Lactoferricins impair the cytosolic membrane of Escherichia coli within a few seconds and accumulate inside the cell

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Abstract
We report the real-time response of E. coli to lactoferricin-derived antimicrobial peptides (AMPs) on length-scales bridging microscopic cell-sizes to nanoscopic lipid packing using millisecond time-resolved synchrotron small-angle X-ray scattering. Coupling a multi-scale scattering data analysis to biophysical assays for peptide partitioning revealed that the AMPs rapidly permeabilize the cytosolic membrane within less than three seconds—much faster than previously considered. Final intracellular AMP concentrations of ~ 80 to 100 mM suggest an efficient obstruction of physiologically important processes as primary cause for bacterial killing. On the other hand, damage of the cell envelope and leakage occurred also at sublethal peptide concentrations, thus emerging as a collateral effect of AMP activity that does not kill the bacteria. This implies that the impairment of the membrane barrier is a necessary but not sufficient condition for microbial killing by lactoferricins. The most efficient AMP studied exceeds others in both speed of permeabilizing membranes and lowest intracellular peptide concentration needed to inhibit bacterial growth.

Introduction
Progress in designing antibiotics with novel key-lock mechanisms is not keeping pace with the worldwide growing number of (multi) resistant bacterial strains, encouraging significant research efforts in promising alternatives such as antimicrobial peptides (AMPs) (Lohner, 2001). AMPs are part of the natural innate immune system and provide a first line of defence against pathogens. Their advantage as compared to conventional antibiotics relies on a rapid impairment of the barrier-function of the bacterial envelope by unspecific physical interactions, often coupled to an ensuing
targeting of bacterial DNA or ribosomes [for review see, e.g., Wimley and Hristova (2011); Lohner (2017); Malanovic et al. (2020); Stellia et al. (2021)].

Membrane-active AMPs contain specific sequences of cationic and apolar amino-acids, granting high affinity to the hydrophobic core of lipid membranes and selectivity towards the negatively charged surfaces of bacterial envelopes. However, despite intense research for several decades, a comprehensive understanding of the specific series of events that pertain to the bactericidal or bacteriostatic activity of AMPs is still elusive. To large extent this is due to the persisting challenge of merging results from in vitro studies with those obtained from lipid membrane mimics, often leading to significant controversies (Wimley and Hristova, 2011). This is nurtured, on the one hand, by difficulties in engineering lipid model systems of sufficiently high complexity to mimic the diverse physicochemical properties of bacterial membranes. On the other hand, the complexity of live bacteria challenges experimental and computational techniques to obtain quantitative results on the molecular level. For example, cryogenic transmission electron microscopy (TEM) provides high subcellular spatial resolution, but might give misleading information due to artefacts that potentially originate from staining or invasive sample preparation. Moreover, structural kinetics occurring in the seconds time scale are yet not accessible to cryo-TEM on cells, but would be needed to unravel the sequence of events induced by AMP activity. Kinetic experiments, using high-speed atomic force microscopy, for example, showed an AMP-induced corrugation of the bacterial outer surface within the first 13 seconds after addition of the AMP, but were limited by the intrinsic time resolution of the technique (Fantner et al., 2010). Additionally, such topological experiments do not provide insight on concurring intracellular changes. Video microscopy, combined with fluorescence labeling schemes for peptides or cellular content, in turn provided the appropriate spatiotemporal resolution to differentiate AMP activity in different cells within several tens of seconds [see Choi et al. (2016) for review]. Importantly, such experiments reported that AMPs often reach the cytoplasm within few minutes, suggesting that the final target for arresting bacterial growth or killing is not the cytoplasmic membrane (Sochacki et al., 2011). However, fluorescence labeling may easily tweak the delicate balance of macromolecular interactions and thus affect experimental observations.

Elastic X-ray and neutron scattering experiments are well-established non-invasive techniques to interrogate structural properties of biological or biobased matter without the need to resort to any bulky labels. However, quantitative analysis of complex biologically relevant systems is challenging and has hampered progress in applying these techniques to live cells (Semeraro et al., 2021a). To this end, a full analysis of cells necessitates to account for structural features at diverse hierarchical levels. We recently refined an analytical multi-scale scattering model of live Escherichia coli, making extensive use of the different sensitivities of X-rays and neutrons to matter, including H/D contrast variation (Semeraro et al., 2021b). This allowed us to detail the bacterial hierarchical structure on lengths scales bridging four orders of magnitude, i.e., spanning from bacterial size to the molecular packing of lipopolysacharides (LPS) in the outer leaflet of the outer membrane. Here we use this model, taking advantage of the fact that the full breath of structural information is encoded in a single scattering pattern, and exploit millisecond time-resolved synchrotron (ultra) small-angle X-ray scattering (USAXS/SAXS) to study the response of E. coli to three well-characterized lactoferricin-derived AMPs: LF11-215 (FWFWRIRIRR-NH₂), LF11-324 (PFFWRIRIRR-NH₂) and O-LF11-215 (octanoyl-FWFWRIRIRR-NH₂) (Zweytick et al., 2011, 2014; Sánchez-Gómez et al., 2015; Marx et al., 2021b).

Joining these elastic scattering experiments with TEM and assays for determining peptide partitioning as a function of peptide activity enabled us to gain unprecedented insight into the peptide-induced sequence of events. We observed that the studied peptides are able to cause a loss of cytoplasmic content just within few seconds. Coupling this finding to the derived final (after one hour) intracellular peptide concentrations at full bacterial growth inhibition (~ 80 – 100 mM) suggests a peptide uptake on similar time scales, and hence much faster than previously reported for other AMPs, such as Rhodamine-labeled LL-37 (Sochacki et al., 2011). The most effective AMP
presently studied, LF11-324, excelled others by most swiftly permeabilizing the cytoplasmic membrane and killing the bacteria at the lowest cytosolic/periplasmic concentration. Consistent with previous studies on these AMPs (Zweytick et al., 2011), we also observed severe damage of the bacterial cell envelope—here defined in terms of loss of LPS packing, loss of positional correlations between outer and inner membranes and vesiculation/tubulation. Interestingly, this structural impairment of the bacterial envelope also occurred at peptide concentrations far below the minimum inhibitory concentration (MIC), but on different (slower) time scales than leakage. Leakage kinetics increased with peptide concentration up to the MIC. Yet, the overall loss of cytoplasmic molecules was not affected by AMP concentration, i.e., it was equal at MIC and below (even at 1% growth inhibition!). The primary cause for bactericidal or bacteriostatic activity of the presently studied peptides is thus not a damage of the structural integrity of the cell-wall, but appears to be due to a fast and efficient impairment of a series of here not further detailed physiological processes occurring within the intracellular space.

Results

Defining structural reference states of AMP activity in *E. coli*

Unravelling the time-line of structural events occurring in *E. coli* due to lactoferricin activity by USAXS/SAXS necessitates a detailed prior characterization of two reference states: (i) neat bacteria before AMP administration ('initial-state'), and (ii) AMP-affected/killed bacteria ('end-state'). Here, end-state refers to one hour of incubation of bacteria at a given AMP concentration. Both states can be treated as quasi equilibrium structures, enabling a detailed characterization through a joint application of X-ray and neutron scattering. This allowed us to constraint parameters in the analysis of the time-resolved USAXS/SAXS data described further below. We have recently reported initial-state structures of *E. coli* ATCC 25922 (and other strains) at different hierarchical length scales—including size of bacteria, distance between inner and outer membranes, and LPS packing density—in terms of a multi-scale analytical model using joint USAXS/SAXS and (very) small-angle neutron scattering (VSANS)/SANS experiments (Semeraro et al., 2021b). Importantly, while this analysis revealed a sensitivity of X-rays/neutrons to structural features of the cellular envelope or bacteria size, we also found that neither flagella or fimbriae, nor macromolecules located in the cytosol (DNA, ribosomes, proteins, etc.) contribute discernible scattering patterns and hence can be detected.

The same methodological concept was applied here to reveal the end-state structure of *E. coli*. Further, and analogously to (Semeraro et al., 2021b), we coupled the USAXS/SAXS and SANS analysis to TEM, in order to remove ambiguities in adjustable parameters originating from ensemble averaging (Figure 1). In order to couple scattering experiments to AMP susceptibility assays it is important to scale peptide concentrations appropriately. Firstly, because SAXS/SANS experiments require up to 10^4 times higher bacterial concentrations than standard growth-inhibition experiments. And secondly, because the total number of AMPs partitioning into bacteria (and hence also their antimicrobial activity) depends on cell concentration in a non-trivial manner (Marx et al., 2021b). We consequently determined the MICs of all here studied AMPs as a function of cell number density prior to scattering experiments; the corresponding data are reported in Figure 1–Figure Supplement 1.

Small-angle scattering (SAS) patterns of initial and end-states showed distinct differences, which are highlighted in panels E and F of Figure 1–Figure Supplement 2. Figure 1–Figure Supplement 2E, in particular, demonstrates a decrease of intensity at very low $q$-values (as approximation of forward scattering), and a faster intensity decay at high $q$ for the end-state, coupled to a loss of two intensity wiggles at $q \sim 0.1$ nm$^{-1}$ and $q \sim 0.3$ nm$^{-1}$ (see also Figure 1A). The comparison of SANS data at 90% D$_2$O (Figure 1–Figure Supplement 2F) instead shows a smoothening of an intensity shoulder at $q \sim 0.13$ nm$^{-1}$ coupled to a slight peak-shift toward lower $q$-values for the end-state. The application of our previously developed analytical model for *E. coli* (Semeraro et al., 2021b)
Figure 1. (A) Mapping the main structural changes in E. coli ATCC 25922 (green symbols) upon 1 h incubation with LF11-324 (red symbols) as observed by USAXS/SAXS and TEM. Scattering data of E. coli ATCC 25922 are from Semeraro et al. (2021b) and have been obtained at 10 fold higher sample concentration, leading to the observed offset of scattered intensities. Black lines are the best fits using Equation 6. Abbreviations: OMV: outer membrane vesicle formation; CWD: cell-wall damaging; SNR: phase separation of the nucleoid region. (B) TEM examples of membrane detachment and OMV formation due to LF11-324, and respective ensemble results from scattering data analysis for the distance distribution between inner and outer membranes. (C) Bacteria upon 1 h incubation with O-LF11-215, showing the formation of tube-like protrusions.

**Figure 1–Figure supplement 1.** Cell number-dependent MIC plots for different peptides.

**Figure 1–Figure supplement 2.** Comparison between USAXS/SAXS and contrast-variation SANS data and details of the scattering data analysis.

**Figure 1–Figure supplement 3.** TEM observations for LF11-215, LF11-324 and O-LF11-215 at the MICs and sub-MICs.

**Figure 1–Figure supplement 4.** Graphical scheme of the adjustable parameters.
Table 1. Change of E. coli structure due to LF11-324 ([P] ~ MIC) as observed from USAXS/SAXS/SANS data analysis. Values are the difference between end- and initial-state. See a graphical scheme of the adjustable parameters in Figure 1–Figure Supplement 4, and Semeraro et al. (2021b) for a more detailed schematic.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
<th>Description</th>
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<tbody>
<tr>
<td>$\Delta R_{CP}$</td>
<td>$10^{-4}$ nm$^2$</td>
<td>$P_{CP} \rightarrow$ SLD of cytoplasmic space</td>
</tr>
<tr>
<td>$\Delta R_{PP}$</td>
<td>$10^{-4}$ nm$^2$</td>
<td>$P_{PP} \rightarrow$ SLD of periplasmic space</td>
</tr>
<tr>
<td>$\Delta \Delta_{OM}$</td>
<td>(nm)</td>
<td>$\Delta_{OM} \rightarrow$ Inter-membranes distance</td>
</tr>
<tr>
<td>$\sigma_{OM}$</td>
<td>(nm)</td>
<td>$\sigma_{OM} \rightarrow$ Std. deviation around $\Delta_{OM}$</td>
</tr>
<tr>
<td>$\Delta R_{PG}$</td>
<td>$10^{-4}$ nm$^2$</td>
<td>$P_{PG} \rightarrow$ SLD of peptidoglycan layer</td>
</tr>
<tr>
<td>$\Delta R_{LPS}$</td>
<td>$10^{-4}$ nm$^2$</td>
<td>$LPS$ packing parameter $R \rightarrow$ Minor radius of the cytoplasmic space</td>
</tr>
<tr>
<td>$\Delta R$</td>
<td>(nm)</td>
<td>$\Delta R_{LPS} \rightarrow$ LPS packing parameter $R \rightarrow$ Minor radius of the cytoplasmic space</td>
</tr>
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(X) from SANS.
(N) from SANS (SLDs were obtained by extrapolating to 0 wt% D$_2$O; see Figure 1–Figure Supplement 2B-D.
Differences in X-ray and neutron SLDs are due to different physical interactions with matter. In case of $P_{LPS}$, this originates from a biological variation of different bacterial cultures.

allowed us to gain detailed insight on the ultrastructural changes pertaining to these differences in scattered intensities. To achieve this goal we jointly analyzed USAXS/SAXS and SANS data at five different contrasts (10, 30, 40, 50, 90% D$_2$O; see Figure 1–Figure Supplement 2). Taking AMP-induced changes of bacterial ultrastructure as observed by TEM into account then leads to the following observations.

Membrane ruffling, mainly originating from increased fluctuations of cytoplasmic membranes clearly seen by TEM, can be modeled by changing the average distance between inner and outer membranes and its fluctuation (Figure 1B), which readily accounts for the above described change of the SANS feature in Figure 1–Figure Supplement 2F. Consistent with previous reports (Sochacki et al., 2011) we also observed an overall shrinking of bacterial size. This correlates with a loss of periplasmic or cytosolic material. Both effects explain the changes in USAXS intensity at very low $q$-values (Figure 1–Figure Supplement 2E).

The scattering shoulder in USAXS/SAXS data at $q \sim 0.07$ nm$^{-1}$ originates from positional correlations between LPS oligosaccharide cores, as revealed by our previous in-depth analysis (Semeraro et al., 2021b). Its smearing out in the presence of peptide consequently indicates a loss of LPS packing correlations, either because of a decrease of the number of LPS molecules in the outer surface, or due to increased membrane roughness or waviness. This effect occurs on sub-nanometer length scales and is thus hidden to TEM. Notably, the end-state scattering patterns in the presence of either LF11-324 or LF11-215 were identical, despite their distinct MIC values (data not shown).

Modelling their scattering data revealed an additional contribution, which we could associate with the help of TEM to the formation of outer membrane vesicles (OMVs), having an average diameter of ~ 30 nm (Figure 1A-B, and Figure 1–Figure Supplement 2E). Instead, the separation of the nucleotide region from the nucleotide-free cytosol revealed by TEM (Figure 1A), and in agreement with a previous report on same AMPs (Zweytick et al., 2011), is not observed in our scattering data. As noted above, SAS is not sensitive to macromolecules and hence aggregates thereof in the cytosol (Semeraro et al., 2021b). Finally, O-LF11-215 led to additional effects. First of all, O-LF11-215, because of its increased hydrophobicity, forms aggregates in buffer as clearly shown by SANS (Appendix 1). In particular, incubating O-LF11-215 with bacteria at concentrations needed for SAS experiments led to the formation of macroscopic aggregates, which impeded the measurements of end-states by SAS. TEM experiments, however, showed the formation of extramembranous tubes (Figure 1C).

All end-state SAS data were fitted with our analytical model using a Monte Carlo genetic-selection.
algorithm; see Figure 1—Figure Supplement 4 for a graphical scheme of all adjustable parameters. Compared to standard least square algorithms, this allowed us to obtain robust parameter values from probability distribution functions and to judge possible parameter correlations (for details, see Methods and Materials). Based on our previous modelling of neat E. coli (Semeraro et al., 2021b), the individual SANS contrasts (Figure 1—Figure Supplement 2A) are motivated as follows: (i) the distance between inner and outer membranes $\Delta_{OM}$ and its fluctuations, $\sigma_{OM}$, are accentuated at 90, 50, and 40% $D_2O$; (ii) 30 to 50% $D_2O$ highlight the scattering length density (SLD) balance between the cytosol $\rho_{CP}$, periplasm $\rho_{PP}$, and the peptidoglycan layer $\rho_{PG}$; (iii) 10% $D_2O$ yields a similar contrast than X-ray and thus provides a direct and independent control for USAXS/SAXS experiments. Table 1 summarizes the results of this analysis for LF11-324 by listing the changes between initial and end-states. As discussed above, our analysis also reports overall bacterial size changes. E. coli are reasonably well represented in reciprocal space using an ellipsoid with multiple shells to account for the different compartments (Semeraro et al., 2021b). Since our smallest probed scattering vector, $q_{\text{min}} \sim 3 \times 10^{-3}$ nm$^{-1}$ (corresponding to distances of about 2 $\mu$m), does not allow to probe the full length of the bacteria, we report size changes in terms of changes of the minor radius of the ellipsoid and in particular, that of the cytoplasmic space $R$. The major radius was coupled to $R$ by fixing the major to minor radius ratio, $\epsilon$, to a value of 2 [see Table 2 in Methods and Material section]. Finally, the USAXS/SAXS scattering shoulder at $q \sim 0.07$ nm$^{-1}$ reports on positional correlations between LPS oligosaccharide cores (Semeraro et al., 2021b). In our analytical model this is captured by the number of oligosaccharide cores (OS), $N_{OS}$. To characterize the peptide-induced changes, we define the LPS packing parameter, $p_{LPS} = N_{OS}/N_{OS}^{0}$, as the ratio between the number of OS (i.e. LPS) in the current and initial-state ($N_{OS}^{0}$). Other parameters of the analytical model, such as the thicknesses of the hydrophobic and hydrophilic layers of the cytoplasmic and outer membranes, as well as their average SLDs, the thickness of the peptidoglycan layer, and the radius of gyration of the oligosaccharides were fixed at the same values as reported in Semeraro et al. (2021b) (see also Table 2 and Table 3 in Methods and Material section). We additionally checked for contributions of peptides to the SLDs of each bacterial compartment, including inner and outer membranes. However, the associated changes were found to be insignificant.

The observed LF11-324-induced changes to E. coli show, upon the application of our model, a decrease of $\rho_{CP}$, along with the increase of $\rho_{PP}$, signifying leakage of mainly low-weight molecules and ions from the cytoplasm (Figure 1—Figure Supplement 2B-C), whose scattering dominate both $\rho_{CP}$ and $\rho_{PP}$ (Semeraro et al., 2021b). Surprisingly, final $\rho_{CP}$ and $\rho_{PP}$ values did not depend on peptide concentration, i.e. were equal within experimental uncertainty at MIC and sub-MICS (see Figure 2B). The overall increase of the distance between inner and outer membranes, $\Delta_{OM}$, corresponds to about 18%; its fluctuations $\sigma_{OM}$ increase by almost a factor of two. The associated disorder in the periplasm then also accounts for the drop of the contrast of the peptidoglycan layer $\rho_{PG}$ by about 2.6%. The LPS packing parameter, $p_{LPS}$, instead drops dramatically by 30–40%. Finally, the observed cell shrinkage amounts to ~5%, which leads to a decrease of cell surface of approximately $2 \times 10^4$ nm$^2$. Apparently this is at least in part compensated by OMV formation, indeed the overall cell-surface decrease is on the same order of magnitude as the estimate of the total surface of all OMVs of $\sim (2−6) \times 10^4$ nm$^2$ as obtained from analysing SAS data (see Appendix 2).

Kinetics: time-resolved USAXS/SAXS
The structural transitions from initial to end-state were followed by USAXS/SAXS at millisecond time resolution. Stopped-flow mixing ensured thorough and rapid re-dispersion (mixing time of 50 ms) of peptides and bacteria (Figure 2—Figure Supplement 1) and led to immediate changes of scattering patterns. Using the initial and end-state values obtained for the adjustable parameters as constraints allowed us to fit kinetic scattering data as detailed in the Methods and Materials section. A close examination of the results obtained for the three LF11s (Figure 2, Figure 2—Figure Supplement 2 and Figure 2—Figure Supplement 3), allowed to discern AMP-concentration independent parameters ($p_{LPS}$, $\Delta_{OM}$, $\sigma_{OM}$ and $\rho_{PG}$) from AMP-concentration dependent parameters.
Figure 2. (A-F) Kinetics of the bacterial structural response to attack by LF11-324; results for three different peptide concentrations are shown. LPS packing (A); cytoplasm and periplasm SLDs (B); minor radius of the cell (C); intermembrane distance (∼ periplasm thickness) (D) and its deviation (E); and peptidoglycan SLD (F). (G-I) Bacterial response to O-LF11-215 at two concentrations. LPS packing (G); cytoplasm and periplasm SLDs (H); and minor ellipsoidal radius of the cell (I). Thick gray bands mark the degree of confidence from bacterial systems w/o peptides [see Table 1 and Semeraro et al. (2021b)], except for (C) and (I), where they refer to the average of the current cell radii at Δt = 0.0175 s. Fluctuations of initial values can be due to biological diversity. The vertical gray grid (A and D-F) indicates the time range of local (A) and macroscopic (D-F) cell-wall damage. Note that this range does not depend on peptide concentration. Colored lines in (B) mark the concentration-dependent lower boundary for the onsets of leakage. Results at Δt = 1 hour refer to end-states, when available.

Figure 2–Figure supplement 1. Schematic of the stopped-flow rapid mixing USAXS/SAXS experiments, including selected scattering patterns.

Figure 2–Figure supplement 2. Kinetics of the adjustable parameters for LF11-215 and O-LF11-215 systems.

Figure 2–Figure supplement 3. Kinetics of the adjustable parameters for LF11-215 systems.

Figure 2–Figure supplement 4. Representative scattering patterns including errors and fitted curves.

Figure 2–Figure supplement 5. Representative distributions of the adjustable parameters at different time points.

Figure 2–Figure supplement 6. Representative series of correlation plots and coefficients of the adjustable parameters.
First, we focus on LF11-324 and AMP-concentration independent parameters. The packing of LPS started to decrease at \( \Delta t \sim 10\) s after mixing (Figure 2A). Changes of \( \Delta_{\text{OM}}, \sigma_{\text{OM}} \) and \( \rho_{\text{PG}} \) in turn are largely decoupled from this remodeling of the outer membrane, with a common onset of 2–10 minutes after peptide addition (Figure 2D – F). AMP-concentration dependent parameters instead showed an increasing delay of changes upon decreasing the amount of administered peptide (Figure 2B, C). In particular, \( \rho_{\text{pp}} \) exhibited a pronounced increase already at \( \Delta t \sim 3\) s at highest peptide concentration shifting to \( \Delta t \sim 2\) minutes at the presently lowest studied peptide concentration. The onset times of decrease of \( \rho_{\text{CP}} \) do not appear to be directly correlated with these changes, but also shifted progressively to later times with decreasing AMPs. Because of the dominant contribution of low-molecular-weight molecules and ions to both \( \rho_{\text{CP}} \) and \( \rho_{\text{pp}} \) \cite{Semeraro2021}, we propose that these changes need to be due to a leakage of inner and outer membranes. Briefly, the cytoplasmic content diffuses first in the periplasmic space and, simultaneously, material from the periplasm leaks out of the cell. This process leads to the rather simultaneous decrease of \( \rho_{\text{CP}} \) and increase of \( \rho_{\text{pp}} \). Because of differences in the individual onsets of these trends at a given AMP concentration, we can only associate a time range for the beginning of permeation of the cytoplasmic membrane: \( 3–10\) s at \( [P] = 1.2 \times \text{MIC} \), \( 10–20\) s at \( [P] = 0.7 \times \text{MIC} \) and \( 50–120\) s at \( [P] = 0.3 \times \text{MIC} \). Despite these differences, and as noted above, \( \rho_{\text{CP}} \) and \( \rho_{\text{pp}} \) reached the same final values for all three AMP concentrations. Finally, the drop of \( R \) started at \( 20–50\) s for \( [P] = 1.2 \times \text{MIC} \) and \( 0.7 \times \text{MIC} \), and \( > 10\) min for \( [P] = 0.3 \times \text{MIC} \) (Figure 2C).

Interestingly, LF11-215 led to almost identical kinetics for the concentration-independent parameters \( \Delta_{\text{OM}}, \sigma_{\text{OM}} \) and \( \rho_{\text{PG}} \) in Figure 2–Figure Supplement 2A-C and \( \rho_{\text{PS}} \) in Figure 2–Figure Supplement 3A. This suggests that LF11-215 and LF11-324 remodel the membranes and affect the macroscopic stability of the cell-envelope in a very similar fashion, although we found no decrease of \( \rho_{\text{PS}} \) for LF11-215 at \( [P] = 1.6 \times \text{MIC} \) (Figure 2–Figure Supplement 3A). Importantly, \( \rho_{\text{CP}} \) and \( \rho_{\text{pp}} \) started to change much later for this peptide \( (\Delta t \sim 20–50\) s at \( 1.6 \times \text{MIC} \), Figure 2–Figure Supplement 3B). The overall changes of \( \rho_{\text{CP}} \) and \( \rho_{\text{pp}} \) indicate, however, that the AMP-concentration induced loss of cytoplasmic content is a common feature of LF11-324 and LF11-215.

The kinetics of the cell-envelope damage caused by O-LF11-215 were found to proceed analogously to both LF11-324 and LF11-215 (Figure 2–Figure Supplement 2D-F). However, while changes of \( \rho_{\text{CP}} \) and \( \rho_{\text{pp}} \) occurred at about similar times than for LF11-324 (Figure 2B, H), \( \rho_{\text{PS}} \) \( (\Delta t \sim 2\) min, Figure 2G) and \( R \) \( (\Delta t \sim 10\) min, Figure 2I) changed much later. The initial increase of \( \rho_{\text{PS}} \) is likely to be an artefact, probably due to the convolution of scattering originating from cells and peptide aggregates (see Figure 2–Figure Supplement 1D).

LF11-215/LF11-324-induced OMV formation was discernible for \( \Delta t > 1–2\) min (Figure 3A) and appears to be peptide- and concentration-independent within the first 10 min. Finally, the peptide cluster term, introduced for the analysis of O-LF11-215 (see Methods and Materials), enabled us to estimate that a large increase of peptide uptake starts after about 2 min, which, however, did not depend on peptide concentration (Figure 3B).

**Peptide partitioning and cooperativity**

We applied a previously detailed assay for AMP partitioning in *E. coli* based on growth inhibition \cite{Marx2021}. A statistical analysis of the corresponding data in terms of cumulative distribution functions (CDF, see Appendix 3) allowed us to derive probability density functions (PDF), describing bacterial growth inhibition as a function of cell concentration \( n_{\text{cell}} \), including the minimum AMP concentration needed for inhibiting a given percentage \( x \) of *E. coli*, \( IC_x \) (Figure 4); note that MIC \( \equiv IC_{99.9}\). We obtained Poisson-like PDF of ‘killing’ events as a function of peptide concentration from the sigmoidal shapes of the measured CDF (Appendix 3). Besides the specific mode of action of a peptide, the shape, width and position of the PDF are a result of intrinsic statistical variations and fluctuations of the systems’ properties, e.g., bacterial size, stage of mitosis, local concentration of partitioned peptides, biological variability within the populations, etc.
Figure 3. (A) Kinetics of the forward intensity of OMV scattering for different concentrations of LF11-324 and LF11-215. The dashed horizontal line represents the detection/visibility limit, below which $I_{cell}(q) + I_{OMV}(q) \approx I_{cell}(q)$ in the entire $q$-range. The dashed exponential curve is a guide for the eyes. (B) Evolution of the number of partitioned peptides per cell for two O-LF11-215 concentrations, as calculated from the analysis of $I_{clu}$.

Figure 4. Amount of LF11-324 or LF11-215 required to attain growth inhibited fractions of either 0.999 (MIC, up triangles), 0.5 (circles) or 0.01 (down triangles) in *E. coli* ATCC 25922 as a function of $n_{cell}$. Lines are fits with Equation 1. These data are overlayed with a surface plot of the associated killing probability density function. The color scales indicate the corresponding magnitudes.

Figure 4–Figure supplement 1. IC as a function of $\phi_{G}$ (inverse CDF).
In agreement with our previous report for *E. coli* K12 (*Marx et al., 2021b*), the MIC of LF11-324 is lower than that of LF11-215 at all cell concentrations. For O-LF11-215, MIC values matched those of LF11-324 at low cell concentrations, but increased strongly with *n*\_cell, finally superseding that of LF11-215 and becoming immeasurably high due to the above described peptide aggregation (Figure 1—Figure Supplement 1). The growth inhibition probabilities for LF11-324 and LF11-215 peak close to the IC\_50 (Figure 4). The distributions are much sharper, i.e. have a smaller full width at half maximum of the probability distributions, \(\sigma_{P}\), for LF11-324 than for LF11-215. This suggests an increased ‘cooperativity’ of killing for LF11-324, in the sense of a two-state transition model between alive and dead bacteria (see Appendix 3). \(\sigma_{P}\) increased with cell concentration, e.g. from \(\sigma_{P} \approx 2.6 \, \mu M\) at \(n_{cell} = 10^7\) CFU/ml to \(\sigma_{P} \approx 13 \, \mu M\) at \(n_{cell} = 10^9\) CFU/ml for LF11-324. Significant noise levels in growth inhibition data for O-LF11-215 impeded a determination of killing probabilities at inhibitory concentrations < IC\_50. However, data retrieved at higher inhibitory concentrations suggest that the probability distributions roughly match those of LF11-324 at low cell concentrations, but become broader than that of LF11-215 at high cell content (Figure 4—Figure Supplement 1).

This is a signature of loss of killing efficacy at high \(n_{cell}\), most likely due to peptide self-aggregation as discussed above.

Next, we derived for each IC\(\_x\) (or growth-inhibited fraction, \(\phi_{IG}\)) the average number of cell-partitioned peptides, \(N_p\), and the effective partitioning coefficient, \(K^{eff}\), applying a previously reported thermodynamic formalism ([*Marx et al., 2021b*]; see also Equation 1). \(N_p\) increased for all three peptides with \(\phi_{IG}\), although the changes of \(N_p\) were smallest for LF11-324, followed by LF11-215 and O-LF11-215 Figure 5A. These results were confirmed independently also by Trp-fluorescence spectroscopy (see Appendix 4).

This partitioning behaviour was also mirrored in the \(\phi_{IG}\)-dependence of \(K^{eff}\). \(K^{eff}\) was nearly constant for LF11-324, increased only slightly for LF11-215 and showed the largest variation for O-LF11-215, reaching about 2.5 times higher levels than the other two peptides Figure 5B. The approximate equal \(K^{eff}\) values of LF11-324 and LF11-215 for \(\phi_{IG} > 0.5\) demonstrate that both peptides partition about equally well into *E. coli*, not only at the MIC, but in a wide range of \(\phi_{IG}\) values.

ζ-potential measurements were performed ~5 min and 1 h (end-state) after adding AMPs. Results did not show significant differences for the two time points. This enabled us to further discriminate the activities of LF11-324 and LF11-215. We observed an initial increase of \(\zeta/\zeta_0\) (\(\zeta_0\) refers to the reference system, i.e. neat bacteria) at low peptide concentrations, reaching plateau-values of \(\zeta/\zeta_0 = 0.80 \pm 0.16\) for LF11-215 and \(\zeta/\zeta_0 = 0.85 \pm 0.17\) for LF11-324 for \(|P| \geq 0.3 x MIC\) (Figure 5—Figure Supplement 1). As detailed in *Marx et al. (2021b)* these data allowed us to calculate the maximum number of peptides associated to the LPS leaflet, \(N_p^{max}\). We found \(N_p^{max} = (8 \pm 2) \times 10^3\) and \((6 \pm 2) \times 10^1\) for LF11-215 and LF11-324, respectively, in the \(\zeta/\zeta_0\)-plateau regions. Normalizing these results by the number of cell-partitioned peptides (\(N_p\)) reveals an overall decrease of \(N_p^{max}/N_p\) with \(\phi_{IG}\) (see Figure 5C). Note that the ζ-potential analysis provides (with the applied mathematical and physical constraints) upper-boundary values for \(N_p^{max}/N_p\) (see Methods and Materials). That is, \(N_p^{true}/N_p \leq N_p^{max}/N_p\). Thus our conservative \(N_p^{max}/N_p\)-ratio strikingly demonstrates that > 98% of the peptides are located within the intracellular compartments of *E. coli* at the MIC. The fraction of outer-leaflet-partitioned peptides increased toward lower \(\phi_{IG}\) and somewhat stronger for LF11-215, but does not exceed 10%. An analogous analysis for O-LF11-215 was impeded by the peptide aggregates, whose sizes were on the same order or even larger than that of bacteria (Figure 5—Figure Supplement 2).

**Discussion**

The entire set of the above detailed results reveals a complex scenario that needs to be evaluated with utmost care. To this end, we stress that all techniques used in this work report *ensemble* averages. That is, they account for the entire bacterial population within the samples. For example, a LPS packing value of \(P_{PS} \approx 0.55\)—observed for growth-inhibited fractions of 1%, as well as 99.9%
Figure 5. (A-B) $N_B$ and $K_{\text{eff}}$ values as a function of inhibited fraction. In the case of O-LF11-215, $N_B \equiv N_B^{\text{eff}}$.

(C) Ratio between the maximum number of peptides on the outer leaflet and total number of partitioned peptides, $N_{\text{max}}^{\text{NP}} / N_B$, as a function of inhibited fraction. Lines are guides for the eye.

**Figure 5—Figure supplement 1.** $\zeta$-potential and size measurements of LF11-215 and LF11-324.

**Figure 5—Figure supplement 2.** $\zeta$-potential and size measurements of O-LF11-215.

after one hour (Figure 2A)—signifies that the ensemble average shows the same loss of LPS-packing under both conditions, irrespective of individual differences in some isolated cells.

A particularly striking result for the LF11-215/LF11-324 end-states is that the peptide-induced effects are similar and independent of peptide concentration (Figure 1, Figure 2). That is, even at growth-inhibited fractions of just 1%, we observed much the same cellular permeabilization and structural changes of the bacterial ultrastructure as at quasi fully growth-inhibited *E. coli* (see also Figure 4 and Figure 4—Figure Supplement 1). Here it is important to bare in mind that, unlike our susceptibility assays, we did not add a growth medium after incubation with peptides in SAS experiments. Our combined SAS and growth inhibition data provide unambiguous evidence that bacteria are able to recover at sub-MIC concentrations from a severe collateral damage of their cell-envelope. Consequently, this damage cannot be the primary killing cause for bacteria. Our structural studies only revealed peptide-specific and concentration dependent effects for the kinetics of $R$, $\rho_{\text{CP}}$, and $\rho_{\text{PP}}$ (Figure 2). It is unlikely though that antimicrobial activity is specific to the rate of bacteria size changes. In turn, the different onsets of changes of $\rho_{\text{CP}}$ and $\rho_{\text{PP}}$ may provide some clues to events that finally inhibit the growth of *E. coli*. In the following we will thus focus on the sequence of events that can be deduced from our study for LF11-324 and LF11-215. Before doing so we note that also O-LF11-215 caused comparable variations of the above mentioned parameters on similar time scales. In this case, however, an equally detailed analysis was impeded by the propensity of O-LF11-215 to aggregate in buffer solution.

**Figure 6** provides a scheme of the time-line of events revealed by the present study, taking also into account our complementary experiments on peptide partitioning. Within a few seconds LF11-324 and LF11-215 cause changes of the LPS packing density ($\Delta t \sim 10$ s), as well as periplasmic and cytoplasmic SLDs. Since ions and small molecules ($<1$ kDa) are the major contributors to its X-ray SLD (Semeraro et al., 2021b), the decrease of $\rho_{\text{CP}}$ will be dominated by a leakage of these entities.
(e.g. ATP). Molecules leaking from the cytosol first diffuse into the periplasm and then further into extracellular space, leading to a net increase of of ρPP. Leaking into extracellular space follows from the observation that final ρPP levels do not reach those of ρCP, despite the much larger reservoir for cytosolic molecules. Further, the initial SLDs of buffer and periplasm are comparable, also explaining why our technique is not directly detecting outer membrane leakage. Hence, either observed change of ρPP or ρCP is due to a permeabilization of both cytoplasmic and outer membranes.

For LF11-324, the permeabilization of the cytoplasmic membrane occurred as fast as 3 – 10 s after mixing at [P] = 1.2xMIC. Dropping peptide concentration led to a slowing down of this effect (10 – 20 s for [P] = 0.7xMIC, and 50 – 120 s for [P] = 0.3xMIC), but did not affect the final cytoplasmic density, i.e. the overall loss of material (Figure 2). AMPs need to translocate all the way through the cell wall in order to induce such effects, implying that peptide translocation of the outer membrane proceeds on time scales faster than ΔρPP or ΔρCP. We stress that the usage of the term ‘translocate’ does not imply that AMPs are able to pass through inner and outer membranes without any noticeable effect on membrane structure, such as the transient formation of pores, nor does it exclude their presence. It merely refers to a generic, unspecified uptake of the peptides beyond the resolution of the present experiments. Further, since we are not able to discriminate membrane peptide adsorption kinetics from the onset of leakage of cellular content, we cannot comment on a potential ‘carpet-mechanism’, proposed from analogous AMP partitioning studies in bacteria (Roversi et al., 2014; Savini et al., 2020). We note, however, that LF11-215 was found to partition into artificial lipid membranes, without noticeable effects on lipid flip-flop (Marx et al., 2021a). We detail this discussion further below.

The drop of R is a natural consequence of the loss of cellular content, but occurs at somewhat later times. This delay is most likely due to the stored elastic energies of the peptidoglycan layer, which will initially resist rapid deformations (Yang et al., 2018). Note, however, that the peptidoglycan properties are likely affected by direct interactions with peptides (Zhu et al., 2019). Remarkably, cytoplasmic membrane leakage in the MIC range occurs ~ 1 min later for LF11-215 (Figure 2–Figure Supplement 3).

Pronounced differences between LF11-324 and LF11-215 were also observed from their efficacies as a function of cell concentration (Figure 4). At equal n_cell, growth-inhibition probability distributions are much narrower for LF11-324. Apparently, this increased 'cooperativity' correlates with the peptide's ability to permeabilize the cytoplasmic membrane faster. It is further illuminating to discuss the total amount of peptide penetrating into the intracellular space. Both peptides saturate the outer LPS leaflet already at concentrations lower than 0.3xMIC (Figure 5–Figure Supplement 1), corresponding to about one AMP per 4 – 5 LPS molecules as upper-bound value (see N_pmax/N_p0 in Methods and Materials section). Thus, consistent with Figure 2A-B, peptides penetrate the outer membrane already when effects on bacterial growth are still very small.

Assuming that the inner leaflet of the outer membrane, as well as the two leaflets of the inner membrane, host to first order similar amounts of LF11-324 and LF11-215 we calculate that ≥ 92% of all peptides are located in the intracellular space at the respective MICs. Using our USAXS/SAXS/DLS data for the average cytoplasmic and periplasmic volumes we further arrive at huge intracellular concentrations of ~ 80 mM for LF11-324, and ~ 100 mM for LF11-215. The difference in cytosolic/periplasmic concentrations between the two peptides corresponds to about the difference in MICs (Figure 1–Figure Supplement 1). Our finding that only a minor fraction of the peptides is located within the membranes remains valid even at sub-MIC values, offering an attractive explanation for the similar and concentration-independent average cell-wall damage induced LF11-324 and LF11-215. Specifically, we propose that this damage (LPS packing, OMV formation, membrane ruffling and detachment) is a consequence of the small fraction of peptides that saturate the membranes and, possibly, the peptidoglycan layer. Following the thermodynamic principles of the partitioning framework, and ζ-potential measurements, this small fraction is supposed to be rather constant above AMP concentrations of about 0.2xMIC. Along these lines, recent in-cell FRET experiments showed that only a small fraction of the peptides partitioned in a cell is actually interacting...
Figure 6. Simplified time sequence of LF11-215 and LF11-324 mode-of-action. The measured time onsets and boundaries refer to LF11-324 at [MIC]×0.3 and [MIC]×1.2, which correspond to measured inhibited fractions of ∼1% and >99.9%, respectively. The outer leaflet is affected by peptides within the first seconds after their attack. Then, depending of AMP type and concentration, a number of rare translocation events, coupled with leakage, takes places over a broad time range. When both membranes are saturated with peptides (exact time not determined), the cell-wall breaks down, leading to OMV formation (Δt >10 s), detachment of outer and inner membranes and waving (Δt >2–5 min). Simultaneously, AMPs accumulate in internal compartments and reach saturation levels within less than 1 h.

<table>
<thead>
<tr>
<th>Inhibited fractions</th>
<th>∆t &lt; 3 s</th>
<th>∆t &lt; 10 s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partitioning on LPS leaflet</td>
<td>∆t &lt; 3 s</td>
<td>∆t &lt; 10 s</td>
</tr>
<tr>
<td>Loosening of LPS lateral packing</td>
<td>∆t ~ 10 s</td>
<td>∆t ~ 10 s</td>
</tr>
<tr>
<td>Translocation/outer membranes permeation</td>
<td>∆t &lt; 3 s</td>
<td>∆t &lt; 50 s</td>
</tr>
<tr>
<td>LPS leaflet saturation</td>
<td>∆t &lt; 5 min</td>
<td>∆t &lt; 5 min</td>
</tr>
<tr>
<td>Crossing the periplasm</td>
<td>∆t &lt; 3 s</td>
<td>∆t &lt; 50 s</td>
</tr>
<tr>
<td>Translocation/inner membranes permeation</td>
<td>∆t ~ 3 s</td>
<td>∆t ~ 50 s</td>
</tr>
<tr>
<td>Outer and inner membrane saturation</td>
<td>∆t &lt; 1 h</td>
<td>∆t &lt; 1 h</td>
</tr>
<tr>
<td>Membrane detachment and waving</td>
<td>Δt = 2-5 min</td>
<td>Δt = 2-5 min</td>
</tr>
<tr>
<td>Outer-membrane-vesicle formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturation of cytoplasm/periplasm</td>
<td>∆t &lt; 1 h</td>
<td>∆t &lt; 1 h</td>
</tr>
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About 10 years ago, Wimley (2010) already discussed the consequences of having $10^7$ to $10^9$ AMPs per cell, suggesting a ‘reservoir’ of non-membrane-bound peptides that would outnumber proteins, ATP and other metabolites. Cytosolic targets were also confirmed by our TEM data [and those of others (Hammer et al., 2010; Scheenstra et al., 2019)] showing a collapsed nucleoid region (Figure 1). For instance this could be due to a competition mechanism with polyamines, as putative stabilizers of the functional architecture of the DNA ring (Hou et al., 2001). In support of the hypothesis of cytosolic targets, recent solid-state $^{31}$P-NMR measurements of *E. coli* in the presence of AMPs revealed increased dynamics of DNA and RNA phosphate groups correlated to TEM observations of collapsed nucleoid volume (Overall et al., 2019). In addition, however, AMP interactions with other negatively charged metabolites and macromolecules in the cytosol, such as ribosomes and proteins, should be considered as potentially detrimental to bacteria (Zhu et al., 2019). The about 1.4 times lower intracellular concentration of LF11-324 then is a signature of a higher potency as compared to LF11-215, possibly hampering a number of physiological functions.
(Scocchi et al., 2015), or inducing an apoptosis-like mechanism (Dwyer et al., 2012) due to interactions with any of the above named cytosolic molecules. The exact killing mechanism of the here studied AMPs, however, evades current experimental design.

It follows from the considerations above that bacteria have an increased probability to recover from the peptides’ attack, if the intracellular concentrations of LF11-324 and LF11-215 fall below those reported above (i.e. $\leq 80$ and $\leq 100 \text{mM}$, respectively). Thus, both the ability of LF11-324 to swiftly permeabilize the cytoplasmic membrane by translocating through the cell envelope and its higher propensity to interfere with physiological processes contribute to its higher ‘cooperativity’ in killing E. coli (Figure 4). Our experimental setup is not sensitive to directly observe either transient membrane pores or other membrane defects. Note, however, that previous studies in lipid-only mimics of bacterial membranes showed only weak membrane remodeling effects of lactoferricin derivatives as compared to other peptides (Zwytick et al., 2011; Marx et al., 2021b). We propose that the here studied LF11s follow the interfacial activity hypothesis, valid for both antimicrobial (Wimley, 2010) and cell penetrating peptides (Kauffman et al., 2015). That is, they are able to locally and transiently induce disorder in the lipids' hydrocarbon chain regime which sufficiently affects the permeability barrier of the bilayer against ions, small polar molecules and, eventually, the peptides themselves. This might be also a consequence of the rather short amino acid sequence of the lactoferricin derivatives, implying a too small length ($\sim 1\text{ nm}$) to span the whole membrane thickness ($3 – 4\text{ nm}$). In turn, the highly flexible secondary structure and an amphipathic moment being aligned along the peptide’s backbone (Zorko et al., 2009; Zwytick et al., 2011, 2014) should allow LF11-324 and LF11-215 to translocate membranes at higher rates than observed for linear peptides (Ulmschneider, 2017; Kabelka and Vácha, 2018). Note that peptide translocation can also lead to transient membrane leakage events (Ulmschneider, 2017), even with negligible AMP-induced lipid flip-flop (Marx et al., 2021a). The higher hydrophobicity of O-LF11-215 should increase the likelihood of remaining membrane bound, which might build up differential membrane curvature stress and lead to the observed formation of membrane tubules. We also note that the different leakage kinetics for the LF11 peptides suggest a coupling to translocation kinetics, which in turn depends on membrane partitioning of the AMPs. Indeed, recently reported data for cytoplasmic membrane mimics of cardiolipin, phosphatidylethanolamine and phosphatidylglycerol show a somewhat faster membrane partitioning for LF11-324 than for LF11-215 (Marx et al., 2021b).

Concluding, the superior time-resolution and sensitivity to ensemble averaged structural changes from cellular size to molecular packing of synchrotron USAXS/SAXS allowed us to demonstrate, upon combination with advanced data modeling and complementary techniques for peptide partitioning, that LF11-based AMPs are able to reach the cytosolic membrane of bacteria on the seconds time scale and subsequently accumulate at high concentrations (80 – 100 mM) in all bacterial compartments, including the cytosol. Based on differences observed for LF11-324 and LF11-215 two factors emerge as key components for their antimicrobial efficacy: (i) a fast translocation through inner and outer membranes, and (ii) an efficient shut-down of here not further detailed vital chemical/biochemical processes, i.e., the lower the number of ‘needed’ AMPs the better (here achieved by LF11-324). The latter conclusion is corroborated by the most striking fact that the final cell-wall damage does not depend on peptide concentration. That is, AMPs inhibiting only 1% of bacteria induce on average—besides some single isolated variations—as much membrane ruffling, detachment of inner and outer membranes, leakage, etc. as AMPs at full levels of growth inhibition. Hence, the collateral damage of the cell-wall structure, including leakage of cellular content, is a ‘by-product’ of AMP activity. Instead we suggest that the primary cause of growth inhibition is driven by interactions of the AMPs with yet-to-be-determined cytosolic/periplasmic molecules. Most likely candidates are the polyanionic DNA, RNA, ribosomes and proteins (Zhu et al., 2019), or charged metabolites. We strongly emphasize that the ability of the here studied peptides to impair the barrier function of bacterial membranes remains an unquestioned highly important property. However, at the same time our study shows that this effect alone is not sufficient to explain all of our data.
It is currently not clear whether the present findings can also be extended to other AMPs. Yet, the importance of the cell-penetrating ability was similarly discussed for, e.g., peptides Sub3 and LL-37 (Torcato et al., 2013; Zhu et al., 2019). Further, a number of AMPs have been reported to show partitioning behavior in bacteria and accumulation in the intracellular volume analogously to the here studied lactoferricin derivatives [see, e.g., (Sochacki et al., 2011; Zhu et al., 2019; Savini et al., 2020; Loffredo et al., 2021; Kaji et al., 2021), and references therein]. Le et al. (2017) gave a more complete account of potential intracellular targets of AMPs—either of fundamental or secondary importance for bactericidal activity. We thus propose, that the combination of membrane translocation speed and efficient impairment of physiological processes are generic factors that should be considered in designing future AMPs to combat infectious diseases. This also implies a widening of the pure focus on membrane-activity of AMPs currently applied in many studies.

Methods and Materials

Samples

Escherichia coli ATCC 25922 were provided by the American Type Culture Collection (Manassas, VA). Freeze-dried peptides powder of LF11-215 (H-FWRIRRR-NH₂), LF11-324 (H-PFFWRIRRR-NH₂) and O-LF11-215 (octanoyl-FWRIRRR-NH₂), purity >95%, were purchased from the Polypeptide Laboratories (San Diego, CA). Lysogeny broth (LB)-agar and LB medium were obtained from Carl Roth, (Karlsruhe, Germany). All the other chemicals were purchased from Sigma-Aldrich (Vienna, Austria).

Bacterial cultures

Bacterial colonies of E. coli ATCC 25922 were grown in LB-agar plates at 37 °C. Overnight cultures (ONCs) were prepared by inoculating a single colony in 3 ml LB-medium in sterile polypropylene conical tubes (15 ml), allowing for growth under aerobic conditions for 12–16 hours in a shaking incubator at 37 °C. Main cultures (MCs) were then prepared by diluting ONCs in 10 ml LB-medium in 50 ml sterile polypropylene conical tubes. Bacteria in the MCs grew under the same conditions applied to ONCs up to the middle of the exponential growth phase. Cells were then immediately washed twice and re-suspended in nutrient-free and isotonic phosphate-buffered saline (PBS) solution (phosphate buffer 20 mM, NaCl 130 mM) at pH 7.4.

AMP samples

LF11-324 and O-LF11-215 peptides displayed a weak solubility in PBS. AMP stocks (including LF11-215) were then prepared by adding acetic acid and DMSO, up to 0.3% and 3% vol/vol, respectively. Peptide stock solutions were diluted for measurements yielding a final concentration of 0.01% acetic acid and 0.1% vol/vol dimethyl sulfoxide (DMSO) (final pH 7.2). Hence, possible effects of DMSO on the cell envelope, as observed for model membrane structures (Gironi et al., 2020) can be neglected. Control USAXS/SAXS experiments adding a similar amount DMSO and acetic acid to E. coli also showed no discernible effect of the organic solvent (data not shown). Similarly, control experiments were performed to exclude effects on bacterial growth. Stock concentrations were determined by measuring the absorption band of the Trp residues with the spectrophotometer NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). Peptide stock solutions were stored in silanized glass tubes until use.

Antimicrobial activity and partitioning modeling

The antimicrobial activity of the AMPs on E. coli was tested in the bacterial concentration range of 5 × 10^2 to 10^9 CFU/ml using a modified susceptibility microdilution assay (Jorgensen and Ferraro, 2009). Cell suspensions were incubated with AMPs in buffer at a given peptide and cell concentration for 2 h at 37 °C (control samples were incubated in buffer only). Cell growth was monitored upon addition of double concentrated LB-medium for about 20 h using a Bioscreen C MBR (Oy Growth Curves Ab, Helsinki, Finland).
Partitioning modeling

The analysis of the inhibited fraction of cells, $\phi_{\text{IC}}$, as a function of the total concentration of peptides, $[P]$, enabled the extraction of IC$_c$ values as a function of $n_{\text{cell}}$, as detailed in Appendix 3. Following previously reported methods to determine peptide partition in liposomes (White et al., 1998) or bacterial systems (Savini et al., 2016), we derived the number of cell partitioned AMPs for a given IC$_c$ value using

$$[P](n_{\text{cell}}) = \frac{N_b [W]}{K^{eff}} + \frac{N_b n_{\text{cell}}}{N_A} \left( \frac{N_A [W]}{K^{eff}} + n_{\text{cell}} \right),$$

(1)

where $[P]_W$ and $[P]_B$ are the concentrations of AMPs dispersed in the aqueous phase and segregated into the cells, respectively; $N_A$ is the Avogadro’s constant; $[W]$ is the concentration of water molecules in bulk (55.3 M at 37 °C); $K^{eff}$ is the effective mole-fraction partitioning coefficient; and $N_B$ is the average number of peptide monomers that are partitioned within a single cell. Details of this analysis are reported in Marx et al. (2021b).

A similar approach was exploited in the case of O-LF11-215 peptide clusters in solution. In this case, the total peptide concentration is given by $[P] = [P]_W + [P]_B + [A]$, where $[A]$ is the molar concentration of aggregates, each of them consisting of an average number of peptides $N$. We also define the aggregate fraction $f_A = [A]/[P]$ and assume the equilibrium state $N[P]_W = [A]$. The definition of the molar partitioning coefficient $K_A \propto K^{eff}$ (Marx et al., 2021b) refers to the balance of concentration of free peptides in bulk and partitioned peptide into the cells. Hence, its bare definition is unaffected by the presence of clusters. Finally, it is trivial to show that in this case Equation 1 becomes:

$$[P](n_{\text{cell}}) = \frac{N_B}{N_A (1 - f_A)} \left( \frac{N_A [W]}{K^{eff}} + n_{\text{cell}} \right) = \frac{N_B^{eff}}{N_A} \left( \frac{N_A [W]}{K^{eff}} + n_{\text{cell}} \right),$$

(2)

where the fitting parameter $N^{eff}_B$ is an upper boundary value for the actual number of partitioned peptides per cell $N_B = N^{eff}_B (1 - f_A)$.

$\zeta$-potential, cell size, and outer leaflet distribution of peptides

$\zeta$-potential and and dynamic light scattering (DLS) measurements were carried out with the Zetasizer Nano ZSP (Malvern Panalytical, Malvern, UK). E. coli suspensions were incubated with different concentrations of AMPs in buffer for 1 hour at 37 °C prior to each measurements. Control samples (no AMPs) were suspended and incubated in buffer. Measurements were also repeated just after mixing with peptides (about 5 min waiting time for sample loading, data not shown), exhibiting negligible variation from samples incubated for 1 h. A concentration of 10$^7$ CFU/ml provides the optimal compromise between high signal-to-noise ratio and low multiple-scattering bias. The AMP concentrations were centered on the MIC values previously determined with the susceptibility microdilution assays, spanning from about 0.2× to 2.5×MIC. For $\zeta$-potential measurements the voltage for the electrodes was set to 4 V, such that currents did not exceed 1 mA, because of the high conductivity of the PBS buffer. Further, measurements were paused between repetitions for 180 seconds. This prevented heat productions leading to sample denaturation and accumulation on the electrodes. The experiments were repeated three times and, due to the low sensitivity of such a set-up, each of them consisted of a minimum of six measurements [see also Marx et al. (2021b)]. For each system, $\zeta$-potential values and associated errors were given by the medians and the median absolute deviations, respectively, averaging over at least 18 repetitions. The same number of scans was also used to obtain mean and standard deviation values for the hydrodynamic diameter, $d_H$, of the cells.

From the measured $\zeta$-potential values we calculated the maximum number of peptides that
partition into the outer LPS leaflet, $N^\text{max}_P$, as reported recently (Marx et al., 2021b)

$$
\frac{N^\text{max}_P}{N^0_P} \approx \left( \frac{z_l}{z_P} \right) \left( \frac{\zeta}{\zeta_0} S \right) - 1,
$$

where $z_l = -6$ (Wiese et al., 1998) and $z_P = +5$ (Zweytick et al., 2011) are the nominal charges of LPS and LF11-215 or LF11-324 AMPs, respectively; $\zeta$ and $S$ are the $\zeta$-potential and total surface values of the system upon addition of peptides; and $\zeta_0$ and $S_0$ are the respective reference values (no AMPs). $N^0_lPS \approx 0.9S_0/A_{PS}$ is the estimated number of LPS molecules, where $A_{PS} \approx 1.6 \text{ nm}^2$ (Kim et al., 2016; Micciulla et al., 2019) is the lateral area per LPS molecule. The prefactor originates from considering a maximum surface coverage of 90% by LPS molecules (Seltmann and Holst, 2002). $S_0$ was derived from DLS measurements, approximating the bacterial shape by a cylinder

$$d_H/2 = \sqrt{(\text{radius})^2/2 + (\text{length})^2/12},$$

considering that the hydrodynamic radius is approximately equivalent to the radius of gyration for micron-sized objects. Then fixing the radius at $\sim 400$ nm (Semeraro et al., 2021b) and retrieving the length from the above relation for $d_H$ one obtains $S_0 \approx 5 \times 10^3 \text{ nm}^2$.

**Fluorescence spectroscopy**

Fluorescence spectroscopy experiments were done with the Cary Eclipse Fluorescence Spectrophotometer (Varian/Agilent Technologies, Palo Alto, CA). The excitation wavelength was set to $\lambda = 280$ nm (which corresponds to the maximum intensity of the absorption/excitation band of Trp), and emission spectra were acquired in the $\lambda$-range between 290 and 500 nm, with the Trp emission band peak being expected to lie around 330 to 350 nm. Samples were loaded in quartz cuvettes of 1 cm path-length. The background was subtracted from every Trp-spectrum prior to further analysis.

**Peptide solubility**

Trp emission allowed determining whether LF11 peptides form aggregates or not in the MIC range. Spectra of LF11-only samples at $[P] = 100 \mu\text{g/ml}$ were fitted with the log-normal-like function (Burstein and Emelyanenko, 1996; Ladokhin et al., 2000)

$$I(I_0, \lambda, \Gamma) = \begin{cases} 
I_0 \exp \left[ -\frac{\lambda^2}{2\sigma^2} \ln^2 \left( 1 + \frac{|\lambda - \lambda_{\text{max}}|}{\sigma} \right) \right], & \lambda > (\lambda_{\text{max}} - y\Gamma) \\
0, & \lambda \leq (\lambda_{\text{max}} - y\Gamma)
\end{cases}
$$

where $\lambda_{\text{max}}$ and $I_0$ are, respectively, wavelength and intensity of the emission peak; $\Gamma$ is the full-width-at-half-maximum (FWHM) of the band; $\sigma$ is a skewness parameter (fixed at an optimum value of 1.36 after testing; and $y = \sigma/\sqrt{2 \ln 2} - 1$).

Both LF11-215 and LF11-324 (see Appendix 1) showed a peak at about $\lambda_{\text{max}} \approx 353 \text{ nm}$ and $\Gamma \sim 63$ nm. This is consistent with a location of the Trp residues in polar chemical environments having full mobility and thus suggests that these AMPs are monomeric (Burstein and Emelyanenko, 1996). In contrast, the acylated O-LF11-215 showed a significant blue-shift related to a location of Trp within apolar surroundings (Ladokhin et al., 2000), indicating the formation of peptide aggregates.

**Peptide partitioning**

Analogously to the partitioning analysis performed for lipid only membranes (Marx et al., 2021b), the Trp emission of bacterial/AMPs mixtures were treated as a two-component signal, one coming from the peptides in the aqueous phase, and the second one from AMPs interacting with the cells.

Bacterial suspensions were incubated with different concentrations of AMPs in buffer for 1 hour at 37 °C (incubator Thermomixer C, Eppendorf, Germany). Reference samples (no AMPs) were suspended and also incubated in PBS. Experiments were performed at cell concentrations of $5 \times 10^6$, $10^6$ and $5 \times 10^6 \text{ CFU/ml}$, and AMPs amounts equal to $0.5x$, $1x$ and $2x$MIC (each experiment was repeated three times). Fluorescence intensities were background-subtracted using the bacteria-only
reference spectra at the corresponding concentrations. This enabled us to subtract the average signal from the aromatic residues in the cells, and the scattering arising from the high cell concentrations of the cell suspensions. Emission spectra of bare bacterial systems surprisingly showed a single and clean Trp-emission band, peaking at $\lambda_{\text{max}} = 334 - 336$ nm ($\Gamma \sim 65$ nm). The peak intensity increased linearly from 4.9 (arb. units) at $10^7$ cells/ml to 122 (arb. units) at $5 \times 10^8$ cells/ml; then turbidity effects had to be accounted for. LF11-215 and LF11-324 showed peaks of comparable intensities even in the high cell-concentration regime, enabling us to retrieve background-subtracted spectra of optimal signal-to-noise ratio. Spectra were analyzed with a linear combination of two independent bands (see Equation 4) $I^W$ and $I^B$, referring to AMPs in bulk (W) and partitioned into the lipid bilayer (B). $\lambda^W$ and $\Gamma^W$ were fixed to the reference values obtained by analyzing spectra from pure AMPs, and $I^B_0$, $I^B_\Gamma$, $\lambda^B$ and $\Gamma^B$ were freely adjusted. LF11-only solutions were measured to calibrate their intensity dependence in buffer. Then, the retrieved $I^W_\Gamma$ values were converted to the concentration of dissociated peptides $[P]^W$. This allowed us to obtain the so-called peptide bound-fraction as $f_\Gamma = 1 - [P]^W/[P]$ (see Appendix 4). The aggregation of O-LF11-215 led to low $\lambda^W_\text{max}$ values (see Appendix 1) and precluded the a similar analysis for these peptides.

**Transmission electron microscopy**

*E. coli* suspensions at $5 \times 10^8$ CFU/ml were mixed with peptides at the corresponding MICs and 0.5xMICs in PBS, and incubated for 1 hour at 37 °C (incubator Thermomixer C, Eppendorf, Germany). Control samples (no AMPs) were suspended and incubated in PBS. Cell suspensions were centrifuged at 1300 $g$ for 4 minutes in 1.5 ml Eppendorf tubes and resuspended (fixed) in 3% vol/vol glutaraldehyde and 0.1 M cacodylate buffer to bring the rapid cessation of biological activity and to preserve the structure of the cell. After fixation the samples were washed and postfixed in 1% vol/vol OsO$_4$ in 0.1 M cacodylate buffer. Dehydration was carried out in an ascending ethanol series followed by two steps with propylene oxide (*Hayat, 1989*) and embedded in Agar Low Viscosity Resin (Agar Scientific, Stansted, UK). Ultrathin sections (70–80 nm) were prepared on an Ultramicrotome UC6 (Leica Microsystems, Vienna, Austria) equipped with a 35° Ultra Diamondknife (Di-atom, Nidau, Switzerland). The grids were poststained with Uranyless (Science Services, Munich, Germany) and lead citrate according to Reynolds (*Hayat, 1989*). Transmission electron microscopy images were acquired with Tecnai T12 at 120kV (TFS, Warmond, Netherlands).

**Small angle scattering**

(Ultra-) Small-angle X-ray scattering

USAXS/SAXS measurements were performed on the TRUSAXS beamline (ID02) at the European Synchrotron Research Facility (ESRF), Grenoble, France. The instrument uses a monochromatic beam ($\lambda = 0.0995$ nm) that is collimated in a pinhole configuration. Measurements were performed with sample-to-detector distances of 30.8 and 3.0 m, covering a $q$-range of 0.001–2.5 nm$^{-1}$ (*Narayanan et al., 2018*). Two-dimensional scattering patterns were acquired on a Rayonix MX170 detector, normalized to absolute scale and azimuthally averaged to obtain the corresponding one-dimensional USAXS/SAXS profiles. The normalized cumulative background from the buffer, sample cell and instrument were subtracted to obtain the final $I(q)$. Bacterial samples ($n_{\text{cell}} \sim 10^8$ CFU/ml) were incubated with peptides in buffer for one hour at 37 °C and directly measured in a quartz capillaries of 2 mm diameter (37 °C), mounted on a flow-through set-up in order to maximize the precision of the background subtraction. Time-resolved experiments were instead performed with a stopped-flow rapid mixing device (SFM-3/4 Biologic, Seyssinet-Pariset, France), with 50 ms mixing of bacterial and peptides stock suspensions (37 °C), and enabling data acquisition after a kinetic time of about 2.5 ms (*Narayanan et al., 2014*). A total of 50 frames was recorded for each experiment with an exposure time of 0.05 seconds and a logarithmic time-spacing ranging from 17.5 ms to about 10 minutes. Radiation damage tests were performed on reference systems prior to setting this X-ray exposure-times. The scattering intensities were further corrected for sedimentation and background scattering from the stopped-flow cell.
Contrast-variation small angle neutron scattering

SANS experiments were performed on the D11 instrument at the Institut Laue-Langevin (ILL), Grenoble, France, with a multiwire $^3$He detector of 256 × 256 pixels (3.75 × 3.75 mm$^2$). Four different set-ups (sample-to-detector distances of 2, 8, 20.5, and 39 m with corresponding collimations of 5.5, 8, 20.5 and 40.5 m), at a wavelength $\lambda = 0.56$ nm ($\Delta \lambda / \lambda = 9\%$), covered a $q$-range of 0.014–3 nm$^{-1}$.

Two distinct E. coli suspensions were incubated with peptides LF11-215 and LF11-324 in buffer for one hour at 37°C. The bacterial concentration during the incubation was 10$^6$ CFU/ml, and both peptide concentrations were in the range of the measured MICs. Samples were then washed and resuspended in five different PBS solutions, containing 10, 30, 40, 50, or 90 wt% D$_2$O. Samples ($n_{cell} \sim 10^{10}$ CFU/ml) were contained in quartz Hellma 120-QS banjo-shaped cuvettes of 2 mm pathway and measured at 37°C. Cuvettes were mounted on a rotating sample holder, which prevented the bacteria from sedimenting. Data were reduced with the Lamp program from ILL, performing flat field, solid angle, dead time and transmission correction. Further data were normalized by the incident neutron flux (via a monitor), and corrected by the contribution from an empty cell. Experimental set-up information and data are available at https://doi.ill.fr/10.5291/ILL-DATA.8-03-910.

Note that the present experimental time (~ 2 h) is much shorter than the onset of cell lysis (Zweytick et al., 2011). As control, SANS measurements were repeated at extended times after mixing with the AMP in order to test for sample stability (data not shown), in terms of shape, cell-wall structure and densities. The scattering intensities after 2, 4, 6 and 8 hours were comparable (with the exception of a weak decrease of $\rho_{CP}$ between 2 and 4 hours). Further a comparison with TEM and SAXS data suggests that the peptide-induced cell-damage does not progress any further after one hour.

Data analysis: peptide clusters

Reference O-LF11-215 samples were measured in the MIC range to investigate the microstructure of the peptide clusters. The SAXS pattern of O-LF11-215 was fitted with the equation

$$I_{clu}(q) = I_0^{\text{AMP}} e^{-qR_g^{2/3}} \left(1 + I_p q^{-f}\right),$$

where the term in brackets is related to the structure of the aggregates, and the exponential Guinier function accounts for the form factor of their subunits of radius of gyration $R_g$ (Zemb and Lindner, 2002), and $I_0^{\text{AMP}}$ is forward scattering intensity. The function $I_p q^{-f}$ is the Porod law that describes the high-$q$ asymptotic trend of scattering signal from the aggregates (Glatter et al., 1982), where $I_p$ is a scaling factor that depends on the surface properties of the aggregates, and $f$ is related to their fractal dimension (Sorensen, 2001) (see Appendix 1).

Data analysis: bacterial modeling

X-ray and neutron scattering data were jointly analyzed with a recently reported analytical scattering model (Semeraro et al., 2021b). USAXS/SAXS patterns of end-states displayed an excess scattering contribution between $q \sim 0.1–0.2$ nm$^{-1}$ in the case of LF11-215 and LF11-324, not visible in the corresponding SANS patterns. Note that SANS experiments were conducted on samples that were washed and resuspended in different D$_2$O-containing buffer, while SAXS data were acquired immediately after one hour incubation with peptides. Together with the observation of OMVs by TEM (Figure 1), this suggests that the additional scattering contribution in SAXS data could be due to freely diffusing OMVs in the suspension medium.

All scattering data were fitted with the analytical functions

$$I(q) = \begin{cases} I_{\text{cell}}(q) & \text{[Neutron data]} \\ I_{\text{cell}}(q) + n_{cell} I_{\text{OMV}}^{g0} \left[3j_1(qR_{\text{OMV}})\right]^2 & \text{[X-ray data]} \\ I_{\text{cell}}(q) + I_{\text{clu}}(q) & \text{[O-LF11-215 data]} \end{cases},$$

(6)
Table 2. List of fixed parameters for the combined analysis of USAXS/SAXS and contrast variation SANS data of *E. coli*.

<table>
<thead>
<tr>
<th>Description</th>
<th>Fixed parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center-to-center distance between the head-group layers in the CM</td>
<td>$D_{CM}$ (nm)</td>
<td>3.73</td>
</tr>
<tr>
<td>Center-to-center distance between the head-group layers in the OM</td>
<td>$D_{OM}$ (nm)</td>
<td>3.33</td>
</tr>
<tr>
<td>Width of the head-group layers for both CM and OM</td>
<td>$W_{ME}$ (nm)</td>
<td>0.75</td>
</tr>
<tr>
<td>Center-to-center distance between the PG layer and the OM</td>
<td>$\Delta_{PG}$ (nm)</td>
<td>16.7</td>
</tr>
<tr>
<td>Width of the PG layer</td>
<td>$W_{PG}$ (nm)</td>
<td>6.0</td>
</tr>
<tr>
<td>Average SLD of the tail group layer in the CM</td>
<td>$\rho_{T1}$ (nm$^{-2}$) x 10$^{-4}$</td>
<td>8.31 x 10$^{-4}$/ 0.022(N)</td>
</tr>
<tr>
<td>Average SLD of the tail group layer in the OM</td>
<td>$\rho_{T0}$ (nm$^{-2}$) x 10$^{-4}$</td>
<td>8.86 x 10$^{-4}$/ 0.012(N)</td>
</tr>
<tr>
<td>Ratio between major and minor radii</td>
<td>$\epsilon$</td>
<td>2.0</td>
</tr>
<tr>
<td>Effective radius of gyration of each OS core</td>
<td>$R_{EOS}$ (nm)</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Where $I_{scl}(q)$ is the scattering form factor for *E. coli*, as reported in Semeraro et al. (2021b), and 3$J_1(qR_{OMV})$ is the form factor of a sphere of radius $R_{OMV}$, with $J_1$ being the normalized spherical Bessel function of order 1. The prefactor $I_{OMV}^0 = N_{OMV}V_{OMV}^2\Delta \rho_{OMV}$ is the OMV forward scattering, where $N_{OMV}$, $V_{OMV}$ and $\Delta \rho_{OMV}$ are, respectively, their number, volume and SLD difference to the buffer. The choice of a simple spherical form factor was driven by its simplicity for checking whether bacteria and OMVs have to be considered as non-interacting scatterers or not. Tests using an interaction cross-term approximating budding OMVs by spheres decorating a larger surface (Larson-Smith et al., 2010), did not result in significant contributions, confirming the dominance of freely diffusing OMVs. Note also that the $I_{OMV}^0$ values were independent of the shape of the normalized form factor, as they include our estimation of $V_{OMV}$ and $\Delta \rho_{OMV}$ (Appendix 2). Finally, in the case of SANS data, instrumental smearing was taken into account. Data were fitted with a convolution of $I(q)$ and a Gaussian function with $q$-dependent width values, as provided by the reduction tools at D11. In USAXS/SAXS data, the smearing effect was negligible.

After thorough testing, the analysis of SAS data (end-states and kinetics) was conducted using only seven adjustable parameters describing $I_{scl}(q)$. These were the number of LPS molecules, $N_{OS}$; the cytoplasmic radius, $R$; the scattering length densities (SLDs) of the cytoplasmic, $\rho_{CP}$, and periplasmic space, $\rho_{PP}$; the periplasmic average thickness, $\Delta_{OM}$, and its deviation, $\sigma_{OM}$; and the SLD of the peptidoglycan layer, $\rho_{PG}$. Additionally $I_{OMV}^0$ and $R_{OMV}$ were fitted for scattering intensities in the presence of LF11-215 and LF11-324, while $I_{AMP}^0$ was used and adjusted in the case of O-LF11-215. Other parameters of $I_{scl}(q)$ were fixed according to the O-LF11-215 alone systems (see Table 1). This allowed us to fully describe the scattering-pattern variations upon peptide activity. Other parameters, including those accounting for the structure of inner and outer membranes, were fixed to the references values [see complete description in Semeraro et al. (2021b); all fixed parameters are listed in Table 2 and Table 3.

The scattering intensities of O-LF11-215-aggregates was comparable to that of bacteria in the high $q$-range (Figure 2–Figure Supplement 1D). While this affected the quality of the ultrastruc-
naturual parameters, it enabled at the same time the investigation of the kinetics of the AMP uptake. Indeed, by assuming that O-LF11-215 is primarily forming aggregates in solution, $I_{\text{AMP}}^{0}$ at $\Delta t = 17.5$ ms can be converted to the total known peptide concentration $[P]$. Hence, the further assumption that peptides leaving the clusters are directly partitioning into the cell allows to convert the difference $[I_{\text{AMP}}^{0}(\Delta t) - I_{\text{AMP}}^{0}(0)]$ to $[P]_{B}(\Delta t)$. It follows that

$$N_{\text{kin}}^{B}(\Delta t) = \frac{N_{A}[P]_{B}(\Delta t)}{n_{\text{cell}}},$$

where $N_{\text{kin}}^{B}$ is the number of O-LF11-215 partitioned within the volume of a single cell that can be obtained time-resolved USAXS/SAXS data.

Finally, time-resolved USAXS/SAXS data were fitted using the parameters of the initial [see Semeraro et al. (2021b)] and end-states as boundaries and guide to refine the $\chi^2$ minimization (see Figure 2–Figure Supplement 4). This was accomplished by means of a genetic selection algorithm exploiting >300 repetitions of converging fittings [see details in Semeraro et al. (2021b)]. Fit results were filtered using an ad hoc threshold of $\chi^2 < 1.15 \times \chi^2_{\text{min}}$, which enabled the exclusion of local minima. Filtered results were used to built up parameter distributions that in turn provided mean values, associated errors (standard deviation) and correlation coefficients. Exemplary distributions are shown in Figure 2–Figure Supplement 5 and Figure 2–Figure Supplement 6. Variations in $\Delta_{\text{OM}}$ and $\sigma_{\text{OM}}$ at $\Delta t = 17.5$ ms are due to lower signal-to-noise ratio available in time-resolved measurements.

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Competing interests
The authors declare that no competing interests exist.

References


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Clusters of acylated peptide O-LF11-215

Peptide clusters formed by O-LF11-215 were investigated by Trp fluorescence and USAXS/SAXS. Trp spectra displayed a $\lambda_{\text{max}} \approx 336$ nm and a FWHM of about 67 nm, which can be related to a heterogeneous distribution of Trp with apolar surroundings (Ladokhin et al., 2000). In addition, O-LF11-215 exhibited Trp phosphorescence emission at about 450 nm, which usually is not measurable due to its dynamic quenching by oxygen and impurities in aqueous suspensions (Cioni and Strambini, 2002). Its presence suggests that a significant portion of Trp residues are buried in hydrophobic cores, with no access to the solvent and with a local high viscosity.

Appendix 1 Figure 1. (A) Trp fluorescence data of LF11-215 (green squares) and O-LF11-215 (blue triangles) at 100 µg/ml (LF11-324 are not shown to avoid redundancy). Data were fitted with Equation 4. Arrows mark the maxima positions of the fluorescence and phosphorescence bands. (B) SAXS data of O-LF11-215 at 400 µg/ml. The fit was performed with Equation 5. Additional lines highlight the obtained intensity decay of slope $\sim 2.4$ (solid line), typical of mass fractals, as opposed to $\sim 2.0$ (dashed line), which would correspond to either Gaussian chains or planar structures. Note that the slope is conserved in a $q$-range larger that one order of magnitude.

USAXS/SAXS data in the low $q$-range ($q_{\text{min}} \sim 0.005$ nm$^{-1}$) exhibited a featureless decay of intensity with a slope of $f = 2.4$. This slope value is typical for mass fractals, i.e. highly branched objects with high surface-to-volume ratio, while $q_{\text{min}}$ suggests a minimum aggregate size of $\sim 2\pi/q_{\text{min}} > 1$ μ. Furthermore, a Guinier term is needed to fit the shoulder at about $q = 0.2$ nm$^{-1}$ corresponding to an average radius of gyration $R_g \approx 10$ nm. Note that this feature also does not vanish for different choices of scaling constants for background subtraction. Interestingly, this value is way too high to describe O-LF11-215 monomers, whose expected radius of gyration would be $< 1$ nm. Possibly, peptide monomers create smaller aggregates of mean size $R_g \approx 10$ nm, which in turn form an heterogeneous and branched supramolecular structure with the characteristics of a mass fractal.
Appendix 2

Estimating the scattering contribution of OMVs

The prefactor of the scattering contribution from extracellular, independent objects used in Equation 6 is $I_{OMV}^0 = N_{OMV}V_{OMV}^2\Delta \rho_{OMV}$, where $N_{OMV}$ is the number of OMVs, $V_{OMV}$ is the volume of an OMV and $\Delta \rho_{OMV}$ is average the SLD difference to the buffer. This definition of forward scattering (per single cell) is valid for every non-interacting object. Hence, to validate the assumption that this scattering contribution is related to OMVs, it is interesting to calculate possible $N_{OMV}$, $V_{OMV}$ and $\Delta \rho_{OMV}$ values. Note that even if modelling OMVs as homogeneous spheres appears as a crude first order approximation, $R_{OMV}$ can be associated to its radius (within ~10% confidence). Assuming the same lipid asymmetry as in the outer membrane, the inner leaflet of OMVs can be mimicked by a 3:1 mole mixture of palmitoyl-oleoyl-phosphatidylethanolamine (POPE) and palmitoyl-oleoyl-phosphatidylglycerol (POPG), respectively (De Siervo, 1969; Lohner et al., 2008; Leber et al., 2018). The SLD membrane profiles of these lipids have been thoroughly investigated (Kučerka et al., 2012, 2015). The outer leaflet might instead be dominated by LPS, whose lipid A possesses about 6 short C14:0 chains (Kim et al., 2016), and the polar region can be approximated as two PG units, in terms of molecular volume and SLD. In addition, LPS inner and outer core volumes and SLDs can be calculated from Heinrichs et al. (1998) and Müller-Loennies et al. (2003), neglecting O-antigen chains for simplicity. Gathering all this information, similarly to the membrane structure estimation in Semeraro et al. (2021b), the vesicles would have a membrane thickness of 4.1 nm and an average SLD of 1.1x10^{-3} nm^{-2} (volume-weighted averages). The lumen of OMVs can be quite diversely composed (Beveridge, 1999). We tentatively assigned the SLD of the periplasmic space of the end-state system, i.e. 9.68x10^{-4} nm^{-2}.

Together with a buffer SLD of 9.47x10^{-4} nm^{-2}, a measured $I_{OMV}^0 = 1500 \pm 200$ nm and a $R_{OMV} = 15.4 \pm 0.6$ nm, for instance, then leads to the estimate $N_{OMV} = 1200 \pm 400$ and a total lipid surface (inner and outer leaflets of all OMVs) of $(4 \pm 2) \times 10^6$ nm$.^2$.
Statistics of bacterial inhibition

Assuming that the AMP-induced delayed bacterial growth is entirely due to a lower number density of survived cells (Marx et al., 2021b), the inhibited fraction of cells, $\phi_{IG}$, as a function of peptide and cell concentrations was fitted with a heuristic approach. Specifically, we used the sigmoidal Gompertz function

$$F([P]; b, c) = \exp[-b \exp(-c[P])],$$

(8)

where $[P]$ is the total peptide molar concentration, and $b$ and $c$ are related, respectively, to the position and width of the sigmoidal. Physically this can be interpreted analogously to a transition between two states; here: alive and dead bacteria. The width of this transition is associated with its cooperativity, i.e. the sharper the transition the higher the cooperativity. This does, however, not imply any cooperative peptide-peptide or peptide-bacteria interaction. It is a mere measure of efficiency to inhibit bacterial growth that will be influenced by several stochastic processes, such as, e.g., cell to cell variations of the average number of partitioned peptides.

Mathematically, $F([P]; b, c)$ can be associated to a cumulative distribution function (CDF), that is, it measures the probability of finding a certain number (or fraction) of inhibited cells at a given peptide concentration. This allows to derive the probability density function (PDF) by calculating the derivative, $f([P]; b, c) = \partial F([P]; b, c)/\partial [P] = \partial \phi_{IG}/\partial [P]$, as well as the inverse CDF, $F^{-1}(\phi_{IG}; b, c)$, i.e., the $\phi_{IG}$-quantiles. This maps $\phi_{IG}$ values to the inhibitory peptide concentrations $IC_x$, where $x$ is the corresponding inhibited bacterial percentage; by definition, $IC_{99.9} \equiv MIC$. In addition, the set of $b$ and $c$ values as a function of $n_{cell}$ can be interpolated to obtain, for example, a continuous trend of $IC_x$ as a function of $\phi_{IG}$.

Note that, by definition, the derived PDFs express the probability of having a “killing” event at a given $[P]$.

**Appendix 3 Figure 1.** (A) Selected $\phi_{IG}$ data for LF11-324 and corresponding fits with the Gompertz function. (B) Corresponding PDFs.
The native fluorescence of the single Trp residue present in LF11 peptides was exploited to validate the partitioning investigation through the equi-activity analysis. For every system, emission signals from partitioned peptides exhibited a weak blue-shift, with \( \lambda_{\text{max}} \) values in the range 346–354 nm for LF11-215, and 340–350 nm for LF11-324. \( \Gamma_B \) values showed no significant variations, instead. Interestingly, these values are consistent with a scenario in which a significant amount of partitioned peptides are heterogeneously dispersed in a polar environment and in a configuration allowing full dynamics of the Trp residues (Burstein and Emelyanenko, 1996; Ladokhin et al., 2000). This is consistent with the \( N^\text{max}_B / N_B \) values calculated via \( \zeta \)-potential measurements, suggesting that a relevant portion of partitioned AMPs are still in monomeric state in the intracellular space.

Given that \( f_B = n_{\text{cell}} N_B / (N_A[P]) \), \( f_B \) values extracted via Trp fluorescence were compared with those obtained from the antimicrobial activity assays at \( [P] = \text{MIC} \) and \( \text{MIC} \times 0.5 \) (LF11-215 data not shown). These two independent methods gave comparable \( f_B \), confirming the validity of \( N_B \) values.
Figure 1—Figure supplement 1. MIC values as a function of $n_{\text{cell}}$ for LF11-215 (green squares), LF11-324 (red dots) and O-LF11-215 (blue triangles) and best fits using Equation 1 and Equation 2.
Figure 1–Figure supplement 2. (A) X-ray and neutron scattering data of bacterial systems after one hour incubation with LF11-324 (end-states) at the MIC (SANS) and 1.2×MIC (SAXS). Lines are the best fits using Equation 6. (B-D) Scattering length contrasts as a function of D$_2$O concentration (wt%) in the medium for the cytoplasm, Δ$_{CP}$, periplasm, Δ$_{PP}$, and peptidoglycan layer, Δ$_{PG}$. Gray symbols are the values reported in absence of peptides [data adapted from Semeraro et al. (2021b)]. Solid and dashed lines correspond to linear regressions. (E) Comparison between SAXS curves from bacterial end-states and a reference sample without peptides [data adapted from Semeraro et al. (2021b)] to match the bacterial concentration. The blue dashed line represents the additional $I_{OMV}$ term whereas the fits refer to Equation 6. (F) Comparison between SANS curves from bacterial end-states and a reference sample without peptides [data adapted from Semeraro et al. (2021b)] in the case of 90 D$_2$O wt%. Lines are the best fits with Equation 6. The inset shows the log-normal probability distribution function (PDF) of the inter-membrane distance with (dashed purple line) and without LF11-324 (solid black line). (E-F) The intensities are on absolute scale and normalized by the cell concentration. Data w/o AMPs in panels (B-D) have been adapted from Figure 5 from Semeraro et al. (2021b). Data w/o AMPs in panels (E-F) have been adapted from Figure 3 from Semeraro et al. (2021b).
Figure 1–Figure supplement 3. TEM images of *E. coli* ATCC (A) and end-states in the presence of LF11-215 (B,E), LF11-324 (C,F) and O-LF11-215 (D,G). All systems were probed at the MICs (B,C,D) and half the MICs (E,F,G).

Figure 1–Figure supplement 4. Graphical scheme of the adjustable parameters.
Figure 2—Figure supplement 1. Schematic of the stopped-flow rapid mixing set-up used for US-AXS/SAXS experiments and kinetic changes of USAXS/SAXS curves of bacterial samples upon mixing with LF11-324 at MIC×1.2 (A), LF11-215 at MIC×1.6 (B) and O-LF11-215 at MIC×1.7 (C). The arrows highlight the most significant variations of intensity, such as the decrease of forward scattering (A-B); the evolution of the low-q oscillation profile (A-C); the disappearance of the feature at \( q \approx 0.3 \) \( \text{nm}^{-1} \) (A-C); the decrease of \( I_{\text{cell}} \) (A-B) and of \( I_{\text{clus}} \) (C) at \( q \approx 0.1 \) \( \text{nm}^{-1} \). (D) Example of the combination of \( I_{\text{cell}} \) and \( I_{\text{clus}} \) in the case of applying O-LF11-215 (see Equation 6).
Figure 2—Figure supplement 2. (A-C) Kinetics of the adjustable parameters upon mixing with two concentrations of LF11-215. These are, the intermembrane distance (~ periplasm thickness) (A), its deviation from the average value (B), and the peptidoglycan SLD (C). (D-F) Kinetics of the adjustable parameters upon mixing with two concentrations of O-LF11-215. The parameters are the intermembrane distance (~ periplasm thickness) (D), its deviation (E), and peptidoglycan SLD (F). Thick gray bands mark the degree of confidence from bacterial systems w/o peptides [see Table 1 and Semeraro et al. (2021b)]. The vertical gray grid in (A-F) is an approximated, concentration-independent time range during which the cell-wall damage occurs.
Figure 2–Figure supplement 3. (A-C) Kinetics of the adjustable parameters upon mixing with two concentrations of LF11-215. These parameters are the LPS packing (A); the cytoplasm and periplasm SLDs (B); and the minor radius of the cell (C). Thick gray bands mark the degree of confidence from bacterial systems w/o peptides [see Table 1 and Semeraro et al. (2021b)], except for (C), where they refer to the average of the current cell radii at $\Delta t = 0.0175$ s.
Figure 2–Figure supplement 4. (A) Representative USAXS/SAXS curves of bacterial samples upon mixing with LF11-324 at \([\text{MIC}] \times 1.2\), highlighting intensity changes and comparison with errors. (B) Best fits of the curves shown in panel (A).
Figure 2–Figure supplement 5. Exemplary distributions obtained from our data analysis for adjustable parameters for LF11-324 system at [MIC]×1.2. Results refer to 0.02 s after mixing (light-blue), 20 s (purple) and 440 s (red). Solid Gaussian curves are guides for the eye and show the center of mass and standard deviation of each distribution, which we report as parameter value and corresponding uncertainty.
Figure 2–Figure supplement 6. Selected example of the list of correlation plots and associated correlation coefficients (defined in the range 0–1). The adjustable parameters refer to LF11-324 system at [MIC]×1.2, 20 s after mixing. Coefficients and blue dots display the results for $\chi^2/\chi^2_{\text{minimum}} < 1.15$, which is the chosen threshold that allows to discard local $\chi^2$-minima. Light-blue squares show the remaining $\chi^2$-landscape.
Figure 4–Figure supplement 1. (A-C) Inhibitory concentration (ICₜₐₜₒ) as a function of inhibited fractions φₜₐₜₒ [inverse CDF, F⁻¹(x; b,c)] for different peptide and cell concentrations. Low φₜₐₜₒ values for O-LF11-215 were not accessible due the high noise-to-signal ratio. Symbols mark the MICs for LF11-324 (circles), LF11-215 (squares) and O-LF11-215 (triangles), and black lines mark the range of ICₜₐₜₒ. The level of confidence is not displayed for sake of clarity. ICₜₐₜₒ values have an about 10% relative error.
Figure 5—Figure supplement 1. ζ-potential (A) and size (B) measurements as a function of peptide concentration (normalized by the respective MICs of LF11-215 and LF11-324 peptides). Lines are guides for the eye.
Figure 5—Figure supplement 2. Comparison between ζ-potential (A,C) and size (B,D) measurements of O-LF11-215 AMP alone and mixed with *E. coli* as a function of peptide concentration (normalized by the MIC of O-LF11-215).