Supporting Information

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Biopolymer Nano-Network for Antimicrobial Peptide Protection and Local Delivery

Natthaporn Klubthawee, Giovanni Bovone, Bruno Marco-Dufort, Elia A. Guzzi, Ratchaneewan Aunpad* and Mark W. Tibbitt*
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Contents
Supporting information on experimental methods.......................................................... 2
Supporting information on the antibacterial activity of PA-13 against Pseudomonas aeruginosa .......................................................... 7
Supporting information on the ratio of CS to DS ........................................................... 7
Supporting information on the characterization of nNPs, pNPs, and sedimenting pNP:nNPs . 8
Supporting information on peptide encapsulation ......................................................... 10
Supporting information of nano-networks with different NP charge ratios and rheology ...... 11
Supporting information on material cytotoxicity ......................................................... 12
Supporting information on the interaction between the released PA-13 and the bacterial membrane .................................................................................................................................................................................................................................................. 13
Supporting information on the release of TAMRA-labelled PA-13 from the nano-network .. 14
Supporting information on the rheology of the 3:2 nano-network formulation ............... 15
Supporting information on the in vitro antibacterial activity of PA-13 loaded nano-network under enzyme-challenged conditions .......................................................... 16
Supporting information on the PA-13 loaded nano-network eliminated P. aeruginosa in an ex vivo infection model .......................................................... 17
Supporting information on the photographs of infected porcine skin after treatment ....... 18
Supporting information on the histological characteristics of porcine skin after nano-network treatment .......................................................................................................................... 19
Supporting information on the hemolytic activity of nano-network .................................. 21
Supporting information on the antibacterial activity after contact with human plasma ..... 22
References .......................................................................................................................... 23
Supporting information on experimental methods

Characterization of sedimenting pNP:nNP aggregates: For the formation of pNP:nNP aggregates, DLS results showed particles in the micrometer range; however, the measurement did not account for the presence of large sedimenting µm to mm-sized aggregates. The size of sedimenting pNP:nNPs was measured with a light microscope (EVOS M5000, ThermoFisher). Approximately 0.3 mL of pNP:nNP aggregate suspension was deposited onto a glass slide. The particle length in 26 pictures was measured with ImageJ. A total of 41 particles were counted and the mean length was calculated.

Quantification of entrapped peptide in nano-network: The encapsulated peptide in the PA-13 loaded nano-network was determined by quantifying free PA-13 in the supernatant following particle and nano-network preparation. PA-13 was detected using the fluorescamine assay following the manufacturer’s instructions. Primary amines present on the peptide react with fluorescamine to form fluorescent products that can be quantified via fluorescence spectroscopy. In brief, 3 mg of fluorescamine was dissolved in 1 mL DMSO. 50 µL of the formed solution was mixed in 150 µL of nano-network supernatant and incubated in the dark at room temperature for 15 min. The fluorescent signal λ_{excitation} = 355/40 nm, λ_{emission} = 460/20 nm was measured using a fluorescent microplate reader (Hidex Sense). To generate a standard curve, a 2-fold serial dilution of PA-13, ranging from 0.49 to 15.63 µg mL\(^{-1}\) was prepared in acetic acid solution (2% v/v). The amount of free PA-13 in the sample supernatant was compared to the standard curve. All measurements were performed in triplicate and data is reported as mean ± SD.

The amount of encapsulated peptide was quantified by subtracting the total amount of loaded PA-13 into the nano-network (2 mg) from the amount of PA-13 present in the nano-network supernatant (~0.5 µg; Table S4). The theoretical loading of peptide, tPL, was defined as the mass of peptide added to the NN, \(m_{\text{peptide loaded}}\), divided by the total dry mass of the material, \(m_{\text{polymer and peptide}}\) (Eq. 1). The latter was measured by lyophilizing (Lyovapor L300, Büchi, CH) the specimen and measuring the residual dry content.

\[
tPL = \frac{m_{\text{peptide loaded}}}{m_{\text{polymer and peptide}}} \cdot 100\%
\]
The effective peptide loading, ePL, defined the nano-network loading of PA-13 while considering the amount of PA-13 which was lost. The mass of non-entrapped peptide remaining in supernatant, \( m_{\text{free peptide}} \), was subtracted from the initial mass of peptide added to the NN (2 mg), \( m_{\text{peptide loaded}} \), and normalized by the remaining dry mass of the sample after lyophilization (Eq. 2).

\[
ePL = \frac{m_{\text{peptide loaded}} - m_{\text{free peptide}}}{m_{\text{residual polymer and peptide}}} \cdot 100\%
\]  

(2)

The efficiency of peptide loading, \( X_{\text{loading}} \), was estimated by the ratio of the effective and theoretical peptide loading (Eq. 3).

\[
X_{\text{loading}} = \frac{ePL}{tPL} \cdot 100\% = \frac{m_{\text{peptide loaded}} - m_{\text{free peptide}}}{m_{\text{peptide loaded}}} \cdot 100\%
\]  

(3)

Flow cytometry analysis: \( P. \ aeruginosa \) at a concentration of \( 10^6 \) CFU mL\(^{-1} \) were incubated with TAMRA-PA13 loaded nano-network at 37 °C for 6 h. The supernatant was collected after centrifugation and the fluorescence signal (\( \lambda_{\text{emission}} = 585/42 \) nm) emitted by TAMRA fluorescence dye was determined by flow cytometry (CytoFlex, Beckman Coulter).

Cryogenic transmission electron microscopy (Cryo-TEM): For cryo-EM analysis 4.0 µL of pNP or nNP suspensions at 0.1% w/v was applied onto Lacey Carbon Film 300 mesh copper grids, which were previously negatively glow-discharged at 25 mA for 30 s. Excess of sample was blotted away for 2 seconds and plunge-frozen in liquid ethane/propane mixture (continuously cooled by liquid nitrogen) using Vitrobot Mark IV (ThermoFisher Scientific) with environmental chamber set to 100% humidity and 22°C.

Vitrified grids were observed under Tecnai F20 (ThermoFischer Scientific) electron microscope using Gatan 626 cryo-holder. Falcon2 integrated micrographs representing cumulative dose of ~45 electrons per Å\(^2\) were collected at approx. −3 µm defocus and 1.32 Å pixel size.
Cryogenic scanning electron microscopy (Cryo-SEM): Around 5 µL of sample were filled into 6 mm aluminium-planchettes and, in each case, two of them where assembled to form a sandwich, frozen in a high-pressure freezer HPM 100 (Bal-Tec/Leica, AT), and stored in liquid nitrogen. Vitrified specimens were then transferred and mounted under liquid nitrogen on the cryo-holder and finally transferred under liquid nitrogen into a precooled (∼130°C) freeze-fracturing system BAF 060 (Bal-Tec/Leica, AT) at 1.10⁻⁶ mbar. Coating was performed with 2 nm tungsten at an elevation angle of 45° followed by 2 nm at 90°. The samples showed in **Figure S3** were directly coated, whereas the sample showed in **Figure 2e** was etched 8 minutes at -100°C and 5.10⁻⁷ mbar prior to coating. Transfer to the precooled cryo-SEM was done under high vacuum (<5 × 10⁻⁶ mbar) with an air-lock shuttle. Cryo-SEM was performed in a field emission SEM Leo Gemini 1530 (Carl Zeiss, DE) equipped with a cold stage to maintain the specimen temperature at −130°C (VCT Cryostage, Bal-Tec/Leica, AT). Inlens-SE- and Everhart-Thornley SE-signals at an acceleration voltage of 2 kV were used for image acquisition.

**Scanning electron microscopy (SEM):** A drop of the 0.1% w/v nano-network suspension was placed on a clean silicon wafer, which was previously glow-discharged. The wafer was mounted onto a stub with conductive silver paint (Plano GmbH - G3692). The specimen was sputter coated with 4 nm Pt-Pd (Safematic CCU-010 Metal Sputter Coater). Images were taken on a FEI Magellan 400 SEM. SE-signals at an accelerating voltage of 3 kV and 50 pA were used for image acquisition.

**Suspension turbidity:** After mixing of pNP and nNP suspension, the formed of pNP:nNP aggregates were left at room temperature to sediment for at least 12h. Subsequently, 1 mL of supernatant was collected, placed in Brand Micro Cuvettes (Polystyrene, path length: 1 cm) and its absorbance was measured with a spectrophotometer at λ = 500 nm (Perkin Elmer, Lambda 35).

**TAMRA PA-13 release from the nano-network:** 270 µL of 2 mg mL⁻¹ TAMRA-labeled PA-13 were mixed with 270 µL of 2 mg mL⁻¹ unlabeled PA-13. The labeled and unlabeled PA-13 were then encapsulated into the nano-network. After centrifugation, 73 mg of the PA-13-loaded nano-network were transferred into an Eppendorf tube. The peptide release experiment was started by adding 400 µL of 1x PBS to the nano-network and mixing the suspension by pipetting
3 times with a 1 mL pipette. The suspension was placed on an orbital shaker (300 RPM) and incubated at 37 °C. At defined timepoints, the nano-network was spun down by centrifuging 1 min at 700 RPM. 200 µL of release supernatant were withdrawn and replaced with fresh 1x PBS. The nano-network was resuspended by pipetting 3 times and incubated on the orbital shaker. The fluorescence of TAMRA-PA-13 was quantified using a plate reader (Hidex Sense, \( \lambda_{\text{excitation}} = 544/20 \text{ nm}, \lambda_{\text{emission}} = 575/20 \text{ nm} \)). The concentration of TAMRA-PA-13 was determined by comparing the results with a calibration curve. The total amount of PA-13 released in the supernatant was calculated with the assumption that the release rate of TAMRA-PA-13 was similar to that for unlabeled PA-13.

**Nano-network cytotoxicity:** Aneuploid immortal keratinocytes from human skin (HaCat) were cultured in DMEM-High glucose medium which was supplemented with 100 U mL\(^{-1}\) Penicillin-Streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum (FBS). The cells were incubated at 37 °C and 5% CO\(_2\). The cytotoxicity of the nano-network and its components was characterized with an Alamar Blue cell metabolic assay. Cells were seeded in 96-well plated at a concentration of \( 30 \cdot 10^3 \) cells/well and incubated for at least 8 h. 180 µL cell culture medium were mixed with 20 µL PBS, or 20 µL 1% acetic acid in pure water, or 20 mg unloaded nano-network, 20 mg PA-13 loaded nano-network, or 20 µL 4.1 mg mL\(^{-1}\) PA-13, or 20 mg Bepanthen®, or 20 µL of 1% Triton-X. The solutions and suspensions were added to the cell culture and incubated. After 12 h, all wells were rinsed at least 3 times with PBS. Subsequently, the Alamar Blue metabolic assay was performed according to the manufacturer protocol. In brief, 90 µL of cell culture medium and 10 µL Alamar Blue were added to the culture and cells were incubated at 37°C and 5% CO\(_2\) for 4 h. For all conditions, cell supernatant fluorescence was quantified with a Hidex Sense at \( \lambda_{\text{excitation}} = 544/20 \text{ nm} \) and \( \lambda_{\text{emission}} = 590/20 \text{ nm} \). The fluorescence of all conditions relative to the untreated culture (100% cell medium) was reported.

**Histological analysis:** For the histological examination, the frozen porcine skin without hair removal was washed and inoculated with *P. aeruginosa* ATCC 27853 at \( \sim 10^6 \) CFU mL\(^{-1}\) at 37 °C for 2 h in a humidified chamber. The formulations were applied once onto the infected side of skins and incubated for 24 h at 37 °C. The porcine skin samples were fixed in 10% neutral-buffered formalin. Preserved tissues were paraffin wax-embedded, sectioned, stained
with hematoxylin and eosin (H&E), and examined microscopically under an Olympus BX53 microscope (Center Valley, PA, USA).

In vitro hemolytic activity analysis: The hemolytic activity of nano-network was assayed in vitro against human red blood cells (hRBCs) by measuring the amount of hemoglobin released after treatment.\[1\] Human whole blood samples were freshly collected from healthy volunteers in polycarbonate tubes containing heparin. The human red blood cells (hRBCs) were collected by centrifugation at 2,000 × g for 5 min, then washed three times in sterile PBS. The hRBCs were diluted to a final concentration of 2% (v/v), then 180 µL of the hRBCs suspension was incubated with 20 ± 1 mg of formed nano-network. After incubation at 37 °C for 1 h and 6 h, the materials and intact hRBCs were pelleted by centrifugation at 2,000 × g for 5 min. The supernatant was transferred to a new 96-well plate and the release of hemoglobin was monitored by measurement of absorbance at 405 nm using a Multiskan FC microplate reader. The hRBCs in PBS only (OD\text{Blank}) and in Hexene skin cleanser solution (OD\text{Hexene}) were employed as negative (0% hemolysis) and positive (100% hemolysis) controls, respectively. The percentage of hemolysis was calculated according to the following equation (Eq. 4):

\[
\% \text{ Hemolysis} = \frac{OD_{\text{Sample}} - OD_{\text{Blank}}}{OD_{\text{Hexene}} - OD_{\text{Blank}}} \times 100\%
\] (4)
Supporting information on the antibacterial activity of PA-13 against *Pseudomonas aeruginosa*

**Table S1.** Minimal inhibitory concentration (MIC) of PA-13 against *P. aeruginosa* in enzyme-challenged condition.

| MIC (µg mL⁻¹) of PA-13 | In enzyme-challenged condition |
|-------------------------|--------------------------------|
| *P. aeruginosa* ATCC 27853 | Trypsin |
|                         | >250 |

**Table S2.** Minimal inhibitory concentration (MIC) of PA-13 compared to the D-isomer of PA-13, dPA-13, against *P. aeruginosa*.

| MIC (µg mL⁻¹) | In normal media |
|---------------|-----------------|
|               | PA-13           | dPA-13*          |
| *P. aeruginosa* ATCC 27853 | 3.91 | 7.81 |

* dPA-13 defined as all L-isomer of amino acids in PA-13 sequence was substituted with D-isomer of amino acids

**Supporting information on the ratio of CS to DS**

Under the experimental conditions, CS carried $5.2 \times 10^{-3}$ positively charged groups per gram and DS $5.8 \times 10^{-3}$ negatively charged groups per gram. The charge of the CS–DS NPs was controlled by tuning the weight ratio of CS:DS used to form the coacervates from 3:5 to 5:3, which corresponded to a ratio of positively to negatively charged groups of ~1.5 and ~0.5, respectively. The nano-network (pNP:nNP, 3:2) corresponded to a positively to negatively charged groups ratio of ~1.
Supporting information on the characterization of nNPs, pNPs, and sedimenting pNP:nNPs.

Cryo-TEM characterization of pNP and nNPs

Figure S1. Representative Cryo-TEM images of nanoparticles. a) Cryo-TEM image of pNPs where NPs are circled in orange. Scale bar, 1 µm. b) Higher magnification of one pNP. Scale bar, 100 nm. c) Cryo-TEM image of nNPs where NPs are circled in orange. Scale bar, 1 µm. b) Higher magnification of one nNP. Scale bar, 100 nm. The EM pictures taken suggest that the NPs are not perfectly spherical but of irregular shape and are composed by numerous smaller discrete entities.

Optical microscopy image of nNP:pNP aggregates which are visible to the eye

Figure S2. Light microscopy characterization of sedimenting pNP:nNP aggregates. a) Representative light microscopy image of sedimenting pNP:nNP aggregates. Scale bar, 750 µm. b) Size estimation from microscopy images suggested that the average length scale of the sedimenting pNP:nNP aggregates was 1.8 mm.
Cryo-SEM of nNP:pNP aggregates

Figure S3. Representative cryo-SEM image of the nano-network. a) The Cryo-SEM image shows the nano-network embedded in an amorphous aqueous solution (1% v/v of acetic acid). The discrete round entities visible in the Cryo-TEM pictures were also visible in the cryo-SEM. Scale bar, 100 nm. b) SEM image of the nano-network. Scale bar, 250 nm.
Supporting information on peptide encapsulation

The concentration of PA-13 was quantified as specified in the Supporting Methods section. By trying to load 9.1% (tPL) of PA-13 into the nano-network, the amount of PA-13 which was retained into the network was 6.9% (Table S4). The loading efficiency was estimated to be 77%.

Table S3. The amount of free PA-13 in supernatant and entrapped PA-13 in nano-network.

| Free PA-13 (µg) | Entrapped PA-13 (mg) | tPL (%) | ePL (%) | Xloading (%) |
|----------------|----------------------|---------|---------|--------------|
| 511 ± 61       | 1.5 ± 0.1            | 9.1 ± 0.1| 6.9 ± 0.3| 77 ± 4       |

Dynamic light scattering and electrophoretic mobility data of pNP and nNPs

Table S4. The particle hydrodynamic diameter ($D_h$), dispersity ($D$), and $\zeta$-potential of unloaded and PA-13 loaded nanoparticles.

|                        | Hydrodynamic diameter ($D_h$; nm) | Dispersity ($D$) | $\zeta$-potential ($\zeta$; mV) |
|------------------------|-----------------------------------|------------------|---------------------------------|
| Unloaded particles     |                                   |                  |                                 |
| pNPs                   | 455 ± 33                          | 0.45 ± 0.04      | +30 ± 2                         |
| nNPs                   | 314 ± 26                          | 0.26 ± 0.03      | -25 ± 1                         |
| PA-13 loaded particles |                                   |                  |                                 |
| pNPs                   | 481 ± 22                          | 0.47 ± 0.05      | +31 ± 3                         |
| nNPs                   | 326 ± 20                          | 0.35 ± 0.02      | -24 ± 1                         |
Supporting information of nano-networks with different NP charge ratios and rheology

The nano-network can be formed by using a variety of different pNP:nNP ratios. In our work, we formed nano-networks with the following charge ratios: 7:3, 3:2, 1:1, 2:3, and 3:7. The different charge ratios influenced the ability of the material to form flakes and sediment (Figure S4a). Turbidity experiments quantified residual scattering caused by NPs that were not able to sediment after at least 12 h (Figure S4b). All samples but the 3:2 pNP:nNP colloidal aggregates showed extensive light scattering due to incomplete sedimentation. The 3:2 pNP:nNP ratio formed large colloidal aggregates that sedimented rapidly. Subsequent rheological characterization of the nano-networks (7% w/v) showed that the nano-network formed with the 3:2 pNP:nNP ratio formed a gel with a storage modulus in the kPa range (Figure S9a). We decided to continue our study with the 3:2 pNP:nNP nano-network, as it exhibited rheological properties that were suitable for the formation of an injectable cream. All other pNP:nNP combinations formed gels that could be used for other applications (Figure 9b-f).

Figure S4. Sedimentation of pNP:nNP colloidal aggregates. a) Pictures showing the extent of colloidal aggregate sedimentation as a function of pNP:nNP ratio. b) Supernatant turbidity of the sedimanted colloidal aggregates. All ratios of pNP:nNP but the 3:2 ratio showed high turbidity, which suggests incomplete particle sedimentation.
Supporting information on material cytotoxicity.

Figure S5 shows the metabolic activity of HaCat cells which came into contact with the nano-network formulations and control samples. An Alamar blue assay was performed to determine the relative metabolic activity of each condition compared to untreated cells (100% cell culture medium). PBS, acetic acid, and the nano-network demonstrated no significant change in HaCat metabolic activity compared with the untreated control. Unencapsulated PA-13 decreased HaCat relative metabolic activity to ~0.44, whereas the relative metabolic activity of cells treated with PA-13 encapsulated within the nano-network remained at ~0.85. The commercial chlorohexidine-based cream Bepanthen, which is commonly used to treat wounds, resulted in no metabolic activity. These results support that the PA-13 loaded nano-network is non-cytotoxic.

Figure S5. In vitro cytotoxicity of the nano-network. The nano-network cytotoxicity was evaluated by measuring the relative metabolic activity of HaCat human keratinocytes after 12 h of treatment. The data is shown as metabolic activity, as assessed by the Alamar Blue assay, relative to the untreated negative control (100% cell culture medium). The values are represented as the mean + SD (N = 3, 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 (****).
Supporting information on the interaction between the released PA-13 and the bacterial membrane

TAMRA-labelled PA-13 was encapsulated into the nano-network and the release of peptide from the material into PBS was quantified. There was only a negligible amount of PA-13 released from the nano-network over 24 h, suggesting that the peptide was strongly attached to the particles (Figure S7). This result was confirmed via flow cytometry by the release of TAMRA-labelled PA-13 from nano-network upon interaction with membrane of *P. aeruginosa* (Figure S6). Untreated bacterial cells were used as a negative control (Figure S6b A). In the absence of TAMRA-labelled PA-13, the unloaded nano-network showed no TAMRA fluorescent signal (0.01%) and served as a background value (Figure S6b B). Release of TAMRA-labelled PA-13 from the nano-network during incubation with bacteria was compared to that with PBS alone. Flow cytometric analysis showed that 8.07% of TAMRA fluorescent signal was observed when the TAMRA-labelled PA-13 loaded nano-network was incubated with bacteria (Figure S6b D). However, only 0.51% of the fluorescent signal was detected from TAMRA-labelled PA-13 loaded nano-network in PBS alone (Figure S6b C). In order to release PA-13 and exert its antimicrobial action, the nano-network should be in close proximity with the target. The small amount of peptide released from the system was enough to display antibacterial activity, based on the low MIC (3.91 µg mL⁻¹) (Table S2).

Figure S6. The interaction of TAMRA-labelled PA-13 released from the nano-network with membranes of *P. aeruginosa*. a) *P. aeruginosa* suspensions were incubated with TAMRA-PA-13 loaded nano-network at 37 °C for 6 h. After centrifugation, the supernatant was analyzed by flow cytometry (CytoFlex, Beckman Coulter). b) Untreated *P. aeruginosa* (A) and unloaded nano-network incubated with bacteria (B) showed no fluorescent signal while the red fluorescence of TAMRA-labelled PA-13 was observed in TAMRA-labelled PA-13 loaded nano-network incubated with bacteria as shown by the positive peak (D), revealing a higher intensity than that of TAMRA-labelled PA-13 loaded nano-network in PBS (C).
Supporting information on the release of TAMRA-labelled PA-13 from the nano-network

Figure S7. PA-13 released from the nano-network. The cumulative mass of TAMRA-labelled PA-13 released from the nano-network over 1 day (0.5, 1, 2, 4, 6, 12, and 24 h) resulted being less than 1% of the total peptide loaded into the nano-network. Therefore, we hypothesized that the diffusion of PA-13 from the nano-network was negligible and the nano-network to work should be in proximity of the bacteria.
Supporting information on the rheology of the 3:2 nano-network formulation

Figure S8. Rheological data for three independent replicates of the 3:2 nano-network. a) The nano-network frequency sweep (ω = 100–0.1 rad s⁻¹, γ = 0.1%) shows that the material exhibited solid-like properties over the whole range of frequency tested and that the properties were reproducible. b) A strain sweep (ω = 1 rad s⁻¹, γ = 0.01–100%) of three replicates of the nano-network showed a reproducible plateau modulus at low strain and a crossover between $G'$ and $G''$ at ~2% strain. c) The shear-thinning behavior of three independent nano-network replicates was characterized via a shear rate ramp (dγ/dt = 0.01–1000 s⁻¹). The replicates demonstrated reproducible results $n = 0.18$.

Figure S9. Rheological data for nano-networks formed by using different ratios of pNP:nNP. a) Effect of pNP:nNP ration on the nano-network storage modulus (ω = 1 rad s⁻¹, γ = 0.1%). b-f) Nano-network frequency sweeps (ω = 100–0.1 rad s⁻¹, γ = 0.1%) at various pNP:nNP charge ratios.
Supporting information on the in vitro antibacterial activity of PA-13 loaded nano-network under enzyme-challenged conditions

Figure S10. Antibacterial activity of PA-13 loaded nano-network under non- or enzyme-challenged conditions in vitro. 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 ANOVA with Tukey’s post hoc test. Significant differences are indicated in GP style: $>0.05$ (ns), $\leq 0.05$ (*), $\leq 0.01$ (**), $\leq 0.001$ (***), $\leq 0.0001$ (****).
Supporting information on the PA-13 loaded nano-network eliminated *P. aeruginosa* in an ex vivo infection model

**Figure S11.** Antibacterial activity of PA-13 loaded nano-network under non- and enzyme-challenged conditions in an ex vivo infection model. Values were log_{10} transformed and presented as the mean ± SD (N = 6, n = 1). *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 (****).
Supporting information on the photographs of infected porcine skin after treatment

![Photographs of infected porcine skin treated with PA-13 loaded nano-network compared with samples treated with unloaded nano-network, PA-13, PBS, Bepanthen, and Hexene skin cleanser after 6, 12, and 24 h of treatment in with and without trypsin-challenged condition.](image)

**Figure S12.** Photographs of infected porcine skin treated with PA-13 loaded nano-network compared with samples treated with unloaded nano-network, PA-13, PBS, Bepanthen, and Hexene skin cleanser after 6, 12, and 24 h of treatment in with and without trypsin-challenged condition.
Supporting information on the histological characteristics of porcine skin after nano-network treatment

The effect of nano-network on porcine skin was evaluated by histology studies (Figure S13). The stratified structures of two layers including epidermis with the overlying stratum corneum and dermis were relatively well preserved in all skin samples. In dermis layer, all treated porcine tissues showed no histological adverse effects. However, morphological change by elongated nuclei were remarkably observed in the epidermis after treatment with Bepanthen and Hexene skin cleanser solution as shown in red arrow (Figure S13g and S13h) compared to untreated skins with and without bacterial inoculation (Figure S13a and S13b). PA-13 loaded nano-network caused less effect comparable to free PA-13 and PBS (Figure S13f). As organic acids are exfoliating agents commonly used in dermatology and cosmetology, its responsible for removing skin discoloration,[3] As expected, the H&E results showed that stratum corneum exfoliation was considerably observed on tissue surface when acetic acid (Figure S13c), as well as the formulations (Figure S13e-f), were applied as shown in black arrow. The stratum corneum exfoliation was also observed in Bepanthen, a commercially antiseptic and wound healing cream, treatment group (Figure S13g). Herein, PA-13 loaded nano-network could serve as a potential antiseptic cream without severe adverse effects on skin.
Figure S13. Histological sections with H&E staining of porcine skin after 24 h single application of nanonetwork. a) Untreated (PBS) skin tissue without bacterial inoculation b) Untreated skin tissue with P. aeruginosa infection c) Treated skin with acetic acid solution d) Treated skin with free-PA13 e) Treated skin with nano-network f) Treated skin with PA-13 loaded nano-network g) Treated skin with Bepanthen antiseptic cream and h) Treated skin with Hexene cleaner solution. The black and red arrow indicates stratum corneum exfoliation and elongated nuclei site, respectively. Each sample was photographed at a magnification of 100. Scale bar, 100 µm.
Supporting information on the hemolytic activity of nano-network

To assess the effect of nano-network on human erythrocyte, hemolytic activity measurement was performed by spectrophotometric measurement of hemoglobin released after exposure to designed materials. The hemolytic activity of the nano-network was calculated relative to the negative control (PBS) and positive control (Hexene skin cleanser solution) (Figure S14). All samples showed time-dependent hemolysis, in which Hexene skin cleanser solution was strongest hemolytic agent. The non-loaded and PA-13 loaded nano-network caused same degree of hemolysis comparable to that of acetic acid. Both PA-13 loaded and non-loaded nano-network washed with PBS showed negligible hemolysis. This suggested that the observed hemolytic activity of our materials resulted from acidic pH. This is not a major concern as acetic acid is one of the alternative topical agents for the treatment of pseudomonal infections of skin, burns, and soft tissue.\(^4\) Enhancing pseudomonal killing activity of PA-13 loaded nano-network with acetic acid could provide a new platform for the treatment of bacterial wound infection.

![Figure S14. Hemolytic activity of nano-network against human red blood cells. Values represented as the mean ± SD (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 (****).](image-url)
Supporting information on the antibacterial activity after contact with human plasma

The results in Figure S15 show that unencapsulated PA-13, also after being exposed to plasma, maintains its antimicrobial activity, indicating that there is not a concern of proteolytic degradation of PA-13 with plasma contact. Both PA-13 loaded and unloaded nano-networks demonstrated a partial loss in efficacy after contact with human blood plasma. There are extensive studies in literature which demonstrate that proteins present can adsorb onto materials and thus forming a shielding protein corona.\textsuperscript{[5]} Given this evidence it is reasonable to hypothesize that the surface of the nano-network, when in contact with human plasma, is covered by a layer of adsorbed proteins. The protein corona could hinder direct contact of the nanomaterial with the substrate thus negatively affecting the antimicrobial effect. This will be an important consideration for clinical translation of these materials, likely further emphasizing the local effect of the material at sites of active infection.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure_s15.png}
\caption{In vitro antibacterial activity of PA-13 loaded nano-network under plasma- and trypsin-challenged conditions. Differences in colony counts ($\Delta \log_{10}\text{CFU mL}^{-1}$) between the untreated (PBS) and treated samples of in vitro antibacterial activity were calculated. Values represented as the mean ± SD (N = 3). 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 (****).}
\end{figure}
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