Antibiofilm Potential of Meropenem-Loaded Poly(ɛ-Caprolactone) Nanoparticles Against Klebsiella pneumoniae

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ABSTRACT

Colonisation of Klebsiella pneumoniae (K. pneumoniae) biofilms on biotic and abiotic surfaces are difficult to treat with traditional methods, prompting investigations on novel treatment strategies. Drug delivery systems show potential for use in biofilm treatment and poly(ɛ-caprolactone) is a polymer emerging as an effective carrier in drug release technology. This study is aimed to synthesise polymeric nanoparticles incorporating meropenem, for investigation against K. pneumoniae biofilms and as a coating over central venous catheters. Meropenem-loaded poly(ɛ-caprolactone) (PCL) nanoparticles were prepared using a modified nanoprecipitation method. Their physical properties and drug incorporation activities were characterised. Planktonic cells and biofilms of K. pneumoniae were treated with meropenem at concentrations corresponding to release profiles of the PCL nanoparticles. Central venous catheter pieces were coated with the nanoparticles and evaluated against K. pneumoniae. Meropenem nanoparticles had negatively charged surfaces and measured 170 - 330 nm. Transmission Electron Microscopy and X-ray diffraction analysis demonstrated spherical and crystalline nanoparticles respectively. The nanoparticles had entrapment efficiencies of 55 - 75% and drug loading percentages of 3 - 4%. In our bioassays, meropenem was released from the PCL nanoparticles at varied concentrations for 7 days. Released meropenem was 100% effective against planktonic cells of K. pneumoniae whilst concentrations higher than 28.6 mg/L were effective against biofilms of the same bacterium. Catheters coated with meropenem-loaded nanoparticles inhibited bacterial growth for 24 hours. As such, we conclude that meropenem-loaded PCL nanoparticles are effective in killing K. pneumoniae planktonic cells and show antibiofilm potential against the bacterium.

Keywords: Meropenem; poly(ɛ-caprolactone) nanoparticles; Klebsiella pneumoniae; biofilms; central venous catheter.

INTRODUCTION

Klebsiella pneumoniae (K. pneumoniae) is a slime producing Gram-negative bacterium frequently associated with community infections and is one of the leading causes of hospital acquired infections in severely ill patients1-4. K. pneumoniae is commonly isolated from biofilms formed on central venous catheters (CVCs) and from patients with intensive care unit (ICU) related infections5-7. Biofilm colonisation and generation of antibiotic-resistant strains have collectively contributed to the complexity of treating Klebsiella infections3. Notably, some antimicrobials are characterised by their poor pharmacokinetics and pharmaceutical properties. The development of drug delivery systems (DDS) has helped reduce these issues with antimicrobials, by acting as reservoirs for the therapeutic agent. With DDS, the drug entity is protected from external factors and the developer is able to tailor the drug release rates to be time and target specific, leading to easier dosing regimens and increased patient comfort and compliance8. As a type of DDS, polymeric nanoparticles have shown to enhance the physicochemical stability of the incorporated drug, increase drug penetration into cells and tissues and protect the drug from enzymatic activities, all of which, help increase drug bioavailability, efficacy and reduce possible toxic effects9,10. In antimicrobial chemotherapy, polymer-based vehicles have been investigated for aerosolised delivery of antibiotics to the lung11 and delivery of ampicillin, gentamicin, ciprofloxacin, penicillin, rifampicin, amphotericin and clarithromycin to target infections involving intracellular organisms12. The bactericidal activity of some antibiotics towards resistant bacteria was found to be enhanced; where antibiotic-tagged nanoparticles were able to increase the concentration of antibiotics at the site of bacterium-antibiotic interaction and facilitate antibiotic binding to cell surface amino acid residues in nonspecific multivalent ways13. Hence, drug loaded polymeric nanoparticles show potential in the treatment and eradication of pathogens, including pathogens commonly associated with biofilm formation and medical device colonisation, such as K. pneumoniae. Drug loaded nanoparticles may also improve the response of this bacteria towards meropenem, an

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antibiotic commonly used against it. Meropenem belongs to a class of antibiotics known as the Carbapenems and is often used as first-line treatment for drug resistant Gram-negative organisms\(^4\). It is one of the antibiotics that have the most consistent activity against extended spectrum beta-lactamase-(ESBL) producing organisms, due to its stability against hydrolysis by ESBLs \textit{in vitro}\(^4\). Meropenem exerts its action by readily penetrating bacterial cells and interfering with the synthesis of vital cell wall components, which eventually leads to bacterial cell death\(^5\). Poly(\(\varepsilon\)-caprolactone) is a biodegradable synthetic polyester polymer, with good hydrophobicity, excellent tissue compatibility and has existing regulatory approval\(^6\). We incorporated meropenem into a poly(\(\varepsilon\)-caprolactone) nanoparticulate drug delivery system and evaluated its potential against \textit{K. pneumoniae} as free living cells and biofilms. The nanoparticles were then coated over central venous catheters and evaluated against the bacterium to address the prevention of biofilm formation over central venous catheters and other invasive medical devices.

**MATERIALS AND METHODS**

**Nanoparticle Synthesis**

Meropenem trihydrate (CAS#119478-56-7) was purchased from PI Chemicals, (Shanghai, China). Poly(\(\varepsilon\)-caprolactone) polymer (Dow Chemicals, USA) was a gift from local suppliers. The content of each nanoparticle formulation is shown in Table 1. It is to be noted that 13 formulations were formulated using different recipes; from which after further evaluation (reported in following sections), four best formulations were selected for discussion and data reported in this paper. Meropenem-loaded nanoparticles were synthesised with a nanoprecipitation method modified from previously reported procedures\(^7\),\(^8\). Briefly, meropenem was dissolved in methanol along with lecithin (Phosphatidylcholine of soybean source, Sigma-Aldrich, USA) to form the drug phase. PCL was dissolved in acetone under ultra-sonication to create the polymer phase. Pluronic F-68 (Sigma-Aldrich, USA) was solubilised in water to make up the aqueous surfactant phase. Under stirring, the drug phase was added drop wise to the polymer phase followed by the drug-polymer phase to the surfactant phase. The resulting formulations were then subjected to high-speed homogenization for 1 minute at 10,000 rpm and high-pressure homogenization at 1,000 Bars for 3 cycles. The suspensions were then subjected to evaporation under pressure to eliminate the solvents. D-mannitol at 20% of the polymer weight (w/w) was added to the formulations as a cryoprotectant before freezing and freeze-drying. Empty (placebo) nanoparticles were synthesised in a similar manner as the drug loaded nanoparticles but with meropenem being omitted from the preparation.

**Nanoparticle Characterisation**

**Light scattering analysis**

Aqueously suspended nanoparticles were characterised with dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments, UK) for their size in nanometers (nm) and surface charge as the zeta-potential in millivolts (mV).

**Transmission Electron Microscopy (TEM) imaging and X-Ray Diffraction (XRD) analysis**

TEM imaging of the nanoparticle morphology were performed at the University Technology of MARA (Faculty of Pharmacy) with a transmission electron microscope (200 KV, FEI Tecnai G2 20S TWIN Model, FEI, USA). The crystallinity of the drug loaded nanoparticles, empty nanoparticles, polymer and pure drug were evaluated with an X-ray Diffractometer (X’pert Pro Panalytical, Philips, Netherlands) at the diffraction angle range of 4 to 90° (\(\theta\), theta) at the Department of Physics, University Putra Malaysia.

**Entrapment efficiency and drug loading determination**

Nanoparticles were suspended in water, vortexed and centrifuged at 10,000 rpm for 10 minutes. Next, 20 ul of the collected supernatant were analysed with HPLC using a method modified from literature\(^9\) and validated. The method utilised 80% (v/v) potassium-dihydrogen phosphate (KH\(_2\)PO\(_4\)) buffer (0.03 M adjusted to pH 3 - 5 with 85% orthophosphoric acid and combined with 0.1%...
Table 2: Size (Z-Average), surface charge, entrapment efficiency (EE) and drug loading (DL) of the meropenem-loaded nanoparticles.

<table>
<thead>
<tr>
<th>Formula-tion (n=3)</th>
<th>Size (nm) ± SD</th>
<th>Zeta Potential (mV) ± SD</th>
<th>EE (%) ± SD</th>
<th>DL (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>210.50 ± 12.83</td>
<td>-33.00 ± 2.30</td>
<td>73.43 ± 16.06</td>
<td>3.19 ± 0.70</td>
</tr>
<tr>
<td>F2</td>
<td>303.42 ± 156.00</td>
<td>-29.50 ± 4.71</td>
<td>55.30 ± 17.89*</td>
<td>4.61 ± 1.49*</td>
</tr>
<tr>
<td>F3</td>
<td>327.57 ± 20.00</td>
<td>-34.88 ± 6.60</td>
<td>70.49 ± 21.73*</td>
<td>3.00 ± 0.92*</td>
</tr>
<tr>
<td>F4</td>
<td>171.13 ± 7.81</td>
<td>-41.75 ± 2.11</td>
<td>75.11 ± 8.70</td>
<td>3.13 ± 0.36</td>
</tr>
</tbody>
</table>

SD is the standard deviation as determined from n=6, * indicates p<0.05.

Figure 2: The combined XRD spectra of the meropenem trihydrate drug substance, poly (ε-caprolactone) polymer and the formulated meropenem-loaded nanoparticles.

Figure 3: In vitro drug release profile of the four meropenem-loaded nanoparticles (n=6) at ten time points, for 7 days.
(v/v) triethylamine and 20% (v/v) acetonitrile at a flow rate of 1 mL/min under a UV wavelength of 298 nm. Encapsulation efficiency (%) was calculated as the ratio between amount of drug in the nanoparticles and amount of drug used in the preparation. Drug loading (%) was determined as the ratio between the amount of drug in the nanoparticles and amount (weight) of nanoparticles.

**In vitro drug release evaluation**

In *vitro* drug release studies were conducted using a modified method reported in the literature\(^\text{18,20}\). Briefly, aliquots of nanoparticle suspensions in phosphate buffered saline (PBS) solution (pH ± 7.4) were incubated at 37°C, with agitation (80 rpm) for 7 days. At ten pre-determined time points, assigned samples were collected, centrifuged at 10,000 rpm for 5 minutes and the supernatant analysed with HPLC (operated at the validated method). All tests were performed using triplicate nanoparticle formulations and repeated.

**Microbiological Studies**

**Biofilm cultivation and evaluation**

*Klebsiella pneumoniae* (ATCC 13883) was purchased from the American Type Culture Collection (ATCC) and grown on tryptic soy agar (TSA) and cultured in tryptic soy broth (TSB). Biofilms for bioassays were cultivated *in vitro* using a previously reported static biofilm assay, modified\(^\text{21,22}\) and optimised. Briefly, *K. pneumoniae* in TSB (10\(^6\) CFU/mL) was pipetted into 96-well microtitre plates (non-surface treated) and incubated at 37°C for 24 hours. Control wells consisted of non-inoculated TSB. After 24 hours, broth suspensions were removed, the plates air dried, the formed biofilms fixed with methanol for 15 minutes, stained with 0.1% crystal violet solution for 15 minutes and washed thrice. Next, the biofilms were treated with 30% (v/v) acetic acid aqueous solution for 5 minutes. The solubilised dye solutions were analysed at 570 nm with a microplate reader. Biofilm forming ability was categorised using a previously reported biofilm grading system\(^\text{21}\).

**Minimal Inhibitory Concentration (MIC) determinations**

MIC determination for planktonic cells was conducted based on guidelines of the British Society for Antimicrobial Chemotherapy (BSAC)\(^\text{23}\). Briefly, meropenem solutions at 0.015 - 4 mg/L and bacterial cells in Iso-Sensi Test broth (ISTB) were incubated at 37°C for ± 18 hours. The wells with the lowest drug concentration without visible turbidity (similar to sterility control) were determined as the MIC. The MICs of meropenem against cells isolated from pre-formed biofilms were determined using a method modified from the literature\(^\text{25}\). Briefly, biofilms were cultivated as previously mentioned and incubated. Then the suspensions containing planktonic and loosely attached cells were removed. Next, the biofilms were dislodged with a cotton swab, transferred onto TSA and incubated. Bacteria from the resulting colonies were then used for the MIC determinations.

**Antibiofilm assay**

Meropenem prepared at concentrations corresponding to the release profile of the nanoparticles were tested against planktonic cells of *K. pneumoniae*. These tests were undertaken similarly to the MIC determinations, where wells with clear broth were determined as the effective inhibitory concentrations. These drug concentrations were then tested against preformed biofilms using procedures modified from the literature\(^\text{26}\). Biofilms were cultivated, broth solutions pipetted out, fresh broth and drug solutions added and the titre plates incubated at 37°C for ± 24 hours. Formed biofilms were dislodged with sterile wooden sticks and suspended in fresh broth, diluted, plated onto TSA plates and incubated for ± 18 hours. Wells containing effective antibiofilm drug concentrations resulted in agar plates with no colony growth. All tests were replicated and repeated.

**Evaluation of nanoparticle coated catheters**

The formulation of nanoparticles with the highest and most stable drug release rate, F2, was selected for further evaluation, as a coating system over central venous catheters. Central venous catheters (single lumen; Certofix Mono S220, B. Braun, Germany) were cut into 1 cm pieces and dip coated thrice with a nanoparticle containing solution (with drying intervals). The coating solution consisted of 1% w/v ethyl cellulose (dissolved in isopropyl alcohol), 4% v/v triacetin (dissolved in the former solution) and F2 nanoparticles (20 mg in 1 mL of coating solution). Antimicrobial susceptibility tests (AST) according to BSAC guidelines (data not shown) were conducted with the chemicals included in the coating solution and were found to not exhibit any inhibitory activity against the growth of *K. pneumoniae* on Iso-Sensi Test Agar (ISTA). The coated catheter pieces were placed in 96-well plates with bacterial suspensions (10\(^6\) CFU/mL) and incubated at 37°C under 150 rpm agitation for ± 24 hours. Control catheters were those coated with placebo nanoparticles, coated with the coating solution alone and uncoated catheters. Post incubation, the turbidity of the wells was visually inspected and compared to controls.

**Statistical Analysis**

Quantitative data are expressed as ± mean standard deviation (± SD). The presence of a significant change or difference was statistically analysed using the Paired-sample Student t-test (SPSS® software Version 18), where p values of less than 0.05 (p<0.05) were considered as significant (as indicated by *).

**RESULTS AND DISCUSSION**

The aggregation of bacterial cells after contact with surfaces *in vivo* promotes the formation of a community of bacteria protected from immune attack and conventional antibiotic treatment. These aggregated communities; collectively termed as biofilms, create complications in hospitalised patients. Novel methods to eradicate clinical biofilms without increasing drug dosing and patient discomfort is under active research. Drug delivery agents have the potential to overcome this technical impasse. A broad-spectrum antibiotic incorporated into a polymeric nanoparticle system was synthesised and investigated for its antibiofilm potential against the commonly isolated bacterium, *K. pneumoniae*.

**Nanoparticle synthesis and characterisation**

In formulating meropenem-loaded PCL nanoparticles, the modified nanoprecipitation method repeatedly synthesised...
nanoparticles with characteristics suitable for use as a drug delivery system. The produced nanoparticles were free flowing, light and reconstituted well in liquids; suggesting minimal aggregation. In the nanoprecipitation method, nanoparticles form by solidification of the polymer (by cooling and/or controlled temperature drop) or by solidification of the polymer (by solidification of the polymer). TEM analysis of the nanoparticle formulations revealed nanoparticles with diameters ranging from 50 nm to 120 nm (Figure 1). Likewise, dynamic light scattering analysis showed that the nanoparticles had a hydrodynamic diameter of approximately 150 nm (Table 2), which have been deemed suitable for the delivery of drugs in vivo. TEM imagery revealed spherical meropenem-loaded nanoparticles and of approximately homogenous morphology. Equal surface area of nanoparticles has been reported to favour even polymer degradation and/or controlled independent diffusion of the incorporated antimicrobial to the surrounding vicinity. XRD analysis suggested incorporation of meropenem into the formed nanoparticles, which were generally crystalline in nature as indicated by the intense peaks (Figure 2); similar to the nature of meropenem and poly(ε-caprolactone). It has been suggested that nanoparticles in the crystalline state have better stability than when in the amorphous state. The nanoparticles demonstrated high entrapment efficiency percentages of 55 to 75% and drug loading percentages of 3 to over 4.5%, in correlation to the meropenem incorporated in the respective formulations (shown in Table 2). This can be attributed to the ingredients used at particular amounts and the formulating technique, which yielded nanoparticles with satisfactory drug loading and entrapment. The drug release profile obtained from the in vitro release study of the nanoparticles show a similar drug release trend in all four formulations. Drug release was generally irregular in all formulations prior to and on the 12 hour mark, followed by a more stable and increasing release trend after 12 hours to Day 7. This irregular drug release is thought to occur due to the rotary incubationary process, which caused more nanoparticles to disaggregate over time and release their drug more controllably. Homogenous distribution of the nanoparticles (in the suspending medium) were achieved only after 12 hours of a fixed rotary movement, then saw more stabilised drug release rates. Delayed but stable release of meropenem was previously reported on meropenem-loaded poly(lactic co-glycolic acid) (PLGA) and poly(lactic acid) (PLA) polymeric nanoparticles. Additionally, Formulation 2 (F2) had the highest drug release concentration over the period of 7 days, attributable to its higher drug load. High drug release rates (like those seen with F2), have been demonstrated with PCL nanoparticles, due to its crystalline matrix and low glass transition temperature ($T_g$). The slight decreases in drug release concentrations between days 5 and 7 are suggestive that limited amounts of the drug loaded in the nanoparticles were beginning to deplete. Further manipulation of parameters involved in nanoparticle preparation to increase drug load, reduce aggregation and stabilise drug release, would collectively help improve the

### Table 3: Response (susceptibility) of *Klebsiella pneumoniae* biofilms towards meropenem concentrations as released from the drug loaded nanoparticles.

<table>
<thead>
<tr>
<th>Time (t)</th>
<th>Nanoparticle, Drug Concentration (mg/L) ± SD (for n=6) and</th>
<th>Colony Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1 DC</td>
<td>C</td>
</tr>
<tr>
<td>30 mins</td>
<td>6.6 ± 0.4</td>
<td>P</td>
</tr>
<tr>
<td>1 hour</td>
<td>12.0 ± 3.0</td>
<td>P</td>
</tr>
<tr>
<td>3 hours</td>
<td>16.0 ± 0.4</td>
<td>P</td>
</tr>
<tr>
<td>6 hours</td>
<td>15.0 ± 0.2</td>
<td>P</td>
</tr>
<tr>
<td>12 hours</td>
<td>19.2 ± 1.9</td>
<td>P</td>
</tr>
<tr>
<td>24 hours</td>
<td>8.1 ± 3.9</td>
<td>P</td>
</tr>
<tr>
<td>48 hours</td>
<td>10.9 ± 1.4</td>
<td>P</td>
</tr>
<tr>
<td>Day 3</td>
<td>28.6 ± 10.8</td>
<td>NP</td>
</tr>
<tr>
<td>Day 5</td>
<td>53.6 ± 2.7</td>
<td>NP</td>
</tr>
<tr>
<td>Day 7</td>
<td>51.0 ± 16.5</td>
<td>NP</td>
</tr>
</tbody>
</table>

DC is drug concentration, C is colonies, P is present and NP is not present.
drug delivering properties of these polymeric nanoparticles.

**Microbiological studies**

*K. pneumoniae* (ATCC 13883) formed biofilms at the bottom of the microtitre plate wells as previously reported\(^5\). Crystal violet staining of the formed biofilms categorise this strain of *K. pneumoniae* as a strong biofilm former. This biofilm forming ability is conferred by production of fimbriae and types 1 and type 3 pilus that help mediate bacterial attachment to and colonisation of surfaces\(^3\). In addition, the mucoid texture of the biofilms produced by *K. pneumoniae* is a characteristic attributable to its slime producing ability\(^35\). Planktonic cells of *K. pneumoniae* were found to be susceptible to meropenem at a concentration of 0.125 mg/L. The MIC of meropenem against cells isolated from the cultivated biofilms was 2 mg/L; 16 times the concentration required to inhibit the planktonic cells. Previously, drug concentrations capable of inhibiting biofilm cells have been reported to be many times higher than the concentrations required for their planktonic counterparts\(^25\). The increased drug resistance of *K. pneumoniae* biofilms has been specifically correlated to thickening of its membrane and capsule and packing of the inner and outer layers of its cell wall with fine and dense fibres\(^34\). Planktonic cells were susceptible to all drug concentrations released from the four nanoparticle preparations (colony growth was nil on all TSA plates). The complete inhibition of planktonic cells was probably caused by the high meropenem concentration, with the lowest concentration being as high as 6.6 ± 0.4 mg/L; which exceeded the MIC required for the planktonic cells. In the clinical setting, targeting planktonic cells and eliminating them prevents initial adhesion and biofilm formation, which is more effective than having to eliminate whole or matured biofilms\(^35\). Furthermore, taking into account that mature biofilms release bacterial cells into the circulation that are free to colonise new niches in the body\(^36\), these cells become important targets for antibiotics. The potential for biofilm metastasis in infected patients makes it vital for DDS to have activity against free-living bacterial cells at the right concentrations. The collective ability of the drug loaded nanoparticles in disrupting the biofilm and inhibiting planktonic cells, may help eradicate the infection faster and better than conventional antibiotic dosage forms. Meropenem concentrations of 6.6 ± 0.4 mg/L to 20.3 ± 5.2 mg/L, as released from the nanoparticles, were not effective against pre-formed biofilms of *K. pneumoniae*, although these concentrations were more than three to 10 times higher than that of the MIC determined for isolated biofilm cells, at 2 mg/L. This is probably due to the components of the external biofilm structure, such as the lipopolysaccharide (LPS) commonly produced by Gram-negative bacteria\(^34\), preventing drug penetration. Released drug concentrations of 28.6 ± 10.8 mg/L and higher were however, disruptive for the biofilms, leaving no surviving cells to multiply and form colonies on the agar plates (data shown in Table 3). To our knowledge, this is the first time meropenem-loaded PCL nanoparticles were coated over polyurethane central venous catheter pieces to target *K. pneumoniae* biofilms in *vitro*. One of our nanoparticle formulations, F2 as a coating over the catheter pieces released effective concentrations of meropenem to inhibit *K. pneumoniae* for up to 24 hours, producing the clear suspensions (see F2C in Figure 4). This comparison was made to untreated blank (placebo) nanoparticles coated, coating solution without nanoparticles coated and uncoated catheters (see F0C, CSC and UC in Figure 4). As visible in Figure 4, F2 coated catheters show no bacterial growth. The 24-hour time period after device insertion is critical, as initial attachment of bacteria to surfaces occurs in less than 24 hours\(^38\). Antibiofilm approaches usually target this stage of biofilm formation, as the colonising bacteria are still susceptible to antibiotics and thus warrant successful treatment\(^39\). Moreover, since twice the concentration of meropenem was released from the nanoparticles between 24 hours and 7 days (as compared to the drug concentration released within 24 hours), it is postulated that the nanoparticle coated catheters would be able to continuously prevent bacterial growth for this time frame, hence preventing adhesion and biofilm formation. Further increasing drug release rates, reducing particulate aggregation and improving catheter coating methods could warrant additional studies on antibiofilm approaches utilising meropenem-loaded polymeric nanoparticles.

**CONCLUSION**

The formulated meropenem-loaded poly(l-caprolactone) nanoparticles had physical characteristics suitable for an efficient drug delivery system targeting bacteria. The nanoparticles released their encapsulated drug at concentrations effective against planktonic cells and biofilms of *K. pneumoniae* for up to 7 days in *vitro*. Additionally, the central venous catheters successfully coated with the nanoparticles were able to inhibit bacterial growth for up to 24 hours. Further improvements in the preparatory techniques could potentiate antibacterial drug-loaded nanoparticles as a more promising tool for the prevention and treatment of biofilm related infections. The incorporation of other novel and efficacious antibacterials and natural compounds in nanoparticles could provide alternative regimens in targeted biofilm therapy and improve clinical outcomes.

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