CHAPTER X

Membrane disrupting peptides: mechanistic elucidation of antimicrobial activity

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Abstract

Membrane-disrupting peptides are promising candidates for the development of anti-infective and anticancer agents. Over the last decade high-resolution time-resolved measurements by in-liquid AFM have enabled direct observation of different modes of membrane disruption, increasing our understanding of peptide systems and informing future design. In this review, we outline the variety of mechanisms, as observed by in-liquid AFM, in the context of peptide sequence, structure and environment. We then discuss how nanoscale imaging can inform the development of synthetic peptide therapeutics, with a focus on supramolecular peptide assemblies.

X.1. Introduction

X.1.1 Membrane targeting peptides as novel antimicrobials

The spread of antibiotic resistance presents a growing threat to global health. Infections that do not respond to clinically available antibiotics continue to increase stimulating the need to develop alternative therapeutic strategies based on molecular classes that have not had an antibiotic before. Antimicrobial peptides (AMPs), a large family of short-to-medium size peptides found in nearly all forms of life as defence against pathogens, have long been proposed as next generation antibiotics.(1) Unlike conventional antibiotics, which target individual intracellular processes, AMPs engage with multiple targets in bacterial cells.(2) Most of these peptides are cationic molecules favouring attack on microbial membranes which tend to be anionic AMPs are primarily membrane disrupting agents, which allows them to disrupt not only bacterial cells, but also fungal, (3–5) neoplastic (6–8) and viral membranes. (9–14). Upon membrane binding, the peptides adopt amphipathic secondary structures, i.e. β-sheets or α-helices, which rapidly arrange into pores or channels causing cell lysis. The peptides do not differentiate between targets in cells they attack and can equally bind to intracellular components, e.g. DNA and ribosomes. To develop resistance against such agents is a formidable challenge for unicellular organisms.(15) Given that membrane binding is the first step in the activity of AMPs, some cells succeed in developing resistance strategies by fortifying their membranes or decreasing their anionic charge. Despite this, a widespread resistance towards AMPs has yet to emerge. Continuous therapeutic interest in these peptides is two-fold. Firstly, it is due to their lower susceptibility to pathogen resistance owing to the ability of the peptides to overcome surface modifications of pathogens. Secondly, it is their versatile activity against membranes – the Achille's heel of unicellular organisms; AMPs do not generally discriminate between susceptible, multi-resistant strains, persister cells or biofilm forming bacteria.(15)

As interest towards AMPs grows, more resistance mechanisms are being discovered. These include proteolytic degradation of the peptides, increased cell membrane rigidity or decreased surface charge, secretion of proteins or lipids that neutralise or complex the peptides reducing access to the membrane surface.(16,17) However,

pathogens tend to develop such resistant mechanisms against particular AMPs, they have exposure to. Other peptides, even structurally related, can successfully overcome these mechanisms, whilst a resistance mechanism universal against most AMPs has not been observed.(18) Therefore, AMPs remain an attractive class of molecules for the development of antibacterial, antifungal, antiviral and anticancer agents.

Broadly speaking, all peptides produced by host defence systems are AMPs. Historically peptide antibiotics of bacterial origin, such as polymyxins and gramicidins, were discovered much earlier than host defence peptides of multicellular organisms. All of these are polypeptide molecules that share similar membranolytic mechanisms of action and vary in their structural topologies and molecular arrangements. Colistin, which has been used for decades in both clinic and agriculture, and remains a last-resort antibiotic against multi-resistant gram-negative infections, is a great example of how difficult it is for bacteria to develop resistance against these evolutionary conserved antimicrobials. Indeed, the first bacteria resistant to colistin were identified within the last two years.

Despite this obvious advantage over intracellular antibiotics, AMPs have not yet reached use in clinic. Nowadays the number of AMPs at different stages of clinical trials is growing, with earlier attempts including pexiganan, an analogue of one of the first AMPs discovered, magainin 2, and iseganan, an analogue of a β-sheet protegrin family.(19)

There is a significant diversity amongst naturally occurring AMPs (Fig 1.1). These are typically ribosomal products of up to 50 amino acid residues in length, are of different amino acid compositions, which adopt a range of secondary structures, and exhibit varied charge and hydrophobicity properties.(20) De novo and enzymatically (e.g. non-ribosomally) synthesised peptides further diversity this class of molecules including sequences incorporating non-proteinogenic amino acids. Irrespective of their origin, in membranes antimicrobial peptides form amphipathic structures segregating hydrophobic and polar amino-acid residues into hydrophobic and polar faces, respectively.(2) Amphipathicity allows AMPs to bind to the anionic phosphate groups of the membrane surface and insert into the hydrophobic interface in the lipid bilayer.(21,22)

Structure-activity relationships in AMPs are improving and the established design principles allow to generate effective broad-spectrum antimicrobials exhibiting improved therapeutic indexes when compared to those of naturally occurring sequences. General physicochemical parameters such as increased cationic charge, increased hydrophobicity and perfect amphipathicity are known to increase antimicrobial potency.(23) For example, increasing net charge of the peptides can promote the binding of cationic AMPs to bacterial surfaces, which are rich in anionic lipids such as cardiolipin (CL) and phosphatidylglycerol (PG)(24). Following peptide accumulation on the surfaces, the hydrophobic faces of AMPs promote peptide insertion deep into the

bilayer.(2) However, increasing hydrophobicity often leads to increased toxicity and hemolytic activities typical of venomous and hemolytic peptides, which are structurally similar to AMPs. By fine tuning biophysical parameters one can improve therapeutic potential of a given peptide sequence. The screening of large libraries offers an advantage over the rational design in that it can correlate biological and physical properties of closely related sequences using unknown modifications or parameters.(15) This approach can produce promising candidates such as LTX-109, a synthetic peptidomimetic currently in phase 3 clinical trials. This compound is based on the pharmacophore sequence of lactoferrin, with additional aromatic modifications introduced to increase its hydrophobicity whilst retaining low toxicity.(25,26)

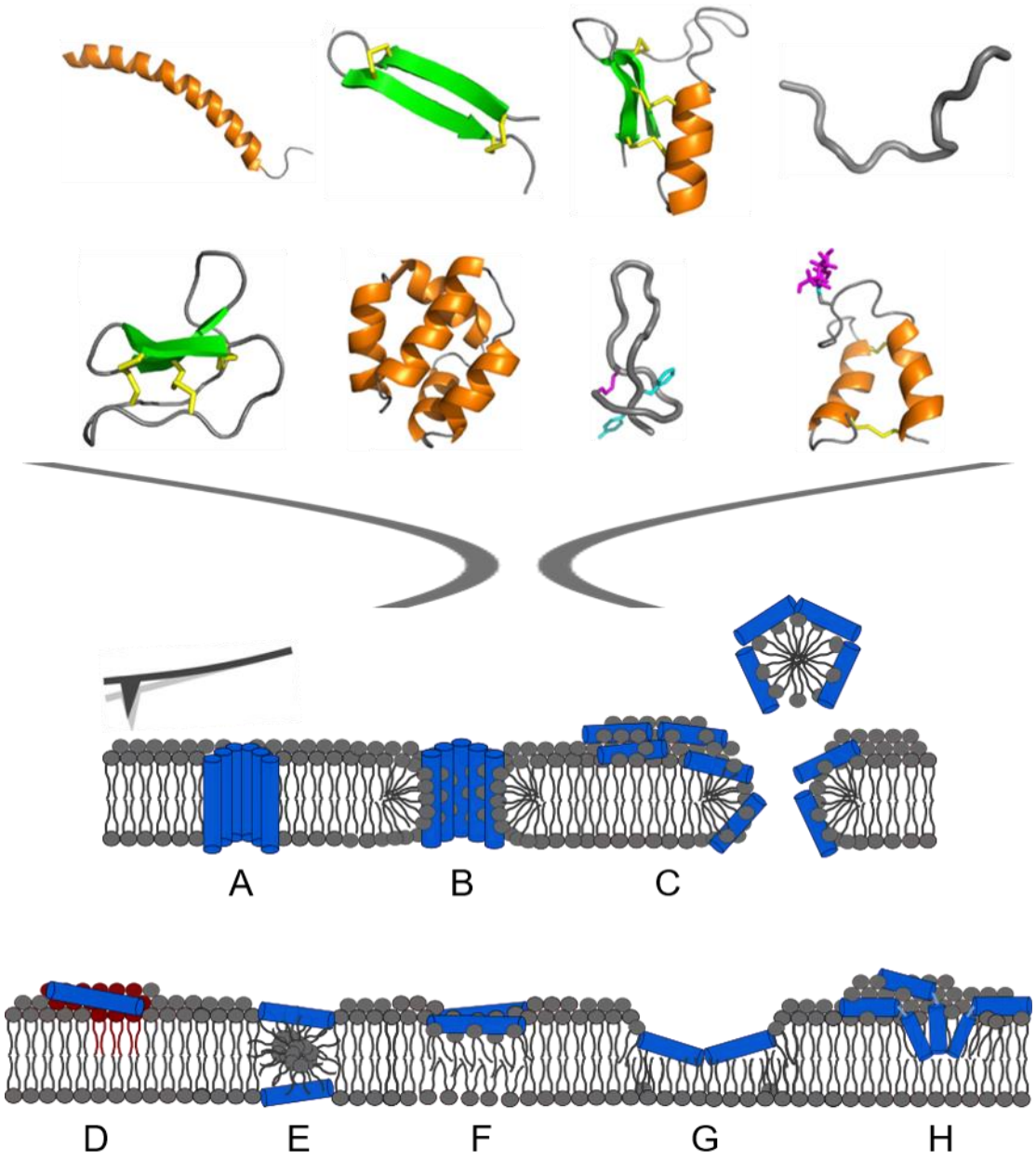

Figure 1.1. AMPs are a structurally diverse family of peptides and small proteins, which includes α-helical, β-sheet, α/β and extended structures as well as more complex topologies such as backbone cyclised peptides, head to side chain cyclised peptides and chemically modified peptides. Different sequences exert different

modes of membrane disruption resulting in (A) barrel-stave pores; (B) toroidal pores; (C) carpet-like disruption leading to micellization; (D) phospholipid clustering; (E) induction of non-lamellar phase; (F) bilayer thinning; (G) monolayer removal; and (H) nanoscale pits. Each mode of disruption can be resolved by AFM. Peptide structures are PDB entries (2K6O, 1KFP, 1ICA, 1G89, 1NB1, 1E68, 1Q71, 2MIJ) rendered by PyMOL: α-helices are shown in orange, β-sheets in green and disulphide bonds in yellow. Adapted from ref. (27) with permission from Elsevier.

X.1.2 Resolving membrane permeabilisation events

Membrane permeabilization by peptides has been studied experimentally using different measurement approaches. Fluorescent dye leakage assays are used to confirm the effect of permeabilised membranes.(28–30) NMR can provide solution 3D structures of peptides in lipid environments.(31) Circular dichroism (CD), linear dichroism (LD) and oriented circular dichroism (OCD) spectroscopy measures peptide conformations and orientation relative to the membrane surface.(32,33) Molecular dynamics (MD) simulations are used to complement experimental results and interpret structural data.(34–38) Data obtained using these techniques underpinned the proposed models of peptide-induced membrane disruption (Fig 1.1A-H).

Common models include barrel-stave poration, where peptides insert into the membrane bilayer and interact with lipid head and tail groups forming pores that do not require rotational rearrangement of the membrane lipids (Fig 1.1A).(39) Toroidal poration models feature peptides that remain bound to the lipid head groups and form pores by pulling the lipid head groups inward. Such pores are lined with both peptides and lipids (Fig 1.1B).(40,41) Non-porating models include the carpet model, in which surface-bound peptide monomers roughen and disrupt lipid packing and upon reaching a threshold concentration cause membrane collapse (Fig 1.1C).(42) Other models proposed are charge-based phospholipid clustering (Fig 1.1D), peptide-induced non-lamellar phases (Fig 1.1E) and localised thinning and thickening effects (Fig 1.1F,G). In all these models, boundary edges are created to compromise membrane integrity introducing discontinuities in the phospholipid matrix through which solutes may more easily pass through. Furthermore, lipid segregation into discrete domains restricts their lateral mobility reducing the ability of the bilayer to self-repair.(43)

No high-resolution structure of a peptide-stabilised pore has been obtained to date, and the nature of most peptide-membrane interactions remains largely unclear.(15) This is in direct contrast to many well characterised pore-forming proteins (PFPs) and pore forming toxins (PFTs).(44) These supramolecular protein assemblies form well-defined structures in lipid bilayers, compatible with high-resolution cryoEM and AFM imaging and the local arrangement of protein subunits, their oligomerisation states and the kinetics of the assembly process are all resolvable. (45–50)

The active state of peptide-induced disruption is more challenging to study. Peptides are smaller and more disordered, and their membrane interactions appear to more dynamic and transient when compare to those of well-defined protein counterparts. Current studies indicate that membrane permeabilization by peptide materials is a dynamic process which is highly dependent on local environments.(51) Defects consistent with different models may be able to form, grow, re-seal and inter-convert. Furthermore, defect formation is highly localised and occurs at the nanoscale. Characterising different peptide-membrane interactions requires high-resolution, time-resolved measurement able to capture the process.

Amongst other techniques, atomic force microscopy (AFM) performed in aqueous buffers, the so-called in-liquid AFM is ideal to measure the impact of AMPs on membranes in real time with a nanoscale resolution. The basic principle of AFM is that it relies on a nanometre sharp tip mounted on a flexible cantilever that is scanned across a sample surface. Distance-dependent interaction forces between the tip and the sample are used to detect the proximity of the surface and to build an image as the tip follows topography contours on the surface.(52) The interaction forces between the tip and the sample cause deflections in the cantilever. These deflections are monitored using a laser beam, reflected off the back of the cantilever onto a photodiode. AFM can achieve a sub-nanometre spatial resolution under near physiological conditions, and advances in high-speed AFM mean imaging can be conducted in real time of rapid molecular events.(53,54) Over the past decade, in-liquid AFM has successfully been used to directly observe different mechanisms of membrane disruption caused by peptides and proteins.

X.1.2.1. Experimental membrane disruption systems and measurements

To investigate the membrane disruption effects as a result of peptide treatment, several approaches can be taken. AFM is a surface technique and requires the sample to be immobilised on a solid substrate. Most mechanistic information to-date has come from model membrane systems such as supported lipid bilayers (SLBs) and supported lipid monolayers (SLMs). In SLBs a lipid bilayer is formed on a hydrophilic surface such as mica or silica. A hydration layer of around 1 nm exists between the lower leaflet lipids and the solid support,(55) which enables SLBs to retain fluidity and dynamics ($D \sim 2\ \mu m^2\ s^{-1}$).(56) These model systems provide a highly controlled environment and enable high-resolution and detailed studies of peptide lipid interactions. Factors such as phospholipid charge, fluidity, thickness and curvature can be independently tuned to assess their respective effects on peptide mode of action.

Extracts of native membranes can also be sued for AFM imaging. They are adsorbed onto mica substrates, such as rod outer segment (ROS) disc membranes and purple membranes.(57,58) These extracts provide the

original content of the native membranes, e.g. membrane proteins and native lipid mixtures, but may lose the native organisation of phospholipid bilayers and in cases lack the structural integrity of the original membranes. For both cases, extracts or reconstituted SLBs, proximity of the underlying solid support can influence lipid dynamics and interactions with peptides. Tethered, polymer-cushioned and floating bilayers are used to mitigate this drawback to introduce a water space between the bilayer and the substrate. (59–61)

Studies have also been conducted on whole bacterial cells. Many have been conducted using fixed samples, or as it is referred to – in air, (62–70). However, these measurements lack dynamic information gained, and may not be completely devoid of artefacts due to drying effects. In-liquid AFM studies with live cells enables direct visualisation of peptide-induced membrane damage under a more physiologically relevant conditions.(71–78) To date, studies have generally focussed on imaging changes across the whole cell, such as peptide induce surface roughening.(78) Improvements in immobilisation protocols and AFM instrumentation can help increase the resolution and reproducibility of live cell imaging(79,80) resulting in the increased number of such studies.

X.1.3. Monitoring peptide-induced membrane effects by direct visualisation

Different membrane disruption scenario induced by peptide treatment can be directly visualised by in-liquid AFM. In this section we discuss known and recently reported disruption modes and correlate these with peptide sequences, structures and environments. We also make an emphasis on how nanoscale imaging can help design AMP sequences with a particular membrane disruption mode, e.g. poration or carpet-like roughening, and describe tuning permeabilisation modes as well as most recent efforts to engineer higher order self-assembling systems based on antimicrobial sequences.

X.2. Imaging peptide action in membrane in real time at the nanoscale

X.2.1 Peptide sequences induce a wide range of membrane disruption events

A major finding from AFM studies is that peptide sequences induce different membrane disruption effects. Over the past decade, observation has been made of membrane topographies consistent with barrel stave poration models,(81,82) carpet-like disruption,(83) toroidal pores,(84) bilayer thinning,(85,86) transitions to non-lamellar phase,(85–87) and formation of localised domains.(83,88–91) Domain formation has been attributed to peptide-induced lipid clustering,(88) localised membrane thinning or thickening(83,89,90) and localised changes to lipid fluidity.(91) Different peptide sequences tend to interact with lipids in distinct ways eliciting disruption and permeabilisation. Even amongst sequences containing the same secondary structure and similar biophysical properties, divergent modes of disruption are induced.

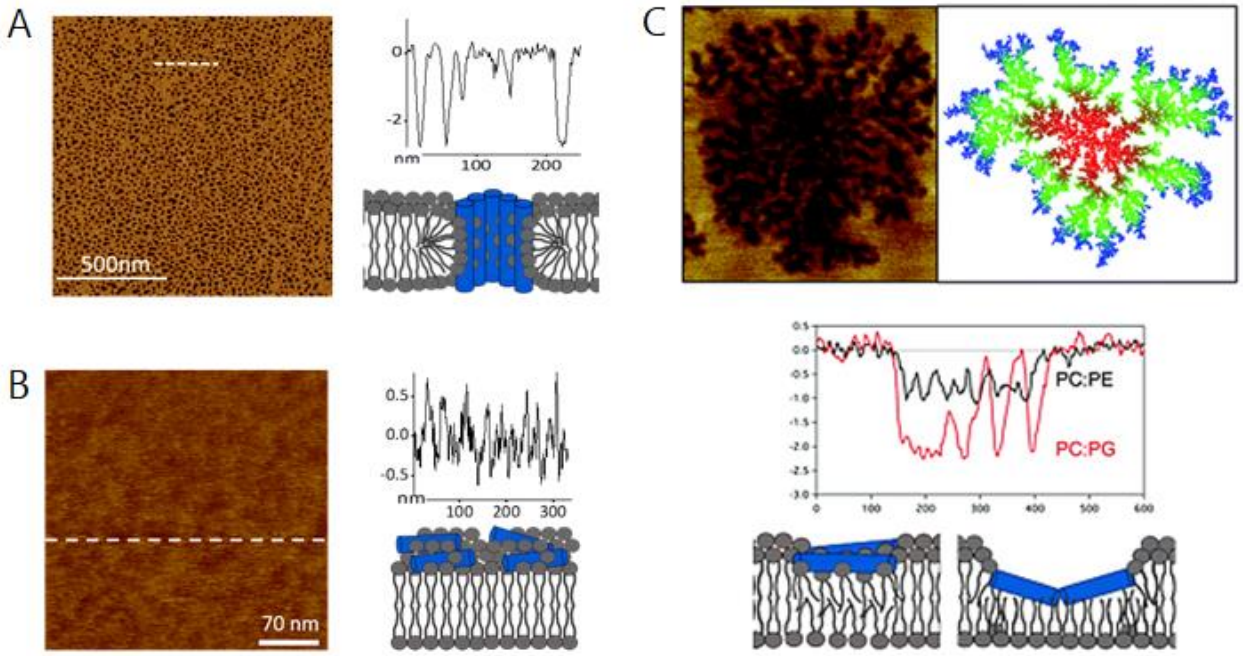

Figure 2.1. Modes of membrane disruption induced by linear amphipathic, cationic peptide α-helices. AFM topography images of SLBs treated with (A) magainin 2 induces transmembrane pores, 3 nm deep and around 20 nm wide, which is consistent with a toroidal pore model; (B) cecropin B inducing surface roughening, with topography variations of +/- 0.5 nm, consistent with the carpet-like disruption mode and (C) Smp-43 inducing fractal-like defects in the upper bilayer leaflet, with defect depths dependent on the lipid composition, consistent with thinning in PC:PE (1 nm deep) and monolayer removal in PC:PG (2 nm deep). Adapted from (84), with permission, © 2019 American Chemical Society; (83), licensed under CC BY 4.0 and (92), licensed under CC BY-NC 3.0, Published by The Royal Society of Chemistry.

Figure 2.1. emphasises the diversity of membrane disruption mechanisms presenting examples observed for peptides with conserved biophysical properties. All peptides used in these examples are linear, cationic, amphipathic α-helices containing only proteinogenic amino acids. Magainin 2 is a 23 amino acid sequence secreted by the African clawed frog *Xenopus laevis* and folds into an amphipathic α-helix with a net charge of +4. It was observed to form pores in SLBs consistent with the toroidal pore model (between 10 and 20 nm in diameter, Fig 2.1A).(84) Cecropin B is a 35 amino acid sequence secreted by the giant silk moth, *Hyalophora cecropia* and folds into an amphipathic α-helix with a net charge of +7. This peptide was found to act via a non-porating mechanism, by roughening the surface of SLBs, which is consistent with a carpet-like model (Fig 2.1B).(83) Smp-43 is a 43 amino acid sequence found in the venom of the Egyptian scorpion *Scorpio maurus palmatus*. This peptide folds into an amphipathic α-helix with a net charge of +4, and found to form defects in SLBs that are confined to the upper leaflet lipids and expand via fractal-like lateral channels (Fig 2.1C).(92) Depending on the lipid composition of the SLB, the depth of these defects is consistent with the domains of peptide induced bilayer thinning and monolayer removal.

Conversely, while sequences adopting similar conformations can exert very different membrane disruption effects, sequences with very different biophysical properties can induce similar effects on membranes. (P)GKY20, a peptide modelled on the Gly271 to Ile290 sequence of the human thrombin, folds into a linear amphipathic α-helix. This peptide induces charge-dependent phospholipid clustering in multi-compositional SLBs.(91) Colistin is an AMP secreted by the bacteria *Bacillus polymyxa*, with entirely different properties (Fig 2.2A). It consists of only 10 amino acids, 7 of which form a peptide ring structure (Fig 2.2A) and 3 of which form an exocyclic chain. A fatty acid tail is coupled to the end of the chain (Fig 2.2A, green), and the peptide contains non-proteinogenic amino acids. Despite that colistin has different sequence and structure when compared to a linear α-helix, it also is observed to act via phospholipid clustering.(88) Addition of colistin to multi-compositional POPC/LPS SLBs yielded LPS-colistin rich domains and LPS-colistin poor domains (Fig 2.2B).

Collectively these results prompt two conclusions. Firstly, membrane permeabilisation can occur through distinct disruption modes. Secondly, subtle and still poorly understood properties of the peptide sequence determine the mode of disruption that is induced.

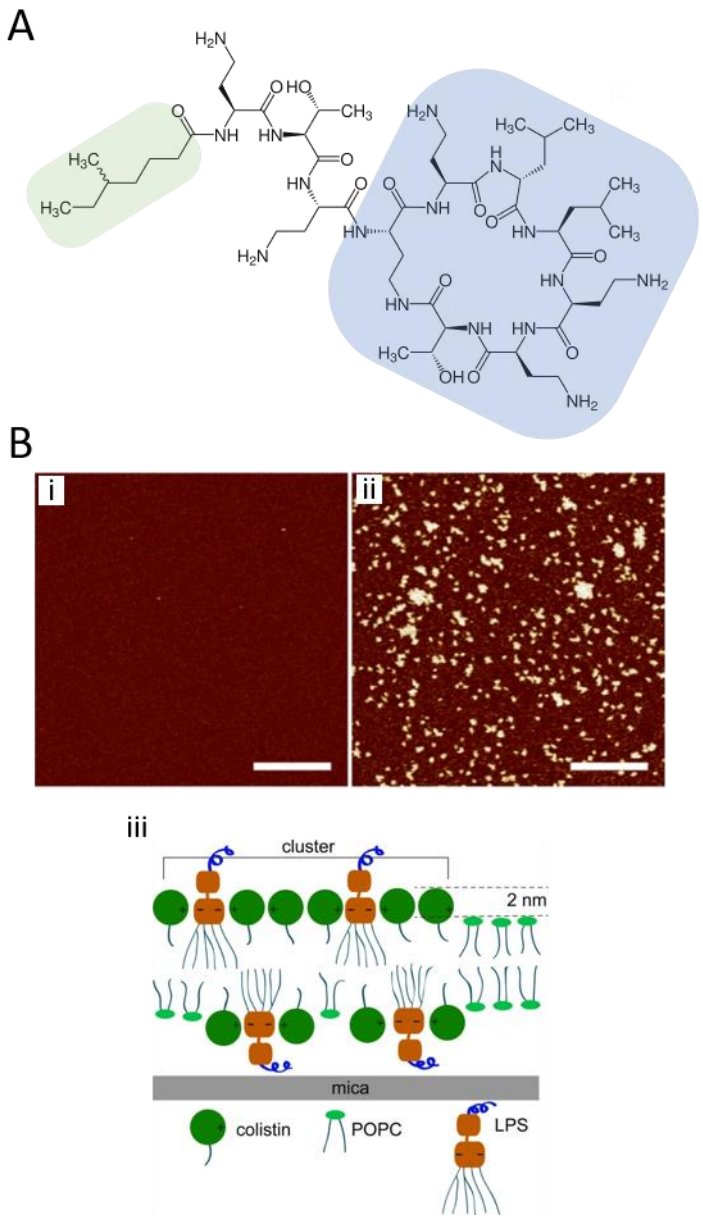

Figure 2.2. Colistin induces phospholipid clustering in mixed SLBs. (A) Chemical structure of colistin: a cyclic ring consisting of seven amino acid residues (blue), an exocyclic chain of three amino-acid residues, and a fatty acid tail (green). (B) AFM topography images of an SLB before (i) and after (ii) treatment with colistin. Note clusters, 2 nm in height, that appear across the membrane surface. A model schematic is shown in (iii). Scale bars are 1 µm. Reproduced from (88), licensed under ACS AuthorChoice, © 2018 American Chemical Society.

X.2.2. External factors that determine disruption modes

Peptide sequence and structure are major determinants of membrane disruption. However, other factors also impact on modes of disruption including the lipid composition of the membrane and a range of external stimuli such as pH, temperature and ionic strength. Membrane fluidity has a significant effect on peptide action as well, with peptide insertion being often more effective in more fluid lipid bilayers (93,94). Lower fluidity

bilayers, such as those containing cholesterol (liquid ordered L_o) or those prepared below the gel-fluid transition temperature (gel-state S_o) have higher lateral order and increased hydrophobic interactions between fatty acyl chains, resulting in an increased energy barrier for peptide insertion.

Higher ratios of anionic phospholipids generally facilitates in the increased activity of cationic peptides.(92,95,96). In most cases of cationic AMPs, membrane disruption activity is only observed when negatively charged phospholipids are present, with no activity observed for zwitterionic, net-neutral bilayers.(84) This is consistent with many studies that show that AMPs are charge selective.(21) It is only for hydrophobic AMPs, whose mode of action is driven entirely by the hydrophobic effect and hence their interactions with aliphatic lipid tails, that lipid charge does not have an appreciable effect on disruption ability and selectivity.(94,97) Peptide concentration also has a significant effect on activity, with higher peptide concentrations increasing the extent of formed defects.(88,96,98)

Whilst different factors can enhance or reduce peptide activity at the membrane surface, a more interesting observation is that different modes of membrane disruption can also be elicited. For example, magainin 2 has been observed to form stable pores in SLBs containing anionic phospholipids and solubilise zwitterionic SLBs in a detergent-like way. Similarly, differences in lipid fluidity can alter the mode of disruption. A synthetic sequence based on Aβ peptides was shown to induce very different effects in the gel-state and fluid-state SLBs.(99) Most surprisingly, the concentration of the peptide itself could alter its mechanism of action. As shown in Figure 2.3, protegrin-1 (PG-1), an 18 amino acid β-hairpin peptide causes boundary remodelling at low concentrations (Fig 2.3B), bulk poration at a higher concentration (Fig 2.3C) and induces a non-lamellar worm-like phase at higher concentrations (Fig 2.3D).(100) Together these results demonstrate that AMPs can adapt their conformations and modes of membrane disruption in direct response to different or changing conditions.

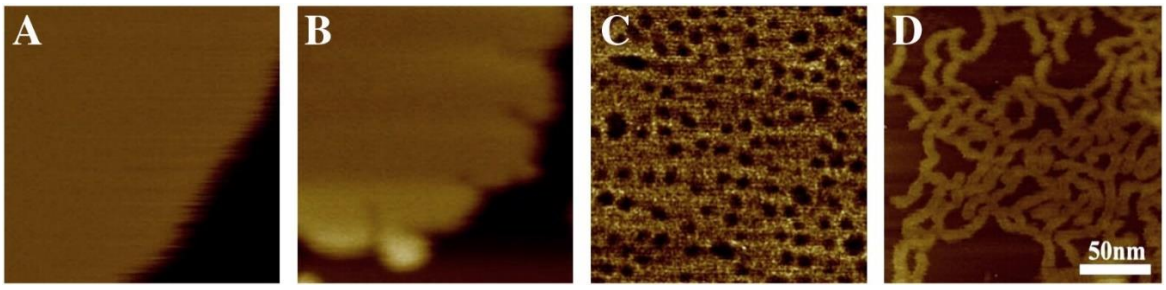

Figure 2.3. Protegrin-1 induces different modes of membrane disruption at different concentrations. AFM topography images of an SLB (A) before peptide addition and (B-C) with increasing peptide concentrations. (A) With no peptide, the SLB is defect free and line-tension maintains a smooth boundary edge; (B) at low concentrations the peptide acts at the SLB edges, elongating the boundary; (C) as concentrations increase

poration becomes to be apparent and (D) at high concentrations of PG-1 the bilayer is transformed into a structure with much-reduced thickness, consistent with worm-like micelles. Reproduced from (100), with permission from Elsevier.

X.2.3. Membrane disruption effects are dynamic and depend on peptide migration through the phospholipid membrane matrix

Peptide disruption mechanisms are highly dynamic. Rakowska et al were the first to show that peptide induced defects can grow over time.(101) A de-novo peptide sequence, termed amhelin, designed to fold into an amphipathic cationic α-helices long enough to span a lipid bilayer, induced transmembrane pores that expanded and merged. Consistent with subsequent studies, defect growth can proceed with pore formation that is not limited to a particular size, form or shaper, i.e. to say the mechanism of action is heterogenous.(94,101,102) It has been proposed that once formed, pores may act as a site of peptide recruitment, driving the migration of peptide from a surface bound state to the pore edge, and connect pores by forming lateral ridges or channels.

Defect growth can also proceed laterally without poration. The scorpion derived α-helical AMP Smp-43, demonstrates fractal-like migration through the peptide matrix (Figure 2.1C).(92) The morphology of these defects is consistent with a two-dimensional diffusion-limited aggregation (DLA) model. In this model diffusing particles that meet aggregates are immediately trapped at the aggregate edge, preventing the particle from diffusing to the aggregate centre which results in fractal-like expansion. Similarly, peptide monomers diffusing across the membrane surface may have a higher probability of sticking to a defect edge and removing lipid than diffusing to the defect centre, leading to fractal-like growth.

Equally, forming defects can get smaller and can even disappear. A synthetic α-helical sequence pHD108, based on the AMP melittin secreted from bee venom, forms metastable pores that fluctuate over time.(103) Sequential line scans with a temporal resolution of 0.3 s were taken of the surface to show pore-like features that appear, disappear and reappear. These observations support previous proposals that peptide induced membrane defects can be irreversible, and that the membrane can undergo recovery and self-repair by pore sealing.(104)

X.2.4. Multi-modal disruption modes by multi-helical peptide folds

Single peptide sequences tend to proceed via a single mode of membrane disruption. The mode itself can change depending on environmental conditions and can develop over time, but single sequences do not generally elicit multiple mechanisms of membrane disruption simultaneously.

This is not the case for more complex peptide folds with multiple domains. Bacteriocins present an exemplar class of antimicrobial folds, which tend to cause more elaborate disruption patterns. A recent study showed that a bacteriocin epidermicin NI01, which folds into a four-helix bundle, induces localised membrane thinning and transmembrane channels in SLBs, and that the two effects network with each other (Fig 2.4A).(105) Each disruption mode can be elicited separately by different subdomains of the peptide, two-helix hairpins: e.g. α1/α2 forms transmembrane channels (Fig 2.4B(i)), whereas α2/α3 induces membrane thinning (Fig 2.4B(ii)) and α3/α4 causes both modes of disruption that appear independent of one another, that is not networked (Fig 2.4B(iii)). Only the full-length form of epidermicin combines the different disruption modes into one synergistic mechanism. Similar observations were made for aureocin A53, an α-helical bundle from the same bacteriocin family.(105) Addition of A53 to SLBs led to an elaborate, flower-like pattern of membrane thinning and transmembrane defects, supporting the conjecture that multi-helical sequences support multi-modal disruption. Inducing more than one type of membrane disruption may give sequence increased plasticity and render it more able to adapt to different membrane environments. Epidermicin NI01 shows potent activity against a broad range of pathogens including superbugs such as MRSA and VRE.(106)

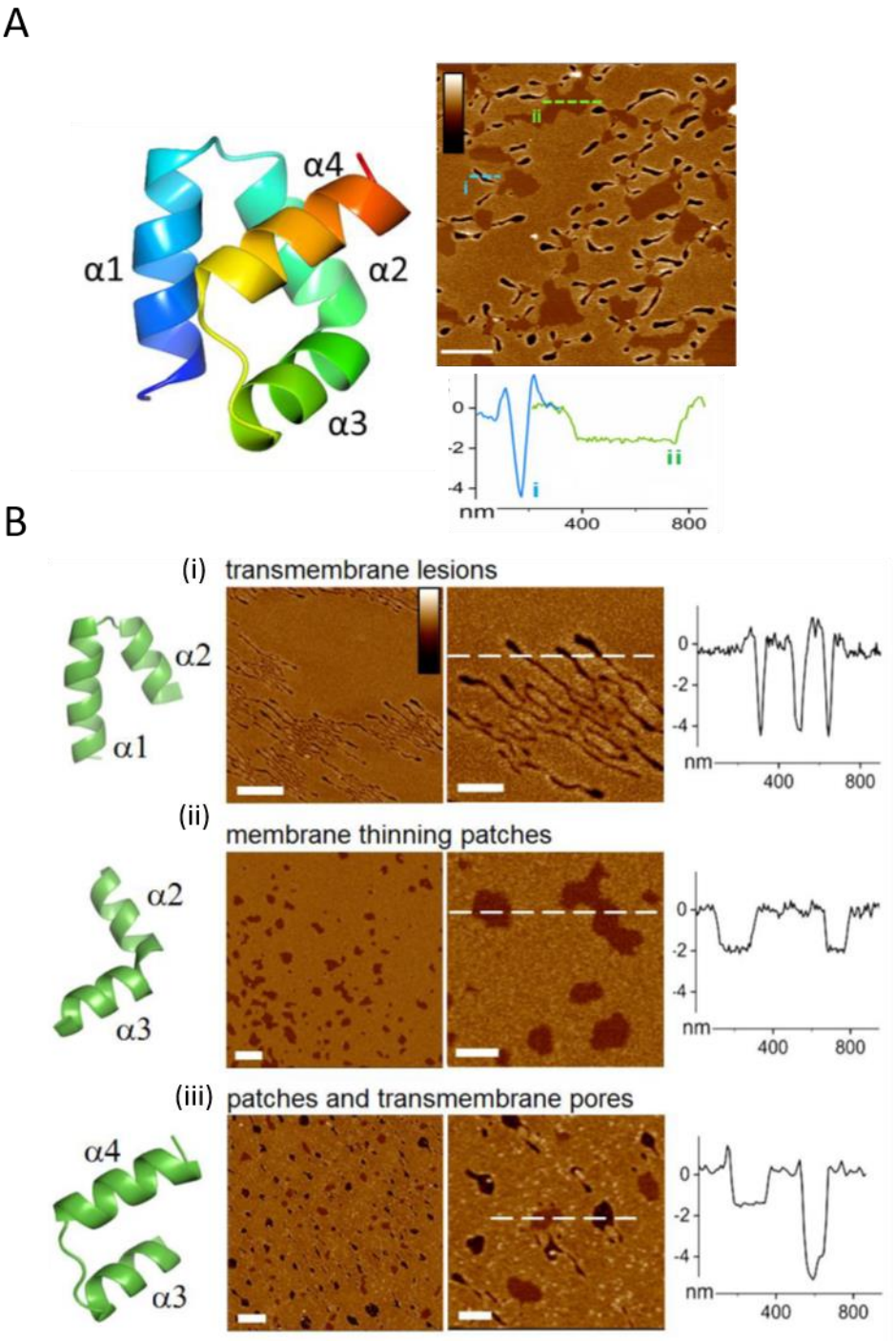

Figure 2.4. Multi-modal membrane disruption induced by a multi-helix bacteriocin, epidermicin NI01. (A) The antimicrobial protein folds as a four-helix bundle and induces membrane thinning, transmembrane circular pores and transmembrane petal-like channels. All these disruption modes network into an elaborate flower-like pattern (B) AFM analysis of constituent parts of the full sequence demonstrate that each disruption modes can be separately induced. (i) transmembrane lesions by α1/α2, (ii) thinned patches by α2/α3 and (iii) dual but non-networking disruption by α3/α4. Scale bars are 500 nm for (A) and 500 nm and 200 nm for left and right images in (B), respectively. Colour bars are 15 nm. Adapted from (105), under CC BY 4.0 licence.

X.2.5. The role of amphipathicity in membrane disruption mechanisms

Imperfect amphipathicity can impart a common mode of action to otherwise diverse peptide sequences and structures. A study by Henderson et al examined the effect of 13 different AMPs of differing charge, sequence and secondary structures.(85) 12 of these peptides fold into imperfect amphipathic structures (either through poor segregation of amino acids, or through breaks in the symmetry of secondary structural elements), whereas the final peptide, alamethicin, shows perfect amphipathicity. Henderson et al demonstrated by using AFM that alamethicin was the only sequence that was not able to lower the line tension of a lipid bilayer. When lipid bilayers are prepared as patches rather than continuous bilayers, the patches spontaneously form compact, round areas, minimising the length of the patch boundaries due to the line tension that arises from the energy cost of re-arranging lipids at the boundary edges. All 12 imperfect amphipathic sequences caused significant elongation of the patch boundary. In contrast, alamethicin showed no line active behaviour.

The authors proposed that imperfect amphipathicity is a prerequisite for peptides to adopt to the curvature of membrane edges if they are to stabilise and propagate the boundary. This has been proposed earlier(107) and is consistent with other AFM studies of imperfect amphipathic sequences inducing the formation of patches in SLBs(86,108) and with studies on phase separated L_o/L_d bilayers (see, e.g. Fig 2.5).(94,109) In phase-separated SLBs there is a hydrophobic mismatch between L_o and L_d phases, while domains form in compact round patches to minimise the boundary length between the two phases (Fig 2.5A). Line-active AMPs cause domains to become extended and heterogenous in shape (Fig 2.5B).

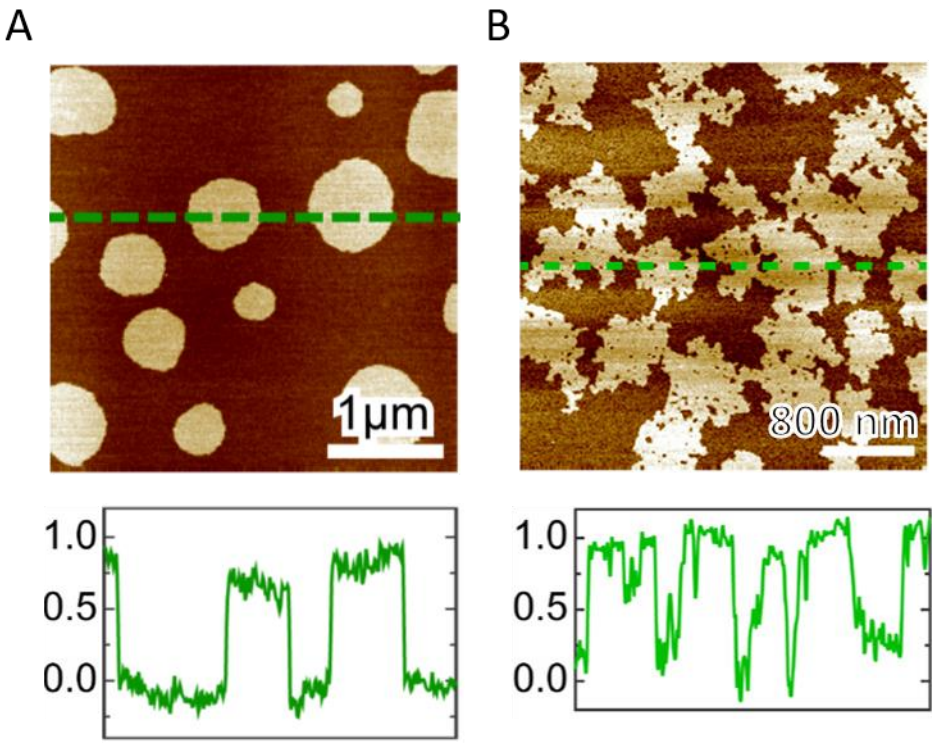

Figure 2.5. Melittin-induced remodelling of lipid boundary edge. (A) AFM topography image of a phase separated L_o/L_d SLB, where domains are formed in compact round patches to minimise boundary length. Depth profile shown across the dotted line shows 1 nm height difference between L_o (brighter) and L_d (darker) domains. (B) Topography image after addition of melittin. Besides causing poration, the peptide induces heterogenous L_o domains with extended boundary edges. Adapted from (94) with permission, © 2016 American Chemical Society.

X.3. Encoding membrane disruption modes by tuning peptide sequences, topologies and assemblies

X.3.1. Sequence mutations

The precise mechanism of antimicrobial action depends on multiple factors including peptide secondary structure, preferential orientation in the bilayer, peptide-peptide oligomerisation, and peptide-lipid and peptide-peptide electrostatic and hydrophobic interactions. The contributions of all factors to different extents produce a favoured mode of disruption. However, this remains challenging to fully predict. Nevertheless, sequence mutations known to enhance or reduce certain interactions can be used in attempt to promote certain modes of membrane disruption. AFM can then be used to reveal whether the intended effect has taken place.

For example, arginyl residues exhibit stronger electrostatic interactions with phospholipid head groups than lysyl residues. Unlike lysine, arginine also remains fully protonated in membranes and can engage with more phosphates and water molecules, resulting in tighter binding to phospholipids upon membrane binding, that is in the upper leaflet of the phospholipid bilayer.(110,111) Recently, it has been shown that exchanging all lysines in a sequence for arginines can switch different modes of membrane disruption including transmembrane poration to membrane thinning effects.(105)

A similar situation can be demonstrated in relation to AGPA motifs. These “hinge moieties” proteins use to introduce kinks in alpha helices.(112) Such a motif can be found in α-helical AMPs as well, e.g. in cecropin B (CecB). The C terminal region of CecB, separated by the AGPA motif, has a net neutral charge, a single cationic residue, minimal hydrophobicity with an abundance of alanines, and consequently has a low ability to interact with membranes. In contrast, the N-terminal region is highly cationic and hydrophobic and pre-disposed to membrane insertion and poration. The full peptide is observed to remain surface bound and cause membrane roughening (Fig 2.1B).(83) Removing the C terminal region removes the barrier to membrane insertion and results in a sequence that inserts into the bilayer in a transmembrane orientation (validated by simulations), and can form transmembrane channels in SLBs (validated by AFM).(83)

In the same study, Pfeil et al demonstrated the effect of promoting helix-helix interactions.(83) Glycine zipper motifs, $G(X)_nG$ where X is any residue and n = 3 to 6, act to weaken α-helicity, and AMP sequences use this motif to tune membrane-responsive folding and reduce helix oligomerisation.(113,114) CecB contains three glycine zippers. As gauged by CD spectroscopy, the peptide exists as a monomeric helix. By removing the first and the third glycine zipper, and further promoting helix-helix interactions by converting i, i+7 spacings of similar residues to i, i +3 and i, i +4,(115) the sequence converts to a coiled-coil. This set up of peptide-peptide interactions also alters the mode of disruption. The surface roughening observed for the native peptide is converted to monolayer poration. The AGPA motif and C terminal domain continue to prevent full insertion, but new helix-helix interactions favour the oligomerisation of peptides into pores.

An alternative approach in tuning the mode of disruption is to use native AMP sequences as templates to produce combinatorial libraries of sequences and identify analogue sequences with more desirable properties (e.g. membrane lysis at lower peptide concentration) using high-throughput screening.(116) Change in mode of action can then be monitored by AFM. An example of this approach can be provided for melittin analogues (Fig 3.1).(117) The first-generation sequence, MelP5, was found to elicit non-localised regions of thinned membrane (~ 0.3 nm deep) and nanoscale pits (~ 1 nm deep), which was in contrast to the second-generation sequence, macrolittin70, which exhibited multifaceted disruption with variable defect depths (Fig 3.1). Intriguingly, both improved analogues exhibited multi-modal membrane disruption, with increases in antimicrobial potency. As discussed earlier, multimodal disruption is uncommon for single helix AMPs. These results indicate that multimodal disruption, albeit heterogenous and variable, may be made favourable and have biological implications.

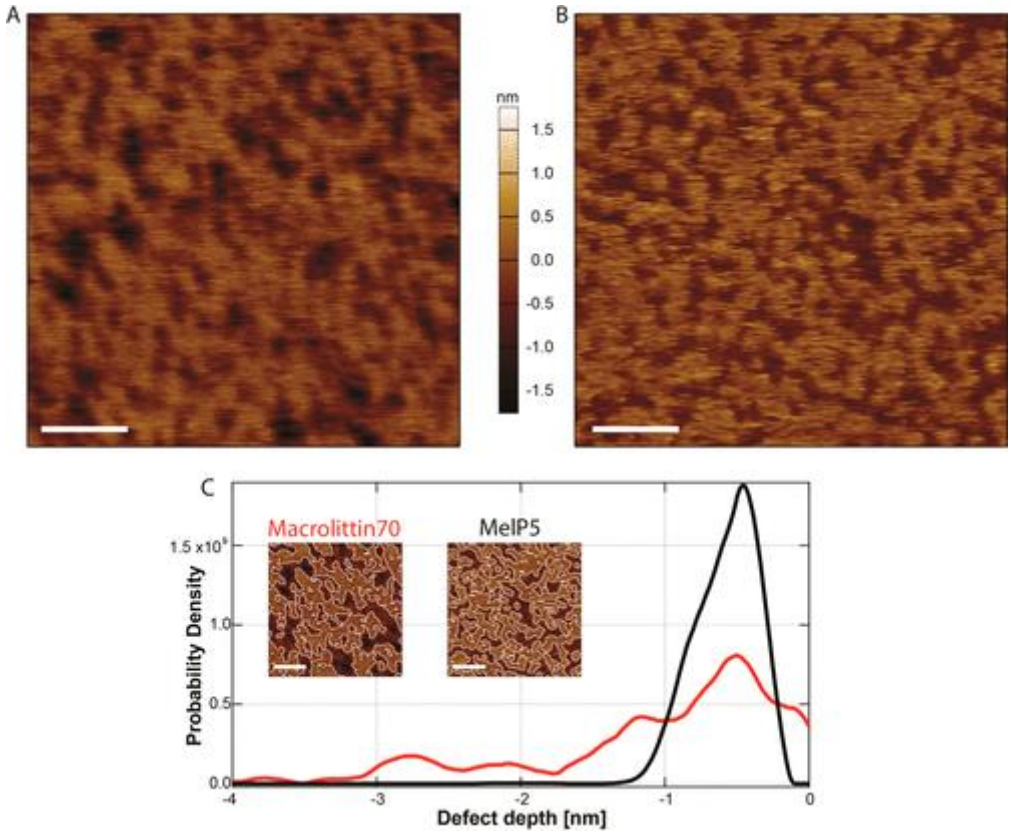

Figure 3.1. Comparison between two sequences derived from high-throughput screening of melittin-like peptides. AFM topography images of SLBs treated with (A) the second-generation sequence, macrolittin70 and (B) the first-generation sequence, MelP5. Scale bars 50 nm. (C) The defect depth distribution for each sequence, with macrolittin70 showing greater variability in membrane disruption patterns. Reproduced from (117), with permission, © 2018 American Chemical Society.

X.3.2. Driving peptide assembly into higher-order nanoscale systems

Peptide sequences can be designed to self-assemble into supramolecular structures such as matrices, hydrogels, nanofibers and nanoparticles. Such higher order peptide systems are increasingly being used to modify and improve the biological activity of AMPs.

In particular, recent studies on AMP based nanoparticles show promising therapeutic potential.(118–121) Liu et al demonstrated that conjugating chol to a cationic AMP combined with a TAT domain led to a lipopeptide template that assembled into micelle nanoparticles. The resulting assembly has potent activity against drug-resistant bacteria, yeast and fungi,(118) as well as in-vivo activity against *C. neoformans*, which induced meningitis in rabbits. The activity was not associated with toxic side effects.(119) Kepiro et al showed that a designed peptide sequence based on the active component of the human AMP lactoferrin but re-engineered to promote the formation of β-sheet-based capsid-like structure, is active against all cell in bacterial populations including susceptible, persister and resistant cells.(121) In this study high-resolution AFM imaging was used

to show how individual capsids bind to the membrane surface and rapidly convert into pores and lesions (Fig 3.2A).(120,121) In contrast, the non-assembled peptide monomer showed no antibacterial activity. The study shows that self-assembly pre-concentrates peptides into a high antimicrobial dose enabling fast and highly localised membrane disruption effects.

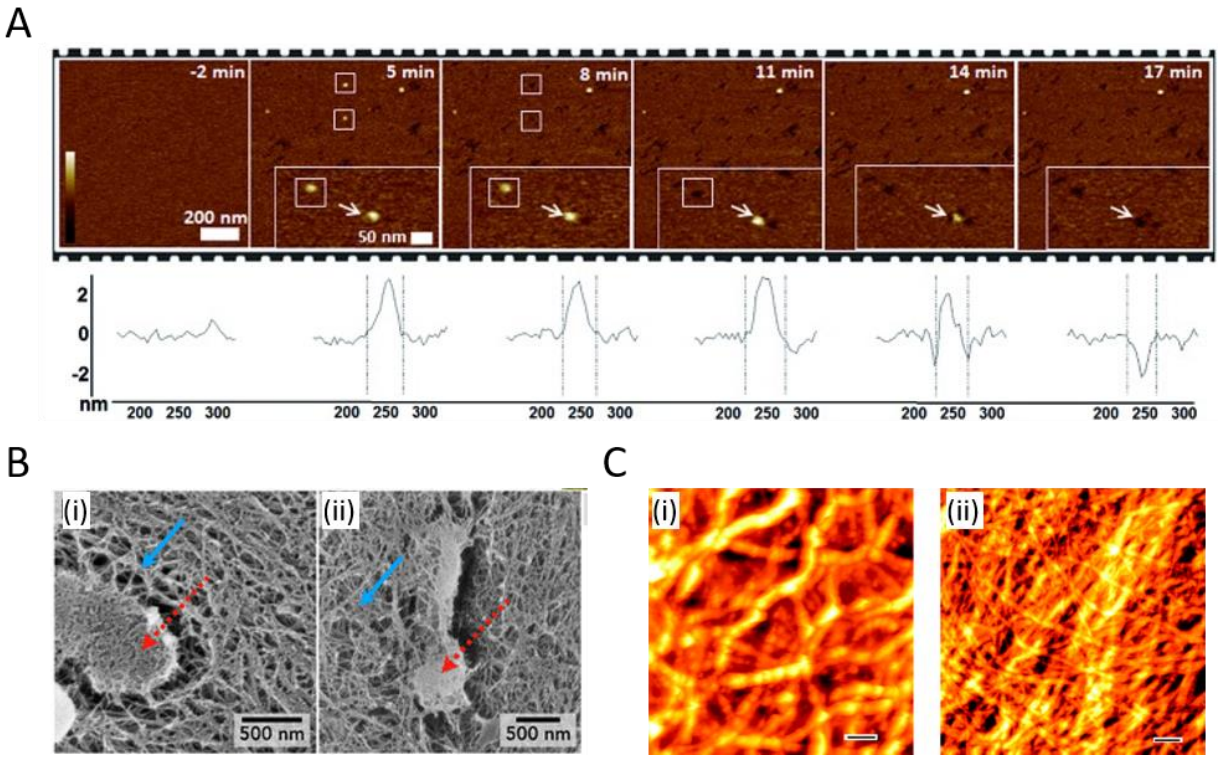

Figure 3.2. Peptide assembly into higher-order peptide materials. (A) AFM topography images of an SLB during treatment with a self-assembling peptide capsid, which can be observed binding to the SLB surface and converting into pores (highlighted by the white arrow and box). Line profiles for the capsule highlighted by the white arrow are given. Colour scale 6 nm. (B) SEM images of a colonies of *Pseudomonas aeruginosa* colonies treated with CASP-K6, a designed self-assembling peptide hydrogel. The fibrillar matrix (blue arrow) coats the bacterial cell (red arrow). (C) AFM topography images of peptide-based hydrogels with tuneable properties. Template (i) can be converted into template (ii) with addition of a single phenylalanyl residue. Scale bars 500 nm. Reproduced from (120), licensed under CC BY-NC 3.0, Published by The Royal Society of Chemistry; (122), with permission, © 2019 American Chemical Society and (123), with permission, © 2010 American Chemical Society.

Peptide hydrogels is another emerging strategy to elicit more potent bactericidal activity to the point of biofilm formation.(122,123) For instance, Sarkar et al designed a self-assembling antimicrobial hydrogel using only three amino acids: a central amphiphilic domain of alternating hydrophobic (L) and hydrophilic (S) residues flanked by terminal cationic lysine residues (K).(122) Hydrophobic interactions between central amphiphilic domains drive nanofiber formation, whilst counter-ion bridging between cationic domains gives rise to cross-

linking and entanglement of individual fibres into a gel. Agar diffusion assays confirmed potent antibacterial activity, and SEM and AFM imaging showed that the hydrogel coats bacterial cells causing cell lysis (Fig 3.2B). In a similar example, Debnath et al designed a self-assembling peptide based hydrogel with bactericidal activity.(123) The construct consisted of a single amino acid or dipeptide functionalised with an N-terminal Fmoc to drive self-assembly through π-π interactions, and a C-terminal pyridinium group to improve potency. The thickness of the resulting fibres can be controlled by changing the identity of the amino acids, demonstrating that peptide-based bactericidal hydrogels can be designed to have tuneable properties (Fig 3.2C).

The therapeutic index of an AMP can be directly modulated by self-assembly. Chen et al combined the toxic AMP melittin with a β-sheet forming peptide to produce peptide nanofibers decorated with melittin.(124) Whereas the melittin monomer cannot distinguish between bacterial and mammalian cells, the higher-order structure showed no cytotoxicity whilst retaining its antimicrobial activity. This demonstrates that by restricting the conformations and interactions of the peptide, its membrane selectivity can be adjusted.

Self-assembled peptide systems have the therapeutic advantage of reduced susceptibility to proteolytic degradation and slower diffusion away from the site of injection when compared to peptide monomers. Combined with their increased local concentration, AMP supramolecular structures have potential to be used as injectable biomaterials. A related approach that is being explored is the design of peptide sequences that assemble into higher order structures only when they reach the membrane surface. The assembly occurs in situ. This distinction helps increase local peptide concentration and subsequently potency, without the delivery issues that can be associated with large pre-assembled systems. For example, FF8, a de-novo eight amino-acid sequence containing a central, hydrophobic Phe-Phe that drives self-assembly, and terminal Lys and Arg residues that promote antimicrobial activity, is a monomer in solution but assembles into nanofibers on negatively charged membranes.(125) No assembly could be observed on zwitterionic membranes. Fibre formation correlated with membrane permeabilisation, with AFM showing poration and defect formation was only elicited on anionic SLBs. A control sequence unable to assemble confirmed the necessity of fibre formation for activity, showing no biological activity and causing no observable disruption of SLBs.

Higher-order systems based on antimicrobial sequences can also be designed as coatings for surfaces such as medical devices to prevent biofilm formation or as substrates that promote selective growth of mammalian cell lines.(126,127) Overall, it is clear that antimicrobial assemblies have potential for development into a range of novel antimicrobial materials.

X.4. Conclusions

Antimicrobial or host defence peptides are promising candidates for therapeutic developments. The design of more effective or selective molecules can be guided by specific modes of membrane disruption correlating with biological activities. In this regard, high-resolution measurements such as those afforded by in-liquid AFM become important to monitor dependencies between peptide structure and membrane disruption in real time. Indeed, over the last decade AFM imaging has revealed how different sequences could elicit a wide variety of membrane disruption mechanisms. Our understanding of specific relationships between peptide sequence and mode of membrane disruption is not complete but improving. The main challenge remains in that seemingly similar sequences can induce very different modes whilst clearly disparate sequences can induce the same modes. A few generic trends have been identified through detailed comparative studies, such as the common role of imperfect amphipathicity in AMP mechanisms. AFM studies have helped to understand that peptides can adopt different modes in response to the changes of external stimuli. This may reflect on that the sequences have an intrinsic ability to circumvent bacterial resistance strategies by activating more effective disruption modes when challenged. Nanoscale imaging will certainly continue to be used to inform better peptide designs with a deliberate choice of specific and more profound antimicrobial mechanisms. Disruption modes tuned using rational peptide design can also be used as research tools to better understand the physics of membrane disruption phenomena, while the assembly of AMPs into higher order structures underpins a fundamentally novel approach to antimicrobial treatments less subject to the counter effects of antimicrobial resistance.

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References*

1. Mookherjee, N., Anderson, M. A., Haagsman, H. P. & Davidson, D. J. Antimicrobial host defence peptides: functions and clinical potential. *Nat. Rev. Drug Discov.* **19,** 311–332 (2020).
2. Nguyen, L. T., Haney, E. F. & Vogel, H. J. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol.* **29,** 464–472 (2011).
3. Vriens, K., Cammue, B. P. A. & Thevissen, K. Antifungal Plant Defensins: Mechanisms of Action and Production. *Molecules* **19,** 12280 (2014).
4. Delattin, N., Brucker, K., Cremer, K., Cammue, B. & Thevissen, K. Antimicrobial Peptides as a Strategy to Combat Fungal Biofilms. *Curr. Top. Med. Chem.* **17,** 604–612 (2016).

5. Ordonez, S. R., Amarullah, I. H., Wubbolts, R. W., Veldhuizen, E. J. A. & Haagsman, H. P. Fungicidal Mechanisms of Cathelicidins LL-37 and CATH-2 Revealed by Live-Cell Imaging. *Antimicrob. Agents Chemother.* **58,** 2240–2248 (2014).
6. Deslouches, B. & Di, Y. P. Antimicrobial peptides with selective antitumor mechanisms: prospect for anticancer applications. *Oncotarget* **8,** 46635–46651 (2017).
7. Felício, M. R., Silva, O. N., Gonçalves, S., Santos, N. C. & Franco, O. L. Peptides with Dual Antimicrobial and Anticancer Activities. *Front. Chem.* **5,** 5 (2017).
8. Roudi, R., Syn, N. L. & Roudbary, M. Antimicrobial Peptides As Biologic and Immunotherapeutic Agents against Cancer: A Comprehensive Overview. *Front. Immunol.* **8,** 1320 (2017).
9. Dean, R. E. *et al.* A carpet-based mechanism for direct antimicrobial peptide activity against vaccinia virus membranes. *Peptides* **31,** 1966–72 (2010).
10. Brice, D. C., Toth, Z. & Diamond, G. LL-37 disrupts the Kaposi's sarcoma-associated herpesvirus envelope and inhibits infection in oral epithelial cells. *Antiviral Res.* **158,** 25–33 (2018).
11. Barlow, P. G. *et al.* Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. *PLoS One* **6,** e25333 (2011).
12. Howell, M. D. *et al.* Selective Killing of Vaccinia Virus by LL-37: Implications for Eczema Vaccinatum. *J. Immunol.* **172,** 1763–1767 (2004).
13. Wiens, M. E. & Smith, J. G. α-Defensin HD5 Inhibits Human Papillomavirus 16 Infection via Capsid Stabilization and Redirection to the Lysosome. *MBio* **8,** (2017).
14. Nguyen, E. K., Nemerow, G. R. & Smith, J. G. Direct Evidence From Single-Cell Analysis That Human {Alpha}-Defensins Block Adenovirus Uncoating to Neutralize Infection. *J. Virol.* **84,** 4041–4049 (2010).
15. Magana, M. *et al.* The value of antimicrobial peptides in the age of resistance. *Lancet Infect. Dis.* (2020). doi:10.1016/S1473-3099(20)30327-3
16. Joo, H.-S., Fu, C.-I. & Otto, M. Bacterial strategies of resistance to antimicrobial peptides. *Philos. Trans. R. Soc. B Biol. Sci.* **371,** 20150292 (2016).
17. Lee, T.-H., Hofferek, V., Separovic, F., Reid, G. E. & Aguilar, M.-I. The role of bacterial lipid diversity and membrane properties in modulating antimicrobial peptide activity and drug resistance. *Curr. Opin. Chem. Biol.* **52,** 85–92 (2019).
18. Peschel, A. & Sahl, H.-G. The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nat. Rev. Microbiol.* **4,** 529–536 (2006).
19. Kosikowska, P. & Lesner, A. Antimicrobial peptides (AMPs) as drug candidates: a patent review (2003–2015). *Expert Opin. Ther. Pat.* **26,** 689–702 (2016).
20. Wang, G., Li, X. & Wang, Z. APD3: the antimicrobial peptide database as a tool for research and

education. *Nucleic Acids Res.* **44,** D1087–D1093 (2016).

21. Sani, M. A. & Separovic, F. How Membrane-Active Peptides Get into Lipid Membranes. *Acc. Chem. Res.* **49,** 1130–1138 (2016).
22. Huang, H. W. Action of antimicrobial peptides: two-state model. *Biochemistry* **39,** 8347–52 (2000).
23. Li, J. *et al.* Membrane Active Antimicrobial Peptides: Translating Mechanistic Insights to Design. *Front. Neurosci.* **11,** 73 (2017).
24. Silhavy, T. J., Kahne, D. & Walker, S. The Bacterial Cell Envelope. *Cold Spring Harb. Perspect. Biol.* **2,** a000414 (2010).
25. Isaksson, J. *et al.* A Synthetic Antimicrobial Peptidomimetic (LTX 109): Stereochemical Impact on Membrane Disruption. *J. Med. Chem.* **54,** 5786–5795 (2011).
26. Bojsen, R., Torbensen, R., Larsen, C. E., Folkesson, A. & Regenberg, B. The Synthetic Amphipathic Peptidomimetic LTX109 Is a Potent Fungicide That Disturbs Plasma Membrane Integrity in a Sphingolipid Dependent Manner. *PLoS One* **8,** e69483 (2013).
27. Koehbach, J. & Craik, D. J. The Vast Structural Diversity of Antimicrobial Peptides. *Trends Pharmacol. Sci.* **40,** 517–528 (2019).
28. Faust, J. E., Yang, P.-Y. & Huang, H. W. Action of Antimicrobial Peptides on Bacterial and Lipid Membranes: A Direct Comparison. *Biophys. J.* **112,** 1663–1672 (2017).
29. Islam, M. Z., Alam, J. M., Tamba, Y., Karal, M. A. S. & Yamazaki, M. The single GUV method for revealing the functions of antimicrobial, pore-forming toxin, and cell-penetrating peptides or proteins. *Phys. Chem. Chem. Phys.* **16,** 15752–67 (2014).
30. Kristensen, K., Henriksen, J. R. & Andresen, T. L. Quantification of leakage from large unilamellar lipid vesicles by fluorescence correlation spectroscopy. *Biochim. Biophys. Acta - Biomembr.* **1838,** 2994–3002 (2014).
31. Haney, E. F. & Vogel, H. J. in *Annu. Reports NMR Spectrosc.* 1–51 (Academic Press, 2009). doi:10.1016/S0066-4103(08)00201-9
32. Avitabile, C., D'Andrea, L. D. & Romanelli, A. Circular Dichroism studies on the interactions of antimicrobial peptides with bacterial cells. *Sci. Rep.* **4,** 4293 (2015).
33. Bürck, J., Wadhwani, P., Fanghänel, S. & Ulrich, A. S. Oriented Circular Dichroism: A Method to Characterize Membrane-Active Peptides in Oriented Lipid Bilayers. *Acc. Chem. Res.* **49,** 184–192 (2016).
34. Lipkin, R. & Lazaridis, T. Computational studies of peptide-induced membrane pore formation. *Philos. Trans. R. Soc. B Biol. Sci.* **372,** 20160219 (2017).
35. Velasco-Bolom, J.-L., Corzo, G. & Garduño-Juárez, R. Molecular dynamics simulation of the membrane binding and disruption mechanisms by antimicrobial scorpion venom-derived peptides. *J.*

Biomol. Struct. Dyn. **36,** 2070–2084 (2018).

36. Zhao, L. *et al.* Molecular Dynamics Simulations of Human Antimicrobial Peptide LL-37 in Model POPC and POPG Lipid Bilayers. *Int. J. Mol. Sci.* **19,** 1186 (2018).
37. Petkov, P., Lilkova, E., Ilieva, N. & Litov, L. Self-Association of Antimicrobial Peptides: A Molecular Dynamics Simulation Study on Bombinin. *Int. J. Mol. Sci.* **20,** 5450 (2019).
38. Lai, P.-K. & Kaznessis, Y. N. Insights into Membrane Translocation of Protegrin Antimicrobial Peptides by Multistep Molecular Dynamics Simulations. *ACS Omega* **3,** 6056–6065 (2018).
39. Ehrenstein, G. & Lecar, H. Electrically gated ionic channels in lipid bilayers. *Q. Rev. Biophys.* **10,** 1–34 (1977).
40. Ludtke, S. J. *et al.* Membrane Pores Induced by Magainin. *Biochemistry* **35,** 13723–8 (1996).
41. Matsuzaki, K., Murase, O., Nobutaka, F. & Miyajima, K. An Antimicrobial Peptide, Magainin 2, Induced Rapid Flip-Flop of Phospholipids Coupled with Pore Formation and Peptide Translocation. *Biochemistry* **35,** 11361–8 (1996).
42. Pouny, Y., Rapaport, D., Mor, A., Nicolas, P. & Shai, Y. Interaction of antimicrobial dermaseptin and its fluorescently labeled analogs with phospholipid membranes. *Biochemistry* **31,** 12416–12423 (1992).
43. Sharma, V. K. & Qian, S. Effect of an Antimicrobial Peptide on Lateral Segregation of Lipids: A Structure and Dynamics Study by Neutron Scattering. *Langmuir* **35,** 4152–4160 (2019).
44. Reboul, C. F., Whisstock, J. C. & Dunstone, M. A. Giant MACPF/CDC pore forming toxins: A class of their own. *Biochim. Biophys. Acta - Biomembr.* **1858,** 475–486 (2016).
45. Salvador-Gallego, R. *et al.* Bax assembly into rings and arcs in apoptotic mitochondria is linked to membrane pores. *EMBO J.* **35,** 389–401 (2016).
46. Yilmaz, N. & Kobayashi, T. Assemblies of pore-forming toxins visualized by atomic force microscopy. *Biochim. Biophys. Acta - Biomembr.* **1858,** 500–511 (2016).
47. Mulvihill, E. *et al.* Mechanism of membrane pore formation by human gasdermin-D. *EMBO J.* **37,** e98321 (2018).
48. Parsons, E. S. *et al.* Single-molecule kinetics of pore assembly by the membrane attack complex. *Nat. Commun.* **10,** 2066 (2019).
49. Hodel, A. W., Leung, C., Dudkina, N. V, Saibil, H. R. & Hoogenboom, B. W. Atomic force microscopy of membrane pore formation by cholesterol dependent cytolysins. *Curr. Opin. Struct. Biol.* **39,** 8–15 (2016).
50. Rudd-Schmidt, J. A. *et al.* Lipid order and charge protect killer T cells from accidental death. *Nat. Commun.* **10,** 5396 (2019).
51. Guha, S., Ghimire, J., Wu, E. & Wimley, W. C. Mechanistic Landscape of Membrane-Permeabilizing

Peptides. *Chem. Rev.* **119,** 6040–6085 (2019).

52. Binnig, G., Quate, C. F. & Gerber, C. Atomic Force Microscope. *Phys. Rev. Lett.* **56,** 930–933 (1986).
53. Ando, T. High-speed atomic force microscopy and its future prospects. *Biophys. Rev.* **10,** 285–292 (2018).
54. Uchihashi, T. & Scheuring, S. Applications of high-speed atomic force microscopy to real-time visualization of dynamic biomolecular processes. *Biochim. Biophys. Acta - Gen. Subj.* **1862,** 229–240 (2018).
55. Zwang, T. J., Fletcher, W. R., Lane, T. J. & Johal, M. S. Quantification of the Layer of Hydration of a Supported Lipid Bilayer. *Langmuir* **26,** 4598–4601 (2010).
56. Guo, L. *et al.* Molecular Diffusion Measurement in Lipid Bilayers over Wide Concentration Ranges: A Comparative Study. *ChemPhysChem* **9,** 721–728 (2008).
57. Rakshit, T. *et al.* Adaptations in rod outer segment disc membranes in response to environmental lighting conditions. *Biochim. Biophys. acta. Mol. cell Res.* **1864,** 1691–1702 (2017).
58. Müller, D. J., Schabert, F. A., Büldt, G. & Engel, A. Imaging purple membranes in aqueous solutions at sub-nanometer resolution by atomic force microscopy. *Biophys. J.* **68,** 1681–1686 (1995).
59. Tanaka, M. & Sackmann, E. Polymer-supported membranes as models of the cell surface. *Nature* **437,** 656–663 (2005).
60. Andersson, J. & Köper, I. Tethered and Polymer Supported Bilayer Lipid Membranes: Structure and Function. *Membranes (Basel).* **6,** 30 (2016).
61. Clifton, L. A. *et al.* Self-Assembled Fluid Phase Floating Membranes with Tunable Water Interlayers. *Langmuir* **35,** 13735–13744 (2019).
62. Liou, J.-W., Hung, Y.-J., Yang, C.-H. & Chen, Y.-C. The antimicrobial activity of gramicidin A is associated with hydroxyl radical formation. *PLoS One* **10,** e0117065 (2015).
63. Torcato, I. M. *et al.* Design and characterization of novel antimicrobial peptides, R-BP100 and RW-BP100, with activity against Gram-negative and Gram-positive bacteria. *Biochim. Biophys. Acta - Biomembr.* **1828,** 944–955 (2013).
64. Alves, C. S. *et al.* Escherichia coli cell surface perturbation and disruption induced by antimicrobial peptides BP100 and pepR. *J. Biol. Chem.* **285,** 27536–44 (2010).
65. Su, H.-N. *et al.* Antimicrobial Peptide Trichokonin VI-Induced Alterations in the Morphological and Nanomechanical Properties of Bacillus subtilis. *PLoS One* **7,** e45818 (2012).
66. Li, A., Lee, P. Y., Ho, B., Ding, J. L. & Lim, C. T. Atomic force microscopy study of the antimicrobial action of Sushi peptides on Gram negative bacteria. *Biochim. Biophys. Acta - Biomembr.* **1768,** 411–418 (2007).
67. Zdybicka-Barabas, A. *et al.* Studies on the interactions of neutral Galleria mellonella cecropin D with

living bacterial cells. *Amino Acids* **51,** 175–191 (2019).

68. Dias, S. A. *et al.* New Potent Membrane-Targeting Antibacterial Peptides from Viral Capsid Proteins. *Front. Microbiol.* **8,** 775 (2017).
69. Oliveira, M. *et al.* Ocellatin-PT antimicrobial peptides: High-resolution microscopy studies in antileishmania models and interactions with mimetic membrane systems. *Biopolymers* **105,** 873–886 (2016).
70. Domingues, M. M. *et al.* Antimicrobial protein rBPI21-induced surface changes on Gram-negative and Gram-positive bacteria. *Nanomedicine Nanotechnology, Biol. Med.* **10,** 543–551 (2014).
71. Overton, K. *et al.* Qualitative and Quantitative Changes to E. coli During Treatment with Magainin 2 Observed in Native Conditions by Atomic Force Microscopy. *Langmuir* **36,** 650–659 (2020).
72. Mularski, A., Wilksch, J. J., Hanssen, E., Strugnell, R. A. & Separovic, F. Atomic force microscopy of bacteria reveals the mechanobiology of pore forming peptide action. *Biochim. Biophys. Acta - Biomembr.* **1858,** 1091–1098 (2016).
73. Mortensen, N. P. *et al.* Effects of Colistin on Surface Ultrastructure and Nanomechanics of Pseudomonas aeruginosa Cells. *Langmuir* **25,** 3728–3733 (2009).
74. Mikuláss, K. R. *et al.* Antimicrobial nodule-specific cysteine-rich peptides disturb the integrity of bacterial outer and inner membranes and cause loss of membrane potential. *Ann. Clin. Microbiol. Antimicrob.* **15,** 43 (2016).
75. Wang, C. *et al.* Real-Time Observation of Antimicrobial Polycation Effects on Escherichia coli : Adapting the Carpet Model for Membrane Disruption to Quaternary Copolyoxetanes. *Langmuir* **32,** 2975–2984 (2016).
76. Pérez-Peinado, C. *et al.* Mechanisms of bacterial membrane permeabilization by crotalicidin (Ctn) and its fragment Ctn(15–34), antimicrobial peptides from rattlesnake venom. *J. Biol. Chem.* **293,** 1536–1549 (2018).
77. Nagy, K. *et al.* Interaction of cysteine-rich cationic antimicrobial peptides with intact bacteria and model membranes. *Gen. Physiol. Biophys* **34,** 135–144 (2015).
78. Fantner, G. E., Barbero, R. J., Gray, D. S. & Belcher, A. M. Kinetics of antimicrobial peptide activity measured on individual bacterial cells using high-speed atomic force microscopy. *Nat. Nanotechnol.* **5,** 280–285 (2010).
79. Benn, G., Pyne, A. L. B., Ryadnov, M. G. & Hoogenboom, B. W. Imaging live bacteria at the nanoscale: Comparison of immobilisation strategies. *Analyst* **144,** 6944–6952 (2019).
80. Heesterbeek, D. A. *et al.* Bacterial killing by complement requires membrane attack complex formation via surface-bound C5 convertases. *EMBO J.* **38,** (2019).
81. Abbasi, F., Leitch, J. J., Su, Z. F., Szymanski, G. & Lipkowski, J. Direct visualization of alamethicin

ion pores formed in a floating phospholipid membrane supported on a gold electrode surface. *Electrochim. Acta* **267,** 195–205 (2018).

82. Abbasi, F., Alvarez-Malmagro, J., Su, Z. F., Leitch, J. J. & Lipkowski, J. Pore Forming Properties of Alamethicin in Negatively Charged Floating Bilayer Lipid Membranes Supported on Gold Electrodes. *Langmuir* **34,** 13754–13765 (2018).
83. Pfeil, M.-P. *et al.* Tuneable poration: host defense peptides as sequence probes for antimicrobial mechanisms. *Sci. Rep.* **8,** 14926 (2018).
84. Hammond, K. *et al.* Helminth Defense Molecules as Design Templates for Membrane Active Antibiotics. *ACS Infect. Dis.* **5,** 1471–1479 (2019).
85. Henderson, J. M., Waring, A. J., Separovic, F. & Lee, K. Y. C. Antimicrobial Peptides Share a Common Interaction Driven by Membrane Line Tension Reduction. *Biophys. J.* **111,** 2176–2189 (2016).
86. Hall, K., Lee, T.-H., Mechler, A. I., Swann, M. J. & Aguilar, M.-I. Real-time Measurement of Membrane Conformational States Induced by Antimicrobial Peptides: Balance Between Recovery and Lysis. *Sci. Rep.* **4,** 5479 (2015).
87. Harrison, P. L. *et al.* Phospholipid dependent mechanism of smp24, an α-helical antimicrobial peptide from scorpion venom. *Biochim. Biophys. Acta - Biomembr.* **1858,** 2737–2744 (2016).
88. Khadka, N. K., Aryal, C. M. & Pan, J. Lipopolysaccharide-Dependent Membrane Permeation and Lipid Clustering Caused by Cyclic Lipopeptide Colistin. *ACS Omega* **3,** 17828–17834 (2018).
89. Mecke, A., Lee, D.-K., Ramamoorthy, A., Orr, B. G. & Banaszak Holl, M. M. Membrane thinning due to antimicrobial peptide binding: an atomic force microscopy study of MSI-78 in lipid bilayers. *Biophys. J.* **89,** 4043–50 (2005).
90. Pyne, A. *et al.* Engineering monolayer poration for rapid exfoliation of microbial membranes. *Chem. Sci.* **8,** 1105–1115 (2017).
91. Oliva, R. *et al.* Membrane disintegration by the antimicrobial peptide (P)GKY20: lipid segregation and domain formation. *Phys. Chem. Chem. Phys.* **21,** 3989–3998 (2019).
92. Heath, G. R., Harrison, P. L., Strong, P. N., Evans, S. D. & Miller, K. Visualization of diffusion limited antimicrobial peptide attack on supported lipid membranes. *Soft Matter* **14,** 6146–6154 (2018).
93. Henderson, J. M. *et al.* Beyond electrostatics: Antimicrobial peptide selectivity and the influence of cholesterol-mediated fluidity and lipid chain length on protegrin-1 activity. *Biochim. Biophys. Acta - Biomembr.* **1861,** 182977 (2019).
94. Pan, J. & Khadka, N. K. Kinetic Defects Induced by Melittin in Model Lipid Membranes: A Solution Atomic Force Microscopy Study. *J. Phys. Chem. B* **120,** 4625–4634 (2016).

95. Leite, N. B. *et al.* PE and PS Lipids Synergistically Enhance Membrane Poration by a Peptide with Anticancer Properties. *Biophys. J.* **109,** 936–947 (2015).
96. Pan, J., Dalzini, A., Khadka, N. K., Aryal, C. M. & Song, L. Lipid Extraction by α-Synuclein Generates Semi-Transmembrane Defects and Lipoprotein Nanoparticles. *ACS omega* **3,** 9586–9597 (2018).
97. Pan, J., Dalzini, A. & Song, L. Cholesterol and phosphatidylethanolamine lipids exert opposite effects on membrane modulations caused by the M2 amphipathic helix. *Biochim. Biophys. Acta - Biomembr.* **1861,** 201–209 (2019).
98. Bodescu, M. A., Rosenkötter, F. & Fritz, J. Time lapse AFM on vesicle formation from mixed lipid bilayers induced by the membrane–active peptide melittin. *Soft Matter* **13,** 6845–6851 (2017).
99. Lei, H.-Z., Tian, T., Du, Q., Hu, J. & Zhang, Y. Sequence-dependent interactions between model peptides and lipid bilayers. *Nucl. Sci. Tech.* **28,** 124 (2017).
100. Lam, K. L. H. *et al.* Mechanism of structural transformations induced by antimicrobial peptides in lipid membranes. *Biochim. Biophys. Acta - Biomembr.* **1818,** 194–204 (2012).
101. Rakowska, P. D. *et al.* Nanoscale imaging reveals laterally expanding antimicrobial pores in lipid bilayers. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 8918–23 (2013).
102. De Santis, E. *et al.* Antimicrobial peptide capsids of de novo design. *Nat. Commun.* **8,** 2263 (2017).
103. Kim, S. Y. *et al.* Mechanism of Action of Peptides That Cause the pH-Triggered Macromolecular Poration of Lipid Bilayers. *J. Am. Chem. Soc.* **141,** 6706–6718 (2019).
104. Lee, T.-H., N. Hall, K. & Aguilar, M.-I. Antimicrobial Peptide Structure and Mechanism of Action: A Focus on the Role of Membrane Structure. *Curr. Top. Med. Chem.* **16,** 25–39 (2015).
105. Hammond, K. *et al.* Flowering poration – a synergistic multi-mode antibacterial mechanism by a bacteriocin fold. *Isc. (in Press.* (2020).
106. Sandiford, S. & Upton, M. Identification, characterization, and recombinant expression of epidermicin NI01, a novel unmodified bacteriocin produced by Staphylococcus epidermidis that displays potent activity against staphylococci. *Antimicrob. Agents Chemother.* **56,** 1539–1547 (2012).
107. Mihajlovic, M. & Lazaridis, T. Charge distribution and imperfect amphipathicity affect pore formation by antimicrobial peptides. *Biochim. Biophys. Acta - Biomembr.* **1818,** 1274–1283 (2012).
108. Lee, T.-H., Hall, K. & Aguilar, M.-I. The Effect of Charge on Melittin-Induced Changes in Membrane Structure and Morphology. *Aust. J. Chem.* **73,** 195 (2020).
109. García-Sáez, A. J., Chiantia, S., Salgado, J. & Schwille, P. Pore formation by a Bax-derived peptide: effect on the line tension of the membrane probed by AFM. *Biophys. J.* **93,** 103–12 (2007).
110. Chan, D. I., Prenner, E. J. & Vogel, H. J. Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action. *Biochim. Biophys. Acta - Biomembr.* **1758,** 1184–1202 (2006).

111. Li, L., Vorobyov, I. & Allen, T. W. The Different Interactions of Lysine and Arginine Side Chains with Lipid Membranes. *J. Phys. Chem. B* **117,** 11906–11920 (2013).
112. Badosa, E. *et al.* Derivatives of the Antimicrobial Peptide BP100 for Expression in Plant Systems. *PLoS One* **8,** (2013).
113. Kim, S. *et al.* Transmembrane glycine zippers: physiological and pathological roles in membrane proteins. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 14278–83 (2005).
114. Senes, A., Engel, D. E. & DeGrado, W. F. Folding of helical membrane proteins: the role of polar, GxxxG-like and proline motifs. *Curr. Opin. Struct. Biol.* **14,** 465–479 (2004).
115. Lupas, A. N. & Bassler, J. Coiled Coils - A Model System for the 21st Century. *Trends Biochem. Sci.* **42,** 130–140 (2017).
116. Krauson, A. J., He, J. & Wimley, W. C. Gain-of-Function Analogues of the Pore-Forming Peptide Melittin Selected by Orthogonal High-Throughput Screening. *J. Am. Chem. Soc.* **134,** 12732–12741 (2012).
117. Li, S. *et al.* Potent Macromolecule-Sized Poration of Lipid Bilayers by the Macrolittins, A Synthetically Evolved Family of Pore-Forming Peptides. *J. Am. Chem. Soc.* **140,** 6441–6447 (2018).
118. Liu, L. *et al.* Self-assembled cationic peptide nanoparticles as an efficient antimicrobial agent. *Nat. Nanotechnol.* **4,** 457–463 (2009).
119. Wang, H. *et al.* The efficacy of self-assembled cationic antimicrobial peptide nanoparticles against Cryptococcus neoformans for the treatment of meningitis. *Biomaterials* **31,** 2874–2881 (2010).
120. Castelletto, V. *et al.* Structurally plastic peptide capsules for synthetic antimicrobial viruses. *Chem. Sci.* **7,** 1707–1711 (2016).
121. Kepiro, I. E. *et al.* Engineering Chirally Blind Protein Pseudocapsids into Antibacterial Persisters. *ACS Nano* **14,** 1609–1622 (2020).
122. Sarkar, B. *et al.* Membrane-Disrupting Nanofibrous Peptide Hydrogels. *ACS Biomater. Sci. Eng.* **5,** 4657–4670 (2019).
123. Debnath, S., Shome, A., Das, D. & Das, P. K. Hydrogelation Through Self-Assembly of Fmoc-Peptide Functionalized Cationic Amphiphiles: Potent Antibacterial Agent. *J. Phys. Chem. B* **114,** 4407–4415 (2010).
124. Chen, W. *et al.* Self-Assembled Peptide Nanofibers Display Natural Antimicrobial Peptides to Selectively Kill Bacteria without Compromising Cytocompatibility. *ACS Appl. Mater. Interfaces* **11,** 28681–28689 (2019).
125. Shen, Z. *et al.* Biomembrane induced *in situ* self-assembly of peptide with enhanced antimicrobial activity. *Biomater. Sci.* **8,** 2031–2039 (2020).
126. Ravi, J., Bella, A., Correia, A. J. V., Lamarre, B. & Ryadnov, M. G. Supramolecular amphipathicity

for probing antimicrobial propensity of host defence peptides. *Phys. Chem. Chem. Phys.* **17,** 15608–15614 (2015).

127. Faruqui, N. *et al.* Differentially Instructive Extracellular Protein Micro-nets. *J. Am. Chem. Soc.* **136,** 7889–7898 (2014).