Biophysical characterization of peptide–membrane interactions

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ABSTRACT
Membrane active peptides directly target membranes rather than receptor proteins and perform their biological functions via cooperative action. To understand the mechanism of peptide–membrane interactions, biophysical characterization of the whole interaction process is essential. The characteristics determined by biophysical methods are briefly summarized and the limitations of these techniques discussed. Circular dichroism (CD), isothermal titration calorimetry (ITC), and fluorescence have been used to determine the binding isotherms of peptide binding to membranes in solution. Lamellar X-ray diffraction (LXD) and small single X-ray scattering (SAXS) have been used to probe peptide-induced thinning of membranes on a substrate and in solution, respectively. The aspiration method has been applied to monitor the area expansion induced by peptide binding to single giant unilamellar vesicles (GUVs). Oriented circular dichroism (OCD) has been used to detect orientation changes of peptides in membranes. The inner water columns of peptide-induced pores in membranes were detected by neutron scattering (NS), and pore structures were reconstructed using anomalous X-ray diffraction. Finally, the time evolution of pore formation induced by peptides binding to single GUVs was monitored by the aspiration method.
1. Introduction

Membrane active peptides can directly target membranes to perform their biological functions [1–3]. For example, antimicrobial peptides (AMPs) interact with membranes and induce uncontrolled membrane permeability to ions and inhibit invading bacteria or microbes [4–6]. Hanatoxin peptide blocks the voltage sensor of the potassium channel Kv2.1 by partitioning into membranes [7–10]. The ion channel peptide gramicidin inserts into the membrane to form a transmembrane dimer, which functions as an ion channel [11,12]. Understanding the mechanism of peptide–membrane interactions is of crucial importance and has applications in non-viral gene delivery, drug design, and disease therapy. Although the proposed models and hypothetical mechanisms have been reported in a number of reviews [13–19], peptide–membrane interaction mechanisms have been the subject of numerous studies and debates in the past decades and are still a puzzle. To understand these mechanisms, biophysical characterization of peptide–membrane interactions is indispensable. In this report, we focus on reviewing the characteristics of peptide–membrane interactions observed by biophysical methods rather than discuss the mechanisms of these interactions.

Biological membranes are mainly composed of lipids in the form of a bilayer as well as other biologically relevant structures, such as monolayers, hexagonal phases, and cubic phase. The bilayer is a ‘sandwich like’ structure with the hydrophobic regions inside and the hydrophilic regions facing the aqueous solution. To interact with membranes, most membrane active peptides have evolutionarily developed as amphiphilic molecules. There are three general steps involved in peptide–membrane interactions: (1) In aqueous solution, the amphiphilic peptides form coiled structures to ‘hide’ their hydrophobic segments inside and ‘encounter’ membranes through diffusion as well as electrostatic attraction. (2) After accumulating around membranes, conformational changes driven by hydrophobic interactions occur. The peptides undergo conformational changes to expose their hydrophobic segments that insert into the interface of the bilayers. (3) The bound peptides carry out their biological functions through cooperative interactions with each other and with membranes. Consequently, peptide-induced functions are highly dependent on peptide concentration, peptide properties (structure, charge, hydrophobicity, etc.), lipid composition, and membrane properties (charge, bending modulus, stretching modulus, etc.).

2. Binding isotherm for peptide–unilamellar vesicle membrane interaction

The inhibition and toxicity functions of membrane active peptides usually show a concentration dependent feature [13,20,21]. The determination of the number of bound peptides rather than the total number of peptides in solution is most important for quantitative analysis and determining not only the minimal inhibitory concentration (MIC) but also the mechanism of peptide-induced inhibition.
2.1. Circular dichroism (CD) spectroscopy

CD spectroscopy is an absorption spectroscopy based on the absorption difference between right-handed and left-handed circularly polarized light. The CD spectrum in the ultraviolet wavelength range is frequently used to determine the secondary structures of a protein in solution. The secondary structures of a protein can be simply clarified as random coil, α-helix and β-sheet as they have distinguishable CD spectra [22]. Amphiphilic peptides form random coil structures in solution but adopt α-helical or β-sheet structures when binding to membranes [23–26]. The change in secondary structure detected by CD measurements has been used to determine the ratio of bound peptide to total peptide. In practical, the normalized CD spectra of free peptides in solution ($S_f$) and binding peptides in membranes ($S_b$) have to be determined. Any measured CD spectrum ($S_m$) can be fit by the linear combination of $S_f$ and $S_b$. The representative CD spectra of $S_f$, $S_b$, and $S_m$ are shown in Figure 1. The A spectrum (black solid line) is the CD spectrum of 25μM melittin in aqueous solution. Successive titrations of small unilamellar vesicles (SUVs) to peptide solution were conducted until CD spectrum didn’t change. The D (gray solid line) shown in Figure 1 is the invariant spectrum of peptide-to-lipid ratio (P/L) equal to 1/250 and B, C are the spectra of P/L = 1/62.5 and 1/125, respectively. In previous studies [27,28], the ratio of bound peptide ($\phi$) was determined by the intensity of CD spectrum at wavelength 222 nm and the simple relation as follows.

$$\phi = \frac{(S_{m}^{222 \text{ nm}} - S_f^{222 \text{ nm}})}{(S_b^{222 \text{ nm}} - S_f^{222 \text{ nm}})}$$  (1)

Figure 1. Normalized CD spectra of melittin in solution and in SUVs solution. Notes: A, B, C, and D are the spectra of pure melittin, P/L = 1/62.5, 1/125 and 1/250, respectively. The dash lines are fitting curves of B and C spectra using spectra A and B as two fitting bases.
According to the relation, the percentages of bound peptide of B and C shown in Figure 1 are 43 and 64%. The red dash line and green dash line shown in Figure 1 are the curves of full-spectrum fittings with 43 and 65% of bound peptide. The result from different data reductions is consistent.

Therefore, CD measurements have been carried out to determine the binding isotherm for peptide binding to membranes, i.e. the ratio of bound peptides to lipids ($P_b/L$) vs. the concentration of free peptides in solution [27–31]. Table 1a lists the specific peptides and lipids for binding isotherm determination in these selected literatures.

### 2.2. Isothermal titration calorimetry (ITC)

ITC is a powerful tool for studying ligand–protein interactions. It detects the total heat transferred during the ligand–protein binding event. Figure 2(a) shows the schematic of ITC. Basically, the temperatures of sample cell and reference cell keep the same by controlling the heat power applied to the cells. The difference of heat power between sample cell and reference cell ($\Delta W$) is recorded with time. Figure 2(b) is the typical titration curve of an exothermic reaction recorded by ITC. Following the assumption of constant binding heat, the number of bound ligands is determined from the total heat change and the constant binding heat. Compared to ligand–protein interactions, peptide–membrane interactions are more complicated due to the multiple interaction forces including hydrophobic interactions, electrostatic forces, entropy, and elastic deformation. There are two approaches to determine the molar reaction enthalpy ($\Delta H^0$) by ITC. (1) To titrate peptides to SUVs in the very low $P/L$ range so that almost all the titrated peptides bind to SUVs. Figure 2b shows the ITC titration curve of titrating 0.14 μM melittin to 1.1 mM DOPC SUVs. $\Delta H^0$ is equal to the peak area divided by the mole of peptides each titration. (2) To titrate SUVs to solution of selected amount of peptides until peak area of the ITC curve close to zero ([30] Figure 1(a and b)). The peak area close to zero means no free peptides in solution so that no additional peptide can bind to SUVs. Therefore, $\Delta H^0$ is calculated by summing all the peak areas and dividing by the mole of peptides. Once $\Delta H^0$ is determined, the peak areas of the measured ITC titration curve (Figure 2c) can be converted to the mole of bound peptides as well as the binding isotherm (Figure 2d). Instead of the single site binding model for ligand–protein interactions, Joachim Seelig's group developed a model based on the Gouy–Chapman theory by taking hydrophobic interactions, electrostatic forces as well as entropic changes into account [32,33]. They successfully determined the binding isotherms for neutral and charged

| Peptide | melittin | magainin2 | Ac-18A-NH₂ |
|---------|----------|-----------|------------|
| Lipid   |          |           |            |
| Vesicles| DOPC     | POPC/POPG | POPC/POPG  |
|         | SUVs     | SUVs      | SUVs       |
| Reference| [27]     | [28]      | [29,30]    |
|         |          |           | Chol/eggPC | [31]      |
|         |          |           | LUVs       |            |
peptides interacting with not only neutral but also charged membranes [34]. Using this model to fit the binding isotherm, the binding constant and enthalpic and entropic changes of the binding process were extracted [35]. Table 1b lists the specific peptides and lipids for the binding isotherm determined by ITC in selected literatures [36,37].

### 2.3. Fluorescence method

A peptide containing phenylalanine (Phe), tyrosine (Tyr), or tryptophan (Trp) amino acid residues exhibits intrinsic fluorescence features that make the label-free fluorescence experiment possible [38]. For example, the lytic peptide melittin

| Peptide       | magainin 2 | PGLa | Nisin Z |
|---------------|------------|------|--------|
| Lipid         | POPC/POPG  | POPC/POPG | POPC/POPG | POPC | POPC/POPG |
| Vesicles      | LUVs | SUVs | SUVs | SUVs | SUVs |
| K (M⁻¹)       | 110 | 55 | 1500 | 500 | 18 |
| Reference     | [30] | [33] | [36] | [37] |
contains 26 amino acid residues with one tryptophan, which is responsible for the blue shift and increased fluorescence emission intensity that accompany peptide–membrane binding [23,39,40]. The number of bound peptides is probed by measuring the intensity increment in fluorescence emission as well as blue shift wavelength. Recently, fluorescence resonance energy transfer (FRET) has been frequently applied for analyzing the interactions between bio-molecules. Using labeled peptide and lipid as the donor and receptor, respectively, in the FRET experiment, the binding isotherm for the peptide–membrane interaction was determined [41,42]. Table 1c lists the binding isotherms of specific peptide–lipid systems measured by fluorescence method in the selected literatures.

In addition to the techniques mentioned above, nuclear magnetic resonance (NMR) [43], monolayer experiments [32], surface plasma resonance (SPR) [44], and X-ray scattering/diffraction [45] have been also used to determine the binding isotherm. Table 1d list the binding isotherms of specific peptide–lipid systems measured by the techniques mentioned above.

In summary, CD is based on the simple assumption of the two-state model but suffers from low resolution given the general property of absorption spectroscopy. ITC has very high resolution but a very complicated model and carries the risk of over interpretation for peptide–membrane systems. The label-free fluorescence experiment is easily carried out but has the intrinsic defect of photobleaching. The usage of labeled fluorescence molecules in FRET is limited by the synthetic techniques available and by photobleaching.

3. The perturbation of membrane structure induced by peptide binding

3.1. Peptide-induced membrane thinning

3.1.1. Lamellar X-ray diffraction (LXD)

X-ray is a powerful tool to study the molecular structures of proteins, DNA as well as membranes. In general, the thickness of membranes varies from 4 to 10 nm depending on lipid composition and environmental conditions, such as temperature, humidity, pH, and so on. In previous studies, X-ray diffraction was used to probe the membrane structure of multilamellar vesicles (MLVs) in aqueous solution [46]. Due to variations in the size of MLVs, membrane undulation and high background noise in water, only four diffraction peaks are available, which limits the resolution of X-ray diffraction [46]. To improve the accuracy of X-ray diffraction, a multilamellar bilayer sample on a substrate was produced and as well

| Peptide       | melittin | magainin 2 |
|---------------|----------|------------|
| Lipid         | eggPC    | DMPC       | eggPC/eggPG | POPC/POPG |
| Vesicles      | SUVs     | SUVs       | LUVs        | SUVs       |
| Reference     | [39]     | [40]       | [42]        |            |

Table 1c. The binding isotherms of specific peptide–lipid systems measured by fluorescence method.
as temperature- and humidity-controlled chamber has been used for diffraction measurements to obtain more than six diffraction peaks, which enabled higher resolution [47–50]. The procedure for data reduction was described in the previous papers [47–49]. Briefly, the procedure started with a background removal followed by the absorption and diffraction volume corrections. Then the integrated peak intensities were corrected for the polarization and the Lorentz factors. The magnitude of the diffraction amplitude was the square root of the integrated intensity. The phases were determined by the swelling method [51]. With their phases determined, the diffraction amplitudes were Fourier-transformed to obtain the trans-bilayer electron density profiles. The profiles were not normalized to the absolute scale; instead they gave the correct phosphate-to-phosphate (PtP) distances, since the latter are independent of the normalization [48]. During the binding process, peptides push the head groups of lipids away and remove the bound water molecules to embed their hydrophobic segments in the hydrophobic regions of the membrane. Because of volume conservation of the hydrophobic region of the membrane, the peptide-induced area expansion of the membrane is equivalent to membrane thinning. LXD was used to precisely monitor not only membrane thickness but also peptide-induced membrane thinning [52–55]. The lipid composition and peptide concentration effects on peptide-induced membrane thinning were systematically studied in previous studies [56–59]. The results indicate that the hydrophobic thickness of the membrane decreases linearly with peptide concentration until it reaches a certain limit. Notably, a 3–8% limit for reduction in the hydrophobic thickness was observed in the different studies. Table 2a shows the peptide-induced membrane thinning of specific peptide–lipid systems measured by LXD in the selected literatures. Despite the advantage of high accuracy, LXD measurement methods involving multi-bilayer sample on a substrate incubated in an almost fully hydrated environment (more than 98% RH) are frequently criticized for not being good models of living cell membranes; the latter in contrast to the former are comprised of a single bilayer in aqueous solution with a lot of solutes.

### 3.1.2. Small angle X-ray scattering (SAXS)

To determine the structure of the membrane, large unilamellar vesicles (LUVs) of approximately 80 nm diameter were produced as a model system to study the structure of membrane in aqueous solution. SAXS was first proposed as a method for determining the membrane thickness of LUVs by Engelman and co-workers [60,61]. The second bump in the scattering curve (Figure 3a), i.e. scattering
intensity vs. moment transfer $Q$, was used to indicate the membrane thickness by following the simple equation:

$$PtP = \frac{4\pi}{Q_{2nd}}$$

where $PtP$, defined as membrane thickness, is the phosphate-to-phosphate distance in the bilayer, $Q$ is the moment transfer, and $Q_{2nd}$ is the $Q$ of the second bump of the scattering curve.

To obtain information about the membrane structure in greater detail, a simple model composed of two positive and one negative Gaussian peaks was constructed to mimic the bilayer structure (Figure 3b) and used to fit the scattering curve of the LUVs. Consequently, not only membrane thickness but also the symmetry or asymmetry between the outer leaflet and inner leaflet of the bilayer structure were probed [62]. Recently, the approach by combining more complex models and simulations to fit scattering curve was developed to obtain more structural details of lipid bilayer [63,64]. Furthermore, SAXS was applied to determine peptide-induced structural changes in the membranes of LUVs, and membrane thinning was probed by SAXS as well as LXD [45,63]. Table 2b shows the peptide-induced membrane thinning of specific peptide–lipid systems measured by SAXS in the selected literatures.

Compared to full spectrum fitting, the method of using the second bump of scattering is suitable for complicated systems for which it is difficult to build models to fit the data. For example, the membranes of living cells are composed of lipids, proteins, and other bio-molecules [61].

### Table 2a. The peptide-induced membrane thinning of specific peptide–lipid systems measured by LXD.

| Peptide | magainin 2 | melittin |
|---------|------------|----------|
| Lipid   | POPC:PS(3:1) | DPhPC | DOPC | DPhPC | DOPC | Di20:1PC | Di22:1PC | POPC |
| P/L     | 1/65 | 1/30 | 1/99 | 1/71 | 1/39 | 1/62 |
| Thinning (Å) | 1.2 | 1.7 | 0.9 | 1.4 | 3.1 | 1.5 |
| Reference | [52] | | | | | |

| Peptide | Alamethicin | melittin | Q25C | penetratin | HaTx1 |
|---------|-------------|----------|------|------------|-------|
| Lipid   | DPhPC | DOPC: PE(2:1) | DOPC | DOPC | SOPC | POPC: DOPG(2:1) |
| P/L     | 1/58 | 1/187 | 1/100 | 1/100 | 1/20 | 1/30 | 1/50 |
| Thinning (Å) | 1.0 | 0.4 | 0.8 | 0.5 | 1.8 | 2.3 | 1.0 |
| Reference | [56] | [53] | [59] | [55] | | |

| Peptide | melittin |alamethicin |
|---------|---------|----------|
| Lipid   | DOPC: lys-oPC (3:1) | DOPC: lys-oPC (8:1) | DOPC: PE (3:1) | DOPC: PE (2:1) | DPhPC: lysoPC (3:1) | DPhPC: PE (9:1) | DPhPC: PE (6:1) |
| P/L     | 1/164 | 1/119 | 1/70 | 1/48 | 1/194 | 1/37 | 1/31 |
| Thinning (Å) | 0.8 | 0.8 | 1.1 | 1.3 | 0.4 | 1.4 | 1.5 |
| Reference | [58] | | | | | | |
Figure 3. (a) The representative scattering curve of lipid bilayer of large unilamellar vesicles (LUVs) in solution. The arrow indicates the Q of the second bump of scattering curve. (b) The model constructed by two positive and one negative Gaussian peaks. Note: Here $\rho_1$, $\rho_2$, $\rho_3$, $\sigma_1$, $\sigma_2$, $\sigma_3$, $\delta_1$, $\delta_3$ are the fitting parameters.

Table 2b. The peptide-induced membrane thinning of specific peptide–lipid systems measured by SAXS.

| Peptide | alamethicin | melittin | Tat |
|---------|-------------|----------|-----|
| Lipid   | Di22:1PC    | Di22:1PC | DOPC| DOPC:PE(1:1) |
| P/L     | 1/100       | 1/50     | 1/15| 1/15         |
| Thinning (Å) | 1.1 | 1.6 | 1.4 | 1.6 |
| Reference | [45] | [64] |       |
3.2. Area expansion induced by peptide binding

3.2.1. Aspiration method for single giant unilamellar vesicles (GUVs)

The aspiration method for single GUVs was developed by E. A. Evans’ group to determine the stretching and bending moduli of membranes [65,66]. Micropipette aspiration was used to monitor the area expansion of the membrane when applying stress to a single GUV. Based on geometrical arguments, the proportionality between area and observed protrusion length inside the micropipette is easily seen in the following first-order approximation [65]:

$$\Delta A \approx \pi D_p (1 - D_p / D_V) \Delta L$$  

Here, $D_p$ is the inner diameter of micropipette, $D_V$ is the diameter of GUV, and $L$ is the protrusion length inside the micropipette. The mechanical properties of membranes including their stretching and binding moduli were extracted from the curve of area expansion vs. applied negative pressure. SAXS measurements on LUVs were based on the averages of large numbers of vesicles but lacked individual and detailed information about peptide–membrane interactions. Moreover, the larger curvature of LUVs compared to that of real cells has been considered a limitation. To understand the nature of the interaction in greater detail and to better mimic the cell membrane in solution, the aspiration method has been applied to single GUVs in peptide solution to monitor the membrane area expansion induced by peptide binding in GUVs. Figure 4 shows a schematic of how the aspiration method can be applied to study the interaction between peptides and single GUVs. The protrusion length inside the micropipette was used as an amplifier to enhance the signal from the less than 5% area expansion induced by peptide binding [67–70]. Table 3 lists the peptide-induced membrane area expansion of specific peptide–lipid systems probed by aspiration method on GUV in selected literatures. In these studies, not only time evolution but also the limits of peptide-induced area expansion were observed and compared to the membrane thinning detected by LXD [69]. Consequently, the membrane expansion limits measured by the aspiration method concur with the results extracted from LXD data. Furthermore, these results indicate that LXD is not only a highly accurate method but also faithfully reflects the properties of the living cell.

4. State transitions of bound peptides in the membrane

4.1. Oriented circular dichroism (OCD)

As mentioned above, CD is frequently used to probe the structural changes of proteins in solution. In such measurements, there is no information of the protein orientation due to the isotropic nature of protein orientations in solution. For peptide–membrane interactions, not only the structure but also the changes in orientation of the membrane-bound peptide are a key issue for understanding
interaction mechanisms. OCD was developed by H. W. Huang’s group to detect the peptide orientation relative to the membrane [71]. In brief, they prepared a multilamellar and orientated membrane sample containing alpha helical peptides for CD measurements and obtained two distinguishable CD spectra corresponding to peptide orientations parallel (S-state) and normal to the membrane surface (I-state). Figure 5 shows normalized OCD spectra of S-state(red solid line) and I-state(gray solid line) of melittin in DOPC:PG(7:3). The spectra of intermediate state(blue and green solid lines) can be fitted by the normalized spectra of S-state and I-state. The fitting curves (black dot lines) are shown in Figure 5 exhibits a good agreement with measured spectra. Using OCD, numerous studies on the interactions of alpha helical peptides with membranes indicated a sigmoidal concentration dependence for the transition between these two states [72].

Table 3. The peptide-induced membrane area expansion of specific peptide–lipid systems probed by aspiration method on GUV.

| Peptide    | wt-20 | melittin | FITC-melittin | daptomycin |
|------------|-------|----------|---------------|------------|
| Lipid      | SOPC  | SOPC     | Di22:1PC      | Di20:1PC   | DOPC:PG(7:3) | DOPC:PG (7:3) |
| ΔA/A       | 0.086 | 0.012    | 0.062         | 0.094      | 0.034       | 0.03 |
| Reference  | [67]  | [67]     | [68]          | [68]       | [69]        | [70] |

Figure 4. The cartoon shows how aspiration method probes the area expansion induced by peptide binding.

Notes: Here $D_V$ is the diameter of GUV, $D_p$ is the inner diameter of micropipette, $L_o$ is the protrusion length without peptide binding and $ΔL$ is the change of the protrusion induced by peptide binding. Based on an assumption of constant volume of GUV, the relation between $ΔL$ and area expansion $ΔA$ is demonstrated as follows: $ΔA \approx \pi D_V (1 - D_p/D_V) ΔL$. The measured $ΔL$ is able to transformed to area expansion $ΔA$. 

![Area expansion induced by peptide binding](image)
evidence suggests that the alpha helical peptides bind parallel to the membrane surface, i.e. the surface state (S-state), at low peptide-to-lipid (P/L) ratios and change their state to perpendicularly insert in the membrane, i.e. the insertion state (I-state), when P/L exceeds the threshold \([54,56,72]\). Table 4 shows the critical concentration \((P/L)^*\) of the peptide orientation change in specific peptide–lipid systems determined by OCD.

**4.2. Lamellar X-ray diffraction (LXD)**

Peptide-induced membrane thinning detected by LXD exhibits state transitions, a feature it shares with OCD. The thickness of the membrane linearly decreases with P/L, increasing when P/L is below the critical concentration \((P/L)^*\) and reaches a limit and remains constant when P/L exceeds \((P/L)^*\) \([54,57]\). The membrane thinning and constant thickness correspond to the S-state and I-state of bound peptides, respectively. Based on elastic theory, a free energy model was proposed to explain OCD data as well as LXD data and clarify the mechanism of transition. A number of studies on the interactions between different peptides and lipids were used to examine the model, and good agreement was achieved \([56–58]\).

**Table 4.** The critical concentration \((P/L)^*\) of the peptide orientation change in specific peptide–lipid systems determined by OCD.

| Peptide    | wt-20 | melittin | FITC-melittin | daptomycin |
|------------|-------|----------|---------------|------------|
| Lipid      | SOPC  | SOPC     | D12:1PC       | DOPC:PG(7:3)| DOPC:PG(7:3)|
| \(\Delta A/A\) | 0.086 | 0.012    | 0.062         | 0.094      | 0.034       | 0.03        |
| Reference  | [67]  | [67]     | [68]          | [68]       | [69]        | [70]        |
5. Pore formation induced by peptides in membranes

5.1. Fluorescence-based leakage experiment

The fluorescence-based experiment has been widely used to monitor the peptide-induced permeability of membrane [73–77]. Its idea is straightforward and easily understood. Briefly, dye-entrapped vesicles were separated from free dyes outside vesicles by gel column. The concentration of dye was chosen in the range of self-quenching. Once membrane leakage induced by peptide occurs, fluorescent dyes leak out so that its concentration decreases below the self-quenching concentration and the fluorescence intensity increases. The intensity of 100% leakage \(F_{100}\) is determined using Triton X-100 to lytic all the vesicles. The leakage percentage can be defined as:

\[
\text{Leakage\%} = 100 \times \frac{(F - F_0)}{(F_{100} - F_0)}
\]

The frequently used dye in leakage experiment is calcein of 50–80 mM [73–77] and other dyes have also been developed [78–80]. The leakage of vesicles have been usually regarded as the evidence of pore formation in membranes. However, the result of leakage comes from the average of all vesicles instead of individual vesicle. We can’t exclude the possibility that partial vesicles were ruptured and the others were intact. To obtain more leakage details of single vesicle, fluorescence-based leakage experiment on single GUV was developed [81–84]. Instead of self-quenching dye, the dye whose fluorescence intensity is proportional to concentration was applied in leakage experiment on GUV. Once leakage occurs, the fluorescence intensity of GUV decreases. Not only the behavior of single GUV but also the time evolution was monitored. Table 5a lists the result of fluorescence-based leakage experiments of specific peptide–lipid systems. Even though leakage experiments gave a lot of evidences for peptide-induced membrane permeability, the structural evidences of pore formation are most direct and urgent for understanding the mechanism of pore formation.

5.2. Neutron scattering

Antimicrobial peptides (AMPs) are known to target membranes directly rather than a receptor protein to kill invading bacteria or microbes and do so by forming pores in membranes. To correlate the pore formation induced by AMPs to the insertion state observed by OCD and LXD in membranes, neutron scattering was used to detect the water columns inside the pores across membranes. The H\(_2\)O was replaced by D\(_2\)O to enhance contrast between membranes and pores for neutron scattering. The scattering curve of pore–pore correlation was obtained and fitted by the constructed model. Consequently, the inside and outside diameters of pore were determined via the fitting. The typical pores in membranes, called barrel-stave and toroidal pores, were probed and the pore sizes determined using
**Table 5a.** The fluorescence-based leakage experiment of specific peptide–lipid systems.

| Peptide       | melittin | magainin2 | Tachyplesin I |
|---------------|----------|-----------|---------------|
| Lipid         | POPC     | POPC/chol | POPG          |
| Dye           | calcein  | POPS      | EYP:PG(1:1)   |
| Vesicle       | LUV      |           |               |
| Reference     | [73]     | [74]      | [75]          |

| Peptide       | magainin2 | PGLa      | pAnpt         |
|---------------|-----------|-----------|---------------|
| Lipid         | eggPC:PG(1:1) | calcein | POPC:PG(7:3) |
| Dye           |          |          | bovine brain PS |
| Vesicle       |          |          | egg PC        |
| Reference     | [76]     | [77]     | [78]          |

| Peptide       | cecropin A(1–8)-melittin(1–18) |
|---------------|---------------------------------|
| Lipid         | bovine brain PS                 |
| Dye           | FITC-dextran                    |
| Vesicle       | LUV                             |
| Reference     | [78]                            |

| Peptide       | penetratin | magainin2 | maculatin | citropin | aurein |
|---------------|------------|-----------|-----------|----------|--------|
| Lipid         | EPC/lysoPC | EPC/DOG   | DOPC      | DOPC/chol | POPC   |
| Dye           | sulfrohodamine B | 5(6)-Carboxyfluorescein | | | |
| Vesicle       | LUV | | | | |
| Reference     | [79] | [79] | | | |

| Lipid         | PC/PE/PV/lysoPC/ cardiolipin |
|---------------|-------------------------------|
| Dye           | 5(6)-Carboxyfluorescein       |
| Vesicle       | GUV                           |
| Reference     | [81]                          | [82] | [83] |

Reference: [73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83]
neutron scattering [85–88]. Table 5b shows the size of peptide-induced pore in membranes determined by neutron scattering. The theoretical model from the point of view of energetics and the mechanism of pore formation in membranes were discussed by integrating these pieces of experimentally obtained structural evidence [57].

### 5.3. Anomalous X-ray diffraction

Determining the pore structure in membranes directly instead of using leakage measurements and neutron scattering are crucial for understanding peptide–membrane interactions. The membrane is a two-dimensional fluid, and therefore, the correlation of pores in membranes is too weak for X-ray diffraction measurements. Another problem in determining the pore structure in membranes by X-ray diffraction is phase determination. Anomalous X-ray diffraction was developed to determine the pore structure in membranes. Two approaches were used for overcoming the barriers mentioned above. For solving the phase problem, the multiple-wavelength anomalous dispersion (MAD) method was applied to membrane samples produced using bromine-labeled lipids. Additionally, pore–pore correlation in membranes was enhanced using dehydration, which makes lipids more ordered. The structures of barrel-stave pores induced by alamethicin as well as toroidal pores induced by the Bax-protein-derived peptide and the bee venom peptide, melittin, were successfully determined by anomalous X-ray diffraction [69,89,90] and provide new insights into pore formation in membranes. The structural result of peptide-induced pore in membranes determined by anomalous X-ray diffraction was shown in Table 5c.

**Table 5b.** The size of peptide-induced pore in membranes determined by neutron scattering.

| Peptide      | alamethicin | maginin2 | melittin |
|---------------|-------------|----------|----------|
| Lipid         | DLPC        | DPhPC    | DMPC:PG (3:1) DLPC POPC |
| Outside diameter of pore (Å) | 40          | 50       | 71       | 78       | 76       |
| Inside diameter of pore (Å)   | 18          | 26       | 37       | 44       | 44       |
| Reference     | [73]        | [74]     | [76]     |

**Table 5c.** The structure of peptide-induced pore in membranes determined by anomalous X-ray diffraction.

| Peptide                  | alamethicin | Bax-protein derived peptide | melittin |
|--------------------------|-------------|-----------------------------|----------|
| Lipid                    | DLPC        | Br-DOPC                     |          |
| P/L                      | 1/30        | 1/30                        | 1/25     |
| Lattice constant (a nm*c nm) | 5.94*4.95 | 6.53*5.13                   | 5.91*5.56 |
| Pore structure           | barrel-stave| toroidal                    | toroidal |
| Reference                | [89]        | [90]                        | [69]     |
5.4. Aspiration method for single giant unilamellar vesicles (GUVs)

The aspiration method was used to probe not only the area expansion induced by bound peptides but also pore formation in membranes. Figure 6(a) shows a cartoon describing the detection of pores induced by peptide binding. Briefly, GUVs were produced with the same osmolarity of sucrose solution inside and glucose solution outside. Then, one GUV, sucked slightly by a micropipette, was transferred to a peptide solution. Initially, peptides bind to the membrane surface and induce area expansion, which is probed by an increase in the protrusion length inside the micropipette when P/L is below the critical concentration (P/L)*. When P/L exceeds (P/L)*, peptides insert into membrane to form transmembrane pores and membrane area is kept constant. Because sucrose has a larger molecular size than glucose and the pores in the membranes are of finite sizes, the glucose molecules outside the GUV can leak into the GUV, but sucrose molecules cannot leak out of the GUV. The decrease in protrusion length, which is equivalent to the increase in the inner volume of the GUV, is observed due to the glucose solution flowing in. Not only fluorescence images but also phase contrast images before and after pore formation were recorded. The upper inset in left side of Figure 6(a) shows the phase contrast image before pore formation. The phase contrast enhancement around the rim of GUV results from the difference of refractive index between glucose and sucrose solutions. When transmembrane pore forms, the phase contrast was reduced by glucose molecules outside the GUV leaking into the GUV. The lower inset in left side of Figure 6(a) shows the phase contrast image corresponding to the decrease in protrusion. Peptide-induced pores in membranes have been observed and the time evolution of pore formation monitored by the aspiration method on single GUVs [68]. In order to obtain the more convincing data, this method was improved using more fluorescence probes [69] and the illustration was shown in Figure 6(b). In short, FITC-melittin was used to evidence the peptide binding to the rim of GUV, which is corresponding to the increase in protrusion. The GUV-entrapped Texas Red dye was applied to deliver leakage measurement. Consequently, the fluorescence intensity decreased with Texas Red molecules leaking out, which is corresponding to the decrease in protrusion.

6. Conclusion

The process of peptide–membrane interactions is complex, and the mechanism has been a puzzle up to now. Therefore, combining different points of view is necessary to understand such a complex system. In the past decades, a number of techniques, including biochemical and biophysical methods, were developed to investigate peptide–membrane interactions. In this short review, biophysical characterization using structural, calorimetric, spectroscopic, and imaging methods were summarized to provide fresh insights into peptide–membrane interactions.
Figure 6. (a) The cartoon reveals how aspiration method detects the pore formation in membrane of GUV. When peptide concentration \((P/L)\) exceeds the critical concentration \((P/L)^*\), peptides insert into membrane for forming pores. At the meanwhile, concentration gradient driven flow-in of glucose and water causes the volume increasing of GUV without any area change. The relation between \(\Delta L\) and volume change \(\Delta V\) is demonstrated as follows:

\[
\Delta V = -\pi \left( \frac{D_p}{2} \right)^2 \left( \frac{D_v}{2} - \frac{D_p}{2} \right) \Delta L.
\]

The decreasing of protrusion length equivalent to volume increasing was observed when pores form. The upper and lower insets in left side of Figure 6(a) shows the phase contrast images corresponding to the increase and decrease in protrusion, respectively. (b) The cartoon reveal how improved aspiration method detects the pore formation in membrane of GUV. FITC-melittin was used to evidence the peptide binding to the rim of GUV, which is corresponding to the increase in protrusion. The GUV-entrapped Texas Red dye was applied to deliver leakage measurement.
Gathering experimental evidence is akin to playing a ‘jigsaw puzzle’ game; any attempt to gather the pieces and put them in the ‘right positions’ represents progress in delineating the mechanism of peptide–membrane interactions.

**Disclosure statement**

No potential conflict of interest was reported by the author.

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