Biopolymer Nano-Network for Antimicrobial Peptide Protection and Local Delivery

Journal Article

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Antimicrobial resistance (AMR) develops when bacteria no longer respond to conventional antimicrobial treatment. The limited treatment options for resistant infections result in a significantly increased medical burden.

Antimicrobial peptides offer advantages for treatment of resistant infections, including broad-spectrum activity and lower risk of resistance development. However, sensitivity to proteolytic cleavage often limits their clinical application. Here, a moldable and biodegradable colloidal nano-network is presented that protects bioactive peptides from enzymatic degradation and delivers them locally. An antimicrobial peptide, PA-13, is encapsulated electrostatically into positively and negatively charged nanoparticles made of chitosan and dextran sulfate without requiring chemical modification. Mixing and concentration of oppositely charged particles form a nano-network with the rheological properties of a cream or injectable hydrogel. After exposure to proteolytic enzymes, the formed nano-network loaded with PA-13 eliminates *Pseudomonas aeruginosa* during in vitro culture and in an ex vivo porcine skin model while the unencapsulated PA-13 shows no antibacterial effect. This demonstrates the ability of the nano-network to protect the antimicrobial peptide in an enzyme-challenged environment, such as a wound bed. Overall, the nano-network presents a useful platform for antimicrobial peptide protection and delivery without impacting peptide bioactivity.

1. Introduction

Multi- and extensively drug-resistant microbes, such as bacteria, parasites, viruses, and fungi, no longer respond to therapeutic interventions that were effective previously. This is a growing public health challenge as there are few options to treat resistant microbes, increasing the risk of infection transmission, the rates of hospitalization, and the associated economic and social costs. Antimicrobial resistance (AMR) is of particular concern in environments with poor hygiene or in countries with less developed healthcare systems. It has been estimated that around 20 million individuals suffer from chronic wounds globally with annual management and treatment costs over 31 billion USD. Further, a retrospective analysis of bacterial wound cultures highlighted that AMR is a growing problem in dermatological and surgical wound infections. Multi-drug resistant bacteria, notably *Pseudomonas aeruginosa* and *Staphylococcus aureus*, impede wound healing, often resulting in prolonged morbidity and increased mortality following post-operative wound infection. *P. aeruginosa* is an opportunistic multi-drug resistant pathogen commonly isolated from wounds, which is classified as a critical pathogen by the Center for Disease Control and Prevention and the World Health Organization. Some strains are resistant to almost all antibiotics and can spread easily in clinical settings, for example on ventilators, resulting in ventilator-associated pneumonia in compromised hosts or life-threatening systemic diseases. Therefore, cost-effective antimicrobial treatments that are simple to administer are needed to mitigate the growing concern of antimicrobial resistance, especially in resource-limited settings.

Antimicrobial peptides possess several advantages over conventional therapies. In addition to exhibiting broad-spectrum antimicrobial activity, high potency, and a decreased likelihood to cause resistance, many antimicrobial peptides also possess antibiofilm and anti-inflammatory properties and induce wound healing and angiogenesis. However, one challenge in the use of bioactive peptides is their vulnerability to proteases present on skin and in wounds, which impede their development as therapeutic agents. Classic peptide stabilization strategies include d-enantiomer substitution and cyclization and...
chemical modification.[17] However, these chemical alterations can negatively impact antimicrobial peptide activity.[16a,18]

Alternative approaches have been explored to stabilize and deliver bioactive peptides without chemical modification to facilitate translation.[19] The activity of proteolytic enzymes, including trypsin, can be minimized by sequestering calcium ions present in the environment.[20] For example, Peppas and co-workers designed calcium-scavenging hydrogels that protected peptides from trypsin degradation and provided pH-tunable biomolecules.[21] Therapeutic peptides have also been trapped physically within hydrogels and lipid- or polymer-based nanocarriers to shield them from enzymatic degradation.[22]

One such approach relies on peptide encapsulation within particles comprised of charged biopolymers. Chitosan (CS), an antimicrobial cationic polysaccharide, and dextran sulfate (DS), an anionic polysaccharide, form colloidal polyelectrolyte complexes via coacervation.[23] Bioactive molecules, including charged antimicrobial peptides, can be encapsulated within CS–DS complexes during formation.[24] The biopolymer molecular weight, pH, CS:DS ratio, and the mixing process control the physicochemical properties (e.g., size and surface charge) of the CS:DS particles.[25] Thus, positively and negatively charged CS:DS particles can be prepared with tunable size.[26]

Following encapsulation of antimicrobial peptides into biomaterials, a method is often needed to apply the protected therapeutic to the site of infection.[27] One way to administer drug-loaded nanoparticles (NPs) to a target site is to assemble them into a colloidal gel that can be applied topically or injected locally.[28] Colloidal gels are composed of particles that form a viscoelastic solid via interparticle interactions, such as electrostatic forces, and have been applied extensively in the biomedical field.[29] Prior examples of antimicrobial colloidal gels required complex preparation techniques, such as polymer functionalization,[30] or exhibited limited efficacy.[31]

Building on these studies, our approach focused on protecting a potent antimicrobial peptide inside biopolymer NPs that were prepared as a colloidal gel for facile topical administration. The antimicrobial peptide, PA-13, was demonstrated to be effective against P. aeruginosa; however, susceptibility to enzymatic degradation and the associated loss of bioactivity limited its translation (Table S1, Supporting Information). To enable effective antimicrobial peptide delivery, we formulated particles from cationic antimicrobial CS and anionic DS without the need for chemical modification (Figure 1a). Positively charged nanoparticles (pNPs) and negatively charged nanoparticles (nNPs) were formed via complex coacervation of DS with CS (Figure 1b). The NP charge was controlled by tuning the CS to DS ratio and the order of polymer mixing. Simple mixing and concentration of positively and negatively charged NPs formed a colloidal nano-network (Figure 1c). The cationic antimicrobial peptide, PA-13, was loaded into the NPs via electrostatic complexation with anionic DS prior to NP formation to shield PA-13 from peptidase degradation (Figure 1d).

The colloidal gel was designed with rheological properties (shear-thinning and self-healing behavior) of a cream or injectable gel for facile topical application (Figure 1e). The nano-network maintained the activity of PA-13 and was able to eradicate P. aeruginosa during in vitro culture and in an ex vivo porcine skin model of infection, even after exposure to proteolytic enzymes. Unencapsulated PA-13 had no effect, suggesting complete enzymatic degradation.

2. Results and Discussion

2.1. Nano-Network Design

Our antimicrobial nano-network was formed by assembling positively charged and negatively charged NPs, referred to as pNPs and nNPs, respectively (Figure 2a). The particles were produced via coacervation of CS and DS, biodegradable and charged polysaccharides. The charge of the CS–DS NPs was controlled by tuning the weight ratio of CS to DS used to form flake-like coacervates and the order of polymer mixing (Figure S1, Supporting Information). A CS:DS ratio of 5:3 formed pNPs with a ζ-potential of 30 mV, based on the abundance of positive amines relative to sulfate groups (Figure 2b). Changing the weight ratio to 3:5 formed nNPs with a negative ζ-potential of ~25 mV. The mean hydrodynamic diameter (Dh) of pNPs was 455 nm with a dispersity (D) of 0.45; nNPs were formed with Dh ≈ 314 nm and D ≈ 0.26 (Figure 2c). The properties of the formed NPs were in agreement with previously published studies.[24] Upon mixing of pNPs and nNPs in a weight ratio of 3:2, the suspension turbidity increased due to the formation of micron- to millimeter-scale colloidal aggregates that were visible to the eye (Figure S2, Supporting Information). The formed aggregates had a negative ζ-potential of ~20 mV.

The pNP:nNP aggregates were centrifuged at 7000 RCF for 30 min to concentrate them into a densely-packed network of particles, forming the colloidal nano-network (~6 wt%; Figure 2d). Scanning electron microscopy (SEM) and cryo-SEM images demonstrated that the nano-network was comprised of discrete colloidal particles (Figure 2e and Figure S3, Supporting Information). Nano-network formation was a simple process which involved only mixing and centrifugation steps without requiring any chemical alteration of the precursor materials used. A variety of other pNP:nNP ratios were used to formulate nano-networks (Figure S4, Supporting Information). In our case, we focused on the 3:2 ratio as this formulation formed colloidal aggregates that sedimented completely and were therefore simple to concentrate.

2.2. Peptide Encapsulation and Release Properties

Recently, a potent antimicrobial peptide, PA-13, was developed to treat antimicrobial resistant P. aeruginosa. However, PA-13 was susceptible to enzymatic degradation and the activity of its α-isomer was too low for effective use (Tables S1 and S2, Supporting Information). Therefore, to protect PA-13 against enzymatic degradation, we encapsulated the antimicrobial peptide within CS:DS NPs to form an antimicrobial nano-network and prevent enzyme access to the peptide. PA-13 was incorporated in both pNPs and nNPs via coacervation with DS prior to NP formation. To image the spatial distribution of the particles in the nano-network, FITC and TAMRA-labeled PA-13 were encapsulated into pNPs and nNPs, respectively. The lack of overlap in the fluorescent signals further indicated that the nano-network was comprised of distinct particles (Figure 2f). In addition, the retention of the labeled peptides indicated that PA-13 was successfully
Figure 1. Formation of a nano-network for antimicrobial peptide protection and delivery. a) The network was composed of chitosan (CS), an antimicrobial positively charged polysaccharide, and dextran sulfate (DS), a negatively charged polysaccharide. b) Complex coacervation of CS and DS formed positively charged particles (pNPs; 5:3 ratio) or negatively charged particles (nNPs; 3:5 ratio). c) A colloidal gel was formed upon simple mixing and concentration of pNPs and nNPs. d) The cationic antimicrobial peptide, PA-13, was complexed with the anionic DS prior to NP formation. DS complexation allowed peptide loading and protease protection within the nano-network. e) Rheological characterization demonstrated that the formed network had the necessary properties for application as a topical cream or injectable material to eradicate antimicrobial resistant *P. aeruginosa*.

The loading efficiency of PA-13 in the nano-network was $\approx 77\%$, based on a fluorescamine analysis, resulting in a final loading in the nano-network of up to $\approx 5.4$ mg mL$^{-1}$ (Table S3, Supporting Information).The peptide loading efficiency in the nano-network was in accordance to literature values for single pNPs and nNPs.[24,25] Peptide inclusion did not alter particle $\zeta$-potential and led to a negligible increase in hydrodynamic diameter and dispersity both for pNPs and nNPs (Table S4, Supporting Information). The nano-network did not affect the metabolism of human skin keratinocytes suggesting that the formulation was not cytotoxic (Figure S5, Supporting Information).

Flow cytometry demonstrated that PA-13 released from the nano-network retained its ability to interact with the membrane of *P. aeruginosa*, which is critical for antimicrobial activity (Figure S6, Supporting Information). Less than 1% of the loaded peptide was released from the nano-network after 24 h, suggesting that the diffusion of PA-13 from the material was minimal (Figure S7, Supporting Information). As designed, attractive electrostatic interactions between PA-13 and the biopolymers maintained the peptide in the nano-network. Based on previous literature, we hypothesized that the nano-network would be effective to protect PA-13 from enzymatic degradation by not allowing the peptide to diffuse out from the material.[21a,32] Thus, we envisioned that the nano-network should be in proximity of the bacteria to exert its therapeutic effect. Given the experimental evidence that both PA-13 and chitosan exhibit antimicrobial activity by lysing the bacterial membrane, we hypothesized that the components of the nano-network could augment the bacterial membrane lysing capability of the peptide.[10b,33]

2.3. Rheological Characterization of the Colloidal Nano-Network

The nano-network was developed to protect PA-13 from enzymatic degradation as well as to deliver the peptide locally to sites of bacterial infection. Therefore, the material was designed to flow out of a tube or syringe upon application of shear (shear-thinning) and reform (self-healing) when the shear was removed (Figure 3a,b). At low strain ($\gamma = 0.1\%$), the colloidal gel exhibited solid-like behavior ($\tan \delta = 0.13–0.2$) with a weak frequency dependence ($\alpha = 0.08$; Figure 3c; Figure S8, Supporting Information). Upon increasing shear, an apparent yield behavior and
flow were observed as the material began to flow beyond $\gamma \approx 2\%$ (Figure 3d). The nano-network exhibited shear-thinning behavior with a shear-thinning index of $n = 0.18$, indicating its suitability for application as a cream (Figure 3e). To characterize the ability of the nano-network to self-heal following extrusion, the material was subjected to alternating high shear strain intervals to break the colloidal network ($\gamma = 100\%$) and low shear strain intervals to enable network reformation ($\gamma = 0.1\%$). The nano-network recovered its mechanical properties and solid-like properties following network disruption (Figure 3f). Gel formation was not limited to the 3:2 pNP:nNP formulation as gels were also formed with other pNP:nNP ratios (Figure S9, Supporting Information). In this work, based on the promising viscoelastic properties of the 3.2 nano-network, we decided to apply the material as a topical cream for local peptide protection and delivery.

2.4. Nano-Network Preserved Antibacterial Activity of PA-13 in a Proteolytic Environment

Our nano-network was designed to protect the encapsulated peptide, PA-13, from enzymatic degradation. The PA-13 loaded nano-network, unencapsulated PA-13, and all controls were incubated with 1 mg mL$^{-1}$ trypsin at 37 °C for 1 h, to simulate a proteolytic environment (Figure 4a). Subsequently, the antibacterial activity of each material was tested against *P. aeruginosa* ATCC 27853, as measured by the difference in viable bacterial counts ($\Delta \log_{10}$CFU mL$^{-1}$) between untreated PBS and treated samples (Figure 4b). We compared the difference in viable bacterial counts ($\Delta \log_{10}$CFU mL$^{-1}$) of non-trypsin-challenged and trypsin-challenged materials. Without contact with trypsin, unencapsulated PA-13 successfully eliminated *P. aeruginosa*. However, PA-13 lost its antibacterial activity in the presence of trypsin due to proteolytic degradation. All controls tested did not show significant differences. When formulated into the nano-network and protected from proteolytic degration, PA-13 eradicated *P. aeruginosa* (reduction of 5 log$_{10}$CFU mL$^{-1}$). The following results highlighted the susceptibility PA-13 against enzymatic degradation and demonstrated the ability of the nano-network to protect and deliver antimicrobial peptides. The commercial Bepanthen antiseptic cream and a chlorhexidine-based solution (Hexene Skin Cleanser) served as positive controls and killed all bacteria. The unloaded nano-network and acetic acid
solution used in NP synthesis exhibited mild antibacterial activity, with ≈2 log10 CFU mL⁻¹ reduction (Figure S10, Supporting Information). This demonstrated that the antibacterial activity of PA-13 loaded nano-network may have been enhanced by the use of acetic acid [34] and chitosan [35] in its design. In summary, these results showed that our engineered nano-network protected PA-13 from enzymatic degradation and retained its bioactivity.

2.5. PA-13 Loaded Nano-Network Eliminated P. aeruginosa in an Ex Vivo Infection Model

Porcine skin serves as a representative model of dermal skin infection, such as in a wound setting. [36] In our study, an ex vivo porcine skin infection model was used to evaluate the antimicrobial activity of the PA-13 loaded nano-network under enzyme-challenged conditions in a dermal environment. We followed a simple and reliable published method as described by Rubinchik et al. (2009). Trypsin-treated porcine skin was infected with a solution of ≈10⁶ CFU mL⁻¹ of P. aeruginosa in PBS followed by treatment with PA-13 loaded nano-network or controls at 0, 6, or 12 h. After 6, 12, and 24 h of incubation, viable bacteria cells were determined using a colony count assay. During log-phase bacterial growth (12 h), the untreated skin (PBS) showed a slight blue-green pigment indicative of P. aeruginosa infection, whereas samples treated with the unloaded nano-network and PA-13 loaded nano-network were colorless (Figure 4d and S11), reflecting inhibition of P. aeruginosa growth. The difference in blue-green pigment was observed most clearly after 24 h of incubation. The positive control, hexene cleaner solution, showed strong antibacterial activity in both non- and trypsin-challenged conditions at all time-points (Figures S11 and S12, Supporting Information). Whereas, Bepanthen cream demonstrated weaker antibacterial activity, especially as compared with PA-13 loaded nano-network (Figure 4e and Figure S11, Supporting Information). The PA-13 loaded nano-network displayed potent bactericidal activity (≥2.7 log10 CFU mL⁻¹ reduction) and was more effective at eliminating P. aeruginosa at 24 h than both the unloaded nano-network and unencapsulated PA-13 (Figure 4e). The bactericidal activity of PA-13 loaded nano-network at 12 h may have been enhanced by the presence of chitosan in nano-network and acetic acid used during its preparation (Figure S11, Supporting Information); however, effective elimination of P. aeruginosa was only observed for the PA-13 loaded nano-network after 24 h of incubation (Figure 4e). In addition, the effect of nano-network on porcine infected skin after 24 h of incubation was evaluated by histology studies. The
Figure 4. Antibacterial activity of PA-13 loaded nano-network under enzyme-challenged conditions in vitro and in an ex vivo infection model. a) Schematic of the workflow for quantification of in vitro antibacterial activity. PA-13 loaded nano-network, unencapsulated PA-13, and control samples were pre-treated with trypsin. After 1 h, a trypsin inhibitor was added to stop the reaction. Samples were then incubated with *P. aeruginosa* for 6 h and viable bacterial cells were quantified using a colony count assay. b) Differences in colony counts ($\Delta \log_{10}$CFU mL$^{-1}$) between the untreated (PBS) and treated samples of in vitro antibacterial activity were calculated. Values represented as the mean ± SD ($N = 3, n = 1$). $p$-values were determined using a two-way
results revealed that the stratified structures of epidermis with the underlying stratum corneum and dermis were well preserved in all conditions (Figure S13, Supporting Information). No adverse effects were observed after treatment with PA-13 loaded nano-network, except stratum corneum exfoliation that may have been caused by the presence of acetic acid (Figure S13c–h, Supporting Information). The skin exfoliation caused by acetic acid was not a major concern as acetic acid is one of the alternative topical agents for the treatment of pseudomonal infections of burns and, skin and soft tissue infections.[14,37] Based on its ability to eliminate *P. aeruginosa* in the porcine skin infection model without severe adverse effects, the PA-13 loaded nano-network comprises a suitable platform for local application and treatment of *P. aeruginosa* at infection sites or wounds.

3. Conclusions

The main obstacle limiting clinical application of the promising anti-pseudomonal peptide, PA-13, is susceptibility to proteolytic enzymes, which results in a loss of antimicrobial activity. To protect PA-13 from proteolysis and improve its stability, we designed a CS–DS nano-network for direct encapsulation and local release of antimicrobial peptides. The nano-network was composed of CD–DS nanoparticles, into which the antimicrobial peptide was entrapped via electrostatic complexation. The antimicrobial nano-network was designed with shear-thinning and self-healing properties for use as a topical cream or injectable gel. The PA-13 loaded nano-network retained potent antimicrobial activity against *P. aeruginosa* in trypsin-challenged conditions in vitro and in an *ex vivo* infection model, implying successful protection of PA-13 from proteolytic degradation and motivating the use of the PA-13 loaded nano-network as an anti-pseudomonal cream for topical applications. In total, the simple design of a biopolymer nano-network presents a potential delivery system for protecting antimicrobial peptides from proteolytic degradation as an additional therapeutic option in the treatment of resistant microbes.

4. Experimental Section

**Materials**: Low MW chitosan (CS; 50–190 kDa), high MW dextran sulfate sodium salt (DS; 500 kDa), glacial acetic acid, fluororescine, and trypsin inhibitor were purchased from Sigma-Aldrich (CH). Trypsin-EDTA was purchased from ThermoFisher (CH). PA-13 and TAMRA-labeled PA-13 peptides were synthesized by Chinapeptide (CN). Ultrapure deionized water (dH₂O) was freshly filtered using a Milli-Q system (Merck Millipore) (CH). All other chemicals and reagents used in this study were analytical grade.

**Preparation of Nanoparticles**: The CS–DS NPs were prepared at room temperature via complex coacervation of two oppositely charged polymers (CS and DS).[24] CS solution (0.1% w/v; pH 2.9) was prepared by dissolving CS in aqueous acetic acid (2% v/v; pH 2.6). DS solution was prepared in dH₂O (0.1% w/v; pH 7.4). To produce pNPs, a 5:3 ratio of CS:DS was used by adding 4.5 mL of DS solution dropwise to 7.5 mL of CS solution. Whereas a 3.5 ratio of CS:DS produced nNPs by adding 3 mL of CS solution to 5 mL of DS solution. The NPs spontaneously formed at RT under stirring at 600 RPM. The particle suspensions were stirred for an additional 15 min after formation. The mean size and zeta potential of both NP suspensions was n=3.2. The peptide, PA-13, was incorporated into both pNPs and nNPs by adding it to the DS solution prior to mixing it with CS. To prepare pNPs, 600 μL of PA-13 solution (2 mg mL⁻¹) was added to 4.5 mL of DS solution. To prepare nNPs, 400 μL of PA-13 solution (2 mg mL⁻¹) were added to 5 mL of DS solution. Formation of PA-13 loaded NPs was performed as described above.

**Preparation of CS-DS Nano-Network**: The nanoparticle network (nano-network) was prepared by adding nNPs to pNPs at a ratio of 3:2 and stirring for 15 min. Subsequently, the particles were centrifuged at 7000 RCF for 30 min. The supernatant was removed. The formed nano-network pellet was collected at the bottom of the centrifuge tube for further studies. The PA-13 loaded nano-network was formed from PA-13 loaded pNPs and nNPs using the same formation procedure as described above. The determination of the nano-network parameters was performed based on three independent experiments. The weight percent of the nano-network was determined after nano-network centrifugation. The material in the hydrated state was lyophilized to determine its dry mass. The weight percent was calculated by the ratio of the dry mass over the hydrated mass of the gel.

**Size of Nanoparticles**: Particle hydrodynamic diameter (Dₜ) and dispersity (D) were characterized via dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern, UK). For each measurement back scattered light at 173° was detected. 1 mL of tenfold diluted NP suspension (1:9 in dH₂O) was analyzed in disposable polystyrene semi-micro cuvettes at 25 °C. For each sample, the z-average of the intensity distribution was taken as the hydrodynamic diameter and mean values were calculated by averaging three DLS measurements.

**Charge of Nanoparticles**: Particle zeta potential, 𝜁, was determined by light scattering using a ZetaSizer Nano ZS (Malvern, UK). The measurement angle was 90° to the incident light. All NP samples were prepared by tenfold dilution in dH₂O followed by addition of 0.1x PBS to achieve a conductivity of 1mS cm⁻². Data were collected over at least ten cycles. The zeta potential was calculated from the electrophoretic mobility using the Smoluchowski approximation. All data are reported as mean ± SD of three independent experiments.

**Quantification of Entrapped Peptide in the Nano-Network**: The encapsulated peptide in the PA-13 loaded nano-network was determined by quantifying unencapsulated PA-13 in the supernatant following particle and nano-network preparation. PA-13 was detected using fluororescine assay following the manufacturer’s instructions. Primary amines present on the peptide react with fluororescine to form fluorescent products that were quantified via fluorescence spectroscopy. In brief, 3 mg of fluororescine was dissolved in 1 mL DMSO. 50 μL of the formed solution was mixed with 150 μL of nano-network supernatant and incubated in the dark at room temperature for 15 min. The fluorescent signal A_emission = 355/40 nm, A_excitation = 460/20 nm was measured using a fluorescent microplate reader (Hidex Sense). The amount of unencapsulated PA-13 in the supernatant was quantified on a standard curve. All measurements were performed in triplicate, and data were reported as mean ± SD. The efficiency of peptide encapsulation was calculated by the ratio of PA-13 mass that was retained in the nano-network and the total mass of PA-13 which was initially added (Supporting Information). The peptide

ANOVA with Tukey's post hoc test. c) Schematic of the experimental setup and workflow of the *ex vivo* infection model in porcine skin. Porcine skin samples were infected with 10⁶ CFU mL⁻¹ of *P. aeruginosa* prior to treatment with saline for 15 min. The PA-13 loaded nano-network was loaded onto the infected PA-13, and control samples were then applied on the infected porcine skin at 0, 6, and 12 h. After incubation, treated porcine skin samples were swabbed to quantify the viable bacteria. d) Photographs of infected porcine skin treated with PA-13 loaded nano-network compared with samples treated with unloaded nano-network or PBS after 6, 12, and 24 h of treatment. e) Differences in viable bacteria (Δlog₁₀ CFU mL⁻¹) between the treated and untreated (PBS) skin at 24 h were calculated. Values were log₁₀ transformed and presented as the mean ± SD (N=6, n=3). *, p-values were determined using a two-way ANOVA with Tukey’s post hoc test. Significant differences are indicated in GP style: >0.05 (ns), ≤0.05 (*), ≤0.01 (**), ≤0.001 (***) , ≤0.0001 (*****).
concentration inside the nano-network was calculated by dividing the retained PA-13 in the nano-network with the total volume of the nano-network.

Rheological Characterization: Rheological characterization of the nano-network was performed on a strain-controlled shear rheometer (MCR 502, Anton-Paar, CH). For all measurements the temperature was maintained at 20 °C by a Peltier stage and, unless stated otherwise, a 20 mm parallel plate geometry was used with 0.5 mm gap size. A passive hold was used to limit water evaporation during the measurements. For each measurement, three independent replicates were tested (n = 3; Figure S8, Supporting Information). The nano-network was characterized with dynamic oscillatory frequency sweeps from 100 to 1 rad s⁻¹ at a constant shear strain, γ, of 0.1%. The frequency dependence was quantified by fitting rheological data to \( G' \propto \omega^n \) over the entire interval tested. The shear behavior of the nano-network was measured with a dynamic oscillatory strain amplitude sweep from 0.01% to 100% at a constant angular frequency, \( \omega \), of 1 rad s⁻¹. The shear-thinning properties of the nano-network were investigated with rotational shear rate measurements (\( \gamma/\dot{\gamma} = 0.01–1000 \) s⁻¹). The measured viscosity was fit to a power law of the form \( n = K_p (\gamma/\dot{\gamma})^{n-1} \), where \( n \) is the shear-thinning index and \( K_p \) is the consistency index. A material is considered shear-thinning if \( n < 1 \), Newtonian if \( n = 1 \), or shear-thickening if \( n > 1 \). The self-healing ability of the nano-network was characterized via oscillatory step strain measurements by alternating intervals of high strain (\( \gamma = 100\% \); \( \omega = 10 \) rad s⁻¹) to break the material network and low strain (\( \gamma = 0.1\%; \omega = 10 \) rad s⁻¹) to monitor network reformation.

Confocal Microscopy: For confocal imaging, TAMRA- and FITC-labeled PA-13 were encapsulated in the NPs and assembled into a nano-network as described previously. The nano-network was deposited on a glass slide and gently covered with a cover slip. The borders of the cover slip were sealed with nail polish to prevent water evaporation. Fluorescent images of the nano-network were collected on a confocal laser scanning microscope (LSM 780, Axio Observer; Zeiss) equipped with an Airyscan detector. FITC was excited with a 488 nm laser and detected between 507 and 571 nm, whereas TAMRA was excited with a 561 nm laser and detected between 674 and 750 nm.

Determination of Antibacterial Activity of Entrapped Peptide in Nano-Network under Proteolytic Enzyme-Challenged Environment: P. aeruginosa ATCC 27853 were cultured and diluted in Mueller Hinton Broth to a concentration of \( 10^6 \) CFU mL⁻¹. Samples of CS–DS nano-network (25 ± 1 mg) containing 200 μg of PA-13 were incubated with 20 μL of 1 mg mL⁻¹ trypsin-EDTA at 37 °C for 1 h. Then, 20 μL of 1 mg mL⁻¹ trypsin inhibitor was added and incubated for an additional 1 h at 37 °C to stop the reaction. After that, 160 μL of diluted bacterial suspension was added to all samples. The bacteria were also exposed to PBS and acetic acid buffer (pH 5.0) as controls. We also tested the antibacterial activity of 20 μL of PA-13 (10 mg mL⁻¹; 200 μg) and 25 ± 1 mg of unloaded nano-network. After 6 h of incubation at 37 °C, the mixtures of bacteria and the formulations were serially diluted in 1× PBS and plated on Mueller Hinton agar. After overnight incubation at 37 °C, the number of viable bacteria was determined and calculated in CFU mL⁻¹.

Ex Vivo Porcine Skin Model of P. aeruginosa Infection in Enzyme-Challenged Conditions: The porcine skin infection model was performed using a modification of a published method. Porcine skin samples were obtained directly from slaughterhouses (Anghong, TH) or from butchers (Zurich, CH). Hairless porcine skin that was free of injuries and redness was pre-swabbed thoroughly with 70% ethanol, further cleaned with sterile 0.85% NaCl and stored at −20 °C. Fat layers were removed from the frozen skin sections with a scalpel and the skin was cut into 2 × 2 cm pieces. These pieces were washed with sterile 0.85% NaCl and sterilized by dipping in 70% alcohol. The skin was dried with sterile paper towels and distributed into experimental groups (one piece per plate). P. aeruginosa ATCC 27853 at \( 10^6 \) CFU mL⁻¹ were applied to each skin surface and incubated at 37 °C for 2 h in a humidified chamber to allow bacterial cells to adhere and establish infection. Afterwards, 1 mg mL⁻¹ of trypsin was applied to the porcine skin pieces for 15 min. The formulations containing 1.54 mg PA-13 were applied onto the infected side of skins at 0, 6, and 12 h, and incubated at 37 °C in a humidified chamber. To control the amount of nano-network added to the porcine skin, we measured the total weight (in mg) of the nano-network before adding the gel to the porcine skin and after. In total, we applied 250 ± 20 mg of nano-network on the ex vivo tissue. After incubation, the treated skin samples were swabbed at three time points (6, 12, and 24 h) by sterile cotton swabs dipped in 0.85% NaCl and CFU counts were determined. Samples without treatment (PBS) served as a control.

Statistical Analysis: Experiments were performed in triplicate and the results, presented as mean ± SD, were analyzed using two-way ANOVA followed by either Tukey or Bonferroni post hoc test. Significant differences are indicated in GP style: >0.05 (ns), ≤0.05 (*), ≤0.01 (**), ≤0.001 (***) and ≤0.0001 (****). Statistical analysis was carried out using GraphPad Prism 7 Software.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

antimicrobial peptides, antimicrobial resistance, biopolymer nanoparticles, nano-networks, peptide stability

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