Antimicrobial and nanoparticle penetration and killing in Infectious biofilms
Rozenbaum, René Theodoor

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Chapter 6

Antimicrobial synergy of monolaurin lipid nanocapsules with adsorbed antimicrobial peptides against *Staphylococcus aureus* biofilms *in vitro* is absent *in vivo*

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Abstract

Bacterial infections are mostly due to bacteria in their biofilm-mode of growth, while penetrability of antimicrobials into infectious biofilms and increasing antibiotic resistance hamper infection treatment. In vitro, monolaurin lipid nanocapsules (ML-LNCs) carrying adsorbed antimicrobial peptides (AMPs) displayed synergistic efficacy against planktonic Staphylococcus aureus, but it has not been demonstrated, neither in vitro nor in vivo, that such ML-LNCs penetrate into infectious S. aureus biofilms and maintain synergy with AMPs. This study investigates the release mechanism of AMPs from ML-LNCs and possible antimicrobial synergy of ML-LNCs with the AMPs DPK-060 and LL-37 against S. aureus biofilms in vitro and in a therapeutic, murine, infected wound healing model. Zeta potentials demonstrated that AMP release from ML-LNCs was controlled by the AMP concentration in suspension. Both AMPs demonstrated no antimicrobial efficacy against four staphylococcal strains in a planktonic mode, while a checkerboard assay showed synergistic antimicrobial efficacy when ML-LNCs and DPK-060 were combined, but not for combinations of ML-LNCs and LL-37. Similar effects were seen for growth reduction of staphylococcal biofilms, with antimicrobial synergy persisting only for ML-LNCs at the highest level of DPK-060 or LL-37 adsorption. Healing of wounds infected with bioluminescent S. aureus Xen36, treated with ML-LNCs alone, was faster when treated with PBS, while AMPs alone did not yield faster wound healing than PBS. Faster, synergistic wound healing due to ML-LNCs with adsorbed DPK-060, was absent in vivo. Summarizing, antimicrobial synergy of ML-LNCs with adsorbed AMPs as seen in vitro, is absent in in vivo healing of infected wounds, likely because host AMPs adapted the synergistic role of the AMPs added. Thus, conclusions regarding synergistic antimicrobial efficacy, should not be drawn from planktonic data, while even in vitro biofilm data bear little relevance for the in vivo situation.
Introduction

Bacterial infections have plagued mankind from the onset of human existence. Early infection control was based on the use of natural products and metals, such as honey, garlic, tree bark extracts, herbs, mercury or silver. The discovery of penicillin in the late 1930ties was considered a major breakthrough in infection control. Currently, it is painfully realized that the discoveries of penicillin and many other antibiotics are insufficient to beat infection.

A major obstacle in the control of bacterial infections is, that more than 60% of all bacterial infections are not due to planktonic bacteria in suspension, but due to bacteria in their biofilm mode of growth\(^1\). In their biofilm mode of growth, bacteria adhere either to other bacterial or mammalian cell surfaces, or the surfaces of bone, teeth or biomaterial implants, and grow while embedding themselves in a self-produced matrix of extracellular polymeric substances (EPS) that protect biofilm inhabitants against the host immune system and antimicrobial treatment\(^2\). The protection offered by the biofilm mode of bacterial growth was already described by Antonie van Leeuwenhoek in the late 17\(^{th}\) century, observing that the vinegar he used to clean his teeth, killed only those bacteria that were on the outside of what we now call “the biofilm”\(^3\). Ever since, the penetrability of infectious biofilms by antimicrobials has hampered effective treatment of infection\(^4\), that is not taken into account when antimicrobials are solely evaluated against planktonic organisms. Nanotechnology offers a number of promising ways to prepare antimicrobials and antimicrobial carriers that break the biofilm barrier and penetrate over considerably larger distances into a biofilm with associated killing of its inhabitants, than conventional antimicrobials on their own\(^5\).

A second major obstacle in the control of bacterial infections, is the continuously increasing occurrence of bacterial strains and species that have become intrinsically resistant to antibiotics, due to their overuse and indiscriminate misuse\(^6\). Antimicrobial peptides (AMPs) are part of the innate immune system and have emerged as novel antimicrobials to treat bacterial infections\(^7\). AMPs are positively charged, amphiphilic molecules that kill bacteria through membrane disruption and pore formation\(^7\). The AMPs positive charge is on the one hand pivotal for antimicrobial action\(^7\), but on the other hand a drawback in their penetration into biofilms, consisting of negatively charged bacteria and EPS\(^8\). Also, many AMPs are subject to hydrolytic and proteolytic breakdown\(^7\). Importantly, though around since the onset of human existence, no naturally developing resistance of bacteria against AMPs has yet been reported, although long-term culturing in presence of AMPs in vitro may create AMP resistance, often associated with a decreased fitness of the mutant\(^7\). Collectively with the AMP mode of action, the natural development of bacterial resistance against AMPs seems improbable\(^9\). However, others anticipate bacterial strategies of resistance to AMPs, especially when used large-scale in clinical infections\(^10,11\).
Both obstacles in the control of bacterial biofilm-associated infections, i.e. biofilm penetrability and intrinsic antibiotic resistance, may be solved by packaging AMPs in suitable nanocarriers\textsuperscript{12}. Various drugs have been successfully encapsulated to eradicate bacterial biofilms \textit{in vitro} and \textit{in vivo}\textsuperscript{5,13}. Lipid nanocapsules (LNCs) are promising, versatile carriers for drugs because they allow drug loading in the core\textsuperscript{14} as well as drug adsorption to their shell\textsuperscript{15}. \textit{In vitro}, AMPs derived from plectasin adsorbed to monolaurin lipid nanocapsules (ML-LNCs), displayed synergistic antimicrobial activity against planktonic \textit{Staphylococcus aureus} of the AMPs and monolaurin, a known antimicrobial surfactant\textsuperscript{15}, although to our knowledge their efficacy against infectious biofilms \textit{in vivo} has never been demonstrated. Whereas bacterial resistance against AMPs seems unlikely to develop under natural conditions, resistance of \textit{S. aureus} against monolaurin could not be achieved by yearlong \textit{in vitro} passage under minimal inhibitory concentrations (MIC) of monolaurin, suggesting resistance of \textit{S. aureus} against monolaurin in combination with AMPs is highly unlikely to develop\textsuperscript{5}. Since \textit{S. aureus} is an opportunistic pathogen, often found in wound infections\textsuperscript{17–20} with the potential to develop resistance against multiple known antibiotics, ML-LNCs carrying adsorbed AMPs may be considered promising to treat \textit{S. aureus} infections, provided ML-LNCs penetrate into infectious \textit{S. aureus} biofilms and maintain their synergistic activity with AMPs both \textit{in vitro} and \textit{in vivo}.

Therefore, the first aim of this study was to determine the mechanism of controlled release of AMPs from ML-LNCs and to establish whether ML-LNCs with adsorbed, AMPs DPK-060 or LL-37 are able to penetrate \textit{S. aureus} biofilms \textit{in vitro}, while maintaining a possible synergistic antimicrobial efficacy with monolaurin. Secondly, this study aims to establish whether possible antimicrobial effects of ML-LNCs and synergistic effects with adsorbed antimicrobial peptides persist \textit{in vivo}. To this end, a therapeutic, murine, infected wound healing model was applied, using a bioluminescent \textit{S. aureus} strain and \textit{in vivo} bio-optical imaging.

**Materials and methods**

**Antimicrobial peptides**

DPK-060 (98.5% purity, molecular weight 2503 Da) was synthesized by Bachem AG (Bubendorf, Switzerland) and provided by Promore Pharma AB (Stockholm, Sweden), while LL-37 (94.7% purity, molecular weight 4491 Da) was synthesized and provided by PolyPeptide Laboratories (Limhamm, Sweden). DPK-060 is a 17-amino acid AMP derived from human endogenous kininogen, with a net charge of +7 at pH 7.0, while LL-37 is an AMP found in human immune cells with a net charge of +6 at pH 7.0.

**Preparation of ML-LNCs with adsorbed AMPs**

ML-LNCs were prepared at a concentration of 200 mg/ml, as previously described\textsuperscript{15}. Briefly, 0.3 g monolaurin (Sigma Aldrich, St. Louis, Missouri, United States), 0.77 g polyethylene glycol (15)-hydroxystearate (Solutol® HS15, kindly provided by BASF,
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Quantitation of non-adsorbed AMPs and AMPs adsorbed to ML-LNCs

Non-adsorbed AMPs were separated from AMPs adsorbed to ML-LNCs using a combined ultrafiltration-centrifugation technique\textsuperscript{22}. To this end, 2 ml of the suspension containing ML-LNCs and AMPs was added to an Amicon Ultra 15 centrifugal filter device with a 10 kDa molecular cut off (Merck, Darmstadt, Germany), and centrifuged (30 min, 4500g) for harvesting of the ML-LNCs with adsorbed AMPs in the filter device, and the non-adsorbed AMPs in the filtrate. The AMPs were extracted from the ML-LNCs suspended in PBS by addition of methanol and centrifuged (30 min, 16000 g) to extract the AMPs.

The amounts of adsorbed and non-adsorbed AMPs were analyzed using high-performance liquid chromatography (HPLC) with a SymmetryShield TM RP18 5 µm, 4.6 x 250 mm column (Waters, Milford, Massachusetts, USA)\textsuperscript{22}. In order to calibrate the HPLC, 20 µl of AMP standard solutions in distilled water (0.001-1 mg/ml) were injected into the column. The mobile phase was delivered in a gradient mode consisting of 0.1% trifluoroacetic acid in ultrapure water (eluent A) and 0.1% trifluoroacetic acid in acetonitrile (eluent B). The eluent flow rate was 1.0 ml/min and the gradient started at 20% of eluent B and was held for 25 min. The gradient was increased to 45% eluent B for 0.1 min and increased to 100% eluent B for 4.9 min. Then, eluent B was decreased to origin value of 20% in 0.1 min, and kept constant for 15 min. UV detection was performed at a wavelength of 200 nm. The AMP peaks had retention times of approximately 16 min for DPK-060 and 30 min for LL-37. Peak areas were plotted against the corresponding AMP concentration to obtain calibration graphs for both DPK-060 and LL-37 ($R^2 \geq 0.9999$). The quantification limits were 0.5 mg/ml for DPK-060 and 1 mg/ml for LL-37.

The AMP adsorption efficiency (AE) was expressed in percentage, calculated as

$$AE= \frac{(M_{AMP} - M_{NA})}{M_{AMP}} \times 100\%$$

where $M_{AMP}$ is the total mass of AMPs added, and $M_{NA}$ is the mass of the non-adsorbed AMPs.

Characterization of ML-LNCs: changes in diameters and zeta potentials during adsorption and desorption of AMPs

The diameters, polydispersity indices and zeta potentials of the ML-LNCs with and without adsorbed AMPs were determined in ultrapure water (ML-LNC concentration 3 mg/ml) using dynamic light scattering with 173 degrees backscatter detection. The electrophoretic mobilities, measured by laser Doppler velocimetry, were converted to zeta potentials using the Smoluchowski equation. Both measurements were conducted on a Zetasizer nano series Nano-ZS equipped with a 633 nm laser (Malvern Instruments,
Worcestershire United Kingdom). First, zeta potentials and hydrodynamic diameters of ML-LNCs (3 mg/ml) suspended in water with a concentration of 1 mg/ml AMP were measured as a function of time. Then, zeta potentials and hydrodynamic diameters were measured as a function of AMP concentration (0.01 to 1 mg/ml), allowing 5 min for equilibration to monitor AMP adsorption. Both experiments were performed at 25°C and 37°C. For three selected AMP concentrations (0.01, 0.1 and 1 mg/ml), desorption at 25°C was monitored by 2-fold diluting their concentration in either water or PBS for 30 min, after which zeta potentials and hydrodynamic diameters were measured again.

**Bacterial strains, culturing and harvesting**

Four staphylococcal strains were used in this study: *S. aureus* 0701A0095, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA-1696 (MRSA) (all infected wound isolates) and bioluminescent *S. aureus* Xen36 (PerkinElmer, Inc., Waltham, USA). Strains were cultured on blood agar plates, except for *S. aureus* Xen36, which was cultured on tryptone soya agar (Oxoid, Basingstoke, UK) with 200 µg/ml kanamycin. One colony was used to inoculate 10 ml of tryptone soya broth (TSB) and incubated aerobically at 37°C for 24 h. For culturing *S. aureus* Xen36, 200 µg/ml kanamycin was added to the TSB. Two ml of each culture was transferred to 40 ml of TSB and incubated again aerobically at 37°C for 16 h under rotary-shaking at 150 revolutions per min, after which the bacteria were harvested by centrifugation (5000 g, 5 min, 10°C). Bacterial pellets were washed two times with 10 ml sterile PBS and the pellet was finally resuspended in 10 ml PBS. Staphylococci were sonicated three times 10 s at 30 W in an ice-water bath to disrupt bacterial aggregates and suspended in TSB supplemented with 0.25% glucose (TSBg), to concentrations as used in the different experiments. Bacterial concentrations were determined by enumeration using a Bürker-Türk counting chamber.

**Minimal inhibitory (MIC) and bactericidal (MBC) concentrations of ML-LNCs with and without adsorbed AMPs**

For determination of the MICs the ML-LNCs (without adsorbed AMPs), DPK-060 and LL-37 were 2-fold serially diluted in demineralized water and 100 µl aliquots were pipetted into 96 wells plates. Hundred µl of bacterial suspension (2 × 10⁶ bacteria/ml) in TSBg (2 times concentrated) was added to each well, adding sterile demineralized water as a control and plates were incubated at 25°C or 37°C for 72 h or 24 h, respectively. The MIC was taken as the concentration at which no visible turbidity was observed. For MBC determination, 30 µl of each solution not showing visible turbidity was plated on tryptone soya agar plates, after which the plates were incubated at 37°C for 24 h. The MBC was taken as the lowest concentration that did not show bacterial growth.
Synergy between ML-LNCs and AMPs

Combinations of ML-LNCs (without adsorbed AMPs) and DPK-060 or LL-37 were evaluated for synergistic antimicrobial action in a checkerboard assay. Two-fold dilutions were made of the ML-LNCs (ranging from 2 to 0.156 mg/ml), LL-37 and DPK-060 (ranging from 8.192 to 0.128 mg/ml) in demineralized water. In a 96 wells plate, ML-LNCs and DPK-060 or LL-37 were combined, after which 100 µl of a bacterial suspension (2 × 10^6 bacteria/ml in TSBg) was added and incubated at 25°C or 37°C for 72 h or 24 h. The fractional inhibitory concentration (FIC) of ML-LNCs and DPK-060 or LL-37 were read out from all wells in which no visual bacterial growth was observed and used to calculate a FIC index according to

\[
\text{FIC index} = \frac{\text{MIC}_{\text{ML-LNC/AMP}}}{\text{MIC}_{\text{ML-LNC}}} + \frac{\text{MIC}_{\text{AMP/ML-LNC}}}{\text{MIC}_{\text{AMP}}} \quad (2)
\]

where MIC_{ML-LNC} is the MIC of ML-LNCs alone, MIC_{AMP} is the MIC of the AMP alone, MIC_{ML-LNC/AMP} is the MIC of ML-LNCs in presence of a given concentration of AMPs, and MIC_{AMP/ML-LNC} is the MIC of AMP in presence of a given concentration of with ML-LNCs. To achieve synergy, the FIC must be < 0.5 and the FIC index is conservatively presented as the minimal FIC index calculated.

Penetration and antimicrobial efficacy against staphylococcal biofilms of ML-LNCs with and without adsorbed AMPs

First, staphylococcal biofilms were grown in flat bottom 96 wells plates in TSBg. Twohundred µl bacterial suspension (1 × 10^6 bacteria/ml) in TSBg was pipetted in each well and incubated for 24 h at 37°C, after which growth medium was removed from the wells and the biofilm was washed once with 200 µl sterile PBS. Next, the ML-LNCs (without adsorbed AMPs), DPK-060 or LL-37 solutions, or ML-LNCs with adsorbed DPK-060 or LL-37 (200 µl) diluted in sterile PBS were added to the biofilm in the wells, and again incubated for 24 h at 37°C. To this end, two concentrations of ML-LNCs were used (50 mg/ml and 100 mg/ml), in combination with two concentrations of DPK-060 or LL-37 (0.5 mg/ml and 1 mg/ml). Sterile PBS was added as a control.

After 24 h, ML-LNCs suspensions and AMP solutions were removed and the biofilms washed once with 200 µl of sterile PBS, after which 200 µl of sterile PBS was added to the biofilms, and biofilms were resuspended by scraping the bottom of the well with pipet tips. A plastic sterile sticker was placed over the 96 wells plate to prevent inter-well contamination, and the plate was sonicated for 5 min in a sonication water bath to further detach the biofilm and break bacterial aggregates. Then, 10-fold serial dilutions were made, and two 10 µl drops of each dilution were spotted onto tryptone soya agar plates and incubated at 37°C. After 1 day, the numbers of colony forming units (CFU) were counted.
and expressed as CFU/ml. Antimicrobial efficacies of the ML-LNCs (with and without adsorbed AMPs) and AMP solutions were expressed as fold reduction in CFU/ml in comparison to PBS as a control.

**Efficacy of ML-LNCs with and without AMPs against a staphylococcal biofilm in a murine model**

Female BALB/c were obtained from Vital River Laboratory Animal Technology Co. (Beijing, China). All animals were housed in the on-site animal facility of Nankai University and all experiments were approved by the Institutional Animal Care and User Committee of Nankai University, Tianjin, China. To suppress the mice immune system, mice were injected with cyclophosphamide four days (150 mg/kg body weight) and one day (100 mg/kg body weight) prior to infection. One day prior to infection, hair on the back of the mice was removed from the wound sites with an electrical clipper and VEET hair removal cream (Reckitt Benckiser, Slough, UK).

Mice were brought under anesthesia by inhalation of 2.5% isoflurane and a 5 mm diameter burn wound was made in the middle of the upper back of each mouse by pressing a circular, hot metal rod (75°C) on the shaved skin for 1 s. Next, a dose of $1 \times 10^8$ bioluminescent *S. aureus* Xen36 was inoculated on the wound to create an infection. Infected wounds were covered with Parafilm®, held in position with surgical tape for 24 h. After 24 h the Parafilm® was removed and bioluminescent images were taken (day 0). Next, mice were randomly assigned into six groups of six animals for treatment of the wound with PBS (control), ML-LNCs in absence of adsorbed AMPs (100 mg/ml), DPK-060 (1 mg/ml), LL-37 (1 mg/ml), ML-LNCs (100 mg/ml) with adsorbed DPK-060 (1 mg/ml), and ML-LNCs (100 mg/ml) with adsorbed LL-37 (1 mg/ml). Treatment included pipetting of 20 µl of a treatment fluid directly onto the infected wound, after which a gauze (0.75 x 0.75 cm) was placed on top of the wound, and another 80 µl of treatment fluid was pipetted on the gauze. The gauze was kept on position with surgical tape and removed for bioluminescence imaging. Bioluminescence intensity and area were recorded using a bio-optical imaging system (IVIS, 30 s exposure time, medium binning, 1 F/Stop, Open Emission Filter). Bioluminescent images were taken after 24 h of treatment, after which a new treatment was applied for 4 consecutive days. After 4 days, the mice were sacrificed, the wound area was cut out for CFU determination.

**Statistical analysis**

Data was first evaluated for normality, after which statistical significance of the difference was determined using analysis of variance (ANOVA), followed by Tukey post-hoc or Dunn’s post-hoc test in Graphpad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA). For *in vitro* experiments, differences were considered significant at p < 0.05, while for *in vivo* experiments differences were considered significant at p < 0.1
Results

Results are divided in two sections: one section dealing with the controlled release of the AMPs from ML-LNCs and a second one demonstrating absence or presence of antimicrobial synergy of ML-LNCs with adsorbed AMPs against *S. aureus* biofilms *in vitro* and *in vivo*.

**Controlled release mechanism of AMPs from ML-LNCs**

**AMP adsorption efficiency to ML-LNCs**

The AMP adsorption efficiency (AE) for DPK-060 increased from 28 to 42% with increasing ML-LNC concentration and for LL-37 ranged between 72 to 77% with no significant effect of ML-LNC concentration (Table 1).

<table>
<thead>
<tr>
<th>AMP (mg/ml)</th>
<th>ML-LNCs (mg/ml)</th>
<th>AE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPK-060</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>50</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>42 ± 2*</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>41 ± 3*</td>
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<tr>
<td>LL-37</td>
<td></td>
<td></td>
</tr>
<tr>
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</tr>
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</tbody>
</table>

Effects of AMP adsorption and desorption on ML-LNCs zeta potentials

In order to more closely monitor the adsorption and desorption of AMPs by ML-LNCs, zeta potentials of the ML-LNCs were measured in presence of different concentrations of AMPs in water to monitor adsorption and subsequently to monitor desorption of AMPs from ML-LNCs upon dilution. First it was established for an AMP concentration of 1 mg/ml that an adsorption equilibrium in water developed for DPK-060 (Figure 2a) and LL-37 (Figure 2b). No zeta potential changes over time were observed, indicating rapid establishment of an adsorption equilibrium. More positive zeta potentials were found at 37°C than at 25°C. Subsequently, zeta potentials were measured as a function of AMP concentration while...
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**Table 1. Adsorption of AMPs on ML-LNCs expressed as adsorption efficiency AE (%). Data represent means ± SD. Experiments were repeated three times with n = 3. Asterisks represent statistically significant differences (p < 0.05) in comparison with other concentrations of the same AMP.**

<table>
<thead>
<tr>
<th>AMP</th>
<th>Concentration (mg/ml)</th>
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<th>AE (%)</th>
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**Effects of AMP adsorption and desorption on ML-LNCs diameters**

A similar series of experiments was carried out with respect to the hydrodynamic diameters of the ML-LNCs (Figure 3). Adsorption or desorption of AMPs from ML-LNCs did not significantly impact their hydrodynamic diameter. However, at 25°C ML-LNCs demonstrated a much smaller hydrodynamic diameter of 32 ± 0.8 nm with a polydispersity index of approximately 0.05 than at 37°C (hydrodynamic diameter 135 ± 20 nm, polydispersity index 0.35).
Figure 2. Zeta potentials of suspended ML-LNCs to monitor adsorption and desorption of AMPs from ML-LNCs.
(a) Zeta potentials of ML-LNCs in water (3 mg/ml) as a function of time in the presence of 1 mg/ml DPK-060 at 25°C and 37°C.
(b) Zeta potentials of ML-LNCs in water (3 mg/ml) as a function of time in the presence of 1 mg/ml LL-37 at 25°C and 37°C.
(c) Equilibrium zeta potentials in water of ML-LNCs as function of DPK-060 concentration in water at 25°C and 37°C.
(d) Equilibrium zeta potentials in water of ML-LNCs as function of LL-37 concentration in water at 25°C and 37°C.
(e) Zeta potentials in water of ML-LNCs after previous equilibration in presence of 0.01, 0.1 and 1 mg/ml DPK-060, and after 2-times dilution in water or PBS to monitor desorption of DPK-060 at 25°C.
(f) Zeta potentials in water of ML-LNCs after previous equilibration in presence of 0.01, 0.1 and 1 mg/ml LL-37, and after 2-times dilution in water or PBS to monitor desorption of LL-37 at 25°C.
All data are expressed as means ± S.D. over triplicate experiments.
Figure 2. Zeta potentials of suspended ML-LNCs to monitor adsorption and desorption of AMPs from ML-LNCs.
(a) Zeta potentials of ML-LNCs in water (3 mg/ml) as a function of time in the presence of 1 mg/ml DPK-060 at 25°C and 37°C.
(b) Zeta potentials of ML-LNCs in water (3 mg/ml) as a function of time in the presence of 1 mg/ml LL-37 at 25°C and 37°C.
(c) Equilibrium zeta potentials in water of ML-LNCs as function of DPK-060 concentration in water at 25°C and 37°C.
(d) Equilibrium zeta potentials in water of ML-LNCs as function of LL-37 concentration in water at 25°C and 37°C.
(e) Zeta potentials in water of ML-LNCs after previous equilibration in presence of 0.01, 0.1 and 1 mg/ml DPK-060, and after 2-times dilution in water or PBS after desorption of DPK-060 at 25°C.
(f) Zeta potentials in water of ML-LNCs after previous equilibration in presence of 0.01, 0.1 and 1 mg/ml LL-37, and zeta potentials in water of ML-LNCs after 2-times dilution in water or PBS after desorption of LL-37 at 25°C.
All data are expressed as means ± S.D. over triplicate experiments.

Figure 3. Hydrodynamic diameters of suspended ML-LNCs after adsorption and desorption of AMPs from ML-LNCs.
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(b) Hydrodynamic diameters of ML-LNCs in water (3 mg/ml) as a function of time in the presence of 1 mg/ml LL-37 at 25°C and 37°C.
(c) Equilibrium hydrodynamic diameters in water of ML-LNCs as function of DPK-060 concentration in water at 25°C and 37°C.
(d) Equilibrium hydrodynamic diameters in water of ML-LNCs as function of LL-37 concentration in water at 25°C and 37°C.
(e) Hydrodynamic diameters in water of ML-LNCs after previous equilibration in presence of 0.01, 0.1 and 1 mg/ml DPK-060, and after 2-times dilution in water or PBS after desorption of DPK-060 at 25°C.
(f) Hydrodynamic diameters in water of ML-LNCs after previous equilibration in presence of 0.01, 0.1 and 1 mg/ml LL-37, and hydrodynamic diameters after 2-times dilution in water or PBS after desorption of LL-37 at 25°C.
All data are expressed as means ± S.D. over triplicate experiments.
**Demonstration of antimicrobial synergy in vitro and in vivo**

**Minimal inhibitory (MIC) and bactericidal (MBC) concentrations of ML-LNCs**

The MICs of DPK-060 and LL-37 were evaluated in TSBg up to a concentration of 8.192 mg/ml without showing inhibitory effects against any of the four staphylococcal strains (see Table 2), while for the ML-LNCs alone, the MICs between 0.125 and 0.500 mg/ml were found depending on the strain involved (see also Table 2). This was regardless of whether carried out at 25°C or 37°C. The staphylococcal MBC for ML-LNCs ranged from 0.250 mg/ml for *S. aureus* Xen36 to 1 mg/ml for the other three staphylococcal strains, also regardless of temperature (data not shown).

**Synergy of ML-LNCs and AMPs against planktonic staphylococci**

The checkerboard assay showed FIC indices < 0.31 at 25°C and 37°C for each of the four *S. aureus* strains when ML-LNCs and DPK-060 were combined in TSBg which is considered as a synergistic effect (Table 2). However, synergy was not observed for the combination of ML-LNCs and LL-37. The MIC of the ML-LNCs used alone and in combination with LL-37 was the same, yielding an FIC index > 1.06. The synergistic effect of DPK-060 combined with ML-LNCs against the planktonic staphylococcal strains was confirmed in a time-kill assay (Supporting Figure S1).

**Efficacy of ML-LNCs with and without adsorbed AMPs against staphylococcal biofilms in vitro**

After 24 h growth, staphylococcal biofilms were exposed for 24 h at 37°C to AMPs or ML-LNCs with and without adsorbed AMPs. Exposure to PBS alone was taken as a control. Exposure of staphylococcal biofilms in presence of either DPK-060 or LL-37 did not result in a substantial reduction of staphylococcal CFUs as compared to exposure to PBS alone (2.1 \( \times \) 10^7 CFU/biofilm), regardless of the strain involved (log-reductions < 0.3; see Figure 4). Exposure to ML-LNCs alone and combinations of ML-LNCs with AMPs resulted in significantly higher reductions in staphylococcal CFUs than when exposed to PBS or AMPs alone. Higher concentrations of ML-LNCs had no significant effects on CFU reduction, neither was there a significant effect of doubling the AMP concentration.

Adapting a significantly larger reduction in biofilm CFUs in presence of ML-LNCs with adsorbed AMPs than observed in presence of ML-LNCs alone as an indication of synergy, it can be seen that synergistic effects occurred for the combination ML-LNCs with DPK-060 for three out of the four staphylococcal strains at the lower and for all four staphylococcal strains, including MRSA *S. aureus* ATCC BAA-1696 (MRSA) at the higher DPK-060 concentration (Figure 4). Similarly, also for the combination of ML-LNCs with LL-37 at the highest concentration synergy occurred for all four strains, although somewhat smaller than observed for DPK-060.

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**Table 2. Minimum inhibitory concentrations (MIC) in TSBg of DPK and LL with limiting values for the fractional inhibitory concentration indices (FIC) quantitating synergy between ML-LNCs and the AMP when the FIC is < 0.5 for different *S. aureus* strains.**

<table>
<thead>
<tr>
<th>Staphylococcus aureus</th>
<th>MIC AMPs (mg/ml)</th>
<th>MIC ML-LNCs (mg/ml)</th>
<th>FIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>DPK-060</td>
<td>&gt; 8.192</td>
<td>0.500</td>
<td>&lt; 0.28</td>
</tr>
<tr>
<td>ATCC 29213</td>
<td>&gt; 8.192</td>
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</tr>
<tr>
<td>Xen36</td>
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<td>0.125</td>
<td>&lt; 0.31</td>
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<td>ATCC BAA-1696 (MRSA)</td>
<td>&gt; 8.192</td>
<td>0.500</td>
<td>&lt; 0.28</td>
</tr>
<tr>
<td>LL-37</td>
<td>&gt; 8.192</td>
<td>0.500</td>
<td>&gt; 1.06</td>
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<tbody>
<tr>
<td>DPK-060</td>
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<tr>
<td>0701A0095</td>
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Figure 4. CFU reduction at 37°C, expressed as a log10 fold reduction of staphylococcal biofilm grown for 24 h and subsequently exposed for 24 h to AMPs and ML-LNCs alone or ML-LNCs with adsorbed AMPs. CFU reduction was expressed relative to exposure to PBS alone and evaluated for different concentrations of ML-LNCs, and DPK-060 and LL-37 equilibrium concentrations against:

(a) S. aureus 0710A0095.
(b) S. aureus ATCC 29213.
(c) S. aureus Xen36.
(d) S. aureus ATCC BAA-1696 (MRSA).

Data are expressed as means ± S.D. over triplicate experiments with separately cultured bacteria, while asterisks represent statistically significant differences (p < 0.05; analysis of variance (ANOVA) with Tukey's post-hoc analysis) compared to exposure to ML-LNCs alone.

Efficacy of ML-LNCs with and without adsorbed AMPs against a staphylococcal biofilm in a therapeutic, murine wound healing model

The effect of AMPs and ML-LNCs with and without adsorbed AMPs was further evaluated in a therapeutic, murine wound healing model, using bioluminescent S. aureus Xen36 to infect the wound. Time series of representative bioluminescence images for one and the same mouse taken daily after the initiation of treatment are presented in Figure 5.

Quantitative analysis of images prior to treatment indicated that the bioluminescence radiance (4.6 × 10^7 ± 2.5 × 10^7 photons/s/cm^2/sr) and the infected wound area (94 ± 29 mm^2) did not vary significantly over the different groups of animals created (Table S1). Treatment with PBS yielded almost full reduction of bioluminescence in the majority of mice already at day 2 (Figure 6), concurrent with a decrease in bioluminescent area (Figure 7). ML-LNCs alone showed a significant reduction in bioluminescence at day 1 after treatment with respect to the PBS control, while at all further days after treatment the differences between ML-LNCs and PBS treatment disappeared. DPK-060 alone also showed reduction in bioluminescence at day 1 after treatment, but LL-37 did not. Moreover, there is no synergy between any of the two AMPs and ML-LNCs. Judged from the bioluminescent wound area, it may even be concluded that treatment with the combination of ML-LNCs and DPK-060 yielded larger bioluminescent areas for longer periods of time after initiating treatment. All mice lost weight after inflicting the wound, but regained weight during the wound healing period (Figure S2a). Culturing of the infected wound tissue after sacrifice at day 4 yielded no significant differences between the different treatment groups (Figure S2b) and amounted 3.3 × 10^5 CFU per wound on average, representing a 300-fold reduction with respect to the number of CFUs applied to infect the wound.
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Figure 5. Time series of bioluminescent images for 6 mice after initiating treatment with
(a) PBS (control).
(b) DPK-060 only (1 mg/ml).
(c) LL-37 only (1 mg/ml).
(d) ML-LNCs alone (100 mg/ml).
(e) ML-LNCs (100 mg/ml) with adsorbed DPK-060 at an equilibrium concentration of 1 mg/ml.
(f) ML-LNCs (100 mg/ml with adsorbed LL-37 at an equilibrium concentration of 1 mg/ml.
Representative mouse images are shown for each group. Treatment was initiated on day 0, directly after making the bioluminescent images. Scale bar represents 0.5 cm.

Figure 6. Relative bioluminescence intensity arising from wounds infected with bioluminescence S. aureus Xen36 as a function of time after treatment. Treatment was carried out daily prior to bioluminescence measurement with
(a) PBS (control).
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Bioluminescence intensity was set at 100% at day 0 before treatment. Data are expressed as individual values with the median. Absolute bioluminescence intensities at day 0 are summarized in supporting Table S1. Asterisks represent statistical significance (p < 0.1) compared against the PBS control group at the same day and was calculated with ANOVA with Dunn’s post-hoc analysis.
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(F) ML-LNCs (100 mg/ml) with adsorbed LL-37 at an equilibrium concentration of 1 mg/ml.
Bioluminescent area was set at 100% at day 0 before treatment. Data are expressed as individual values with the median. Absolute bioluminescent areas at day 0 are summarized in supporting Table S1. Absence of statistical significance was calculated with ANOVA with Dunn’s post-hoc analysis.
Discussion

Release of AMPs from ML-LNCs is controlled by shifting the adsorption-desorption equilibrium in reply to changes in AMP concentration in suspension, as demonstrated here through the measurement of zeta potentials and unlike operative in most drug release systems, that are pH controlled or light activated\textsuperscript{24,25}. ML-LNCs without AMPs were effective in killing staphylococci in a planktonic and biofilm mode of growth, but DPK-060 and LL-37 on their own did not. Both AMPs demonstrated synergistic, antimicrobial efficacy with ML-LNCs at their highest concentration applied against all four staphylococcal biofilms. In a therapeutic, infected-wound healing model in mice, ML-LNCs provided faster initial wound healing than PBS, but neither AMPs DPK-060 nor LL-37 yielded faster wound healing nor displayed any antimicrobial synergy with MC-LNCs in speeding up wound healing. This is likely because host AMPs, as found in neutrophils and epithelial cells\textsuperscript{26}, took over the role of the AMPs added through adsorption to monolaurin capsules.

**Controlled release of AMPs**

Assuming adsorption of AMPs will be established in water at temperatures around 37°C, while desorption would take place under ionic strength conditions resembling PBS at temperatures closer to body temperatures, we measured zeta potentials during adsorption in water at 25°C and 37°C and during desorption in both water and PBS at 25°C. After diluting the ML-LNC suspension with dissolved AMPs, there were little differences in zeta potentials measured in water or PBS, which all hovered slightly above 0 mV. However, zeta potentials of the ML-LNCs were significantly more positive at 37°C than at 25°C (see Figure 2c and 2d). This is because 25°C is below the phase inversion temperature of ML-LNCs (37°C)\textsuperscript{15}, at which temperature hydrogen bonds between the liposomes and water are increasingly broken\textsuperscript{21} making monolaurin less hydrophilic, forming the on-set of their disassembly with increasing temperature. The on-set of disassembly is evident already from the increased hydrodynamic radius of the nanocapsules at 37°C (32 nm and 135 nm at 25°C and 37°C, respectively; Figure 3c and 3d).

**In vitro antimicrobial studies**

The antimicrobial mechanism of action of monolaurin is by non-specifically targeting bacterial surface signal transduction systems through interaction with the plasmatic membrane\textsuperscript{16,27}, which explains the antimicrobial synergy constituted by addition of AMPs to an ML-LNCs suspension, that also operate on the basis of membrane disruption. Antimicrobial efficacies of AMPs depend on the medium in which the evaluation is carried out. Because AMPs are susceptible to ions, with a negative impact on their antimicrobial efficacy\textsuperscript{12,28} and causing e.g. aggregation of LL-37 in growth medium\textsuperscript{29}, MIC and MBC evaluations in the current study were carried out in full medium, as common in microbiology\textsuperscript{30}. Evaluation in full medium did not yield any antimicrobial efficacy against
planktonic staphylococci, likely due to the above. Evaluations by others of the antimicrobial activities of AMPs, including also DPK-060 and LL-37, have been done in diluted media\textsuperscript{31–33} or buffer\textsuperscript{33–36}. In diluted medium (1% BHI, 99% water), MICs of 0.004 mg/ml and 0.016 mg/ml were found against \textit{S. aureus} for DPK-060 and LL-37, respectively. Use of diluted medium or buffer however, can on its own already inhibit growth, and hence these low values may not be directly associated with the AMPs themselves, which is the reason why we preferred to evaluate MICs in full medium.

 Whereas for the combination of ML-LNCs with two other AMPs, synergy was demonstrated against planktonic \textit{S. aureus} ATCC 25923 and \textit{S. aureus} 0702E0196\textsuperscript{15}, antimicrobial synergy in the current study was only observed for ML-LNCs with DPK-060 against planktonic \textit{S. aureus} (Table 2 and Figure S1). However, when in a biofilm mode of growth, DPK-060 and LL-37 combined at their highest concentration with ML-LNCs, demonstrated synergistic antimicrobial efficacy against \textit{S. aureus} biofilms \textit{in vitro} (Figure 4). No concentration dependence of the antimicrobial efficacy of ML-LNCs with or without adsorbed AMPs against staphylococcal biofilms was observed, as opposite to observations on planktonic staphylococci. Likely, the biofilm mode of growth presents a barrier, that controls antimicrobial penetration and therewith their efficacy over the concentration range involved.

\textit{In vivo antimicrobial studies}

\textit{In vivo}, healing of wounds infected with bioluminescent \textit{S. aureus} Xen36, treated with ML-LNCs alone in a murine model, was faster after infection than when treated with PBS. At sacrifice, i.e. four days after infection, bioluminescence intensities and the number of staphylococcal CFUs isolated from per wound area were similarly low in all groups. Accordingly, conclusions about antimicrobial efficacy and synergy can only be drawn with respect to the kinetics of pathogen clearing. This is quite common in infection models in animals. Either, the animals die after pathogen inoculation when the dose is too high, or manage to fully cure the infection themselves\textsuperscript{37–39}. This not only leaves a narrow window for the inoculation dose, but also implies that at end-stage, without clinical signs of infection, bacterial contents from the wounds are similarly low. In far most all animal studies that are known to us, the advantage of antimicrobial treatment is concluded from a faster recovery than the control\textsuperscript{38,40–43}, as we have done.

While our \textit{in vivo} study adapted a therapeutic approach to infection control, other \textit{in vivo} studies investigated the ability of monolaurin to prevent infection, which is against common clinical practice in which antimicrobial treatment is only initiated after the occurrence of infection, as in our therapeutic wound healing model. In murine studies, preventive oral\textsuperscript{44,45} administration of monolaurin successfully reduced bacteremia due to \textit{S. aureus} injection, while prophylactic injection of monolaurin increased survival of rabbits due to induced \textit{S. aureus} toxic shock syndrome\textsuperscript{46}. Opposite to planktonic and \textit{in vitro} biofilm
studies, we observed no therapeutic antimicrobial synergy in healing of infected wounds, when ML-LNCs were combined with an AMP, likely because host AMPs, such as cathelicidin related antimicrobial peptide in mice\textsuperscript{47} took over the role of the AMPs added.

Conclusions
1. Controlled release of AMPs from ML-LNCs occurs through an adsorption-desorption equilibrium.
2. ML-LNCs were effective in killing planktonic staphylococci, and staphylococci in a biofilm mode of growth. In a therapeutic, infected wound healing model in mice, ML-LNCs provided faster initial wound healing than a PBS control.
3. The AMPs DPK-060 and LL-37 did not display antimicrobial efficacy against staphylococci regardless of their mode of growth, but ML-LNCs with adsorbed DPK-060 and LL-37 demonstrated synergistic, antimicrobial efficacy at their highest concentrations applied against all four staphylococcal biofilms involved in this study. \textit{In vivo} wound healing was not sped up by the AMPs.
4. Synergistic antimicrobial efficacy of ML-LNCs with DPK-060 and LL-37 as \textit{observed in vitro}, could not be demonstrated to persist in a therapeutic, murine infected wound healing model, likely because host AMPs took over the synergistic role of AMPs added.

For future experiments, this study highlights that conclusions drawn regarding antimicrobial efficacy and synergy of antimicrobials should not be drawn from planktonic data, while even biofilm data \textit{in vitro} may bear little relevance for the much more complex \textit{in vivo} situation, especially with regards to synergy.


**Funding information**

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**Competing financial interests**

HJB is also director of a consulting company, SASA BV (GN Schutterlaan 4, 9797 PC Thesinge, The Netherlands). The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article. Opinions and assertions contained herein are those of the authors and are not construed as necessarily representing views of their respective employers.

**References**

Supporting information

Figure S1. Number of colony forming staphylococcal units (CFUs) as a function of time in the time-kill curves of DPK-060, LL-37 and ML-LNCs used alone and in combination against the four *S. aureus* strains. Data expressed as mean ± S.D., experiments were repeated three times. Experiments were done by adding 20 µl of a staphylococcal suspension (5 × 10⁷ bacteria) to 2 ml of either ML-LNCs, DPK-060, LL-37, or a combination of ML-LNCs and DPK-060 or LL-37, dispersed in TSBg medium, and incubated at 37°C. As a control, growth medium without antimicrobials but with bacteria was taken. An amount of 100 µl was withdrawn from each tube after 0, 3, 6 and 24 h and serial 100-fold dilutions were prepared in demineralized water. An amount of 20 µl of the diluted bacterial suspension was pipetted and spotted on tryptone soya agar plates. After incubating the agar plates overnight at 37°C, the colonies were counted. Synergy was defined as an increase of at least 2 log₁₀ in killing caused by the combination of the antimicrobials compared to the most active antimicrobial agent¹.
Figure S2.
(a) Weight of the mice before inflicting the wound at day -1 and during the wound healing period, starting at day 0, for the different treatment groups. Weight was expressed as a percentage with respect to the weight of the mice when entering the experiment (on average, 26.2 g).
(b) The average numbers of CFUs isolated from the wound area after sacrifice at day 4 for the different treatment groups.
Error bars represent standard deviations over 6 mice per group.

Table S1. Absolute bioluminescence radiance and absolute bioluminescence area from untreated mice, prior to participating in the different treatment groups on day 0. Standard deviations in the table are calculated over 6 mice per group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radiance (p/s/cm²/sr) (× 10⁷)</th>
<th>Bioluminescence area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>3.6 ± 2.9</td>
<td>78 ± 16</td>
</tr>
<tr>
<td>DPK-060</td>
<td>5.8 ± 1.9</td>
<td>104 ± 27</td>
</tr>
<tr>
<td>LL-37</td>
<td>6.2 ± 2.3</td>
<td>97 ± 30</td>
</tr>
<tr>
<td>ML-LNCs</td>
<td>3.7 ± 2.3</td>
<td>98 ± 38</td>
</tr>
<tr>
<td>DPK-060 adsorbed ML-LNCs</td>
<td>4.6 ± 3.3</td>
<td>103 ± 34</td>
</tr>
<tr>
<td>LL-37 adsorbed ML-LNCs</td>
<td>3.8 ± 1.6</td>
<td>82 ± 32</td>
</tr>
</tbody>
</table>

References