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Encapsulation in oleyl-modified hyaluronic acid nanogels substantially improves the clinical potential of the antimicrobial peptides SAAP-148 and Ab-Cath

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ABSTRACT

Antimicrobial peptides (AMPs) are promising alternatives to antibiotics for treatment of antimicrobial resistant (AMR) bacterial infections. However, their narrow therapeutic window due to in vivo toxicity and limited stability hampers their clinical use. Here, we evaluated encapsulation of two amphiphilic AMPs, SAAP-148 and snake cathelicidin Ab-Cath, into oleyl-modified hyaluronic acid (OL-HA) nanogels to improve their selectivity index. The AMP-loaded OL-HA nanogels ranged 181–206 nm in size with a PDI of 0.2, highly negative surface charge (∼47 to ∼48 mV) and moderate encapsulation efficiency (53–63%). The AMP-loaded OL-HA nanogels displayed similar activity in vitro as AMP solutions against AMR Staphylococcus aureus and Acinetobacter baumannii, with a dose-dependent effect over time. Importantly, the AMP-loaded OL-HA nanogels showed decreased cytotoxicity towards human erythrocytes and primary skin fibroblasts, thereby improving the selectivity index of SAAP-148 and Ab-Cath by 2- and 16.8-fold, respectively. Particularly, the selectivity of Ab-Cath-loaded OL-HA nanogels has great clinical potential, with an index that reached >300 for S. aureus and >3000 for A. baumannii. These findings indicate that OL-HA nanogels are a promising drug delivery system to reduce the cytotoxicity of AMPs without substantially affecting their antimicrobial activity, thereby increasing their selectivity index and potential as therapeutics to combat AMR bacterial infections.

1. Introduction

The introduction of antibiotics is one of the biggest medical advances of the last century, saving millions of lives every year by treating and preventing bacterial infections. However, the occurrence of antimicrobial resistant (AMR) bacterial strains is rising globally, due to over-prescription and inadequate use of currently available antibiotics [1,2]. The emergence of antibiotic-resistant pathogens, such as Staphylococcus aureus and Acinetobacter baumannii, is increasingly encountered and constitute a major health threat [3]. Moreover, biofilm formation by these pathogens additionally hampers the effective use of antibiotics by further increasing the resistance of bacteria to antibiotics up to 1000 times [4–6]. Such biofilms are involved in approximately 80% of chronic and recurrent bacterial infections [6]. Additionally, several difficult-to-treat subpopulations exist within these biofilms, such as metabolically less active persister cells that reside in deeper layers of the biofilm [7,8], further emphasizing the tremendous need for novel antibiotics and antibiotic therapies to combat AMR bacterial infections.

Antimicrobial peptides (AMPs) are promising candidates for further development into antibiotic therapies. For example, the synthetic antimicrobial and antibiofilm peptide (SAAP)-148 has shown potent broad-spectrum antimicrobial activity against a range of AMR bacteria, planktonic, and in biofilms, without inducing resistance development [9]. The same study showed that SAAP-148 treatment successfully eradicated AMR bacteria from superficial skin wound infections in mice. Importantly, SAAP-148 was also effective against persister cells in mature AMR St. aureus biofilms [10]. Another promising example is the novel snake cathelicidin Ab-Cath. This AMP also showed to be potent...
unsuitable for use as a DDS. Chemical modification of HA can improve tissue and skin due to a fast-enzymatic degradation [19], making it its anticancer activity encapsulation of paclitaxel with 70% loading efficiency and improved stability, shelf-life, and viscoelasticity [20]. As a result, HA-based infections. Hydrogels, and in particular nano-sized hydrogels known as hydrophilicity were calculated using https://www.bachem.com/knowledge-center/peptide-calculator/.

Amino acid sequence, molecular weight, net charge and hydrophilicity of SAAP-148 and Ab-Cath.

Table 1

<table>
<thead>
<tr>
<th>Sequence</th>
<th>MW (g/mol)</th>
<th>Net Charge</th>
<th>Hydrophilic residues (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAAP-148</td>
<td>acetyl-LKRWVKEFVKLLKEYWQILKPVPR-amide</td>
<td>3269.6</td>
<td>+11</td>
</tr>
<tr>
<td>Ab-Cath</td>
<td>acetyl-KRPFKFKMKVKKVWYRLKCVYFKYKXKFIAYTIPY-amide</td>
<td>4159.6</td>
<td>+12</td>
</tr>
</tbody>
</table>


2. Materials and methods

2.1. Materials

Chemicals were obtained from commercial suppliers and used without further purification. SAAP-148 and Ab-Cath (see Table 1 for peptide characteristics) were synthesized using standard Fmoc chemistry and purified to > 95% by the Department of Immunology (LUMC, the Netherlands) and Enzynepe (Geleen, the Netherlands), respectively (see Figs. S1 and S2). Sodium oleyl hyaluronate (17 kDa, 12% degree of substitution) was obtained from Contripio (Dolní Dobrouč, Czech Republic). Isopropanol, trifluoroacetic acid (TFA) and Triton™ X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade solvents for UPLC analysis included ultrapure water (Veolia Purelab Chorus 1, ELGA Labwater, High Wycombe, UK) and acetonitrile (VWR, Radnor, PA, USA). Ultrapure water for sample preparation and in vitro assays was obtained from a MilliQ system, and phosphate buffered saline (PBS) from Fresenius Kabi (Graz, Austria). Trypsin stock broth (TSB) and Mueller-Hinton (MH) agar were purchased from Oxoid (Basingstoke, UK). Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1% (v/v) GlutaMAX™, penicillin-streptomycin (pen/strep) and trypsin-EDTA were obtained from Gibco (Waltham, MA, USA) and human serum from Merck (Darmstadt, Germany). Inactivated fetal bovine serum (FBS) was purchased from Corning (Corning, NY, USA) and human plasma from Sanquin (Leiden, the Netherlands). Human erythrocytes were isolated from fresh blood of anonymized healthy donors obtained after written informed consent (LUMC Blood Donor Service, LeuVDS, Leiden, the Netherlands). Human primary skin fibroblasts were kindly provided by dr. A. El Ghalbzouri (Department of Dermatology, LUMC, the Netherlands).

2.2. Preparation of OL-HA nanogels

OL-HA nanogels were produced at room temperature. The AMP (SAAP-148 or Ab-Cath) and OL-HA were dissolved in ultrapure water to a concentration of 300 µg/mL and 1000 µg/mL, respectively. These solutions were mixed in a 1:1 (v/v) ratio and vortexed for 30 sec before an equal volume of isopropanol was added and the mixture was vortexed for another 30 sec. This mixture was evaporated overnight, rehydrated in the same volume of ultrapure water and vortexed for 30 sec to yield the AMP-loaded OL-HA nanogels. The OL-HA nanogels were frozen in liquid nitrogen and lyophilized overnight using an Alpha 1-2-3 (Edwards, Burgers Hill, UK) freeze dryer (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) with RV3 vacuum pump and EMF10 oil mist filter (Edwards, Burgess Hill, UK). Lyophilized OL-HA nanogels were stored at −20 °C until use. For all experiments lyophilized samples were rehydrated in the relevant media before use, where the concentration of peptide is given as the total peptide concentration present in the sample.

2.3. Size, polydispersity index and zeta potential of nanogels

The average size, polydispersity index (PDI) and zeta potential (ZP; measure for surface charge) of the nanogels were determined using dynamic light scattering. The size, PDI and ZP measurements were performed in ultrapure water at 25 °C using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. Malvern DTS v.6.20 software was used for...
data acquisition and analysis. Measurements were performed in triplicate for three independent sample batch replicates.

2.4. Quantification of SAAP-148 and Ab-Cath

Quantification of SAAP-148 and Ab-Cath was performed using reverse phase ultra-high performance liquid chromatography (UPLC) on a Shimadzu Prominence system (Kyoto, Japan). Chromatographic separation was carried out using a Kinetex XB-C18 column (50 × 2.1 mm, 2.6 um, Phenomenex, Torrance, CA, USA) with eluent A (95.5% (v/v) water/acetonitrile) and eluent B (5:95% (v/v) water/acetonitrile), both containing 0.1% (v/v) trifluoroacetic acid. Samples were run with a gradient of 0 to 100% eluent B over 8 min at 0.8 mL/min at 40 °C and data was analyzed using the LabSolutions software version 5.82 with SAAP-148 or Ab-Cath calibration curves (0.5–45 μg/mL).

2.5. Encapsulation efficiency and drug loading of SAAP-148 and Ab-Cath in nanogels

The amount of SAAP-148 or Ab-Cath loaded in the nanogels was determined by measuring the residual amount of AMP present in the aqueous bulk phase after nanogel production. The aqueous bulk phase was obtained by centrifugation of the nanogels at 500,000 g for 30 min to ensure sedimentation of the nanogels. Quantification of encapsulation efficiency (EE) was performed in triplicate for three independent sample batch replicates. The EE is calculated as follows:

\[
EE (\%) = \frac{Total \ AMP (\mu g) - unencapsulated \ AMP (\mu g)}{Total \ AMP (\mu g)} \times 100\%
\]  

(1)

The drug loading was calculated similarly:

\[
DL (\%) = \frac{Total \ AMP (\mu g) - unencapsulated \ AMP (\mu g)}{Total \ weight \ nanogels (\mu g)} \times 100\%
\]  

(2)

2.6. Circular dichroism

The secondary structure of AMP solutions, AMP-loaded nanogels and placebo nanogels was investigated using circular dichroism (CD). Spectra were recorded using a Chirascan CD spectrometer (Applied Photophysics, Leatherhead, Surrey, UK) with a 1 mm path length quartz cuvette. Spectra were obtained at a peptide concentration and/or polymer concentration of 150 μg/mL and 500 μg/mL, respectively. Spectra were recorded from 190 to 260 nm at an interval of 0.1 nm and at 25 °C, and were corrected for background contributions.

2.7. Bacterial strains and culturing

This study makes use of AMR strains of S. aureus (LUH14616; NCCB100829) and A. baumannii (RUH875). The bacterial strains were stored in glycerol at –80 °C until use. Briefly, bacteria were cultured on blood agar plates (BioMérieux SA, Marcy-l’Etoile, France) overnight at 37 °C. Prior to the experiment, 3–5 colonies were cultured to mid-log phase in TSB for 2.5 h under continuous rotation. Mid-log phase bacterial cultures were centrifuged at 1,000g for 10 min, washed with PBS and resuspended in the preferred medium to the required concentrations based on the optical density at 600 nm.

2.8. In vitro bacterial activities

Mid-log phase bacterial cultures were resuspended in PBS to a concentration of 5 × 10^8 colony forming units (CFU)/mL. Next, 30 μL of PBS containing increasing concentrations of AMP, AMP-loaded nanogels or placebo nanogels were pipetted into wells of a polystyrene V-shaped microplate (Greiner BioOne, Alphen a/d Rijn, the Netherlands) and mixed with 50 μL of pooled human plasma and 20 μL of the bacterial suspension. The microplate was sealed with an AmpliStar adhesive plate seal (Westburg, Leusden, the Netherlands) and incubated for 2, 4, 6, 24 or 48 h at 37 °C under rotation at 200 rpm. Finally, the number of viable bacteria was assessed by plating dilution series on MH plates. Results were expressed as the lethal concentration (LC)_{99.9}, i.e. the lowest concentration of AMP killing 99.9% of the inoculum.

2.9. In vitro hemolysis assay

Fresh blood from anonymous healthy donors was collected in citrate tubes (BD Vacutainer Systems, Plymouth, UK), centrifuged at 1,811g, washed three times with PBS and diluted to obtain a 2% (v/v) human erythrocyte suspension in PBS. Next, 25 μL of PBS containing increasing concentrations of AMP, AMP-loaded nanogels or placebo nanogels were mixed with 50 μL of pooled human plasma or PBS and 25 μL of 2% (v/v) human erythrocytes in wells of a polystyrene V-shaped microwell. A 5% (v/v) Triton™ X-100 solution in PBS was included as positive control and a PBS solution as negative control. The microplate was incubated for 1 h at 37 °C and 5% CO2 after which the human erythrocytes were pelleted by centrifugation for 3 min at 290g. Finally, 75 μL of the supernatant was transferred to a 96-well flat-bottom polystyrene microplate and the optical density was measured at 415 nm. The percentage hemolysis was calculated as follows:

\[
\text{Hemolysis} (\%) = \frac{OD_{415} \text{sample} - OD_{415} \text{negative control}}{OD_{415} \text{positive control} - OD_{415} \text{negative control}} \times 100\%
\]  

(3)

Results were expressed as the effective concentration (EC)_{50}, i.e. the concentration of AMP resulting in 50% hemolysis. Non-linear regression curves with bottom and top restrictions at 0 and 100% were fit to each individual experiment to determine the medians (and ranges) of the EC_{50} values.

2.10. In vitro cytotoxicity assay using human primary skin fibroblasts

Human primary skin fibroblasts were cultured in DMEM supplemented with 1% (v/v) GlutaMAX™, 1% (v/v) pen/strep and 5% (v/v) FBS. The skin fibroblasts were harvested using 0.05% (v/v) trypsin-EDTA, washed with PBS and resuspended to 2 × 10^5 cells/mL. Finally, 20,000 cells were seeded in 96-wells flat-bottom culture plates (Corning, NY, USA) to form monolayers after overnight incubation at 37 °C and 5% CO2. The monolayers were exposed for 4 h to increasing concentrations of AMP, AMP-loaded nanogels and placebo nanogels in DMEM supplemented with GlutaMAX™, 1% (v/v) pen/strep and 0.5% (v/v) human serum. A 1% (v/v) Triton™ X-100 solution was included as positive control and medium as negative control. Lactate dehydrogenase (LDH) release into the medium was detected by the CytoToxicity Detection Kit (Roche, Basel, Switzerland) and the metabolic activity of the cells was measured using the water-soluble tetrazolium (WST)-1 cell proliferation reagent (Roche), both according to manufacturer’s instructions. Results were expressed as the effective concentration (EC)_{50}, i.e. the concentration of AMP resulting in 50% cytotoxicity or metabolic activity. Non-linear regression curves with bottom and top restrictions at 0 and 100% were fitted for each individual experiment to determine medians (and ranges) of the EC_{50} values.

2.11. Calculation of the selectivity index

The selectivity index, i.e. the ratio between cytotoxicity and bactericidal activity, was determined at the 4 h time point and calculated as follows:

\[
\text{Selectivity index} = \frac{EC_{50} \text{ human primary skin fibroblasts (μM)}}{LC_{99} \text{ AMR bacteria (μM)}}
\]  

(4)

2.12. Ethics statement

For this study we used fresh human blood (LUMC Blood Donor...
Both AMPs showed encapsulation efficiencies above 50% and a drug in solution and after loading in OL-HA nanogels. The AMPs were present in sizes ranging from 100 to 200 nm, but not exceeding 500 nm, are preferred for AMP-loaded OL-HA nanogels to target bacterial biofilms and to prevent clearance from the blood stream by the complement system [33,34]. Thin film hydration was used to prepare OL-HA nanogels loaded with SAAP-148 or Ab-Cath. The resulting nanogels were monodispersed and negatively charged, with sizes of 181 and 206 nm for SAAP-148- and Ab-Cath-loaded nanogels, respectively (Table 2). In comparison, placebo nanogels, not loaded with peptide, were larger in size, more polydisperse and more anionic than AMP-loaded nanogels. Both AMPs showed encapsulation efficiencies above 50% and a drug loading ranging from 24 to 27%.

### 3.2. Circular dichroism of SAAP-148- and Ab-Cath-loaded OL-HA nanogels

The secondary structures of SAAP-148 and Ab-Cath were determined in solution and after loading in OL-HA nanogels. The AMPs were present in a random coil conformation when in solution, as indicated in Fig. 1 by very low ellipticity above 210 nm combined with a minimum near 195 nm [35,36]. However, after nanogel formulation SAAP-148 showed a distinctive minimum around 210 nm and maximum at 195 nm, indicating that the peptide assembled into an α-helix-like structure upon formulation in OL-HA nanogels [35,37]. In comparison, Ab-Cath nanogels only showed minor differences compared to placebo OL-HA nanogels. These observations indicate an interaction between the amphiphilic AMPs and the amphiphilic polymer leading to conformational changes in the peptides.

### 3.3. Antimicrobial activities of SAAP-148- and Ab-Cath-loaded OL-HA nanogels

The antimicrobial activities of SAAP-148- and Ab-Cath-loaded OL-HA nanogels were compared to AMP solutions and placebo nanogels against planktonic AMR S. aureus and A. baumannii in presence of 50% plasma. Human plasma is known to reduce the activity of AMPs, as it is bioavailable [15,38]. A previous study on the antimicrobial activities of SAAP-148 demonstrated that this peptide was more efficient in killing bacteria under physiological conditions in vitro than many known pre-clinical- and clinical-phase antimicrobial peptides [9]. Here, antimicrobial activities of SAAP-148 as well as Ab-Cath remained low in the micromolar range, indicating excellent antimicrobial activities of both AMPs in presence of plasma. The AMP-loaded OL-HA nanogels were as effective as the AMP solutions, demonstrating that the antimicrobial activity was not affected by encapsulation in the carrier (Table 3 and Figs. S3 and S4). Placebo nanogels did not show any antibacterial activity in the tested concentration range. In addition, Ab-Cath was more effective than SAAP-148 against S. aureus at the 6 h and 24 h time points and showed marginally better performance (within the 2-fold dilution sensitivity of the method) at the remaining timepoints. Ab-Cath was substantially more potent towards A. baumannii in comparison to SAAP-148 at all the tested time points, with a 10-fold lower concentration required to kill these Gram-negative bacteria.

### 3.4. Cytotoxic activities of SAAP-148- and Ab-Cath-loaded OL-HA nanogels

Next, the hemolytic and cytotoxic activities of SAAP-148- and Ab-Cath-loaded OL-HA nanogels were compared to AMP solutions and placebo nanogels towards human erythrocytes and human primary skin fibroblasts. The cell-disrupting activity of AMPs is dependent on their interaction with plasma membranes [9,13], therefore, the release of hemoglobin or LDH into the medium upon AMP treatment was used as measure for membrane disruption and/or perturbation of human erythrocytes or primary skin fibroblasts, respectively. Encapsulation of the peptides in OL-HA nanogels substantially reduced both the hemolytic and cytotoxic effect of the AMP, with an observed decrease of > 4-fold, depending on the cell type (Fig. 2 and Table S1). As expected, the presence of 50% plasma noticeably reduced the hemolytic activities of SAAP-148 and Ab-Cath, both in solution as well as loaded in nanogels. In addition, it was shown that Ab-Cath was less hemolytic and less cytotoxic than SAAP-148.

### 3.5. Selectivity indices of SAAP-148- and Ab-Cath-loaded OL-HA nanogels

Finally, the selectivity index, i.e. the ratio between cytotoxicity and bactericidal activity, was determined at the 4 h time point. Loading SAAP-148 or Ab-Cath in OL-HA nanogels improved the overall selectivity index of the AMPs by 2-fold and 16.8-fold, respectively (Table 4). In particular, the selectivity of Ab-Cath-loaded OL-HA nanogels against Gram-negative A. baumannii was impressive with an index of 3490. In addition, the selectivity index of Ab-Cath was much higher than that of SAAP-148, especially against A. baumannii. These results highlight the benefits of formulating AMPs into OL-HA nanogels, where Ab-Cath-loaded OL-HA nanogels showed most promise for future clinical development.

### 4. Discussion

Modification of HA with hydrophobic side chains allows for the self-assembly of HA into nanogels and has shown to improve stability, shelf-life, and viscoelasticity of HA-based nanogels [20]. Previously, OSA-modified (8-carbon lipid chain) HA nanogels have been used to encapsulate amphiphilic AMPs thereby reducing cytotoxic activities of these peptides and improving their selectivity index towards AMR bacteria over mammalian cells [11,25-28]. However, very little is known about nanogels formed using HA modified with different or longer lipid chains. A molecular dynamics study has indicated that using a much longer (18-carbon) lipid chain may result in a more stable nanogel system due to a stronger hydrophobic collapse of the lipid side chains [30]. This could potentially allow higher stability of the DDS and substantially affect the
represent SAAP-148 solution (— — —), SAAP-148-loaded nanogels (—) and placebo nanogels (—). B) Spectra represent Ab-Cath solution (— — —), Ab-Cath-loaded nanogels (—) and placebo nanogels (—). The amount of placebo nanogel was equal to that of the dose of AMP-loaded nanogel. All spectra were obtained at a peptide and or polymer concentration of 150 µg/mL and 500 µg/mL, respectively. Data are shown as median of at least one experiment.

Fig. 1. Circular dichroism spectra of AMP solutions, AMP-loaded OL-HA nanogels and placebo nanogels.

Table 3
Time-dependent antibacterial activities of SAAP-148 and Ab-Cath-loaded OL-HA nanogels against planktonic AMR S. aureus and A. baumannii. compared to AMP solutions and placebo nanogels.

<table>
<thead>
<tr>
<th>Species (strain)</th>
<th>Exposure</th>
<th>SAAP-148 (µM)</th>
<th>SAAP-148 nanogel (µM)</th>
<th>Ab-Cath (µM)</th>
<th>Ab-Cath nanogel (µM)</th>
<th>Placebo nanogel (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus (LUH14616)</td>
<td>2 h</td>
<td>6.4 (6.4–12.8)</td>
<td>12.8 (12.8–25.6)</td>
<td>3.2</td>
<td>3.2</td>
<td>&gt;51.2</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>6.4</td>
<td>12.8</td>
<td>3.2 (1.6–6.4)</td>
<td>3.2 (1.6–3.2)</td>
<td>&gt;51.2</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>6.4</td>
<td>6.4 (6.4–12.8)</td>
<td>1.6 (1.6–3.2)</td>
<td>1.6 (0.8–6.4)</td>
<td>&gt;51.2</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>3.2</td>
<td>3.2 (3.2–6.4)</td>
<td>0.8 (0.4–1.6)</td>
<td>0.8 (0.8–1.6)</td>
<td>&gt;51.2</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>1.6 (1.6–3.2)</td>
<td>3.2 (1.6–3.2)</td>
<td>0.8 (0.8–1.6)</td>
<td>0.8</td>
<td>&gt;51.2</td>
</tr>
<tr>
<td>A. baumannii (RUH875)</td>
<td>4 h</td>
<td>3.2</td>
<td>3.2 (3.2–6.4)</td>
<td>0.3 (0.2–0.4)</td>
<td>0.3 (0.2–0.4)</td>
<td>&gt;25.6</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>3.2</td>
<td>3.2</td>
<td>0.3 (0.2–0.4)</td>
<td>0.1 (0.05–0.2)</td>
<td>&gt;25.6</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>3.2</td>
<td>3.2 (3.2–6.4)</td>
<td>0.2</td>
<td>0.2</td>
<td>&gt;25.6</td>
</tr>
</tbody>
</table>

Planktonic bacteria were exposed for 2, 4, 6, 24 or 48 h to AMP, AMP-loaded nanogel or placebo nanogel in PBS in presence of 50% (v/v) plasma. The amount of placebo nanogel was equal to that of the dose of AMP-loaded nanogel. Data are expressed as lethal concentration (LC)ₜ₀₀₀, i.e. the lowest peptide concentration (in µM) eliminating 99.9% of the bacteria. Results are shown as median (and range) of at least two independent experiments performed in duplicate.

The physicochemical properties of SAAP-148- and Ab-Cath-loaded OL-HA nanogels (Table 2) were compared to the previously studied OSA-HA nanogels loaded with the same AMPs (Table S2). The SAAP-148 and Ab-Cath-loaded OL-HA nanogels ranged 181–206 nm in size, very similar to sizes found for these AMPs encapsulated in OSA-HA nanogels [11,27]. The size dispersion of OL-HA nanogels (PDI = 0.2) was higher than of OSA-HA nanogels (PDI = 0.03–0.06) likely due to the properties of the lipid chain as well as to differences in production method (thin film hydration versus microfluidics). Due to the higher hydrophobicity of the OL-HA polymer, microfluidics – a method that allows precise control over the production process thus resulting in very monodisperse samples [40] - did not result in the production of stable nanogels, hence the thin film hydration method was used. The SAAP-148- and Ab-Cath-loaded OL-HA nanogels had a more anionic outer surface charge compared to the same AMPs loaded in OSA-HA nanogels, possibly because the AMPs were less efficiently encapsulated in OL-HA nanogels (EE = 53–63%) than in OSA-HA nanogels (EE = 99–100%), leaving more unbound peptide in solution yet not associated with the surface of the nanogels. These results suggest that SAAP-148 and Ab-Cath are less efficiently incorporated in the OL-HA nanogels, but that the percentage that is encapsulated most likely resides deep inside the OL-HA nanogels resulting in excellent shielding of the cationic charges of these AMPs. Contrastingly, these AMPs were very efficiently encapsulated in the OSA-HA nanogels, but with a higher proportion of AMP associated close to the nanogel surface, resulting in less anionic nanogels.

Although SAAP-148 and Ab-Cath are both highly cationic AMPs, differences in peptide characteristics (peptide length, secondary structure, charge and hydrophobicity) will certainly influence the physicochemical properties of the resulting OL-HA nanogels. SAAP-148 has a shorter peptide length, lower molecular weight, lower net charge and higher hydrophilic content compared to Ab-Cath (Table 1). Peptide nanogel complex formation is mediated by a combination of electrostatic and hydrophobic interactions and is accompanied by a reduction in size and change in surface charge [41]. Indeed, we observed that encapsulation of SAAP-148 and Ab-Cath in OL-HA nanogels resulted in smaller nanogels compared to placebo nanogels and was accompanied with an increase in surface charge. Moreover, the binding affinity of cationic AMPs to anionic nanogels has shown to increase with peptide length [42], and with higher hydrophobicity [43]. Based on peptide length and hydrophobicity, it can thus be expected that the bigger and more hydrophobic Ab-Cath will bind more strongly to the OL-HA nanogels compared to SAAP-148. Although the Ab-Cath-loaded OL-HA nanogels were very similar in size to SAAP-148-loaded OL-HA nanogels, the encapsulation efficiency of Ab-Cath (63%) was significantly higher than that of SAAP-148 (53%). This may be due to stronger binding of Ab-Cath to OL-HA nanogels, possibly due to its longer peptide length and higher hydrophobic content. Finally, charge and hydrophobicity of the AMP affect cell selectivity. In general, the specificity of AMPs towards
bacterial cells over mammalian cells is mediated through electrostatic interactions, thus higher cationic charge of AMPs leading to higher selectivity towards bacteria [44]. At the same time, less hydrophobic AMPs are known to be substantially less cytotoxic to mammalian cells, although the positions of hydrophobic residues can be of influence. In this study, the more cationic and more hydrophobic Ab-Cath showed the highest selectivity index, with highest antimicrobial activities and lowest cytotoxic activities.

Importantly, the AMP-loaded OL-HA nanogels exhibited excellent antimicrobial activities against planktonic bacteria of AMR S. aureus and A. baumannii in biologically relevant medium. Time-killings revealed that the AMP-loaded OL-HA nanogels became as effective as the AMP solutions from 24 h onwards, indicating release-mediated kinetics. Importantly, hemolysis and cytotoxicity of both AMPs were reduced substantially upon formulation in OL-HA nanogels, resulting in an improved selectivity index for SAAP-148 and in particular Ab-Cath. The antimicrobial activity, cytotoxicity and selectivity index of OL-HA nanogels can be compared to previously studied OSA-HA nanogels (Table S3). The observed trends for OL-HA nanogels are similar to those observed for these AMPs loaded in OSA-HA nanogels [11,27]. Nonetheless, it is important to note that the OL-HA nanogels reduced the cytotoxic activities of these AMPs more strongly than OSA-HA nanogels, indicating its superior host cell protective activity. Therefore, a higher selectivity index can be obtained with OL-HA nanogels compared to OSA-HA nanogels. A direct comparison of SAAP-148 and Ab-Cath in solution indicated that Ab-Cath was more effective than SAAP-148.
especially against the Gram-negative *A. baumannii*. Additionally, Ab-Cath showed lower cytotoxicity compared to SAAP-148. These findings were even more pronounced when Ab-Cath was formulated in the OL-HA nanogels, resulting in a very high selectivity index of ≥ 300 and ≥ 3000 against AMR *S. aureus* and *A. baumannii*, respectively. Therefore, Ab-Cath-loaded OL-HA nanogels show great promise for further clinical development as pharmaceutical product to combat AMR bacterial infections.

5. Conclusion

Together, SAAP-148 and Ab-Cath were successfully encapsulated in OL-HA nanogels with excellent physicochemical properties, e.g. small size, monodispersed size distribution, anionic surface charge, moderate encapsulation efficiency and high drug loading. Encapsulation of these AMPs in OL-HA nanogels maintained their antimicrobial activities against AMR bacteria, while reducing their hemolytic and cytotoxic activities substantially. This novel formulation thus improved the selectivity index of SAAP-148 and Ab-Cath by 2-fold and 16.8-fold, respectively. Therefore, Ab-Cath-loaded OL-HA nanogels show great promise for further clinical development as a delivery system to combat AMR bacterial infections.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2023.11.005.

References


Table 4

Selectivity index of SAAP-148- and Ab-Cath-loaded OL-HA nanogels compared to AMP solutions.

<table>
<thead>
<tr>
<th></th>
<th><em>S. aureus</em> (LUH14616)</th>
<th><em>A. baumannii</em> (RUH875)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAAP-148</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>SAAP-148 nanogel</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Fold increase</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Ab-Cath</td>
<td>19.5</td>
<td>208.3</td>
</tr>
<tr>
<td>Ab-Cath nanogel</td>
<td>327.2</td>
<td>3400.0</td>
</tr>
<tr>
<td>Fold increase</td>
<td>16.8</td>
<td>16.8</td>
</tr>
</tbody>
</table>

Selectivity index was determined by dividing EC50 values against human primary skin fibroblasts by LC50 values against *S. aureus* (LUH14616) and *A. baumannii* (RUH875), both at the 4 h time point.


