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Cooperative Function of LL-37 and HNP1 Protects Mammalian Cell Membranes from Lysis

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ABSTRACT LL-37, cleaved from human cathelicidin, and human neutrophil peptide-1 (HNP1) from the defensin family are antimicrobial peptides that are occasionally co-released from neutrophils, which synergistically kill bacteria. We report that this couple presents another type of cooperativity against host eukaryotic cells, in which they antagonistically minimize cytotoxicity by protecting membranes from lysis. Our results describe the potential of the LL-37/HNP1 cooperativity that switches from membrane-destructive to membrane-protective functions, depending on whether the target is an enemy or a host.

SIGNIFICANCE A mixture of different types of biomolecules sometimes boosts or suppresses their activities or even generates a new function known as cooperativity. We report a unique cooperative function between two well-known antimicrobial peptides (LL-37/HNP1) that kills bacteria more efficiently while minimizing the host damage by suppressing mammalian cell membrane lysis. Such a "double cooperativity" may be used in our immune system and may help with developing efficient and safe antimicrobial agents in the future.

INTRODUCTION

Synergy among antimicrobial peptides (AMPs) (1–19), in which mixing different types of AMPs boosts their antimicrobial efficiency, has garnered attention as a possible approach to improve their potency and because of its underlying interesting mechanism (2,20–25). Particularly, synergy between the cathelicidin-derived peptide (26), LL-37, and human neutrophil peptide 1 (HNP1) from the α-defensin subfamily (27–30) is important because they are among major human AMPs. LL-37 and HNP1 are mainly produced in the bone marrow during neutrophil maturation, which are then occasionally co-released into the blood and tissues for synergistically combating pathogens (10). However, LL-37 is known to exhibit cytotoxicity at high concentrations because of its membrane-destructive properties (31). How the host eukaryotic cells escape from their attack is unknown.

In this work, to study the effect of their cooperativity on host cells, mammalian cells and mammalian cell membrane mimics were challenged by LL-37, HNP1, and their mixture, and their responses were observed by biophysical assays based on supported and pore-spanning bilayers, such as fluorescence recovery after photobleaching (FRAP), quartz crystal microbalance with dissipation (QCM-D), electrochemical impedance spectroscopy (EIS), and single-channel conductance measurement, which we have routinely employed for membrane-active compound characterization for the past years (32–38), combined with toxicity test by calcium-sensitive dye, several spectroscopic methods, and electron-beam microscopy. The result showed that this couple cooperatively protects mammalian cell membranes from lysis for minimizing the cytotoxicity in contrast to known synergistic effect against bacteria.

MATERIALS AND METHODS

For a detailed summary of the materials and methods used in this study, see Supporting Material.

RESULTS

HNP1 unexpectedly suppresses LL-37 cytotoxicity in MDCK cells and HUVEC

Apart from its antimicrobial activities (39), LL-37 is a well-known key player of several biological processes in the host, such as immunomodulation (40), the promotion of cell

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motility, and wound healing (39–42), whereas it becomes cytotoxic at high concentrations (>2.5–13 μM (40,43,44)). This cytotoxicity of LL-37 toward mammalian cells could be visualized by calcium-sensitive dyes in a time-dependent manner. The addition of LL-37 to Madin-Darby canine kidney (MDCK) cells at 29 μM resulted in a fluorescence increase from the intracellular calcium reporter, Fluo-3, within 3 min (Fig. 1 *a*, *first row*), indicating its cytotoxicity. The rapid response time implies defect formation in the plasma membrane and the subsequent induction of a large calcium influx from the extracellular space into the cytosol, as has been reported previously (45). This pore-induced cytotoxicity was inhibited by the mixture of LL-37 and HNP1 at a 1:1 molar ratio (Fig. 1 *a*, *third row*). The control experiment showed that HNP1 individually did not induce noticeable cytotoxicity at 29 μM (Fig. 1 *a*, *second row*). The similar HNP1-related inhibition of LL-37 cytotoxicity was observed in human umbilical vein endothelial cells (HUVEC), as shown in Fig. 1 *b*. Note that the timescale required to induce the LL-37 cytotoxicity in HUVEC was 50 times longer; thus, this might have involved other mechanisms than a simple pore formation. These results show that HNP1 neutralizes LL-37 cytotoxicity in MDCK cells and HUVEC in contrast to the previously reported synergy against bacteria (10).

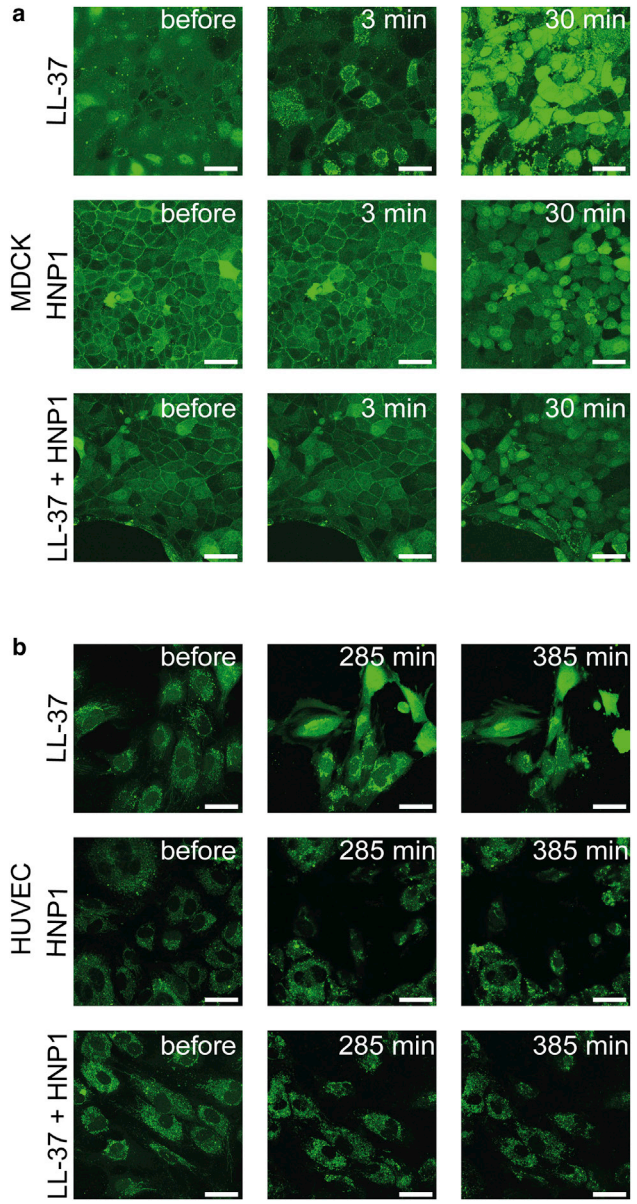

FIGURE 1 Cytotoxic activity of LL-37 and its inhibition by HNP1, studied with calcium sensitive dye, Fluo-3. (*a*) MDCK and (*b*) HUVEC with Fluo-3 are monitored by confocal laser scanning microscopy over time, where after 350 s for MDCK and 1292 s for HUVEC, LL-37, HNP1, and their mixture at 1:1 molar ratio were added all at 29 μM (in the case of the mixture, it is 29 μM LL-37 + 29 μM HNP1). Scale bars, 10 μm. To see this figure in color, go online.

LL-37 and HNP1 did not interact in solution

To study whether LL-37 and HNP1 already bind in solution, their interactions in a physiological HEPES buffer solution (150 mM NaCl (pH 7.4)) were monitored using isothermal titration calorimetry (ITC), tryptophan fluorescence spectroscopy, and circular dichroism (CD). No clear evidence for their binding was observed in ITC, as seen by the constant endothermal peaks that come from heats of dilution during the titration of LL-37 into HNP1 in a HEPES buffer solution (Fig. 2 *a*). To confirm this result, we measured the HNP1 tryptophan fluorescence emission peak both in the presence and absence of LL-37. HNP1 has a single tryptophan (Trp) residue, which causes a blue shift in its emission peak in a hydrophobic microenvironment. We observed no change in the tryptophan emission peak position between with or without LL-37 (Fig. 2 *b*), indicating no interaction. This was further validated by CD, in which the double-dip at 208 and 222 nm characteristic for α-helical structures (46) in LL-37 remained unchanged after the addition of HNP1 (Fig. 2 *c*), suggesting the absence of structural rearrangements often observed upon binding (17).

ITC results show no significant effect of HNP1 on the LL-37 adsorption onto the POPC bilayers

Because bilayer disruption is one of the well-known mechanisms of LL-37 toxicity, its suppression by HNP1 might be taking place in the membranes. As a first step toward better understanding such a cooperative function in membranes, we studied their adsorption to 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), which is one of the major lipids in eukaryotic plasma membranes. We titrated peptides with POPC vesicles in ITC. LL-37 bound to POPC vesicles (Fig. 3 *a*). The binding is strongly entropic ($-T\Delta S = -6.258$ kcal/mol $<< \Delta H = -0.194$ kcal/mol, where $\Delta G = \Delta H - T\Delta S = -6.452$ kcal/mol), indicating that the hydrophobic interaction was the main cause of the binding rather than the electrostatic interaction. Note that these values are rough

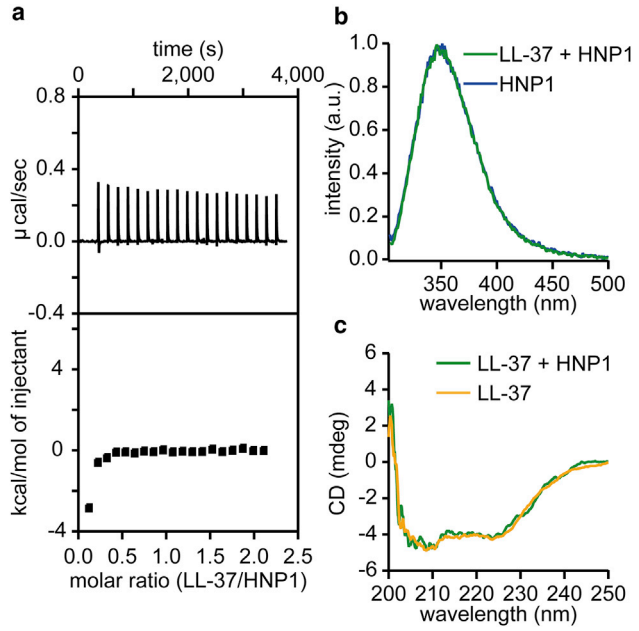

FIGURE 2 LL-37 and HNP1 did not interact in solution, evidenced by isothermal titration calorimetry (ITC), tryptophan (Trp) fluorescence spectroscopy, and CD. (*a*) Heat flow and the integrated heat from ITC when 40 μM HNP1 in HEPES buffer solution in a chamber volume of 200 μL was titrated with 400 μM LL-37 in HEPES buffer solution at 2 μL each time for 20 times. (*b*) Shown are Trp fluorescence emission spectra of HNP1 alone in HEPES buffer solution at 2.9 μM and in combination with LL-37 at 1:1 molar ratio (LL-37 signal subtracted). (*c*) Shown are CD spectra of LL-37 in HEPES buffer solution at 29 μM and in combination with HNP1 at 1:1 molar ratio (HNP1 signal subtracted). All measurements were done in 10 mM HEPES and 150 mM NaCl at pH 7.4. To see this figure in color, go online.

estimations as the ITC signals also contain the information on peptide arrangements in the bilayers, such as pore formation, in addition to binding (47,48). Titration of vesicles to HNP1 did not produce any detectable amount of heat from binding as no titration curve was observed (Fig. 3 *a*). Fluorescence microscopy and quartz crystal microbalance with dissipation monitoring confirmed the adhesion of HNP1 to POPC bilayers, as we will discuss later (Fig. 3, *c* and *e*). Thus, this lack of significant heat production upon binding is the result of a too entropic interaction, which was below the ITC sensitivity at this concentration. The mixture of LL-37 and HNP1 yielded a titration curve similar to that of LL-37 (Fig. 3 *a*). This suggests that the LL-37 adhesion to POPC vesicles was affected little by the presence of HNP1. The vesicle sizes, estimated by dynamic light scattering, were not altered significantly by the addition of peptides (Fig. 3 *b*).

FRAP results demonstrate LL-37-induced POPC bilayer destruction, rescued by the addition of HNP1

The first evidence for the interference of LL-37 and HNP1 was observed during FRAP, as shown in Fig. 3 *c*. FRAP is a well-established tool for the characterization of the lipid bilayer integrity and fluidity (49). Supported POPC + 0.2% mol 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl lipid bilayers were assembled on glass coverslips by vesicle fusion as confirmed by full FRAP recovery with a diffusion coefficient of $D = 3.38 \pm 0.25$ $\mu m^2/s$ (Fig. 3 *d*). These results were comparable with previously published values (49). LL-37 disrupted the lateral continuity of these bilayers at 2.9 μM, as demonstrated by a lack of FRAP recovery (Fig. 3, *c* and *d*). The decreased fluorescence intensity from the bilayer indicated the detachment of lipids from the substrate upon the addition of LL-37 (Fig. 3 *c*). HNP1 induced membrane protrusions, demonstrated by the appearance of bright dots in the fluorescence images (Fig. 3 *c*). Nevertheless, the bilayer maintained the lateral continuity as FRAP showed a full recovery with a diffusion coefficient of $D = 2.14 \pm 0.48$ $\mu m^2/s$, which was 37% reduced compared to the POPC bilayers without peptides. This illustrates that HNP1 inserted into the bilayers and increased the surface area of the membranes, where the excess area folded into structures such as tubes are visible as bright spots, without disintegrating the bilayer. The mixture of LL-37 and HNP1 interacted with the bilayer as some bright dots were also observed after the incubation, yet the bilayer continuity was maintained, confirmed by the full FRAP recovery with a reduced diffusion coefficient of $D = 2.14 \pm 0.81$ $\mu m^2/s$ (Fig. 3, *c* and *d*). These FRAP data provided evidence to the LL-37-induced POPC bilayer destruction, whereas adding HNP1 rescued this effect. Such a cooperative function between LL-37 and HNP1 in synthetic POPC bilayers might be linked to the observed neutralization of cytotoxicity shown in Fig. 1.

QCM-D indicates that LL-37 removes lipids from bilayers, HNP1 creates membrane protrusions, and their mixture suppresses both effects

To confirm the observed peptide-bilayer interactions, we next used QCM-D. QCM-D enables the detection of wet mass (including the water mass) on the sensor crystal and the viscoelastic properties of the deposited film by the changes in the resonance frequency Δf and the decay of the sensor oscillations ΔD (50), frequently used to study AMP-bilayer interactions (51). Supported POPC bilayers were formed on silicon dioxide-coated quartz crystal microbalance crystals by vesicle rupture, as confirmed by the typical frequency and dissipation change in QCM-D during this process. The initial decrease in Δf (increase in mass) and the increase in ΔD indicated the vesicle adsorption, whereas the following increase (decrease in mass) and the stabilization of Δf at around 24 Hz and the decrease in ΔD illustrated the vesicle rupture and the corresponding release of water mass (Fig. 3 *e*, bilayer formation; (52)). LL-37 partially removed lipids from the

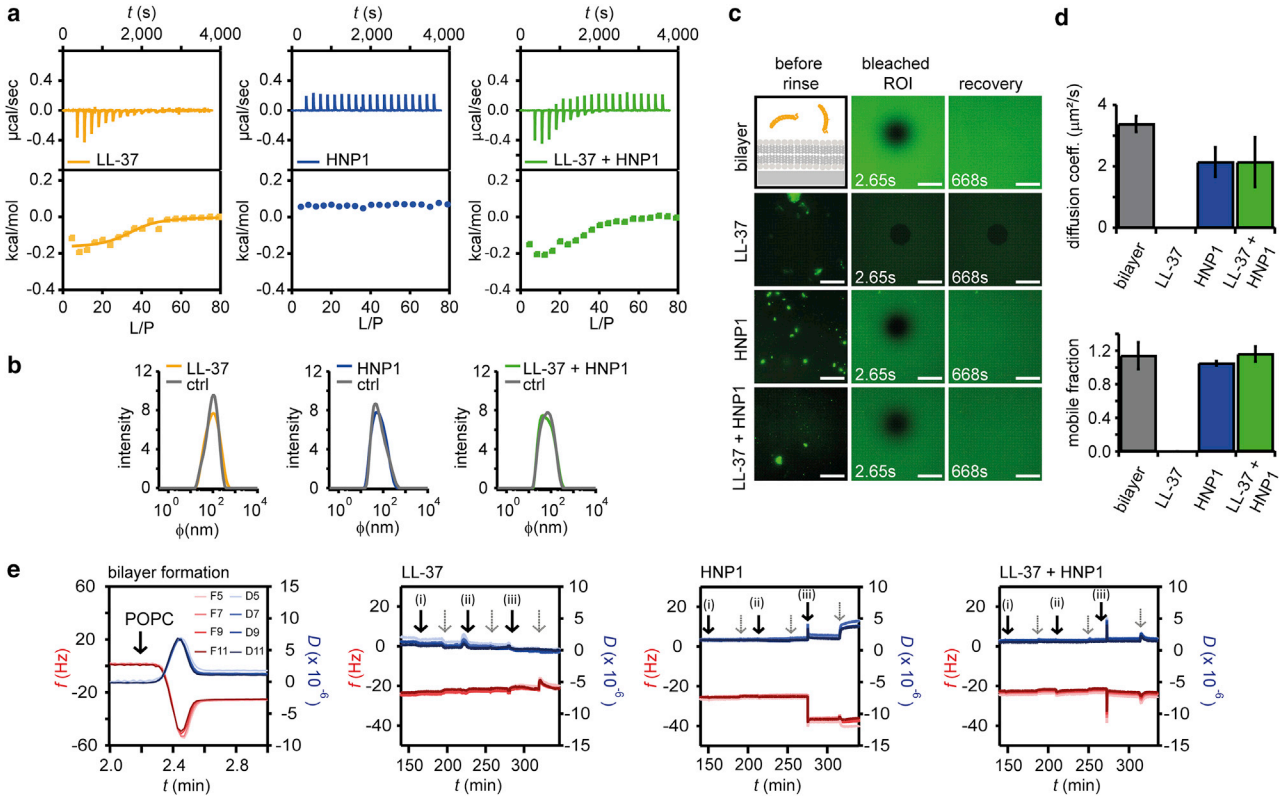

FIGURE 3 Peptide-lipid interactions monitored by ITC, fluorescence recovery after photobleaching (FRAP), and QCM-D. (*a*) Heat flow and the integrated heat from ITC when LL-37, HNP1, and LL-37 + HNP1 at 1:1 molar ratio all at 40 μM (in case of the mixture 40 μM each) in the chamber volume of 200 μL were titrated with POPC vesicles at 15 mM with 2 μL each time for 20 times. The reverse titration is shown in Fig. S1. (*b*) Vesicle size after each ITC experiment was determined by dynamic light scattering. (*c*) Shown are fluorescence images of supported POPC lipid bilayers with 0.2% mol 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl after incubation with each peptide at 2.9 μM before rinsing and subsequent FRAP images after rinsing. Note that the enhancement is different between before and after the rinse to visualize the bright dots in the images before rinse. Scale bars, 50 μm. (*d*) Diffusion coefficients were calculated from FRAP experiments as follows: 3.38 $\pm$ 0.25 $\mu m^2/s$ for the POPC bilayers, no recovery was observed for LL-37, 2.14 $\pm$ 0.48 $\mu m^2/s$ for HNP1, and 2.14 $\pm$ 0.81 $\mu m^2/s$ for the peptide mixture. Experiments were repeated for three times and their average and the standard deviation are plotted. (*e*) Shown are QCM-D data for the bilayer formation by vesicle rupture and the subsequent addition of LL-37, HNP1, and their mixture at (i) 0.29 μM, (ii) 2.9 μM, and (iii) 29 μM. The gray dotted arrows indicate the rinse by injecting HEPES buffer solution. Their overtone analysis is shown in Fig. S2. To see this figure in color, go online.

sensor surface and made the bilayer slightly more rigid, implied by the increase in frequency (a loss in mass) and the decrease in dissipation (Fig. 3 *e*). The mass of LL-37 is too small to be detected because even a monolayer membrane coverage would have resulted only in Δf = 2–4 Hz (53,54). Further overtone analysis provided a resolution in a *z*-direction as acoustic wave penetration depths decrease along with the increasing overtone numbers (55,56). No significant overtone dependency except for a slight variation in Δf at the highest concentration (29 μM, Fig. S2, *a* and *b*) was observed, in agreement with the previously reported detergent-like mechanism (57). HNP1 induced a reduction in Δf by 13.6 Hz (increase in mass) and increase in dissipation by 2.4×10^{-6} after the last rinse (Fig. 3 *e*), indicating the lipid bilayer morphological change and the associated increase in the coupling of water mass typical for the membrane protrusion or vesicle adsorption (52). This is in agreement with the appearance of bright dots in fluorescence images (Fig. 3 *c*). A slight overtone dependence (Fig. S2, *c* and *d*), visible at 29 μM, both in Δf and ΔD also suggests that the main mass increase took place at least tens of nanometers away from the sensor surface (58), which is compatible with the membrane protrusion. The mixture of LL-37 and HNP1 showed no significant signal change (Fig. 3 *e*; Fig. S2, *e* and *f*), indicating that the effect of LL-37 (destruction) and HNP1 (protrusion) on supported bilayers are both suppressed when they are mixed.

EIS confirms that HNP1 suppresses LL-37 defect formation

Next, to monitor the bilayer destruction process with higher sensitivity, we performed EIS. We used supported POPC lipid bilayers formed on highly doped (metallic) silicon wafers as a working electrode in a three-electrode setup. The

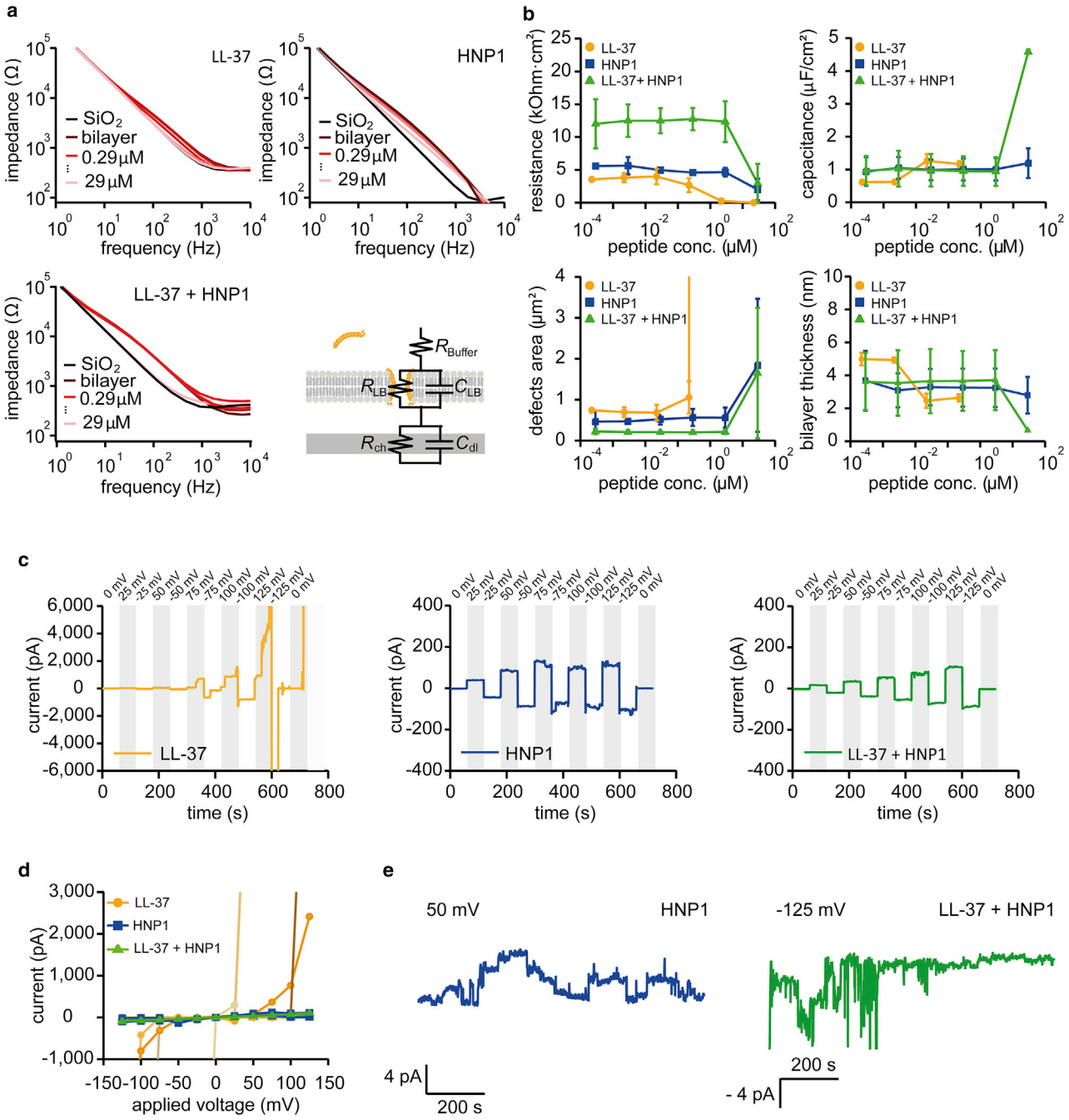

FIGURE 4 EIS with supported lipid bilayers and conductance measurements with porespanning bilayers confirm that HNP1 suppresses LL-37 membrane disintegration. (*a*) Shown are EIS spectra of supported POPC bilayers at different peptide concentrations. An equivalent circuit used for fitting is also shown. (*b*) Extracted bilayer resistance and capacitance and their conversion into defect area and bilayer thickness were plotted against peptide concentrations. Experiments were repeated twice and their average and the standard deviation are plotted. (*c*) Shown are current recordings through free standing lipid bilayer with the addition of LL-37 at 8 μM, HNP1 at 8 μM, and LL-37+HNP1 at 1:1 molar ratio with the total peptide concentration of 16 μM at different applied voltages and (*d*) extracted *I-V* plot. For each peptide, multiple runs were analyzed and presented in the *I-V* plot. (*e*) Shown are conductance measurements of pore-spanning lipid bilayers exposed to HNP1 at 15 μM at 50 mV holding potential and LL-37+HNP1 mixture at 1:1 molar ratio with the total concentration of 26 μM at -125 mV holding potential. To see this figure in color, go online.

top surface of silicon is silicon dioxide, which facilitates the self-assembly of bilayers by vesicle fusion. From the obtained impedance spectra (Fig. 4 *a*), a bilayer resistance R_{LB} and a capacitance C_{LB} were extracted (Fig. 4 *b*) by fitting them with an equivalent circuit presented in Fig. 4 *a*. Both R_{LB} and C_{LB} were further converted into the total defect area and the average bilayer thickness. LL-37 destroyed bilayers at 2.2 μM, indicated by an abrupt increase

in the defect area (Fig. 4 *b*, defect area). LL-37 is known to form defects in a concentration-dependent manner, both in bacterial and eukaryotic cell membrane mimics, previously shown by vesicle leakage assay (59). In contrast, HNP1 created a total defect size of 1.6 μm^2 at 29 μM (Fig. 4 *b*, defect area), suggesting that HNP1 also forms small defects at such a high concentration. When they were mixed, the total defect area became 0.3 μm^2 at 29 μM, indicating that HNP1 suppressed the LL-37-induced bilayer destruction. The average bilayer thickness started to decrease at the LL-37 concentration of 0.22 μM (Fig. 4 *b*, bilayer thickness). Beyond 2.2 μM, the thickness could not be estimated because the bilayer was destroyed. For HNP1 and the mixture, the average bilayer hydrocarbon thickness was 3.2 and 3.1 nm, both at 2.9 μM. These impedance data further confirm that LL-37 forms large defects in bilayers, whereas the mixture of HNP1 rescues it.

Single-channel conductance indicates that LL-37 alters the behavior of the HNP1 pores

Next, the bilayer conductance was measured at a fixed (DC) voltage with lateral pore-spanning bilayers as described previously (36,38). After a giga-Ohm seal was achieved, LL-37, HNP1, and their mixture were added to the *cis* chamber, and step voltages from 0 to 125 mV/−125 mV were applied. For LL-37, transmembrane currents exceeded 6000 pA at ±125 mV (Fig. 4 *c*), or the bilayer often ruptured. These dramatic effects on the bilayers were in agreement with the previously proposed carpet-like mechanism for LL-37-bilayer interaction (60). For HNP1 and its mixture with LL-37, the transmembrane currents never exceeded 200 pA at ±125 mV (Fig. 4 *c*). These results further confirm that LL-37 forms large defects in bilayers, whereas the addition of HNP1 suppresses it. In between these conductance measurements, we ran impedance spectroscopy to monitor the change in the bilayer thickness. The standard POPC bilayers have a capacitance density of 0.6–1.0 $\mu F/cm^2$ (61–63), which corresponds to the thickness of around 4 nm. In the case of LL-37, defects in bilayers were observed even at a capacitance as low as 0.3 $\mu F/cm^2$ (Fig. S3), which corresponds to the bilayer thickness of 10.4 nm because of the remaining organic solvent sandwiched between the two monolayer leaflets. This indicated that LL-37 formed defects independent of the bilayer thickness as in the carpet-like model (60). Bilayer conductance measurement at a fixed voltage captured single channels for HNP1. The size of these pores was around 1.2 ± 0.2 Å at −50 mV (Fig. 4 *e*), although the pore size seemed to fluctuate as other channel conductance were also seen. This single pore conductance appeared to be disturbed when LL-37 was mixed (Fig. 4 *e*). These results suggest that LL-37 destabilizes bilayers, HNP1 forms small stable pores at high concentrations (15 μM), and their mixture forms small defects without rupturing bilayers.

Cryo-electron microscopy visualized that LL-37 destroys POPC vesicles into small fragments, HNP1 creates a stable opening in vesicles, and their mixture forms bilayer sheets or nanodisks

To visualize the peptide-induced lipid structural change, POPC vesicles incubated with LL-37, HNP1, and their mixture were imaged by cryo-transmission electron microscopy (cryo-TEM). LL-37 disintegrated lipid membranes, captured by small fragments of lipid-peptide composites (Fig. 5 *a*) with a size ranging from 4.8 to 19.4 nm (the average was 9.7 ± 2.8 nm) that did not exist before adding LL-37 (a control image shown in Fig. S4). These fragments were not observed with POPC vesicles incubated with HNP1 (Fig. 5 *b*). Instead, a stable perforation of bilayers by HNP1, which created openings in vesicles, was observed (*arrows* in Fig. 5 *b*). The mixture of LL-37 and HNP1 produced objects that resembled nanodisks and sheets (Fig. 5 *c*). Recently, several amphiphilic proteins have been shown to spontaneously assemble into lipid-protein nanodisks, in which these proteins wrap lipid bilayer disks like a belt to reduce the line tension at the bilayer edge (64–66). Although we acquired these images at rather high concentrations (lipid concentration = 1.5 mM, peptide concentration = 150 μM, L/P = 10) to adjust the density of the objects compatible with cryo-TEM, the LL-37-induced bilayer destruction and its partial inhibition by HNP1 is in agreement with our other data.

DISCUSSION

LL-37 is a 37 residue, amphipathic, human helical peptide, expressed in epithelial cells of the testis, skin, gastrointestinal tract, and respiratory tract, as well as in leukocytes, such as monocytes, neutrophils, T cells, natural killer cells, and B cells. It has been found to have antimicrobial (10), antiviral (67), and anticancer (68) activities, as well as immunomodulatory roles comprising both anti- (69) and proinflammatory functions (70), chemotactic (71), and cytotoxic effect (44), and it also promotes cell migration and wound closure (72). HNP1 is a 30-amino acid human peptide adopting a triple-stranded β-sheet structure (73). It is released from cells upon stimuli or, in some cases, constitutively, for example from neutrophils, natural killer cells, and monocytes, and exhibits antimicrobial activity (74,75), neutralizes bacterial toxins (76), and modulates the adaptive immune response (77). Bilayer membranes are the primary targets of LL-37 and HNP1; thus, these peptides affect fungi (78,79) and enveloped viruses (67,80) besides bacteria. LL-37 and HNP1 are frequently co-expressed. In 2000, Nagaoka and co-workers reported direct evidence of their synergistic antimicrobial effect against *Escherichia coli* and *Staphylococcus aureus* (10). In this study, we observed the opposite, an antagonistic effect toward MDCK cells and HUVEC, in which the LL-37 cytotoxicity is suppressed by HNP1 (Fig. 1). The previous report, in addition to our result,

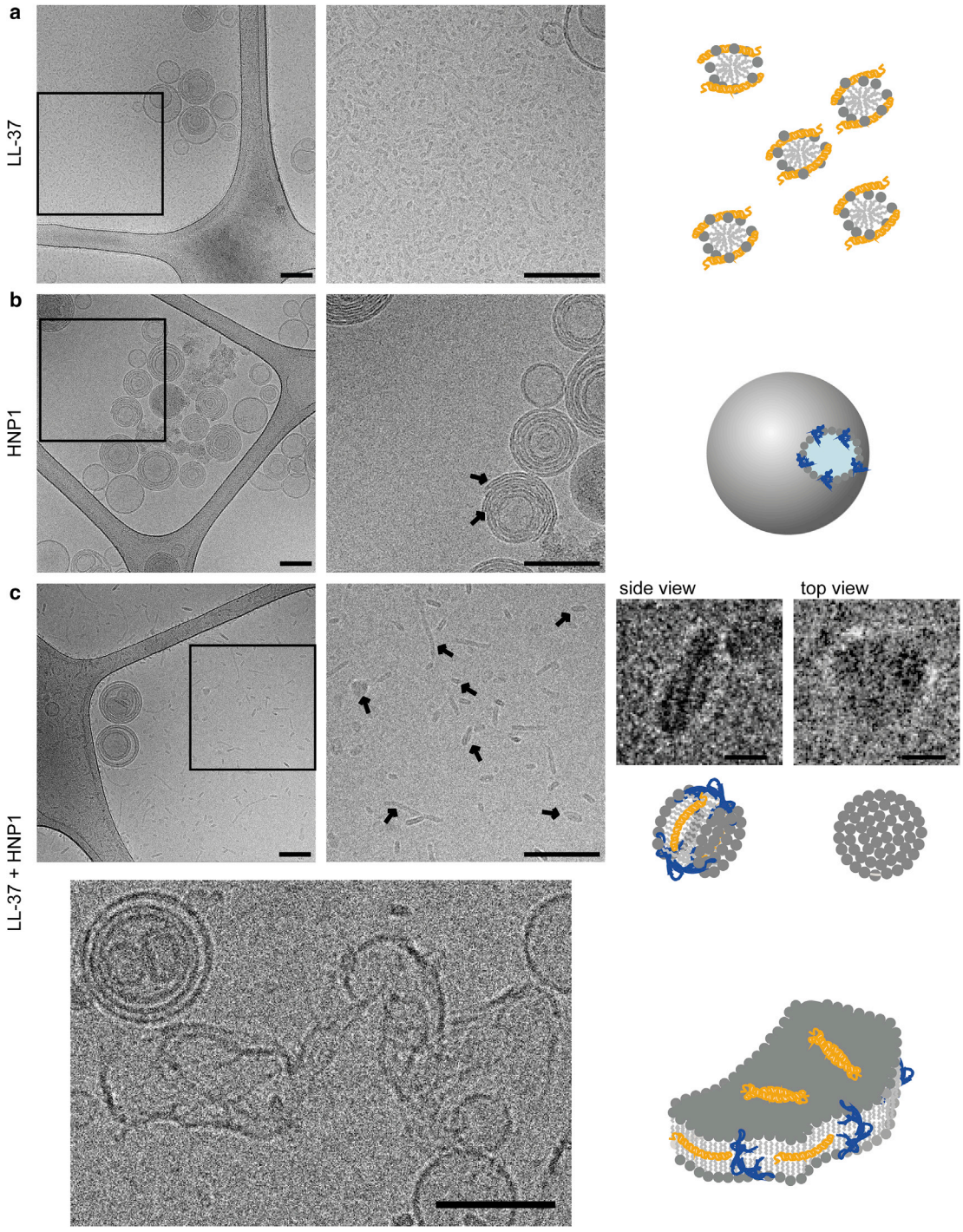

FIGURE 5 Cryo-electron microscopy visualized that LL-37 destroys POPC vesicles into small fragments, HNP1 creates stable opening in vesicles, and their mixture forms bilayer sheets or nanodisks. Shown are cryo-electron microscopy (cryo-TEM) images of POPC vesicles after incubation with (*a*) LL-37, (*b*) HNP1, and (*c*) LL-37 + HNP1 mixture. Peptides at 150 μM were incubated with vesicles at 1.5 mM for 30 min in physiological HEPES buffer solution. In the case of the mixture, 300 μM was the final concentration of both peptides combined (150 μM LL-37 + 150 μM HNP1). Possible interpretation of these images is drawn on the right side. All images were taken with Talos TEM 200 kV, except the one at the bottom, which was taken by Titan Krios G3i 300 kV. All the scale bars represents 100 nm except for the zoom-in top and side-view images of nanodisks, which are 10 nm. To see this figure in color, go online.

implies that the cooperative function of the LL-37/HNP1 pair might switch from destructive to protective, depending on the target. Our data showed that LL-37 and HNP1 did not bind in solution, as confirmed by ITC, tryptophan fluorescence spectroscopy, and CD (Fig. 2). Previously LL-37 has been reported to form dimers, trimers, or tetramers

(81–83) in solution, whereas HNP1 has been shown to form dimers (84). Their oligomerization in aqueous solution is driven by the minimization of their free energy to hide their hydrophobic residues from the surrounding water. Once their hydrophobic parts have been already concealed as oligomers, their net positive charge would induce an electrostatic repulsion between LL-37 and HNP1. This partially explains the lack of their binding in solution. LL-37 adhesion to POPC bilayers was not significantly affected by the presence of HNP1 either, indicated by ITC (Fig. 3 *a*). We found evidence for their interaction only after they bound to bilayers. LL-37 formed large defects or destroyed bilayers that were visible in FRAP (Fig. 3, *c* and *d*), QCM-D (Fig. 3 *e*), EIS (Fig. 4, *a* and *b*), transmembrane currents (Fig. 4, *c* and *d*), and cryo-TEM (Fig. 5 *a*). The mechanism of the LL-37 interaction with membranes is still debated. However, several models have been proposed, depending on the applied method. Carpet/toroidal membrane disintegration has been proposed based on solid-state NMR studies for LL-37 interacting with different membranes (85). The parallel orientation of peptide to membrane surface was confirmed by attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) (82), supporting a carpet-like mechanism. Some studies suggest that LL-37 could distinguish anionic and zwitterionic lipids (86). However, other studies have shown a lack of such discrimination (82). HNP1 induces protrusions in bilayers, as we observed by QCM-D and fluorescence images (Fig. 3, *c–e*), yet the bilayer continuity was maintained, as highlighted by the full recovery in FRAP (Fig. 3, *c–e*). The single pore conductance shows that HNP1 forms transmembrane pores (Fig. 4 *e*), as it has been previously reported (87) and further supported by solid NMR, in which HNP1 dimers line the pore with the hydrophilic part of the peptide facing the water column (88). However, their total pore size observed by EIS (Fig. 4 *b*) was 1.6 μm^2 at 29 μM, which corresponds to 1.7×10^{-6}% of the total lipid bilayer area, implying that the pore formation might be only a minor function of HNP1. When LL-37 and HNP1 were mixed, both the destruction of bilayers induced by LL-37 and the membrane protrusion induced by HNP1 were inhibited, as seen by a lack of significant mass change in QCM-D (Fig. 3 *e*; Fig. S2), full recovery in FRAP (Fig. 3, *c* and *d*), and only minor defects or pores observed by EIS and transmembrane conductance (Fig. 4, *a–e*). Cryo-TEM demonstrated bilayer sheets and objects that resemble nanodisks, which also support that mixing HNP1 partially suppressed the destruction of bilayers by LL-37 because LL-37 alone fragmented vesicles into much smaller particles.

These functional studies demonstrated that the cooperative activities of LL-37 and HNP1 start only in lipid bilayers. Both LL-37 and HNP1 form homo-oligomers in aqueous solution without binding each other, yet upon incorporation into bilayers, the unique hydrophobic environment triggers their interactions and initiates the cooperative effect (Fig. 6). To further link the function to the structure, a set of additional experiments were performed by CD and fluorescence spectroscopy. When LL-37 was titrated by POPC vesicles, the amount of helix in LL-37 increased as the intensity of the double-dip structure became enhanced as a function of lipid to peptide ratio (L:P) until it reached saturation at L:P = 10 (Fig. S5 *a*). The ratio between the dip at 222 and 208 nm (CD222/CD208), which has been previously associated with the aggregation of the peptides (89), did not change significantly as a function of L/P ratio. When we titrated the mixture of LL-37 and HNP1 by POPC vesicles, a similar result was obtained (Fig. S5 *b*), implying that the secondary structure of LL-37 was not significantly altered by the presence of HNP1 in bilayers. Next, we monitored the titration by fluorescence spectroscopy to follow the emission spectra from the solvatochromic tryptophan in HNP1. When HNP1 was titrated by POPC vesicles, a blue shift was observed, suggesting that the tryptophan is in contact with the hydrophobic carbon chains in lipids (Fig. S6). When the LL-37/HNP1 mixture was titrated by POPC vesicles, the emission spectra blue shifted similarly after the subtraction of the spectra from LL-37 (Fig. S6). This suggests that the exposure of the tryptophan, which is near the C-terminal of HNP1, to the lipid environment was also not altered by the presence of LL-37. This lack of clear evidence toward peptide-peptide interactions in membranes suggests that the observed cooperative function might originate from a lipid-mediated interaction without strong direct peptide-peptide contact.

In conclusion, we report that LL-37 and HNP1 exhibited an unexpected antagonism that prevented LL-37

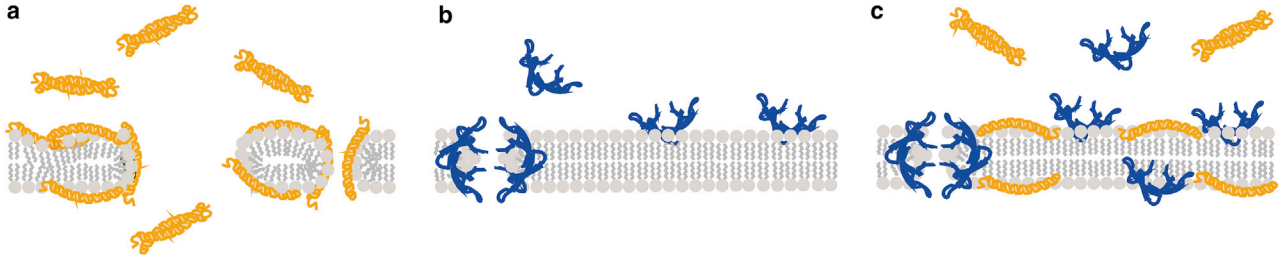

FIGURE 6 Schemes presenting the possible model of interactions between (*a*) LL-37, (*b*) HNP1, (*c*) LL-37 + HNP1, and POPC lipid bilayers. To see this figure in color, go online.

cytotoxicity in MDCK cells and HUVEC. LL-37 and HNP1 did not bind in physiological buffer solution, partially because their initial oligomeric states in solution concealed their hydrophobic residues, whereas their positive charge induced electric repulsion between them. The LL-37 binding to POPC bilayers was only moderately affected by the presence of HNP1. However, once they adhered to bilayers, this hydrophobic environment triggered their interactions and generated the cooperative effects, in which LL-37 could not destroy bilayers anymore. This explains the observed neutralization of cytotoxicity as LL-37-induced membrane destruction was the origin of the toxicity. These biophysics assays based on supported and pore-spanning bilayers used in this work (FRAP, EIS, QCM-D, and bilayer conductance measurements) in addition to other vesicle-based spectroscopy and ultrahigh-resolved cryo-imaging techniques are proven to be informative tools for studying the cooperative functions of AMPs.

SUPPORTING MATERIAL

Supporting Material can be found online at https://doi.org/10.1016/j.bpj.2020.10.031.

AUTHOR CONTRIBUTIONS

E.D. contributed to the design of the experiments, performed these experiments, analyzed data, and drafted the manuscript. K.S. contributed to the design of the experiments, analysis, and interpretation of the data and the drafting of the manuscript.

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