrobials and outsmart multi-drug resistant bacteria. Exploiting a basic survival mechanism of bacteria, lipase production, monoolein liquid crystal nanoparticles (MO-LCNP) were investigated as a bacterial-triggered drug delivery system for three different antimicrobial compounds and compared with model sn-1/3 regiospecific and non-regiospecific lipases via pH-stat titration, proton nuclear magnetic resonance and in situ synchrotron small-angle X-ray scattering. The release of model hydrophobic (rifampicin) and macromolecular (alginate lyase) antimicrobials were triggered from MO-LCNPs at 82-fold and 7-fold higher rates (respectively) due to bacterial lipase digestion of MO-LCNPs, which could not be stimulated with a small hydrophilic antibiotic (ciprofloxacin HCl) or non-digestible, phytantriol-LCNPs. While sn-1/3 regiospecific lipase rapidly digested MO-LCNPs in a two-phase process, the single-phase digestion kinetics of the non-regiospecific lipase steadily digested the cubic Im3m structure and gave rise to lamellar structures that ultimately stimulated the triggered antibiotic release. Accordingly, MO-LCNPs have an application for localised Pseudomonas aeruginosa and Staphylococcus aureus infections that produce non-regiospecific lipases and for concentration-dependent antibiotics that have macromolecular (MW ~ 30 kDa) or hydrophobic (logP ~ 4) chemistries, as a triggered bolus release would be clinically efficacious for improved bacterial eradication.
**Graphical Abstract**

Bacterial lipase triggers the release of hydrophobic and macromolecular antimicrobials

**Key words:** liquid crystalline nanoparticles, antimicrobials, triggered drug delivery

**Abbreviations:** Antimicrobial resistant (AMR), monoolein (MO), phytantriol (PHY), liquid crystalline nanoparticle (LCNP), ciprofloxacin hydrochloride (CIP), rifampicin (RIF), alginate lyase (AL), synchrotron small angle X-ray scattering (sSAXS), proton- nuclear magnetic resonance (H\(^1\)-NMR), monoglycerides (MG), free fatty acids (FFA).

3
1. Introduction

Antimicrobial resistant (AMR) bacteria or “superbugs” pose one of the biggest threats to global health. Within the next 30 years, the annual mortality in humans is expected to rise to 10 million deaths due to AMR bacterial infections, which will be more than the predicted deaths from cancer and diabetes combined [1]. The golden era of antibiotics, in which antibiotics effectively eradicated bacterial infections, is over and intensified efforts are required to develop new strategies to combat “superbug” infections.

Excessive use of antibiotics in humans, companion animals and livestock, increased international travel and poor hygiene/sanitation are major contributors to AMR bacteria [2]. Incorrect use of antibiotics for infections that are not bacterial (i.e. viral, parasitic or fungal) have also contributed to the development AMR bacteria [3, 4]. It is imperative that new antibiotics are developed and that the few remaining active compounds are safeguarded. The most critical bacteria as per World Health Organisation include: *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae*. Methicillin-resistant *Staphylococcus aureus* (MRSA) is another high priority target bacteria [5]. Beyond AMR bacteria, bacterial biofilms and intracellular infections are more challenging types of infections, which permit bacterial survival in highly noxious environments. Biofilms are classified as intricate communities of bacteria, protected by an extracellular matrix that houses bacteria of different species, metabolic states, and highly active communication [6, 7]. Bacteria in biofilms are able to tolerate up to 1000-fold higher concentrations of antibiotics due to the biofilms creating additional virulence factors, in addition to a physical protective barrier (extracellular polysaccharide matrix, EPS), which excludes antimicrobial entry [8]. Intracellular infections have evolved from bacteria escaping phagocytosis, the key defence mechanism for bacterial removal in humans. By adapting an intracellular lifestyle, bacteria can deviously hide inside the host cells phagocytic cells (i.e. macrophages), where the bacteria are protected from the innate immune system as well as from antibiotics [9, 10].

The development of new antibiotics is a tediously long and expensive process, with present efforts being insufficient to fight the looming post-antibiotic era [11]. Re-purposing and re-formulating currently available medicines could be a faster and more economically viable strategy to provide effective antimicrobials. Since bacteria constantly evolve, drug delivery systems can offer favourable alternatives to renew the efficacy of existing antibiotics. Through loading antibiotics into a vehicle, drug delivery
systems can improve the availability of antibiotics at the site of bacterial infection [12], impart covertness or stealth behaviour [9], and enhance the antimicrobial action through synergy with the formulation excipients [13]. Drug delivery systems can additionally improve the therapeutic use of peptide and proteins, which have recently received much attention as alternative antimicrobial therapies [14, 15]. Novel proteins and peptides of interest include proteins that degrade the biofilm extracellular polymeric matrix (i.e. biofilm degrading enzymes [14]) and peptides that down regulate key cellular processes within bacteria (i.e. anti-microbial/biofilm peptides [16]). However, these macromolecules are highly labile compounds, susceptible to degradation and loss of activity upon administration to the human body, all of which may be circumvented by protection using encapsulation and drug delivery systems [17].

Antimicrobial release from the delivery system can be controlled by exploiting the microenvironment of bacteria (e.g. toxin production, pH and temperature variations) as a stimulus to initiate a bacterial-triggered release. Such triggered release provides the benefits of on-demand delivery and reduces unnecessary exposure to antimicrobials, thereby reducing the potential emergence of resistance. Bacterial virulence factors including lipases, esterases, phospholipase A2 and alpha toxin are enzymes secreted by bacteria in abundance to elicit host damage, exert dominance and increase the survival rate of bacteria [18, 19]. These toxins have been explored as stimuli to trigger the release of drugs from systems such as liposomes [20-22] and polycaprolactone polymeric (nano)particles [23], amongst other combinations of polymers with ester or amine linkers or stabilisers [24-26]. Since antimicrobial drugs can possess a range of physiochemical properties (e.g. hydrophilicities and molecular weights), a desirable bacterial-triggered drug delivery system should be able to encapsulate and trigger the release of a variety of antimicrobials.

Liquid crystalline nanoparticles (LCNPs) are a diverse drug delivery system, capable of encapsulating hydrophobic, hydrophilic small molecules, peptides and proteins. They are produced from self-assembling amphiphilic lipids, such as monoolein (MO) and phytantriol (PHY) (Figure 1), with internal liquid crystalline structures driven by the molecular shape of the amphiphilic lipids when dispersed in excess water [27]. LCNPs are gaining extensive interest as drug delivery vehicles due to their thermodynamic stability, ease of preparation and sub-micron size that permits diffusion across biological barriers [28, 29]. Both MO and PHY are generally recognised as safe (GRAS) compounds and are used in cosmetic and food products [28, 30]. However, MO is generally preferred over
phytantriol due to a lower propensity to cause haemolysis at dose-relevant concentrations [31]. MO is a monoglyceride that contains a lipase-labile ester functional group, which has previously limited the use of MO-LCNPs as an oral drug delivery vehicle due to gastrointestinal digestion resulting in precipitation and limited absorption of poorly water soluble drug [32].

LCNPs can be derived from the bulk liquid crystal (LC) formed through a physical mixture of an amphiphilic lipid with water or biological fluids. The mixture is then typically fragmented in a larger aqueous volume in the presence of a stabiliser, such as poly(ethylene oxide)-poly(propylene oxide) -poly(ethylene oxide) (PEO-PPO-PEO)[28]. The structures of LCNPs used in drug delivery are the inverse hexagonal (H₂), inverse bicontinuous cubic (Im3m, Pn3m, Ia3d) and discontinuous micellar cubic phases (Fd3m) [28, 33]. We have previously reported on the bulk MO LC gel as a bacteria-responsive topical drug delivery system [34]. Pseudomonas lipase enhanced the release of AL and gentamicin from the bulk MO-LC, however, complete release was not achieved. This was hypothesised to be due to the limited surface area of the gel that restricted the action of the interfacially-active lipases which has in part motivated the present study of LCNPs with much greater surface area. Moreover, the specificities of the triggered release from the bulk material was not explored across lipases, bacteria and other antimicrobials.

Therefore, the aim of this study was to determine the feasibility of MO-LCNP as a bacterial-triggered drug delivery system for a range of antimicrobials and different lipases. It was hypothesised that the release of different antimicrobial compounds, i.e. hydrophilic, hydrophobic and macromolecular compounds could be triggered from MO-LCNPs by bacterial lipase-mediated digestion and disruption of the liquid crystalline structures. The hydrophilic model compound used to test this theory was ciprofloxacin hydrochloride (CIP), which is a water-soluble salt (logP = 0.3) and an important drug
candidate against top priority pathogens such as *Pseudomonas aeruginosa*. Rifampicin (RIF) was chosen as the model lipophilic small molecule with a high partition coefficient (logP = 4.2). It is the last resort treatment against highly prevalent MRSA infections. Finally, alginate lyase (AL) was chosen as a model macromolecule, as it has demonstrated effectiveness in degrading bacterial biofilm extracellular polymeric matrix materials. The three major aims of the current study were: (1) to form LCNPs loaded with the model antimicrobial compounds, in particular ensuring the activity of the AL protein was maintained; (2) to examine the release of the model compounds from LCNPs both with and without the presence of bacterial lipase; and (3) to compare the kinetics and LC structure during MO-LCNP lipolysis mediated by porcine pancreatin (hereafter referred to as pancreatic lipase) as a model sn-1/3 regiospecific lipase, and lipase from *Pseudomonas cepacia* (hereafter referred to as *Pseudomonas* lipase) as a model non-regiospecific lipase.

2. Methods

2.1. Materials

Alginate lyase (AL) from *Sphingobacterium multivorum* (≥10 units/mg solid, EC 4.2.2.3, pI = 10.2 [35]), ciprofloxacin hydrochloride (European Pharmacopoeia Reference Standard), 0.01 M phosphate buffered saline (PBS) tablets, 0.05 M phosphate-citrate buffer tablets, Pluronic F-127, Trizma® maleate, sodium chloride (NaCl), sodium hydroxide (NaOH), magnesium sulfate (MgSO4), calcium chloride dihydrate (CaCl2·2H2O), propylene glycol, lipase from *Pseudomonas cepacia* (≥30 units/mg), 4-bromophenylboronic acid, glyceryl tributyrate (≥99%), chloroform (>99.9% HPLC), deuterated chloroform (CDCl3) (99.8% D) and dichloromethane (99.8%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rifampicin (>98%) was purchased from Chem Supply (Adelaide, SA, Australia). Porcine pancreatin extract (activity equivalent to 8× USP specification) was supplied by MP Biomedicals (Seven Hills, NWS, Australia). Tryptic Soy Broth (TSB) media, agar, bacteriological peptone and yeast extract were purchased from Oxoid Limited (Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia). Myverol 18-99K (part number: 5D01253, composed of 93% unsaturated monoglycerides. The specific composition was unknown, although previous report account for 58.3-86.6 % monoolein [36, 37]) was kindly donated by Kerry Ingredients and Flavours (Egham, Surrey, UK) and phytantriol was kindly donated by DSM (Heerlen, the Netherlands).
2.2. Formation of liquid crystal nanoparticles (LCNP)

To determine the appropriate method to form LCNPs loaded with AL, the traditional fragmentation method (pre-loading and post-loading of the protein) was compared to the hydrotrope dilution method (Supplementary Material). In preliminary studies, the hydrotrope dilution method was determined to be most appropriate to maintain protein activity and loading [38]. Therefore, all subsequent experiments used this method as detailed below.

2.2.1. The hydrotrope dilution method for loading AL, CIP and RIF LCNPs

To form LCNPs via hydrotrope dilution, MO (100 mg), Pluronic F-127 (15 mg) and propylene glycol (0.270 g) were weighed in a glass scintillation vial and dissolved in 10 mL chloroform. The chloroform was then evaporated under a stream of nitrogen gas. Thereafter, Tris-NaCl (50 -150 mM, 100 µL) buffer was added to the lipid mixture. The lipid mixture was then diluted up to 5 mL in water and vortex mixed until a homogenous milk-like mixture was formed (approximately 2 minutes).

To load AL and CIP, the 100 µL of Tris-NaCl buffer was charged with either 3 mg of AL or 6 mg of CIP. For RIF, 6 mg of the drug was dissolved together with MO, propylene glycol and Pluronic F-127 in 10 mL of chloroform. As a non-digestible control sample, PHY-LCNPs were also formed by substituting 100 mg of PHY for the MO in the previous formulations.

2.2.2. Quantification of particle size and zeta potential

A diluted sample of the LCNP in water supplemented with 1 mM KCl (to offset pure water double layer artefacts) (final concentration 0.00175 % w/v MO) was analysed via dynamic light scattering and phase analysis scattering at 25°C (assuming the viscosity of water) using a Zetasizer Nano ZS (Malvern, Worcestershire, UK) to determine the particle size and zeta potential, respectively. The built-in software enabled determination of the average particle size (z-average) and the particle size distribution (as poly dispersity index (PDI)) over 15 triplicated measurements, with unimodal distributions.

2.3. In vitro release of antimicrobials

Pressure ultrafiltration (Amicon® Stirred Cell), equipped with a pre-soaked 0.05 µm mixed cellulose pore filter membrane (MF-Millipore, both Merck Millipore, Bayswater, Victoria, Australia) was used to determine the release of AL, CIP and RIF from MO-LCNPs. The conditions of the in vitro release studies were performed to mimic the physiological environment of generalised topical bacterial infections.
2.5 mL of each LCNP dispersion was added to 10 mL of 0.01 M PBS buffer (pH 7.4) in the ultrafiltration cell to mimic physiological conditions. The mixture was magnetically stirred and at specific time points (0, 5, 10, 15, 30, 45, 60, 90, 120, and 240 mins) 0.5 mL samples were collected by applying 100 kPa of nitrogen gas. The liquid removed was replaced with 0.5 mL of fresh 0.01 M PBS (pH 7.4). Released AL, CIP and RIF were all confirmed to pass through the membrane with < 1.5 % losses during filtration. While CIP and RIF release was determined to plateau after 4 hours, further release samples were collected for AL at 360, 480, 1440 and 2880 minutes (until a plateau was reached). At these plateau time points, the extracted sample was replaced with 0.5 mL of *Pseudomonas* lipase in 0.01 M PBS (pH 7.4) to a final concentration of 1 mg/mL (30 units/mL). The release of AL, CIP and RIF was continually monitored at the same specific time points as aforementioned. Samples were quantified for AL content using a commercially available protein assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia), with 0.0045 mg/mL AL as a lower limit of quantification and strong linear sensitivity [coefficient of determination (R²) = 0.998 – 0.999]. 98% of the total colorimetric signal from the BCA assay was determined to be attributable to AL, with < 2% of the signal resulting from *Pseudomonas* lipase. The total amount of AL released over time was computed by rescaling to 98%, through subtracting the calculated contribution of *Pseudomonas* lipase from the total sample and then against a known standard concentration curve.

CIP and RIF concentrations were determined using high-performance liquid chromatography (HPLC) with UV detection (Shimadzu, Kyoto, Japan). Separation was carried out on a Phenomenex Luna 5 µm C18 100 Å (250 x 4.6 mm) (Torrance, USA) column equipped with a column guard. The system was maintained at 40 °C, with an injection volume of 10 µL and elution with a mobile phase at a flow rate of 1 mL/min. For CIP, the mobile phase contained 84% aqueous acetic acid (2% v/v) and 16% acetonitrile. Each sample was analysed over 8 min at a detection wavelength of 278 nm, with a retention time of 4.6 minutes for CIP. For RIF, the mobile phase consisted of 55% potassium phosphate buffer (0.03 M, pH 3) and 45% acetonitrile. Samples containing RIF were analysed over 12 minutes at a detection wavelength of 230 nm, with a retention time of 7.8 minutes for RIF. The samples were quantified against known concentrations of the respective compounds (calibration curves for CIP 0.025-250 µg/mL; RIF 0.1-100 µg/mL).
Previous studies have demonstrated a linear relationship between the percentage of drug released from the LCNPs and the square root of time, which corresponds to diffusion-controlled (Fickian) release according to the following [39, 40]:

Equation 1: \[ Q = D_m C_d (2A - C_d)t^{1/2} \]

Where Q is the amount of drug released per unit matrix (mg/mL), \( D_m \) is the diffusion coefficient of the drug in the matrix, A is the initial amount of drug loaded in the matrix, \( C_d \) is the solubility in the drug matrix and t is time.

2.4. In vitro lipolysis

In vitro lipolysis experiments were carried out using the Titralab 854 pH-stat titration apparatus (Titranuo, Metrohm, Herisau, Switzerland) as described previously [41, 42]. In a jacketed vessel maintained at 37°C, the LCNPs were diluted 1:4 in 20 mL standardised digestion buffer (50 mM Tris, 150 mM NaCl, 5 mM CaCl\(_2\)), pH 7.5, to a final concentration of 100 mg lipid. The diluted LCNPs were equilibrated for approximately 5 minutes at pH 7.5 before 2 mL of the lipase solution was added (Pseudomonas lipase = 30 units/mL and pancreatic lipase ~ 2000 TBU). The generated free fatty acids were titrated with 0.6 M NaOH to maintain a pH of 7.50 ± 0.01 over 60 minutes. At time 0, 5, 30 and 60 minutes a 2 mL aliquot of the digesting mixture was removed, and the lipase activity was arrested via the addition of 10 µL of 0.5 M 4-bromophenylboronic acid in methanol. The aliquot was then centrifuged, and the supernatant collected for further analysis via proton nuclear magnetic resonance.

The total amount of titrated fatty acids was subsequently calculated from the volume of NaOH required to maintain the pH at 7.50 ± 0.01 during lipid digestions, minus the volume of NaOH required during blank (no lipid) digestions. In preliminary studies, after 60 minutes of digestion, the pH was rapidly increased to pH 9 by the NaOH titrate to ionise any remaining unionised fatty acids. However, as the titre at pH 9 was equivalent between blank (no lipid) and lipid digestions, all fatty acids were assumed ionised at pH 7.5. The percentage of hydrolysis was calculated by dividing the moles of fatty acids titrated by total moles of lipid added [43].

Lipase-mediated hydrolysis has been described as a pseudo first-order reaction [44], where there is an excess of lipase present and the rate of reaction is dependent on the concentration of the lipid substrate.
In the present study, mono- and bi-exponential equation were fitted to the digestion, as appropriate to the curve fitting;

Equation 2: (a) Monoexponential: \( \%H = H_{\text{max}}(1 - e^{-k_1t}) \), (b) Bi-exponential: \( \%H = H_{\text{max}}(1 - (e^{-k_1t} + e^{-k_2t})) \),

Where \( \%H \) is the percentage of lipid hydrolysis, \( H_{\text{max}} \) is the maximum extent of hydrolysis, \( K_1 \) and \( K_2 \) are the hydrolysis rate constants for the first and second phase, respectively and \( t \) is time.

The extent of MO hydrolysis was then correlated to the total concentration of antibiotics released from the MO-LCNPs 1 hour after *Pseudomonas* lipase was added into the *in vitro* release study, where the \( R^2 \) value was determined via Graphpad Prism version 7.00 for Windows (GraphPad Soft- ware, La Jolla, CA) linear-regression software.

### 2.4.1. Quantification of lipolytic products by NMR

The aliquots of the *in vitro* lipolysis studies were analysed by proton nuclear magnetic resonance (\(^1\)H NMR) spectroscopy. As previously described by Joyce *et al.* [43], the aliquots were subjected to a liquid-liquid extraction using 3 mL of dichloromethane to dissolve the lipid components and remove the aqueous phase. The dichloromethane was then evaporated under vacuum and the lipolytic products were dissolved in 0.8 mL deuterated chloroform (CDCl\(_3\)) for \(^1\)H NMR analysis using a Bruker 500 MHz Avance III HD NMR Spectrometer (Billerica, MA, United States). The acquisition parameters were as follows: spectral width 6410 Hz, relaxation delay 3 s, number of scans 32, acquisition time 3.28 s and pulse width 90°. The lipolytic species were then determined using methods and equations previously derived [43, 45, 46], according to the chemical shift assignments in Table 1.

<table>
<thead>
<tr>
<th>Signal</th>
<th>Chemical shift (ppm)</th>
<th>Multiplicity</th>
<th>Types of protons</th>
<th>Lipid Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>0.88–0.89</td>
<td>t</td>
<td>-CH(_3)</td>
<td>All</td>
</tr>
<tr>
<td>(b)</td>
<td>1.19–1.42</td>
<td>m</td>
<td>-(CH(_2))(_n)-</td>
<td>All</td>
</tr>
<tr>
<td>(c)</td>
<td>1.61–1.72</td>
<td>m</td>
<td>-OCO–CH(_2)--CH(_2)-</td>
<td>All</td>
</tr>
<tr>
<td>(d)</td>
<td>2.26–2.38</td>
<td>m</td>
<td>-OCO–CH(_2)--</td>
<td>All</td>
</tr>
<tr>
<td>(e(_1))</td>
<td>1.92–2.15</td>
<td>m</td>
<td></td>
<td>Unsaturated</td>
</tr>
</tbody>
</table>

*Table 1. Chemical shift assignments and multiplicities of the \(^1\)H NMR signals in CDCl\(_3\) of the main protons of glycerides and fatty acids present during the samples obtained during lipolysis of MO-LCNP. Further details on the analysis can be found elsewhere [43, 45]. The signal letters agree with those given in Figure 7. Abbreviations: d: doublet, t: triplet, m: multiplet*
### 2.4.2. SAXS liquid crystalline structure analysis and in situ digestion:

The liquid crystalline (LC) structures of the LCNPs were determined by synchrotron small angle X-ray scattering (sSAXS). All sSAXS measurements were performed at the Australian Synchrotron on the SAXS/WAXS beamline[47]. The LCNP suspensions were measured in glass capillaries tubes, with a sample to detector distance of 1689 mm, giving a q range 0.010–1.148 Å⁻¹. The wavelength of the X-rays λ was 0.954 Å with a photon energy of 13 keV. The 2D SAXS patterns were acquired for 1.0 sec on a Pilatus 2 M detector.

In situ digestion titrations were performed as per the in vitro lipolysis section below using a pH-stat titration apparatus (Metrohm 902 STAT titration system). The digesting sample was circulated from the thermostated digestion vessel through a capillary mounted in the X-ray beam and back into the digestion vessel to allow for continuous monitoring of the scattering patterns of the digesting mixture [48-50]. Scattering patterns were recorded using a Pilatus 2M detector and acquisitions were taken for 5 s every 20 s (three exposures per minute). The computer program ScatterBrain was used to convert 2D scattering images into plots of scattered X-ray intensity I(q) versus q [\(=\frac{4\pi}{\lambda}\sin\theta\) where 2θ is the scattering angle]. Bragg peaks from the LC phases were indexed to corresponding structures and the corresponding lattice parameters were calculated using known relationships between them and the peak positions as described by Hyde [51]. The lattice parameters were used to calculate the size of the water channels, as per Briggs et al. [52] and Bisset et al. [40], based on geometric considerations.

<table>
<thead>
<tr>
<th>(\varepsilon_2)</th>
<th>2.77</th>
<th>t</th>
<th>(-\text{CH}_2\text{=CH=}), Polyunsaturated</th>
<th>(=\text{HC=CH}_2\text{=CH=})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(f)</td>
<td>3.65</td>
<td>d</td>
<td>ROCH(_2)–CHOH–CH(_2)OH</td>
<td>1-MG</td>
</tr>
<tr>
<td>(g)</td>
<td>3.73</td>
<td>d</td>
<td>ROCH(_2)–CH(OR(′))–CH(_2)OH</td>
<td>1,2-DG</td>
</tr>
<tr>
<td>(h)</td>
<td>3.84</td>
<td>d</td>
<td>HOCH(_2)–CH(OR)–CH(_2)OH</td>
<td>2-MG</td>
</tr>
<tr>
<td>(i)</td>
<td>3.94</td>
<td>m</td>
<td>ROCH(_2)–CHOH–CH(_2)OH</td>
<td>1-MG</td>
</tr>
<tr>
<td>(j)</td>
<td>4.05–4.21</td>
<td>m</td>
<td>ROCH(_2)–CHOH–CH(_2)OR(′)</td>
<td>1,3-DG</td>
</tr>
<tr>
<td>(k)</td>
<td>4.28</td>
<td>ddd</td>
<td>ROCH(_2)–CH(OR(′))–CH(_2)OH</td>
<td>1,2-DG</td>
</tr>
<tr>
<td>(l)</td>
<td>4.93</td>
<td>m</td>
<td>HOCH(_2)–CH(OR)–CH(_2)OH</td>
<td>2-MG</td>
</tr>
<tr>
<td>(m)</td>
<td>5.08</td>
<td>m</td>
<td>ROCH(_2)–CH(OR(′))–CH(_2)OH</td>
<td>1,2-DG</td>
</tr>
<tr>
<td>(n)</td>
<td>5.13–5.28</td>
<td>m</td>
<td>(-\text{CH=CH=})</td>
<td>Unsaturated</td>
</tr>
</tbody>
</table>
Equation 3: (A) \((Im3m)\) \(r = 0.305a - l\); (B) \((Pn3m)\) \(r = 0.391a - l\)

Equation 4: \(H_2 \) \(r = \frac{a - 2l}{2}\)

Where \(r\) is the water channel radius, \(a\) is the lattice parameter and \(l\) is the lipid chain length, all distances being in nm.

2.4.3. Quantification of bacterial lipase activity

The endogenous lipase activity of different bacterial strains was determined via the tributyrin agar assay, as previously described by Carrazco-Palafox et al. [53]. Briefly, tributyrin agar plates were prepared with 15 g agar, 3 g yeast extract, 5 g peptone, 1\% (v/v) tributyrin, 2.5 mM CaCl\(_2\) and 5 mM MgSO\(_4\). The lipid forms an opaque appearance in the agar, into which 6 mm diameter holes were punched in the dried agar and filled with 20 µL bacterial suspension (0.10 ± 0.01 optical density at 600 nm (OD600)) prepared from a freshly streaked agar plate in tryptic soy broth (TSB) or 0.9\% sodium chloride, as a control. Strains including Methicillin-resistant *Staphylococcus aureus* (MRSA strain USA300), *Staphylococcus epidermis* ATCC 14990, *Pseudomonas aeruginosa* strain PA01 (American Type Culture Collection, Manassas, VA) and mucoid *Pseudomonas aeruginosa* clinical isolate (alginate-producing) were obtained from the institutional culture collection (UniSA, Adelaide). The inoculated agar plates were then incubated at 30°C for 3 days. Lipase activity was determined by a distinctive halo (zone) surrounding the bacterial strain indicating lipid digestion.

To correlate the tributyrin lipase activity to the lipid content in MO-LCNP, the agar plates were also prepared as previously described with a final concentration of 1 mg/mL MO-LCNPs. As before, the bacterial strains, MRSA (USA300), *S. epidermis* ATCC 14990, *P. aeruginosa* PA01 and *P. aeruginosa* mucoid clinical isolate were loaded into the holes punched into the dried agar. The distinct halo surrounding the bacteria, indicating lipase activity was determined after incubation at 30°C for 3 days. As a control, 20 µL of *Pseudomonas* lipase (30 units/mL) was added into the agar punched holes.

2.5. Statistical analysis

Data is reported as mean ± standard deviation. Student t-test was used to compare the difference in hydrolysis mediated by the lipases. One-way analysis of variance (ANOVA) assessed the difference in the digestion products observed from H\(^1\)-NMR and the lipase activity in the lipid agar tests. Statistical
significance was evaluated at the 95% confidence interval. All tests were performed using GraphPad Prism (version 7.00 for Windows; GraphPad Soft- ware, La Jolla, CA).

3. Results

3.1. Characterization of LCNP

As AL is an enzyme, the traditional fragmentation method to form LCNP from the bulk LC was unable to be used, due to the excessive sonication that destroyed the enzyme’s activity. The hydrotrope dilution method is an alternative preparation method for LCNPs, in which a hydrophilic co-dispersant facilitates the fragmentation of the lipid by low energy dispersal techniques such as vortex mixing [38, 54]. Preliminary experiments confirmed that the hydrotrope dilution method maintained the activity and an efficient loading capacity of the enzyme, AL, in comparison to the excessive energy fragmentation method (Supplementary Figure 1). While post loading the macromolecule after LCNPs were formed via fragmentation has previously maintained the activity of macromolecules [55], this resulted in a lower loading of AL (1.20% w/w, Supplementary Figure 1), which may be attributable to steric hindrance of the stabiliser [38, 54]. As the hydrotrope dilution method maintained the highest drug loading (2.82% w/w) and maintain the activity of the protein, this method was continued with for the rest of the present studies.

3.1.1. Size and drug loading

The size, zeta potential and antimicrobial loading of LCNPs are summarised in Table 2. The sizes of the LCNPs generated by the hydrotrope dilution method were comparable before and after loading with CIP, RIF and AL. The negative surface charge on the LCNPs containing no antimicrobial was likely due to the interaction between Pluronic F-127’s hydroxyl ions with water, as observed previously [38, 55]. The surface zeta potential of the LCNP became less negative upon loading of AL, CIP and RIF, differentiated by an increasing positive charge of each compound, respectively. Higher antibiotic loadings were achieved with CIP and RIF than with AL.
Table 2. Particle size characterisation and antimicrobial loading of LCNP. Represented as mean ± standard deviation, n = 3.

<table>
<thead>
<tr>
<th>LCNP</th>
<th>Particle size (Z-average) (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
<th>Antibiotic load (% wt/lipid wt)</th>
<th>Encapsulation efficiency (%)</th>
</tr>
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<tbody>
<tr>
<td>MO no drug</td>
<td>180 ± 1</td>
<td>0.07 ± 0.02</td>
<td>-22.4 ± 0.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MO-AL</td>
<td>226 ± 4</td>
<td>0.29 ± 0.01</td>
<td>-11.2 ± 1.1</td>
<td>2.8 ± 0.3</td>
<td>94.0</td>
</tr>
<tr>
<td>MO-CIP</td>
<td>171 ± 2</td>
<td>0.25 ± 0.01</td>
<td>-9.5 ± 0.5</td>
<td>5.1 ± 0.5</td>
<td>84.8</td>
</tr>
<tr>
<td>MO-RIF</td>
<td>153 ± 8</td>
<td>0.15 ± 0.03</td>
<td>-4.6 ± 1.6</td>
<td>5.5 ± 0.5</td>
<td>91.0</td>
</tr>
<tr>
<td>PHY no drug</td>
<td>180 ± 3</td>
<td>0.35 ± 0.13</td>
<td>-21.1 ± 0.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PHY-AL</td>
<td>200 ± 4</td>
<td>0.36 ± 0.23</td>
<td>-8.8 ± 1.3</td>
<td>2.6 ± 0.4</td>
<td>85.8</td>
</tr>
</tbody>
</table>

3.1.2. Liquid crystalline structures

Small angle X-ray scattering (SAXS) diffractograms for the prepared LCNP are displayed in Figure 2. The LC structures, as established from the Bragg peak spacing ratios, along with the lattice parameters and calculated water channel diameters are shown in Table 3. MO-LCNPs containing no drug had a hexagonal (H2) phase structure with Bragg peaks at spacing ratios 1: √3: √4 and a lattice parameter of 52.6 Å. The H2 structure was largely unchanged upon loading AL, with similar lattice parameter of 53.5 Å. The diffraction peaks shifted towards lower q values upon CIP and RIF loading, indicating higher lattice parameters of 58.4 Å and 58.9 Å, respectively. PHY-LCNP containing no drug also had a H2 phase structure before and after loading with AL, with a lower lattice parameter to MO-LCNPs of 42.1 Å and 42.9 Å, respectively.
Figure 2. Small angle X-ray scattering (SAXS) profiles of various LCNPs formed.

Table 3. The structure and corresponding lattice parameters calculated from the positioning of Bragg peaks [56] and calculated water channel diameter of the LCNP formed (as calculated from Equation 3-5).

<table>
<thead>
<tr>
<th>LCNP</th>
<th>Phase</th>
<th>Lattice Parameter (Å)</th>
<th>Water Channel Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO no drug</td>
<td>H₂</td>
<td>52.6 ± 0.1</td>
<td>3.9</td>
</tr>
<tr>
<td>MO-AL</td>
<td>H₂</td>
<td>53.5 ± 0.3</td>
<td>3.9</td>
</tr>
<tr>
<td>MO-CIP</td>
<td>H₂</td>
<td>58.4 ± 0.1</td>
<td>4.3</td>
</tr>
<tr>
<td>MO-RIF</td>
<td>H₂</td>
<td>58.9 ± 0.1</td>
<td>4.3</td>
</tr>
<tr>
<td>PHY no drug</td>
<td>H₂</td>
<td>42.9 ± 0.2</td>
<td>3.1</td>
</tr>
<tr>
<td>PHY-AL</td>
<td>H₂</td>
<td>42.1 ± 0.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

3.2. Bacterial lipase-triggered drug release from LCNP

3.2.1. Alginate Lyase

The in vitro release profile of AL from the MO-LCNPs is shown in Figure 3A. In 0.01 M PBS, AL was released steadily, at a rate of 0.09 mg/h^{1/2} (Table 4), as derived from fitting the release data (prior to plateau, Figure 3C) to equation 3. After 8 h the release plateaued with 30% of AL being released while the rest of AL remained within the MO-LCNPs (data not shown).
The release of AL from (A) MO-LCNPs and (B) PHY-LCNPs over time. The rates of release were determined from plotting the total amount of AL released (prior to the plateau region, where the subsequent data points have been excluded from the linear regressions and not shown) against the square root of time ([C] MO-LCNPs and [D] PHY-LCNPs), and are shown in Table 4. The blue circles indicate release in 0.01 M PBS, pH 7.4, while the green squares indicate the addition of 1 mg/mL (30 Units/mL) Pseudomonas lipase to the release media. For some data points, the error bars are smaller than the corresponding symbol. Data is represented as mean ± standard deviation, n = 3. Linear regression analysis, R² > 0.95.

The addition of *Pseudomonas* lipase significantly increased the release of AL from MO-LCNPs, with a further 50% of the remaining AL being released within 1 h, at an 82-fold higher release rate of 7.50 mg/h^{1/2} (Figure 3C). Following the action of the *Pseudomonas* lipase, the release of AL plateaued, with AL being completely released after 1 day (Figure 3A). As a control, non-digestible PHY-LCNP were loaded with AL. The release of AL from PHY-LCNP was faster than MO-LCNP, reaching 20% release within 2 h, at a rate of 0.12 mg/h^{1/2} (Figure 3B and D), which then plateaued at 2 h. Although, after *Pseudomonas* lipase was added, there was no further increase in the release of AL for 24 h.
Table 4. Rates of release in mg/h$^{1/2}$, as determined using the graphs of the amount released versus the square root of time (Figure 3 and Figure 4), in accordance with equation 1.

<table>
<thead>
<tr>
<th></th>
<th>Rate of release (mg/h$^{1/2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MO-LCNP</td>
</tr>
<tr>
<td>AL</td>
<td>0.09</td>
</tr>
<tr>
<td>CIP</td>
<td>1.57</td>
</tr>
<tr>
<td>RIF</td>
<td>0.77</td>
</tr>
</tbody>
</table>

3.2.2. Small molecules: ciprofloxacin and rifampicin

CIP's diffusion-controlled release from MO-LCNPs resulted in 100% being released within 2 h (Figure 4A). As all drug was released from MO-LCNPs, the addition of Pseudomonas lipase at 4 h could not further increase the release. The diffusion of CIP occurred at a rate of 1.57 mg/h$^{1/2}$ (Figure 4C, Table 4).

For RIF, the diffusion-controlled release was lower than for CIP, with 43% of RIF being released from the MO-LCNPs within 45 mins in 0.01 M PBS media (rate of release 0.77 mg/h$^{1/2}$, Figure 4D) leaving the remaining lipophilic drug inside the LCNP (Figure 4B). The addition of Pseudomonas lipase triggered the release of the remaining 57% of rifampicin within 1 h after lipase addition. The rate of release was also increased to 5.56 mg/h$^{1/2}$ by the addition of the bacterial lipase, which was approximately 7-fold greater than the release rate in the buffered physiological media.
3.3. Lipase-mediated digestion of LCNPs

3.3.1. pH stat titration

Lipase-mediated hydrolysis is a pseudo-first order reaction, with the rate dependant on the concentration of digestible lipid when an excess of lipase is present [57]. Upon digestion of MO-LCNPs, as monitored via pH-stat titration, the pancreatic lipase achieved a similar total extent of hydrolysis (78%) as the Pseudomonas lipase (81%) (P = 0.16) (Figure 5A).

A better fit to a pseudo first-order function was obtained through splitting the pancreatic lipase digestion into two phases (Figure 5B). Using the bi-exponential equation (Equation 2B), the lipolysis rate
constants for pancreatic lipase was faster initially (0.059 min⁻¹), compared to the slower second phase of digestion (0.011 min⁻¹). In contrast, *Pseudomonas* lipase digestion corresponded to a single-phase pseudo first-order reaction, at a rate of 0.029 min⁻¹, and was fitted with the mono-exponential equation (Equation 2). As expected for the non-digestible lipid, there was no appearance of fatty acids upon the addition of lipase to PHY-LCNPs.

Correspondingly, there was a strong linear correlation between the extent of lipolysis and the amount of AL ($R^2 = 0.977$) and RIF ($R^2 = 0.959$) released in the presence of the *Pseudomonas* lipase (Figure 6A and B, respectively).

---

**Figure 5.** In vitro lipolysis of MO-LCNPs and PHY-LCNPs. (A) The percentage of MO-LCNP lipolysis occurring over time with pancreatic lipase (purple triangles) and *Pseudomonas* lipase (green squares), compared to PHY-LCNP digestion with pancreatic lipase (black circles) as determined by pH stat titration at pH 7.0. (B) Pseudo-first order fit for MO-LCNPs digestion with pancreatic lipase, (purple triangles) and *Pseudomonas* lipase (green squares). Data represented means ± standard deviation, $n = 3$. 

(Add relevant equations and references here if necessary.)
Figure 6. Influence of MO-LCNPs digestion on the release of encapsulated antibiotics. Correlation between the percentage of lipolysis of MO-LCNPs with pseudomonas lipase and total amount released of (A) AL, (B) RIF and (C) CIP corresponding to the extent of MO-LCNP digestion by Pseudomonas lipase. Data represented as mean ± standard deviation.
3.3.2. Proton nuclear magnetic resonance

To further quantify the lipase-mediated hydrolysis of MO-LCNPs, proton nuclear magnetic resonance ($^1$H NMR) was used to determine the relative molar ratios of the glyceride and fatty acid products formed [43]. Representative $^1$H NMR spectra are provided in Figure 7. The spectral signals of interest lie within the ranges of 2.26 to 2.40 ppm and 3.20 to 4.50 ppm, representing the protons specifically from the acyl groups and the glyceryl backbone of diglycerides, monoglycerides and fatty acids (Table 1). The Myverol 18-99K used to form the MO-LCNPs has a 93% monoglyceride composition, as stated by the manufacture (Kerry Ingredients) with the exact lipid composition not disclosed. As supported by the $^1$H NMR spectra of the MO-LCNP at digestion time = 0 s, the relative molar percentages of lipidic components were: 84% of 1-monoglycerides, 8% 2-monoglycerides, 8% 1, 2-diglycerides and less than 0.1% 1, 3-diglycerides (mol/mol). As lipolysis proceeded following the addition of lipase, a reduction in the intensity of the peak signals at 3.20 to 4.50 ppm were observed due to the decrease in protons indicative of an acyl glyceryl backbone. Meanwhile, the integrated area of the spectral peak signals corresponding to fatty acids (2.26 to 2.40 ppm) increased in intensity.

![Figure 7](image)

*Figure 7. Representative $^1$H NMR spectrum obtained from in vitro lipolysis of GMO-LCNP by Pseudomonas lipase from time 0 (bottom) up to 60 minutes.*

The similarity in the extent of lipolysis by both lipase types was further supported by the quantification of the relative molar proportions of fatty acids produced at 60 minutes, which were not statistically
different (P = 0.221). However, as highlighted in Figure 8, there were differences in the increasing molar percentage of fatty acids produced over time for both lipases. Upon initiation of digestion, pancreatic lipase yielded 65% of free fatty acids after 5 minutes of digestion, 77% after 30 minutes and 78% (mol/mol) after 60 minutes. In comparison, *Pseudomonas* lipase liberated 49% after 5 minutes of digestion, 61% at 30 minutes and 73% (mol/mol) at 60 minutes.

Unlike pancreatic lipase, *Pseudomonas* lipase demonstrated a more gradual decrease in 1-monoglycerides (46, 34 and 22% [mol/mol] after 5, 30 and 60 minutes of digestion, respectively). For pancreatic lipase, the digestion of 1-monoglycerides was more rapid, moving from 20% to 9% [mol/mol] at 5 and 30 minutes of digestion, respectively. Moreover, due to the sn-1/3 regio-specificity of pancreatic lipase,
lipase, a significant 10% increase of the 2-monoglyceride stereoisomer was observed and remained after 5 minutes of digestion, compared to Pseudomonas lipase (P = 0.001).

3.3.3. Bacterial lipase production and activity

The lipase production from various bacteria was determined through incubating the bacteria in lipid doped agar plates containing either MO-LCNPs or tributyrin (as a control). Partial phase separation of the lipid in the agar causes an opaque appearance that is removed upon digestion by bacterial lipases. If lipase was produced by the bacteria, a distinctive halo was observed, as demonstrated in Supplementary Figure 2. MO-LCNP digestion was demonstrated after incubation with different bacterial species, including; Gram-positive (MRSA (USA 300) and S. epidermidis) and strains of gram-negative P. aeruginosa (i.e. clinical isolate and PA01 strain), as denoted in Table 5 by the size of the distinctive halos surrounding the bacteria in the lipid doped agar. The size of the inhibition zone observed around the bacteria in the agars indicates the activity of the lipases released by the bacteria. In comparison to tributyrin-doped agar (a triglyceride), the 4 bacterial strains produced lipases that generated insignificant differences in the distinctive halos in MO-LCNP-doped agar (P = 0.949, 0.947, 0.874 and 0.724, relative to the (commercial) Pseudomonas lipase for MRSA, S. epidermidis, P. aeruginosa clinical isolate and PA01, respectively). In addition, an equivalent distinctive halo and therefore lipase activity was demonstrated between all bacterial strains and the commercial Pseudomonas lipase inoculated in agar plates (P = 0.536, 0.538, 0.396 and 0.627, respectively).

Table 5. Distinctive halos (cm) surrounding bacterial culture or lipase solution in lipid doped agar plates, compared to NaCl control (no halo). Represented as mean ± standard deviation, n = 3

<table>
<thead>
<tr>
<th></th>
<th>Pseudomonas (Commercial)</th>
<th>PA Clinical</th>
<th>PA01</th>
<th>S. epidermidis ATCC 14990</th>
<th>MRSA USA300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tributyrin 1% (v/v) agar</td>
<td>1.35 ± 0.07</td>
<td>1.58 ± 0.21</td>
<td>1.77 ± 0.15</td>
<td>1.70 ± 0.07</td>
<td>1.70 ± 0.07</td>
</tr>
<tr>
<td>MO-LCNP 0.1% (w/v) agar</td>
<td>1.28 ± 0.07</td>
<td>1.69 ± 0.16</td>
<td>1.60 ± 0.11</td>
<td>1.63 ± 0.17</td>
<td>1.63 ± 0.17</td>
</tr>
</tbody>
</table>

3.3.4. Liquid crystalline structures formed during lipolysis: in situ small-angle X-ray scattering

The diffractograms mapping the in-situ digestion of MO-LCNPs loaded with AL by both Pseudomonas and pancreatic lipase are displayed in Figure 9A and C. When MO-LCNPs containing AL were diluted
In buffered media, there was a shift from the H₂ phase originally determined to a mixed primitive bicontinuous cubic (Im3m and Pn3m) structure with Bragg peaks at spacing ratios of $\sqrt{2}: \sqrt{4} : \sqrt{6}$ (Im3m) and $\sqrt{2}: \sqrt{3}$ (Pn3m). After 200 s equilibration (digestion time 0), pancreatic lipase was added and there was a reduction in the intensity of the Bragg peaks related to the Im3m cubic phase (Figure 9A). After 100 s of digestion, there was an appearance of a Bragg peak at $q = 0.203 \text{ Å}^{-1}$, which by 180 s could be associated with an inverse hexagonal phase with weaker Bragg peaks also observed at $q = 0.350$ and $0.401 \text{ Å}^{-1}$, corresponding to peak ratios of $\sqrt{1}: \sqrt{3}: \sqrt{4}$. The intensity of the Bragg peaks of the hexagonal phase increased linearly with the amount of fatty acids titrated (extent of digestion, Figure 10A). The lattice parameter of the hexagonal phase remained constant at 35.7 Å and was smaller than that of the original hexagonal phase structure of MO-LCNPs containing AL (non-diluted) prior to digestion (53.5 Å).

For comparison, PHY-LCNP loaded with AL in situ digestion diffractograms are displayed in Figure 9B. The 1:5 dilution of PHY-LCNPs containing AL slightly increased the lattice parameter of the hexagonal phase structure from 42.1 Å to 49.1 Å. Upon lipase addition (200 s into SAXS measurement), there was an abrupt change in LC structure from the initially observed hexagonal phase to a co-existing Pn3m cubic phase with Bragg peak spacing ratio $= \sqrt{2}: \sqrt{3}: \sqrt{4}$. As digestion proceeded, the intensity of the Bragg peaks associated with the hexagonal phase gradually merged with the Pn3m and were no longer detected at 100 s of digestion. The Pn3m cubic phase remained for the 45 minutes of digestion but two sets of Pn3m peaks lying between $q = 0.142/0.174/0.200$ and $0.119/0.145/0.168 \text{ Å}^{-1}$ were observed corresponding to phases with lattice parameters between 62.6 and 74.7 Å. As the digestion progressed, the intensity of the Bragg peaks for the Pn3m phase with the smaller lattice parameter increased as the peaks moved towards lower q values (increasing lattice parameter) until they coalesced with the lower q Bragg peaks. This indicated the tendency of the system to equilibrate to a phase with larger lattice parameter over time.
A. Pancreatic lipase – MO-LCNP (AL)

B. Pancreatic lipase – PHY-LCNP (AL)

C. Pseudomonas lipase – MO-LCNP (AL)
The X-ray scattering pattern observed during the *in-situ* lipolysis of AL doped MO-LCNPs with *Pseudomonas* lipase was contrastingly different to that with pancreatic lipase. When *Pseudomonas* lipase was added (200 s into SAXS measurement), the Bragg peaks from the prominent Im3m cubic phase (Bragg peak ratios = $\sqrt{2}$: $\sqrt{4}$: $\sqrt{6}$) began to shift towards lower q values indicating an increase in the lattice parameter from 122.3 to 241.1 Å and their intensity decreased over time with the phase apparently absent after 2100 s (35 mins) of digestion (Figure 9C). This shift of the Im3m cubic phase corresponded to a linear increase in the lattice parameter as digestion progressed (Figure 11C). Meanwhile, there was a linear increase in the intensity of a weak Bragg peak at q = 0.142 Å$^{-1}$, beginning from 280 s into digestion which is consistent with previous reports on the diffraction from the calcium soaps of fatty acids liberated during lipolysis [49]. The spacing ratio of the Bragg peaks associated with lamellar phases is $\sqrt{1}$: $\sqrt{2}$: $\sqrt{3}$ and towards the end of digestion the weak second order diffraction peak could be observed around q = 0.27 Å$^{-1}$. The lamellar phase therefore had a lattice parameter of around 49.4 Å, which is commensurate with the calcium soaps of fatty acids containing C18:0 chains [49]. The increase in the intensity of the Bragg peak associated with the lamellar phase therefore also correlated with the amount of fatty acids titrated as digestion proceeded ($R^2 = 0.995$, Figure 10B).
Figure 10. Variations in the liquid crystalline phase properties of MO-LCNPs as a function of the extent of digestion. Correlations between the amounts of free fatty acid (FFA) titrated from MO-LCNPs digestion with (A) pancreatic lipase and (B) Pseudomonas lipase with the major peak intensity of respective phases. The maximum peak intensity is the highest intensity value of the first Bragg peak minus the base line intensity value.
Figure 11. Changes in lattice parameters of LCNs as digestion proceeds for (A) MO-LCN with Pseudomonas lipase, (B) MO-LCN with pancreatic lipase and (C) PHY-LCN with pancreatic lipase.
4. Discussion

4.1. LCNP phase characterisation

LCNPs are an attractive drug delivery system, able to load compounds of varying physicochemical properties and bioactivity. In this study, MO-LCNPs were explored as a bacterial-triggered drug delivery system for 3 antimicrobial compounds with different chemistries (AL, CIP and RIF). In excess water, MO is reported to form a bicontinuous cubic phase structure at physiological temperatures [58], whereas in the present study, the inverse hexagonal phase was formed by MO-LCNPs using the hydrotrope dilution method. While the presence of an inverse hexagonal phase is often due to MO hydrolysing in excess water, which results in a rising concentration of oleic acid [59], \(^1\)H NMR data demonstrated small quantities of fatty acids present in freshly prepared samples that were used for the sSAXS measurements. The addition of the co-solvent, propylene glycol can change the phase structure of the MO system [60], and is postulated to be incorporated at least partially into the lipid bilayer, increasing the critical packing parameter, favouring the hexagonal phase observed. The hexagonal phase structure phenomena was also demonstrated with PHY, which is also widely known to form a bicontinuous cubic structure in excess water [61]. In line with previous reports, PHY-LCNPs demonstrated smaller water channels than the MO-LCNP systems [33].

4.2. Release of antimicrobials from LCNP

Drug release from LCNPs is controlled by diffusion from the water channels that are intercalated within the lipid bilayers, forming the matrix as described by Higuchi [62]. The nuances in release profiles of AL, CIP and RIF from MO-LCNPs in buffered medium can be explained by the different physicochemical properties of the compounds. CIP is a 368 Da water-soluble salt (up to 35 mg/mL), with a clogP of 0.3 (on-line Lipophilicity/Aqueous Solubility Calculation Software, ALOGPs). It completely diffuses (at a rate of 1.57 mg/h\(^{1/2}\)) from the water channels of the MO-LCNPs within 2 h, as previously described for small hydrophilic compounds [63]. In comparison, RIF (823 Da; clogP of 4.2 at pH 7.4) is only slightly soluble in water (up to 1.20 mg/mL), and the diffusion-controlled release from MO-LCNPs plateaued at 43% in non-sink conditions (at a rate of 0.77 mg/h\(^{1/2}\)). Sink conditions were unachievable with RIF release studies, due to the limited water solubility and sensitivity of the quantification measurement, in addition to maintaining an equivalent experimental setup as was used for the CIP release studies. Despite the differences in polarities of CIP and RIF, an antibiotic loading levels of 5.09 and 5.46 % (w/w),
respectively, did not alter the hexagonal phase structure of MO-LCNPs. Although, a similar 0.4 nm expansion in the water channels diameter occurred after loading the antibiotics compared to MO-LCNPs containing no drug [59]. The release of RIF was different to CIP due to RIF’s higher partition coefficient (clogP), which has been previously known to control drug release from LCNPs, as drug must first partition from the lipid bilayer into the water channels, before it is released by diffusion [64]. Generally, drug partitioning occurs faster than diffusion [65], where RIF’s release plateaued within 45 mins, compared to 2 h for CIP. However, as limited RIF was expected to partition from the lipid bilayer and more CIP could diffuse out of the LCNPs water channel, the rate of CIP’s release was twice that of Rif.[1]

For larger molecular weight, AL (35 kDa), complete release was also not achieved, with only 30% of AL being released after 8 h. The hydrophobic and hydrophilic regions of AL anchors it at the interface of the water channels and lipid bilayers [28]. Depending on the size of the molecule, the anchoring at the lipid bilayer interface restricts the diffusion of the macromolecules [66]. Bisset et al. [40] demonstrated the extent of drug release increased from 15% to 40% for 21 kDa and 4 kDa fluorescein isothiocyanate (FITC)-dextran, respectively, from bulk PHY-LC with a cubic phase structure (water channels diameter 6 nm). Then, upon switching from the cubic phase to the hexagonal phase structure, the release was halted for all molecular weights of FITC-dextran. While the hexagonal phase structure of MO-LCNPs containing no drug remained unchanged when AL was loaded, upon 1:5 dilution (as performed during in situ-sSAXS and release studies), the phase structure shifted towards the Im3m bicontinuous cubic phase, which explains the release profile of AL from MO-LCNPs. In comparison, the release of AL from PHY-LCNPs (20%) was still lower due to the smaller water channels of PHY-LCNP (3.1 nm) compared to 4.6 nm water channels of MO-LCNPs after dilution [33, 67]. In addition, the LCNPs (~200 nm) have a substantially higher surface area compared to the bulk LC containing FITC-dextran, which according to the Higuchi [62] (Equation 1), increases the diffusion-controlled drug release by 200,000 times compared to a single bulk LC unit with one exposed surface [64]. This further explains the increased rate in AL released from the MO-LCNP (0.09 mg/h$^{1/2}$), compared to previous studies with bulk MO-LC, where the rate of AL released was 3.06 x 10$^{-7}$ mg/h$^{1/2}$ [34].
4.3. LCNP digestion leading to triggered drug release

Despite the limited diffusion of AL and RIF from the MO-LCNPs, *Pseudomonas* lipase triggered their release (in separate systems), which correlated strongly with lipase-mediated digestion of MO-LCNP. This triggered release did not occur from non-digestible PHY-LCNPs, where the release of AL remained unchanged in the presence of bacterial lipase. The MO-LCNP digestion triggered released was also notably greater than observed in the previous reports with the bulk MO-LC [34]. This is related to the hydrolytic activity of the lipases that occurs at lipid-water interface, hydrolysing the ester functional group of the glycerides [41, 68, 69]. Due to the increase in surface area and change in geometry of the LCNP compared to the bulk, the lipase has greater potential to act at the lipid-water interface [70]. While the surface stabiliser, Pluronic F-127, may also reduce the lipid-water interface exposed and the activity of the lipases, this was not determined and all LCNP studied were formed with the surface stabiliser.

Given that CIP was completely released from MO-LCNP due to its small molecular size and hydrophilic properties, digestion of MO-LCNPs did not further increase the release. However, the intrinsic rate of CIP diffusion (1.57 mg/h\(^{1/2}\)) was less than the triggered release rates of AL and RIF, which were similar in magnitude (7.50 and 5.56 mg/h\(^{1/2}\), respectively). While it was not explored, bacterial lipase-mediated digestion may increase the rate of CIP released (if exposed to bacterial lipase immediately) as sSAXS demonstrated the digestion of MO-LCNP linearly increased the water channel dimensions, which is likely to permit a higher release rate of the hydrophilic compound. In the presence of *Pseudomonas* lipase, the bicontinuous Im3m cubic phase of MO-LCNPs swelled and became less prevalent in the system as AL’s release was triggered, suggesting that the triggered release is caused by the degradation of the LCNP liquid crystalline (cubic) structure. Meanwhile, a lamellar phase became the dominant phase in the system and was attributable to the removal of the produced fatty acids as calcium soaps [49, 50]. While RIF loaded MO-LCNPs were not measured by sSAXS in the diluted state, a similar shift to the cubic phase structure, as observed in AL containing MO-LCNP, is expected. This is due to the undiluted MO-LCNP being at a phase boundary of the tertiary phase diagram of MO-propylene glycol-water system [71]. Thus, the rapid release after the addition of *Pseudomonas* lipase is postulated to correspond to the swelling and disruption of the RIF MO-LCNP structure.
The swelling of the lattice parameter for the cubic structure was similar to previous reports by Warren et al. [48], in which MO-LCNPs were digested by pancreatic lipase within 20 mins. However, under lipolysis by *Pseudomonas* lipase it took 35 mins for the cubic phase to become undetectable by sSAXS. Lipases are not all equal in activity and can demonstrate regiospecificity for the glyceryl carbons (denoted as *sn*-1, 2, or 3) [72]. Despite similarities in the extent of MO-LCNP digestion from the model lipases explored in the present study, *Pseudomonas* lipase displayed different digestion kinetics to pancreatic lipase. This is explained by the *sn*-1 and 3 regiospecificity of pancreatic lipase [43, 73], which produced more 2-monoglycerides (2-MG) as the major lipolytic product of the 1, 2-diglycerides in the Myverol 18-99K mixture. In comparison, the *Pseudomonas* lipases had no regiospecificity and a larger proportion of 1-monoglycerides (1-MG) was present in the products, which would eventually be consumed, as represented in Figure 12. Thus, triggered release from *Pseudomonas* lipase would be expected to be greater due to complete digestion, dissimilar to pancreatic lipase.

![Figure 12. Schematic representation of the different digestion processes between pancreatic lipase (A. *sn*-1/3 regiospecific) and *Pseudomonas* lipase (B. non-regiospecific) and the different structures formed after digestion.](image)

The inability of pancreatic lipase to digest 2-MG is commensurate with the plateau observed in digestion after 15 mins, as highlighted by the slower second phase of digestion kinetics. The initial digestion by
Pancreatic lipase was faster (0.059 min\(^{-1}\)) than *Pseudomonas* lipase (0.029 min\(^{-1}\)) and resulted in a 1.5-fold higher free fatty acid (FFA):MG ratio. Salentinig *et al.* [74] demonstrated that at a specific pH, the FFA:MG ratio is indicative of the crystalline structure formed in lipid mixtures. Where at pH 7.5, the hexagonal phase was demonstrated at a 4:1 ratio of oleic acid to monoolein [75]. This is similar to the ratio of FFA:MG observed from H\(^{1}\)-NMR after 5 mins of pancreatic lipase digestion. Coincidentally, an inverse hexagonal phase arose 2 minutes post pancreatic lipase injection, as the bicontinuous cubic phase of MO-LCNP expectedly swelled and disappeared within 20 minutes [48]. Weak Bragg peaks corresponding to calcium soaps were eventually observed after 11 mins of pancreatic lipolysis, but not to the same intensity as the corresponding *Pseudomonas* lipolysis, which achieved a lower, 2:1 ratio of FFA:MG at 30 minutes, also favouring the lamellar phase observed [74].

Under physiological intestinal digestion and in previous studies examining the digestion of MO-LCNP, pancreatic lipase is facilitated by mixed micelles comprising of bile salts and phosphatidylcholine [48, 68]. This continually favours a hydrophilic surface at the lipid interface, assisting in the emulsification and removal of lipolytic products from the lipid interface, promoting the action of pancreatic lipase [68]. Preliminary data (not shown) demonstrated that the extent of MO-LCNP digestion increased by 10% upon the addition of mixed micelles. Bile salts/phosphatidylcholine mixed micelles are secreted by the liver to the small intestines and are not presumably available during a local bacterial (skin, sinus or lung) infection [76]. As the absence of the mixed micelles reduces the binding of the lipase, digestion products may position onto the lipid surface, particularly indigestible 2-MG, increasing the critical packing parameter that favours a hydrophobic interface and the hexagonal phase structure [77].

Despite PHY-LCNP not being digested and demonstrating no change in release of AL upon the addition of lipase, the inverse hexagonal phase structure changed to a bicontinuous cubic phase (Pn3m) upon lipase addition. This was considered to be due to the inability of the lipase to digest the lipid, thus in this case, the lipase can position at the lipid interface, which instead lowers the critical packing parameter of the lipid, forming the bicontinuous cubic phase. While the 1:5 dilution in the digestion media only slightly increased the lattice parameter of PHY-LCNP containing AL, the additional 10% dilution from the lipase addition could also be attributable to the change in phase structure observed. The pancreatic lipase used (pancreatin) further contains a cocktail of lipases, proteases, amylase, trypsin and ribonuclease [41]. While it was not examined in the present study, the proteases in porcine pancreatin will undoubtedly begin to digest the proportion of AL released. This could give rise to structural
differences through displacing AL from the aqueous-lipid interface, decreasing the steric hindrance and further allowing the lipase or digestion products to migrate to the interface, altering the phase structure of the lipid.

As previously mentioned, the hexagonal phase structure constricts the diffusion-controlled release from LCNPs due to smaller water channels obtained with a closed state geometry from the lipid structure [33]. Bisset et al. [40] demonstrated an “on-off” triggered release of FITC-dextran from the PHY-oleic acid LC structures through switching from the cubic to hexagonal phase structure. Due to the narrower water channels’ geometry of hexagonal phase structure, large molecular weight compounds, such as AL, experience a slower release relative to the corresponding cubic phase [33, 40]. While the release of AL was not quantified in the presence of pancreatic lipase, our previous study with the bulk MO-LC demonstrated an initial difference in release between pancreatic and Pseudomonas lipase. The release of AL rapidly spiked after the inclusion of pancreatic lipase (plus bile salt/phosphatidylcholine mixed micelles) that then slightly decreased, whereas Pseudomonas lipase resulted in a steady incline of AL released, obtaining an equivalent extent of the release after 48 h [34]. The change to the hexagonal phase from pancreatic lipase digestion of MO-LCNP, as observed from the sSAXS, is thus not favourable for triggered release of the antibiotics. However, whether such antibiotics would remain loaded in the hexagonal phase requires further exploration.

In the presence of both Gram-positive and Gram-negative bacterial strains, equivalent MO-LCNP digestion was observed to the (commercial) Pseudomonas species lipase. Nonetheless, the methods used did not determine the specific kinetics and lipolytic products derived from active bacterial digestion, thus depending on the regiospecificity of the bacterial lipases, different triggered release profiles may be expected. Staphylococcus aureus and some Pseudomonas species (i.e. P. cepacia and P. aeruginosa) lipases have previously demonstrated no regiospecificity, while different Pseudomonas species (i.e. P. fragi, P. fluorescens and P. geniaulata) lipases have proved to be sn-1 and-3 regiospecific, analogous to porcine pancreatin [69, 78-81]. Since non-regiospecific lipases lead to more complete digestion that was proportional to the triggered release of AL and RIF, they are preferable for MO-LCNPs triggered antibiotic delivery.

Former investigations into bacterial-triggered delivery systems have relied on the bacterial lipase to cleave an ester/amine bond between the antibiotic and delivery system to trigger the antibiotic release.
For example, a lipase-sensitive ester linkage between ciprofloxacin and a polyethylene glycol surface coating triggered the release of the antibiotic in the presence of *P. aeruginosa* [24]. This approach is highly specific to the chemical structure of the antibiotic and requires chemical modification of compounds, which may alter the compounds antimicrobial efficacy and safety profile. In contrast, the present study describes a trigger from the bacterial lipase digesting the delivery system itself, requiring no chemical modification of the loaded antimicrobials. MO-LCNPs are not an "on-off" bacterial-triggered delivery system, but rather respond to the presence of bacteria, increasing the rate and extent of antibiotic release. The triggered release of antimicrobials from MO digestion is predicted to be beneficial for topical delivery to bacterial infections by producing an effective bolus of the antimicrobial at the site of infection. As such, the experimental conditions of the release studies (isotonic buffered solution and 10 mL volume) were designed in this case to mimic a localised environment of a wound or lung. The concentration and activity of the bacterial lipase was consistent with previous reports in literature [82-84] and was also confirmed to have equivalent activity to *P. aeruginosa* strains PA01 and a clinical isolate. The observed bolus antimicrobial dose from the LCNPs would be particularly favourable for biofilm-related bacterial infections, where high concentrations are required due to the high antibiotic tolerance observed [8]. For antimicrobials that are concentration-dependent in their effect, including CIP and RIF, an immediate bolus release at or above the therapeutic concentration is also beneficial. Furthermore, limiting the release of the antibiotic in the absence of bacteria aims to reduce unnecessary exposure of antibiotics, preventing further development of antimicrobial resistance. Future studies will aim to address the clinical efficacy of the MO-LCNPs as a topical bacteria-triggered drug delivery system.
5. Conclusion

The release of antibiotics from MO-LCNPs can be triggered in the presence of bacteria, by lipase production digesting the lipid carrier vehicle. However, it is reliant on the molecular size and hydrophobicity (i.e. partition coefficient) of the loaded compound and the regiospecificity of the bacterial lipase. The triggered release was particularly effective from macromolecules (i.e. enzymes and proteins) and hydrophobic antimicrobial drugs, which are otherwise confined inside of the nanoparticles liquid crystalline structure, as observed with AL and RIF. In contrast, a low molecular weight hydrophilic compound (CIP) was rapidly released from MO-LCNPs from the moment of incorporation into buffered physiological media. The digestion of the MO-LCNP was more favourable by non-regiospecific lipases due to the single-phase digestion kinetics of MO that swelled and degraded the bicontinuous cubic structure, proportional to the release of AL and RIF. Conversely, sn-1/3 regiospecific lipase’s two-phase digestion kinetics resulted in faster removal of the bicontinuous cubic phase yet an undesirable rising inverse hexagonal phase structure. Thus, MO-LCNP is a bacterial-triggered release system recommended for topical bacterial infections where non-regiospecific lipases are produced, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and for delivering macromolecular and hydrophobic antibiotics.

Acknowledgements

This research is supported by the Australian Government and the Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology. The SAXS studies were conducted on the SAXS/WAXS beamline at the Australian Synchrotron. CRT is a recipient of a Research Training Program Scholarship. AJC is the recipient of an Australian Research Council Discovery Early Career Research Award (DE190100531) funded by the Australian Government. NT is supported by a Mid-Career Fellowship from the Hospital Research Foundation.

Declarations of interest

None

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