A comparison of different strategies for antimicrobial peptides incorporation onto/into lipid nanocapsules

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Aim: Over the last decade, antimicrobial peptides (AMPs) have emerged as a promising alternative for the treatment of various infections. The aim of this work is to explore the potential of lipid nanocapsules for the delivery of AMPs. Three approaches were compared in terms of encapsulation efficiency, peptide activity and protection against proteases: peptide encapsulation, surface adsorption or covalent attachment of three selected AMPs.

Results: A potentiation of the antimicrobial activity and a partial protection of the peptides after adsorption were demonstrated compared with native peptides. Conversely, encapsulation allowed better peptide stability, correlated with higher encapsulation efficiencies and a preservation of the activity. Finally, the covalent attachment strategy turned out to be less conclusive due to peptide inactivation.

Conclusion: In brief, a lipid nanocapsule-based platform appears suitable to deliver AMPs.

Graphical abstract:
There is a continuous increase in the number of bacterial strains that are resistant for almost all marketed antibiotics [1], which drastically limit treatment options. These include methicillin-resistant Staphylococcus aureus (MRSA) [2], multiantibiotic resistance in clinical isolates of Pseudomonas aeruginosa [3], and cause hundreds of thousands of infections annually [4-6].

This rapid increase in drug-resistant infections coupled with declining antibiotic discovery [7] presents an acute problem in the healthcare sector, generating interest in novel antimicrobial strategies. Antimicrobial peptides (AMPs) represent a promising new class of antimicrobial agents [8]. AMPs in general and those selected in this study are molecules with a high potential to kill bacteria rapidly and at low concentrations [9]. Many efforts have been made to their development and the improvement of their stability and efficacy. Nevertheless, their clinical development is often hindered by their lack of chemical stability and stability in the biological environment due to the presence of human and bacterial proteases [10-14]. By protecting the integrity and improving the bioavailability of AMPs, new formulations may improve their therapeutic index. Hence, it is important to develop delivery systems that are suitable for their antimicrobial activity and stability. Potent and safe AMP-based therapeutics could be developed with these delivery systems, through allowing a better stability to chemical and biological degradation of AMPs in formulation and after administration, by controlling AMP release, reducing adverse side effects, promoting biofilm penetration, or through achieving co-localization with intracellular bacteria [15]. Novel formulations for AMPs in the nanomedicine include liposomes, micelles, polymer and lipid nanoparticles or nanocapsules. These nanocarriers may be loaded with AMPs, and transport them to reach the intracellular pathogens usually found in the phagocytic cells [16]. This study is a part of AMP nanoformulation approach, based on lipid nanocapsules (LNCs). LNCs are well-described nanocarriers. LNCs are obtained by a phase inversion temperature (PIT) process [17]. They are composed of an oily core surrounded by a tensioactive rigid membrane. Their structure closely resembles a hybrid between polymeric nanocapsules and liposomes [18]. The oily phase is composed of medium-chain triglycerides: triglycerides of capric and caprylic acids (Labrafil® WL 1349). The surfactant shell is made of lecithin and PEGylated surfactants [19]. Since their development [17], they were investigated to deliver a broad range of drugs. First, LNCs have been used to render lipophilic drugs more prone to be administered. In this context, the encapsulation of different lipophilic drugs was conducted with success covering a variety of area [20-23]. Recently, their use was extended to antimicrobial therapy through the encapsulation of aromatic and terpenic compounds [24,25]. Following these encouraging results [26], a challenge was launched, the nanoformulation of AMPs. Despite the hydrophilic nature of these peptides, LNCs are interesting nanocarriers, which have previously been used for the encapsulation [27,28] or association of hydrophilic molecules [29-31].

Knowledge about the nanocarrier structure and the previous attempts to deliver hydrophilic drugs has prompted us to explore different ways to incorporate the peptides, the presentation of the AMPs on the surface of the LNCs and AMP encapsulation inside the lipidic core of the LNCs.

In this study, AA230, LL-37 and DPK-060 were investigated. These AMPs are short (20-40 amino acids residues), cationic and hydrophilic peptides; however, present different properties in terms of selectivity, physical properties, amphipathicity and secondary structure. AA230, an arenicin-3 derivative is a newly developed AMP composed of 21 amino acid residues with a β-hairpin structure conferring a high amphipathicity to this AMP. DPK-060 is a random coil AMP of 20 amino acids derived from human kininogen. Finally, LL-37 also known as hCAP18, is the C-terminal part of the only human cathelicidin identified to date. LL-37 is one of the most widely investigated AMPs in the literature [32-35] and has served as a benchmark molecule in our study.

In brief, the purpose of this study is to investigate for the first time the potential of LNCs, as effective nanocarriers to deliver peptides by using different strategies for peptide loading. Based on the core–shell structure of the LNCs, three approaches were tested: the adsorption of the cationic AMPs on the surface of LNCs; covalent attachment of AMPs to the shell of LNCs; and their encapsulation in the core of the LNCs. These approaches will be compared in terms of feasibility, encapsulation efficiency, the antimicrobial activity of the AMPs after association and their stability toward proteases.

Materials & methods

Materials
Labrafac WL1349 (caprylic/capric acid triglycerides) and Oleic Plurol® CC497 (polyglyceryl-3 dioleate) was obtained from Gattefossé S.A. (Saint-Priest, France). Dioctyl sulfocinate sodium salt was purchased from...
Sigma-Aldrich (MO, USA). Lipoid® S75-3 (soy bean lecithin at 70% phosphatidylcholine and 10% phosphatidylethanolamine) and Kolliphor® HS15 (a mixture of free PEG 660 and PEG 660 hydroxy stearate) were obtained from Lipoid GmbH (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. NaCl was purchased from Prolabo VWR International (Fontenay-sous-Bois, France). Purified water was obtained from a MilliQ® System (Millipore, Paris, France). LL-37 (94.7% purity) was synthetized and provided by PolyPeptide Laboratories (Limhamn, Sweden). DPK-060 (98.5% purity) was synthetized by Bachem AG (Bubendorf, Switzerland). AA230 was synthetized by PolyPeptide Laboratories and provided by Adenium Biotech ApS (Copenhagen, Denmark). The structure and properties of the peptides and excipients are summarized in Table 1. Trypsin from porcine pancreas was purchased from Sigma-Aldrich (Steinheim, Germany). Other chemicals used were of analytical grade.

**Nanocarrier formulations**

Depending on the incorporation strategy and for peptide stability reasons, two types of lipid nanoparticles were prepared.

**Lipid nanocapsules**

Standard LNCs (described by Heurtault *et al.* [17]) were prepared for AMP association on LNC surface. The 21.48, 1.48, 1.75 and 58.62% (w/w) of Labrafac WL1349, Lipoid S75-3, Kolliphor HS15, sodium chloride and water, respectively, were mixed together and homogenized under magnetic stirring. Three cycles of temperature between 60 and 90°C were applied to the mixture. The oil in water emulsion, formed at low temperature, underwent a phase inversion and formed a water-in-oil emulsion at high temperature. At the fast cooling, when the temperature achieved 78°C, corresponding to the phase inversion temperature (PIT), rapid dilution with 2°C purified water led to the formation of LNCs of about 50 nm. The peptide was associated with blank LNCs by either adsorption or transacylation strategies.

**Adsorption strategy**

After formulation the blank LNCs were incubated with AMP solutions at a final weight ratio of 1:180 (AMP:particles) during 3 h at room temperature. The adsorption efficiency was determined by HPLC method after the separation of free and loaded AMP by ultrafiltration centrifugation method through Amicon Ultra filter devices with a molecular weight cutoff (MWCO) of 100 kDa.

**Transacylation**

The blank LNCs were then transacylated, according to the method described by Messaoudi *et al.* [30], where transacylation has used to fix covalently chitosan on the surface of LNCs [30]. In our study, the transacylation reaction is postulated to occur between the pegylated hydroxy stearate from the LNC shell and the functional amino groups of basic residues (mainly, lysine) as displayed in Figure 1. Briefly, 2 ml of the blank LNCs were mixed with 100 μl of NaOH 10N and 5 mg of each AMP. The reaction was performed at room temperature for 15 min to avoid LNCs crosslinking in a basic environment. The reaction is stopped by adding 2 ml of glycine–HCl buffer at pH 2.2 and the pH was then adjusted to pH 7.4 ± 0.5. After neutralization, the transacylated LNCs were purified by dialysis using float-a-lyzer® dialysis devices with an MWCO of 100 kDa (Repligen GmbH Manufacturing, Ravensburg, Germany). This allowed the removal of the free AMP by diffusion in the dialysis water.
### Table 2. Structure and properties of the antimicrobial peptides and excipients.

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>Denomination</th>
<th>Chemical structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labrafac® WL 1349</td>
<td>Caprylic/capric acid triglycerides</td>
<td><img src="image1" alt="Chemical structure" /></td>
</tr>
<tr>
<td>Oleic Plurio® CC 497</td>
<td>Polyglyceryl-3 dioleate</td>
<td><img src="image2" alt="Chemical structure" /></td>
</tr>
<tr>
<td>Kolliphor® HS-15</td>
<td>Free PEG 660 (30%) and PEG 660 hydroxystearate (70%)</td>
<td><img src="image3" alt="Chemical structure" /></td>
</tr>
<tr>
<td>Lipoid® S75-3</td>
<td>Soy bean lecithin at 70% phosphatidylicholine and 10% phosphatidylethanolamine</td>
<td><img src="image4" alt="Chemical structure" /></td>
</tr>
<tr>
<td>AOT®</td>
<td>Dioctyl sulfosuccinate sodium salt</td>
<td><img src="image5" alt="Chemical structure" /></td>
</tr>
</tbody>
</table>

B: Excipients; GRAVY: Grand average of hydropathicity; MW: Molecular weight.

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**Reverse micelle-loaded lipid nanocapsules**

Reverse micelles formation in Labrafac

Peptide-loaded reverse micelles (RMs) were prepared according to the method developed by Groo *et al.* [36]. Blank RMs were obtained by mixing Labrafac oil and a lipophilic surfactant. In this study dioctyl sulfosuccinate sodium...
salt (AOT) was retained as a surfactant. Blank RMs were prepared by incubating AOT with Labrafac at a weight ratio of 1:10 for 3 h at 45°C. The suspension was then centrifuged at 4000 r.p.m. for 5 min in order to eliminate nonsolubilized AOT. The supernatant was used for the preparation of peptide-loaded RMs. AMP–RMs were prepared by incubating AA230, LL-37 and DPK-060 with blank RMs, at an initial concentration of 6 mg/g of RMs, in the presence of water (<2% volume/volume) for 2 h at 45°C. The suspension was centrifuged at 4000 r.p.m. for 30 min at 20°C, to remove free peptide (i.e., nonsolubilized remnants). According to Vrignaud et al. method, RMs were considered to be formed when a transparent, homogeneous and stable mix was obtained [28]. The supernatant was used for the preparation of RM-loaded LNCs (RM–LNCs). The encapsulation efficiency into RMs corresponds to the peptide remaining in the oily supernatant after the centrifugation step.

**RM–LNCs formation**

RM–LNCs were prepared following a previously described method [36], corresponding to the PIT method [37]. Briefly, a low PIT, suitable for the entrapment of thermolabile peptide [27], was obtained thanks to the use of oleic Plurol instead of Lipoid in the formulation. The RMs were added to the LNCs, before the final dilution with water. RM–LNCs were made with 3.7% (w/w) oleic Plurol (polyglyceryl-3 dioleate), 5.6% (w/w) Kolliphor HS-15, 14.1% (w/w) lipophilic Labrafac WL 1349, 74.3% (w/w) water, 1.9% (w/w) NaCl and 0.5 % (w/w) AOT.

Briefly, the Labrafac WL1349, oleic Plurol, Kolliphor HS15, sodium chloride and water, were mixed by magnetic stirring. The same process as for standard LNCs was used. The temperature cycles are carried out between 30 and 50°C. Previously formed blank or loaded RMs were introduced in the water phase of emulsion to form RMs–LNCs, during the last cooling, when the temperature reached 43°C. When the PIT was achieved (at around 38°C), the mixture underwent a fast cooling-dilution process with water at 0°C, leading to generate nanocarriers. Then, the LNC suspension was stirred at 300 r.p.m. for 5 min at room temperature. To determine the encapsulation efficiency, free and loaded AMPs were separated by a 24-h dialysis using float-a-lyzer dialysis devices with an MWCO of 100 kDa, and loaded AMP was quantified by HPLC. The structures of the RMs and RM–LNCs are represented in Figure 2.

**Particle size & ζ-potential determination**

The size, polydispersity index and ζ-potential of the blank and loaded particles were determined by dynamic light scattering using a NanoZS® (Malvern Instruments S.A., Malvern, UK). All measurements were performed in triplicate at 25°C after dilution with deionized water.

**HPLC**

The peptide-loading efficiency was determined by the direct quantification of loaded AMP after purification. Purified formulations were diluted in MeOH (LNC–AMP) or MeOH:brine (LNC–RM–AMP) to solubilize the lipidic constituents. The apparatus was composed by injector (Waters 717 plus), pump (Waters 660 E), detector (Waters 2487), controller (Waters 600), software: Millenium 32 version 3.2 (Waters, Saint Quentin-en-Yvelines, France). The column used was a Waters Symmetry Shield®, RP18 column (250 x 4.6 mm, 5 μm) column (Waters Corporation, MA, USA). Injected volume and the detection wavelength were respectively 20 μl and 200 nm.
The flow rate was set at 1.2 ml/min. Mobile phase was initially composed of a mixture of 90% water/0.1% trifluoroacetic acid (A) and 10% 0.085% trifluoroacetic acid in acetonitrile/water (4:1; B). Then, it reached 35% B after 5 min, 60% after 14 min, 100% after 14.1 min, by applying a linear gradient, maintained for 2 min and returned to its initial conditions. It was linear with respect to the area under the curve between 5 and 100 mg/l, with a correlation coefficient above 0.99. The AMPs peaks had a retention time of approximately 11.0 min, 12.2 min and 17.2 min for AA230, DPK-060 and LL-37, respectively. Data collection and integration were performed using Empower®3 software. The encapsulation efficiency into RMs (EERM) was calculated using the following equation:

\[
EERM(\%) = \frac{\text{experimental AMP concentration}}{\text{theoretical AMP concentration}} \times 100
\]

The experimental AMP concentration corresponds to the total AMP in RM–LNCs. The theoretical AMP concentration relies on the quantity of the AMP weighed to prepare the AMP–RMs. Encapsulation and association efficiencies were calculated after the separation of free and loaded AMP. The encapsulation/association efficiency (EAE) was calculated using the following equation:

\[
EAE(\%) = \frac{A}{B} \times 100
\]

where A is the mass of encapsulated or associated AMP, and B the total amount (mass) of AMP.
**Bacterial strains**

*Staphylococcus aureus* (reference strain ATCC 25923), MRSA (clinical strain no. 0702E0196), *P. aeruginosa* (reference strain ATCC 27853 and clinical strain no. 0704C0134), *Escherichia coli* (reference strain ATCC 25922), extended-spectrum β-lactamase (ESBL) *E. coli* (clinical strain ATCC 9007550201) and *Acinetobacter baumannii* (AYE reference strain ATCC BAA-1710) were all obtained from the bacteriology department (CHU d’Angers, France). The strains were cultured on Columbia agar supplemented with sheep blood.

**Minimum inhibitory concentrations**

The *in vitro* antibacterial activity of the free and formulated AMPs was evaluated using the broth microdilution method. Overnight colonies were suspended into 2 ml of 0.85% NaCl solution to a turbidity of 1.1 McFarland (*S. aureus*) or 0.5 McFarland (other isolates). The suspensions were further diluted 100-fold (*S. aureus*) and tenfold (other isolates) in either brain heart infusion medium (BHI; AA230) or BHI diluted 100-times with water (LL-37 and DPK-060) to obtain an inoculum of approximately $3.3 \times 10^6$ colony-forming unit/ml and approximately $1.5 \times 10^7$ colony forming unit/ml, respectively. Serial twofold dilutions of the samples in BHI medium were prepared to obtain the desired concentration range between 32 and 0.25 µg/ml. Next, 50 µl of the bacterial suspension in BHI broth was added to each well of a sterile 96-well plate that already contained 50 µl of the tested sample or control. The positive control wells contained only BHI and the bacterial suspension, whereas the negative control wells contained exclusively BHI and the tested sample. The plates were finally incubated at 37°C for 24 h. The minimum inhibitory concentrations (MIC) was defined as the lowest concentration that prevents the visual growth of a microorganism after 24-h incubation time. MIC values were considered different if they varied by more than one dilution.

**Protease sensitivity assay: trypsin, *P. aeruginosa* elastase & human neutrophil elastase**

To inactivate AMPs, bacteria produce peptidases and proteases. Thus, peptide integrity against proteolysis should be evaluated at an early stage, for native and nanoformulated peptides. The model enzyme used, was the trypsin. The proteolytic concentration of trypsin for each peptide was determined by testing different ratio trypsin/peptide/phosphate buffered saline (PBS). Native and nanoformulated AA230, DPK-060 and LL-37 were incubated with an appropriated trypsin ratio at 37°C for up to 4 h. In parallel, AMP sensitivity to bacterial and human elastases was assessed. 200 µg of AMP or nano-AMPs were incubated with 4 µg of *P. aeruginosa* elastase (PE) or 5 µg of human neutrophil elastase (HNE) enzymes at 37°C for maximum 4 h. Negative controls were performed by the incubation of the peptides in PBS without proteases in the same conditions of incubation. Aliquots were taken after 10, 20, 30 and 45 min and 1, 2 and 4 h, and the amount of peptide remaining was determined via HPLC, as described previously above.

**Results**

**Formulation**

AMP-loaded LNCs were developed according to the three strategies described above and characterized for particle size, polydispersity index, ζ-potential and encapsulation efficiency. Table 1 described structure and properties of the AMPs (A) and excipients (B). AA230, LL-37 and DPK-060 were evaluated for the encapsulation in the core of the RM–LNCs and the results were compared with the results obtained with adsorption or transacylation strategy. Table 3 summarizes the result obtained using the three evaluated strategies. The data are heterogeneous and were dependent on the tested AMP. AA230 did not show encapsulation or transacylation efficiency, but the adsorption strategy was better allowing 26.4% association efficiency to the LNCs. The encapsulation of LL-37 after its solubilization into RMs succeeded, demonstrating > 90% encapsulation efficiency. The LNCs obtained were around 60 nm with a good monodispersity. The ζ-potentials of the RM–LNCs were more negative than the LNCs. Besides, the peptide adsorption and transacylation were also achieved, demonstrating 34.6 and 82.5% association efficiency, respectively. DPK-060 was also efficiently encapsulated (88% encapsulation efficiency) and adsorbed on the surface of the LNCs whereas its transacylation on the surface of the LNCs failed.

**Antimicrobial activity**

One of the most important parameters to ensure, when AMPs are formulated, is to preserve the antimicrobial activity of the peptide after association. The MICs of the native peptides were first determined against seven selected bacterial strains. The results are depicted in Tables 4–6 below. AA230 has shown to be active only against
Table 3. Particle characteristics and encapsulation/association efficiency after formulation.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>ζ (mV)</th>
<th>Final peptide concentration (μg/ml)</th>
<th>EERM (%)</th>
<th>EAE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>59.3 ± 1.1</td>
<td>0.042</td>
<td>-7.8 ± 1.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AA230</td>
<td>60.1 ± 0.4</td>
<td>0.047</td>
<td>-3.7 ± 0.5</td>
<td>498.0 ± 7.2</td>
<td>26.4 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>LL37</td>
<td>76.5 ± 1.4</td>
<td>0.043</td>
<td>-1.7 ± 0.3</td>
<td>496.2 ± 6.5</td>
<td>34.6 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>DPK-060</td>
<td>76.8 ± 1.5</td>
<td>0.034</td>
<td>-0.8 ± 0.2</td>
<td>500.2 ± 4.2</td>
<td>33.8 ± 3.5</td>
<td></td>
</tr>
</tbody>
</table>

Transacylation strategy

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>ζ (mV)</th>
<th>Final peptide concentration (μg/ml)</th>
<th>EERM (%)</th>
<th>EAE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>56.42 ± 0.8</td>
<td>0.040</td>
<td>-7.3 ± 2.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AA230</td>
<td>76.97 ± 0.7</td>
<td>0.029</td>
<td>-14.8 ± 0.8</td>
<td>32.0 ± 1.2</td>
<td>3.2 ± 1.4</td>
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</tr>
<tr>
<td>LL37</td>
<td>61.17 ± 0.9</td>
<td>0.090</td>
<td>-16.8 ± 2.1</td>
<td>583.1 ± 34.9</td>
<td>82.5 ± 10.5</td>
<td></td>
</tr>
<tr>
<td>DPK-060</td>
<td>96.93 ± 0.0</td>
<td>0.090</td>
<td>-3.3 ± 0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Reverse micelle strategy

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>ζ (mV)</th>
<th>Final peptide concentration (μg/ml)</th>
<th>EERM (%)</th>
<th>EAE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>57.9 ± 0.4</td>
<td>0.040</td>
<td>-37.0 ± 1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AA230</td>
<td>58.9 ± 1.1</td>
<td>0.043</td>
<td>-33.1 ± 0.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LL37</td>
<td>58.6 ± 0.1</td>
<td>0.035</td>
<td>-36.3 ± 1.0</td>
<td>297.6 ± 6.3</td>
<td>99.4 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>DPK-060</td>
<td>58.9 ± 0.6</td>
<td>0.049</td>
<td>-37.3 ± 4.8</td>
<td>266.8 ± 11.2</td>
<td>87.7 ± 3.7</td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as mean ± standard deviation (n = 3).
EAE: Encapsulation/association efficiency; EERM: Encapsulation efficiency into reverse micelle; PDI: Polydispersity index.

Table 4. Minimum inhibitory concentrations of free or nanoformulated AA230 (in micrograms of peptide per milliliter).

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Free AA230</th>
<th>Peptide-LNCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Adsorption strategy</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> (S1)</td>
<td>64–128</td>
<td>64–128</td>
</tr>
<tr>
<td><em>S. aureus methicillin resistant</em> (S2)</td>
<td>64–128</td>
<td>64–128</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (S3)</td>
<td>2–4</td>
<td>1</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (S4)</td>
<td>4–8</td>
<td>2</td>
</tr>
<tr>
<td><em>Escherichia coli ATCC 25922</em> (S5)</td>
<td>2–4</td>
<td>1</td>
</tr>
<tr>
<td><em>E. coli clinical strain</em> (S6)</td>
<td>1–2</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em> (S7)</td>
<td>2–4</td>
<td>0.25</td>
</tr>
</tbody>
</table>

LNC: Lipid nanocapsule; ND: Not determined.

Table 5. Minimum inhibitory concentrations of free or nanoformulated LL37 (in micrograms of peptide per milliliter).

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Free LL37</th>
<th>Peptide-LNCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Adsorption strategy</td>
</tr>
<tr>
<td>S1</td>
<td>8–16</td>
<td>8–16</td>
</tr>
<tr>
<td>S2</td>
<td>8–16</td>
<td>4</td>
</tr>
<tr>
<td>S3</td>
<td>8–16</td>
<td>8–16</td>
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<tr>
<td>S4</td>
<td>8–16</td>
<td>8</td>
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<td>S5</td>
<td>16</td>
<td>4–8</td>
</tr>
<tr>
<td>S6</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>S7</td>
<td>16</td>
<td>2–4</td>
</tr>
</tbody>
</table>

LNC: Lipid nanocapsule; ND: Not determined.

gram-negative bacteria, while LL-37 and DPK-060 have demonstrated a broad spectrum activity.

The MICs of the nanoformulated peptides were then determined and compared regarding the strategy used. The results are summarized in Tables 4–6. Except for AA230, for which encapsulation has failed, the encapsulated peptides (LL-37 and DPK-060) have shown MICs that are comparable to the MICs of the unformulated peptides, which confirms the preservation of the antimicrobial activity of the AMPs within the formulations. However, an
increase of the MICs values was observed for the encapsulated LL-37 and DPK-060 against certain strains (E. coli, A. baumannii with both peptide and S. aureus and ESBL E. coli with DPK-060). Conversely, the transacylation strategy resulted in a total inactivation of LL-37 (the only peptide that has been efficiently transacylated). Finally, the adsorbed AMPs have demonstrated at least a preservation of the antimicrobial activity of the native peptides (e.g., adsorbed LL-37 against S. aureus, and adsorbed DPK-060 against E. coli). Moreover, the MICs were significantly decreased in some cases, especially with adsorbed AA230 against gram-negative bacteria.

**Stability against proteolysis**

The most promising formulations (demonstrating a preserved or enhanced antibacterial activity) have been selected for the evaluation of AMPs stability against the proteolytic effect of trypsin, selected because it is a highly potent and model enzyme, and then against HNE and PE, usually involved in AMP inactivation in a biological environment. The kinetics of degradation by the proteases were studied for each peptide in order to evaluate the intrinsic susceptibility of AA230, LL-37 and DPK-060 at the predefined ratio. The graphs in Figure 3 display the AMPs stability after incubation with trypsin at 50% (w/w) for AA230 and 5% (w/w) for LL-37 and DPK-060, PE at 2% (w/w) and HNE at 2.5% (w/w). For all peptides, no degradation was observed after incubation at 37°C in PBS without peptidases (data not shown).

AA230 demonstrated a high resistance against proteolytic degradation. Indeed, a high amount of trypsin is needed to degrade native and LNC-adsorbed AA230 peptide. Moreover, no degradation of AA230 was observed after a 4-h incubation with PE and HNE (Figure 3B & C). The stability against HNE and PE of nanoformulated AA230 was not evaluated due to resistance of AA230 to these peptidases.

DPK-060 demonstrated a higher susceptibility to trypsin compared with AA230. 5% (w/w) of trypsin was sufficient for the total degradation of the peptide after 4-h incubation. When adsorbed or encapsulated, DPK-060 demonstrated a partial but significant protection against trypsin degradation compared with the native peptide in solution (Figure 3D). However, the ability of the formulation to protect DPK-060 seems to be higher when the AMP is encapsulated within the core of RM–LNCs.

Second, the stability of LL-37 was assessed in the same conditions. The results demonstrated a poor stability of LL-37 toward the three enzymes. However, the nanoformulations succeeded to ensure protection of the peptide. The kinetics of LL-37 degradation (in the presence of trypsin, PE and HNE) was significantly slower, when adsorbed on the surface or encapsulated in the core of LNCs. Once again, the protection was more pronounced when the LL-37 was encapsulated, which is correlated with peptide encapsulation efficiency.

Finally, in solution DPK-060 demonstrated a relatively high resistance against PE, 60% of the peptide remained stable after 4-h incubation. No additional protection was shown when DPK-060 was adsorbed. Encapsulated DPK-060 displayed improved stability against PE, allowing almost 100% protection over 4 h. Incubation with HNE did not affect DPK-060 (Figure 3F). The stability against HNE of nanoformulated DPK-060 was not evaluated due to the resistance of DPK-060 against HNE.

**Discussion**

**Formulation**

LNCs are versatile nanocarriers, a much-appreciated property to adapt to a broad range of therapeutic compounds. The development of the different formulations relied on the previous attempts to deliver amphiphilic or hydrophilic
Figure 3. Protease degradation assay. The kinetics of degradation of native and nanoformulated AA230 (A–C), DPK-060 (D–F) and LL-37 (G–I) in the presence of trypsin, Pseudomonas aeruginosa elastase (PE) and human neutrophil elastase (HNE) at 37 °C. Mean ± standard deviation (n = 3).

AMP: Antimicrobial peptides; LNC: Lipid nanocapsules; RM: Reverse micelle; PE: Pseudomonas aeruginosa elastase; HNE: Human neutrophil elastase.

Drugs and then optimized to be compatible with the AMPs delivery [19]. LNCs, which are able to efficiently encapsulate hydrophilic molecules, have been already developed [38]. Moreover, due to their large area for the purposes of adsorption, LNCs are promising vehicles for hydrophilic molecules [39]. The adsorption of peptides on the surface of LNCs has been already investigated for the formation of LNC coated with a cell-internalizing peptide [29,40]. These successful attempts have motivated the development of AMP-based nanoformulations for a therapeutic purpose, where the peptides can be either adsorbed on the surface, or covalently linked to the hydroxystearate chains present at the LNCs surface or encapsulated within their lipid core. The comparison of the different strategies will help to determine the more promising formulations of LNCs that are suitable for AMP delivery. The three AMPs selected for the study are molecules with distinct structure and origin (Table 1), which predicated different behavior and hence the importance to investigate different approaches to associate them with LNCs.

Drug delivery through adsorption onto nanoparticles is more and more investigated [41,42]. This strategy is an interesting alternative to explore when: drug encapsulation is incompatible with the drug-delivery system; there is no need for encapsulation (burst release); or when a combination of adsorption and encapsulation demonstrated benefit (tailored release or co-treatment). The advantages of this strategy are the high surface area of the nanoparticles allowing AMPs nanopresentation, rapid drug availability and protection of the drug against the conditions of the...
process of nanoparticle fabrication that can be harsh for labile drugs. The ability of the AMPs to be adsorbed on the surface of LNCs was then assessed. The relatively high proportion of cationic residues, mainly arginine and lysine, distributed along the AMPs backbone, has led to the investigation of the potential of these AMPs to be adsorbed on the surface of the LNCs. The results have shown a good adsorption efficiency of the three tested peptides. The adsorption of LL-37 and DPK-060 on the surface of the LNCs demonstrated an increase in particle size of approximately 20 nm, whereas the increase in the size of LNCs was less pronounced when particles are associated to AA230. An increase in the ζ-potential has been noticed in all the cases, which is in favor of the surface localization of the AMPs. The HPLC results for peptide association (AA230 < LL–37 = DPK–060) were in accordance with the size and the ζ-potential measurements. Moreover, the level of AMP structure packing could in part explain the different behaviors. In fact, the β-hairpin structure of AA230 is more packed compared with more extended structures of LL-37 (linear α-helix conformation) and DPK-060 (random coiled). In the study of Boge et al., LL-37 showed a much higher degree of association than DPK-060, with >60% of added peptide [43]. However, these different results were obtained for the adsorption of LL-37, DPK-060 and another AMP into cubosomes, with different surface properties than LNCs. The interaction between the AMPs and the LNCs may imply electrostatic, dipole-ion, Van der Waals or hydrophobic interactions. The slightly negative charge of the LNCs could be attributed to the presence of a negative dipole on PEG [44]. This negative charge and the positive charge of the AMPs may lead to dipole–ion interactions. In addition, protein adsorption may be also driven by hydrophobic interactions. Kolliphor HS15, a PEG 660 hydroxystearate, is composed of a hydrophobic moiety covalently linked to the hydrophilic PEG-containing moiety. Furthermore, due to their amphiphilic structure, AMPs have surface active properties. As a consequence, some AMPs could be also localized at the nanocapsule surface with the PEGylated surfactant. Depending on their structure and composition, AMPs could be localized at the interface, and hydrophobic interaction could be occurred. Therefore, Umerska et al. have demonstrated the predominant role of electrostatic interactions concerning AMPs adsorption on LNCs [31]. Moreover, particle concentration and ionic strength may influence peptide association because electrostatic forces primarily govern it [31]. Similar trends in adsorption of bovine serum albumin were found on spherical polyelectrolyte brushes [45].

As a consequence, an increased peptide association may be expected with the LNCs.

Then, the transacylation strategy was investigated. The objective of this strategy was the establishment of stronger binding of the peptides on the surface of the LNCs and its consequence on peptide activity. The transacylation is based on the transfer of acyl groups between the PEG-hydroxystearate chains forming the shell of LNCs and the functional amino groups of the basic residues of the AMPs. AA230, LL-37 and DPK-060 possess lysine residues (which are the residues with the most reactive functional amino groups) and were postulated to react with surface components of the LNCs. Curiously, only LL–37 has shown a good transacylation efficiency, with 80% of the peptide remaining attached to LNCs after purification (48-h dialysis). AMPs structure could be the factor governing the transacylation success. Indeed, it was suggested that the level of peptide folding would be responsible for such results, rendering the residues more or less accessible for the transacylation reaction. Moreover, AA230 and DPK-060-transacylated LNCs have demonstrated a change in particle size with a high polydispersity index, suggesting another hypothesis, in which the transacylation can be in favor of a destabilization of the system. In fact, the peptides interactions can displace the hydroxystearate chains from the surface of LNCs and hence destabilize their structure. At the same time, it is also important to underline that neither LNCs nor AMP stability was affected by exposure to NaOH during the transacylation process. Moreover, the activity of the free peptides remains intact after these conditions (data not shown).

Finally, the encapsulation strategy was investigated. AA230, LL-37 and DPK-060 are known to be hydrophilic peptides and hence fail to be efficiently solubilized in the oily core of the LNCs. For that, RM-loaded LNCs were developed according to Anton et al., method [37] and optimized for the incorporation of labile AMPs. In fact, the addition of polyglyceryl-3 dioleate (instead of lecithin) and increased amount of sodium chloride allow the lowering of the PIT and hence the protection of the peptides against a possible thermal denaturation. RMs composed of triglyceride and AOT were successfully developed. The ζ-potentials of RM–LNCs were more negative than LNCs (Table 3). The presence of this sulfosuccinate type anionic surfactants (Table 2) into the core of LNCs seems to influence ζ-potential. Its adsorption on inner surface of the shell could lead to a rearrangement and interfacial competition between AOT and PEG hydroxystearate. This could conduce to the presence of a part of AOT in the shell of LNCs and not only in the oily core. LL–37 and DPK–060 were efficiently loaded within the hydrophilic compartment of the RMs. Nevertheless, the addition of a small amount of water (<2 %v/v) was required to solubilize the hydrophilic peptides within the RMs. Moreover, the incorporation of the AMP-loaded RMs into the
LNCs has resulted in a high encapsulation efficiency observed for LL-37 and DPK-060, which demonstrated the ability of these AMPs to be encapsulated within the optimized LNCs. The stability in ζ-potential and size upon peptide incorporation is in favor of the localization of the cationic AMPs in the particle's core. Inversely, AA230 have shown no encapsulation efficiency within the LNCs. This AMP is an arenicin-3 derivative with a β-hairpin structure conferring a high amphipathicity [46], a property that can confers a high instability in the hydrophilic compartment of the RM composed of Labrafac and AOT. Moreover, peptide stability could be compromised by manufacturing processing conditions, and excipients [47]. In general, the preparation of nanovehicles could affect peptide morphology or denature it, because of their drawbacks, such as an organic solvent exposure, a shear stress, high temperature or very low temperature [48]. The physical and chemical modification of peptides can lead to differences in bioperformance. From our results, it appears that the RM–LNC formulation process keeps both LL-37 and DPK-060 intact but compromises AA230.

Even though they seem similar (small cationic AMPs), AA230, LL-37 and DPK-060 have demonstrated different results. As a function of their conformation and amino acids composition, their interaction with LNCs has shown to be completely different. The adsorption showed limited association efficiency (around 30%) but was compatible with the three peptides. Then, transacylation is adapted only for LL-37, probably due to the flexibility of such peptide (free of S–S bridges and residues with huge side chain as tryptophan), but leads to an inactivation. In a second hand, this study has shown the ability of LNCs to be tailored to load AMPs. The encapsulation was very successful but was limited to AMPs that shows a low amphipathicity.

**Antibacterial activity**

The development of AMP-loaded LNCs, in which AMPs maintain their intrinsic activity, is an indispensable prerequisite. For that, the MICs of the native and nanoformulated AMPs were determined. The more interesting results were obtained with adsorbed peptides, for which the antimicrobial activity of the peptides was at least preserved or significantly enhanced (more than one dilution difference in the MIC value) after adsorption compared with unformulated peptides. Similar results were obtained with DPK-060-loaded cubosomes, but the reduced effect was observed for LL-37-loaded cubosomes [43].

After transacylation, the covalently linked LL-37 was inactivated. All together, these data suggested a difference in the release of the peptides when they are encapsulated (more sustained) or adsorbed, whereas transacylated peptides seem to not be released at all, which will significantly affect their activity. Moreover, conformational switches of LL-37 occur upon interaction with the bacterial membrane. The disordered random coil conformation in aqueous solution changed to a pronounced α-helix. This peptide displayed the propensity for α-helix structure upon the binding to anionic phospholipid of the membranes and anionic polysaccharides [49]. This secondary structure may be impeded by the covalent link with LNCs and interfere with LL-37 activity.

The encapsulated peptides have shown a preservation of their antibacterial activity against *P. aeruginosa* and MRSA. However, the encapsulated LL-37 and DPK-060 have shown a tendency to be less potent against certain strains (e.g., *E. coli*, *A. baumannii* for both peptides, and *S. aureus* and ESBL *E. coli* for DPK-060). These results are in accordance with a recent study, where the level of entrapment of LL-37 and DPK-060 in cubosomes and hexosomes was inversely proportional to their antimicrobial activity [50]. The decrease in antimicrobial activity for the encapsulated peptide may be explained by the trend of the peptide to remain in RM environment resulting in a slow release from these particles. DPK-060 is the most hydrophilic tested peptide (Table 1), and its property explains the fact that its release is more hindered by lipid core of LNCs. As demonstrated by Nordström et al., LL-37 incorporated into oppositely charged microgel undergoes dramatic conformational changes [51]. On contrary to findings for microgel, encapsulation into negative RMs did not decrease drastically LL-37 activity. In all cases, the decrease of AMP activity did not exceed more than a two dilution step difference in the MIC test.

Thus, further studies are necessary in order to clarify peptide conformation, and the impact into release rate, membrane interactions and antimicrobial activity.

**Stability against proteases**

Protease stability is a pivotal consideration in the development of peptide-based drugs. One of the greatest limitations to peptide therapeutics is their degradation by peptidases and proteases found in different levels of the human body (e.g., in the skin, blood, liver and kidneys) [52]. In order to enhance peptide half-lives, various modifications of peptides have been studied [53]. Nevertheless, the alteration of chemical structure of the peptide may potentially

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affects its therapeutic activity [54,55]. Pseudomonas aeruginosa and S. aureus are well known to develop resistance to AMPs due to their proteolytic degradation [13].

One of the objectives to deliver AMPs through LNCs is to protect them against proteolytic degradation. For this reason, the stability of the native and the formulated AMPs has been tested with trypsin, a serine protease, which cleaves proteins at the C-terminal of lysine and arginine amino acid residues; PE, a bacterial proteases and the HNE, an endogenous serine proteinase were secreted by neutrophils and macrophages during inflammation.

In line with previously reported data, LL-37 was degraded by infection-related enzymes from either bacteria (PE) or human defense cells (leukocyte elastase, HNE) [43,56]. In contrast, AA230 and DPK-060 have demonstrated no or moderate susceptibility to degradation, respectively, by both PE and HNE. This is due to the previous effort made on AA230 and DPK-060 precursors namely, arenicin-3 and GKH17 to improve their resistance to proteolytic inactivation [57]. All three AMPs are degraded by trypsin.

Adsorption on LNCs protects LL-37 against trypsin and PE. However, this protection was moderate and protected DPK-060 only from proteolytic attacks by trypsin but not by PE. In a previous study, an AMP was protected from trypsin degradation by its adsorption onto lipid-disks particles [58]. It may be attributed to the PEG on the surface of the particles, which provided steric hindrance and thus blocked interactions between AMP and enzyme. Similarly, Kolliphor HS15 provides a PEG shell around LNCs, which protect AMP adsorbed onto LNCs. Interestingly, AMPs loaded on RM–LNCs were shown to protect considerably DPK060 and LL-37 against attack from PE, HNE and trypsin. This is in line with the preferential localization of AMP within lipid core of RM–LNCs. The encapsulated peptides demonstrated the highest resistance, which confirms that encapsulated peptides are more importantly buried within the particles and less accessible to proteolytic degradation compared with adsorbed peptides. Previously, an enzymatic protection has resulted in increased antibacterial effect after exposure to enzyme [43]. As a consequence, an improvement of AMP activity could be expected in vivo when it was nanoformulated.

The native peptides demonstrated a difference in susceptibility when exposed to proteases. In fact, AMPs intrinsic resistance can be classified as followed AA230 > DPK-060 > LL-37. Whereas, when adsorbed or encapsulated the opposite trend is observed, that is, nanoformulated LL-37 demonstrated the higher improved resistance against proteolysis compared with the free peptide in solution, followed by DPK-060, while no additional resistance has been shown with the nanoformulated AA230.

Conclusion
Taken together, these data have shown the ability of LNCs to load AMPs of different nature. The adsorption method has demonstrated a great advantage in terms of AMP antimicrobial activity and a protection against degradation by proteolytic enzymes while encapsulation presents an even better protection against proteolytic degradation and good entrapment efficiency but is not suitable for peptides with high amphipathicity. In brief, this study indicates that a development of an LNC-based platform to deliver AMPs may be feasible. Depending on the nature of the peptide, the therapeutic application, the target site and the route of administration, one or other strategy will be preferred.

Future perspective
This study showed an important impact of peptide properties on the strategy of association with LNCs. Depending on the nature of the peptide, the therapeutic application, the target site and the route of administration, adsorption or RM strategy will be preferred. So, the intention at present is to evaluate if the bacterial penetration of AMPs is enhanced by LNCs, and to determine the impact of association strategy on bacterial uptake. Indeed, bacterial penetration of AMPs could be improved by lipid nanocarriers, due to an effective fusion with microorganism. In the study of Theerthagiri et al., a fusion between drug-loaded liposomes and infectious microbes lead to the drug release into the bacteria, directly [59]. In many types of infection, biofilms, formed by the aggregations of infectious bacteria, protect the infected area against antibiotics and other therapies. They are current and difficult to penetrate. Thus, it will be necessary to study antimicrobial penetration in biofilms [60]. It is also possible to evaluate the antimicrobial activity of formulations on biofilms. Moreover, the modification of the surface of LNC-based nanocarriers may influence the nanovector elimination. Pharmacokinetic and biodistribution should be evaluated for each strategy. Finally, in long term, other peptides could be encapsulated or adsorbed on LNCs to allow their protection and to improve their activity.
Lipid nanocapsule-based platform to deliver antimicrobial peptides

- Lipid nanocapsules are able to load antimicrobial peptides of different nature but strategy of association depends on peptide properties.

The adsorption method has
- A great advantage in terms of antimicrobial peptide antimicrobial activity.
- A protection against proteolytic degradation.
- But a limited association efficiency (around 30%).

Encapsulation presents
- An even better protection against proteolytic degradation.
- A good entrapment efficiency (> 87%).
- But is not suitable for peptides with high amphipathicity.

Financial & competing interests disclosure

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Papers of special note have been highlighted as: ● of interest; ●● of considerable interest


**The predominant role of electrostatic interaction concerning AMP adsorption on lipid nanocapsules.**

**Encapsulation of AP138, an AMP, on reverse micelle-lipid nanocapsules.**
•• Crucial evidences showing amphipathicity of AA230, an arenicin-3 derivative.
•• Key paper showing that LL-37 undergoes conformational changes upon interaction with bacterial membrane.