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Molecular simulations of venom peptide-membrane interactions: progress and challenges

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Abstract

Due to their wide range of biological activities venom peptides are a valuable source of lead molecules for the development of pharmaceuticals, pharmacological tools and insecticides. Many venom peptides work by modulating the activity of ion channels and receptors or by irreversibly damaging cell membranes. In many cases, the mechanism of action is intrinsically linked to the ability of the peptide to bind to or partition into membranes. Thus, understanding the biological activity of these venom peptides requires characterising their membrane binding properties. This review presents an overview of the recent developments and challenges in using biomolecular simulations to study venom peptide-membrane interactions. The review is focused on i) gating modifier peptides that target voltage-gated ion channels, ii) venom peptides that inhibit mechanosensitive ion channels and iii) pore-forming venom peptides. The methods and approaches used to study venom peptide-membrane interactions are discussed with a particular focus on the challenges specific to these systems and the type of questions that can (and cannot) be addressed using state-of-the-art simulation techniques. The review concludes with an outlook on future aims and directions in the field.

Keywords: venom peptides, biomolecular simulations, molecular dynamics simulations, peptide-membrane interactions, molecular interactions, gating modifiers, antimicrobial peptides, structure-activity relationship, mode of action.

1. Introduction

Animal venoms are a rich source of biologically active peptides that show a remarkable range of pharmacological activities including analgesic, antiarrhythmic, anti-inflammatory, anti-microbial and antiparasitic, cytolytic and haemolytic as well as enzymatic activities. Combined with their structural diversity this makes venom peptides a valuable source of lead molecules for the development of pharmaceuticals, pharmacological tools and insecticides [1-3]. While their molecular targets and mechanisms of action (MOA) are diverse, many venom peptides exert their biological activity by inhibiting ion channels or receptors. In many cases the binding site is fully or partially membrane-embedded. As a result, the biological activity of the peptide is often directly or indirectly linked to its ability to bind to or partition into membranes. Other venom peptides work by irreversibly disrupting cell membranes. In this case, the MOA of the peptide is intrinsically linked to its membrane-binding properties. For the purpose of this review, venom peptides are categorised into the following three groups based on their target and MOA. The first group are gating modifiers (GMs) that modulate the activity of voltage-gated ion channels by binding to their membrane-embedded voltage-sensing domains (Figure 1). Most GMs are 20-60 amino acids long and fold into a well-defined secondary structure that is stabilised by multiple, highly conserved disulfide bonds [4]. Some of the most studied examples include the spider venom peptides VsTx1 [5-6], SGTx1 [7], Hanatoxin [8], ProTx-I [9] and ProTx-II [10]. The second group are disulfide-rich peptides that act on mechanosensitive channels (MSCs). These channels are activated by changes in tension or curvature of the surrounding membrane [11-12] (Figure 2). Interestingly, MSC-directed peptides can act as inhibitors without being in physical contact with the channel. These peptides work by reducing the local stress in the membrane which in turn prevents channel activation [12]. Like GMs, MSC-directed peptides are mostly disulfide-rich peptides with a well-defined secondary structure. Examples include the spider venom peptides GasFII, GsMTx-2 and GsMTx-4 [13-16]. The third group are pore-forming peptides that irreversibly damage cell membranes by a pore-forming mechanism (Figure 3). Many pore-forming venom peptides are part of the well-studied family of antimicrobial peptides (AMPs) [17]. Compared to GMs and MSC-directed peptides, pore-forming venom peptides show a larger structural diversity ranging from short α-helices (e.g. melittin), β-sheets (e.g. gomesin) to large disulfide-rich peptides (e.g. cobra cytotoxins).

For peptides acting by one of the mechanisms described above, establishing a structure-activity relationship includes characterising their interactions with membranes. Biomolecular simulations and related approaches have been extensively used for this. Molecular dynamics (MD) simulations in particular, is still one of the few techniques that allows us to study the structure and dynamics of biomolecules with atomistic or near atomistic resolution [18-20]. The recent growth in computing power combined with improvements in enhanced sampling algorithms and biomolecular force fields has led to a significant increase in the size and complexity of the systems that can be simulated. These developments have also extended the feasible time-scales of such simulations from a few tens of nanoseconds to hundreds of nanoseconds, and more recently, a few microseconds. As a result, we can now carry out simulations that are more realistic; both in terms of the biologically-relevant environment as well as in describing complex processes. It is becoming increasingly feasible to reach simulation times that enable the calculation of (sufficiently converged) macroscopic and structural properties and thus allow a semi-quantitative or quantitative comparison to experimental data [20]. At the same time advances in experimental techniques means we now have more information on the structure and spatio-temporal dynamics of membranes and their interactions with peptides. We are increasingly able to carry out simulations that go beyond providing fundamental insight into peptide-membrane interactions but help us understand the MOA for a given peptide, and use this knowledge to inform the design of peptides with specific biological activities or physiochemical properties.

This review provides an overview of the recent developments in using biomolecular simulations to study venom peptide-membrane interactions and highlight what can (and cannot) be achieved with current state-of-the-art simulation methods. For computational scientists that are new to the field of venom peptides this review aims to outline the commonly used simulation approaches and give an overview of the challenges, some of which are specific to venom peptide-membrane systems. The review, however, does not cover the theoretical background of the methods used and the reader is referred to the appropriate literature. For readers that are ‘wet-lab’ scientists this review aims to present an overview of the type of systems that can be studied and the questions that can (and cannot) be addressed with simulations. These points are illustrated using selected examples of simulation studies of GMs, MSC-directed and pore-forming peptides isolated from animal venom. This review does not cover simulations to study the interactions of venom peptides with ion channels, which have been reviewed elsewhere [21-23]. Also, AMPs isolated from sources

other than venom are not discussed and the reader is referred to some of the many available reviews on AMPs [17, 24-28]. Finally, challenges and limitations that are common to all biomolecular simulations [20] such as the choice of force field [29-31], the use of enhanced sampling methods [32-33] and the problem of sampling errors and convergence [34-36] are only discussed in the context of how they affect the accuracy and reliability of venom peptide – membrane simulations.

The remainder of this review is organized as follows. Section 2 outlines the simulation approaches commonly used to study venom peptide-membrane interactions with a focus on challenges and considerations that are specific to these simulation systems. In section 3 selected studies of GMs and MSC-directed peptides are reviewed based on the questions they aim to address. Similarly, section 4 provides a review of recent studies of pore-forming venom peptides. The review concludes in section 5 with a summary and outline of the future aims and trends in the field.

2. Computational approaches to study venom peptide – membrane interactions

Before taking a closer look at the specific methods and the types of simulation systems studied, it is worth emphasizing that the accurate description of peptide – membrane interactions is, for many reasons, one of the most challenging tasks in bimolecular simulations. Firstly, membranes are complex and heterogeneous systems and their structure and dynamics is affected by a range of environmental factors (e.g. pH, ionic strengths and temperature). Secondly, compared to most proteins, there is no distinct 'binding site' and a peptide binding to a membrane surface can adopt multiple, interchangeable binding modes. The number of different binding modes and their relative stabilities depends, among others, on the conformation of the peptide and the local structure of the membrane as well as its lipid composition. The 'binding site' is also not spatially unique and peptides can continue to bind until the membrane surface is saturated. Furthermore, the binding of the peptide will likely induce changes to the local structure of the membrane while the peptide might undergo conformational changes upon binding to the membrane. Consequently, to accurately describe peptide-membrane interactions and thus obtain macroscopic properties that are in agreement with experiments, the simulation needs to sample a large conformational space . This so-called 'sampling problem' is still one of the biggest challenges in simulations of peptide-membrane systems (and biomolecular simulations in general). Related to this is the fact that modelling peptide – membrane systems results in large simulations systems thus limiting the

feasible simulation times to hundreds of nanoseconds or a few microseconds, even with the most state-of-the-art supercomputing facilities. Yet the biological process of interest might take place on much longer timescales ranging from microseconds to milliseconds, or even seconds [9-10, 37-40].

2.1. Simulation methods

The most commonly used simulation technique for studying venom peptide – membrane interactions is atomistic MD simulations, either using an all-atom or united atom force field. To extend the accessible simulation times and increase the conformational space explored, coarse-grained (CG) MD simulation are also used. While CG simulations have been successfully used for membrane systems [25, 41-42] it is important to consider the details of a CG model before using it for simulations of venom peptide – membrane systems. For example, in many CG models the backbone of the peptide has limited conformational freedom. While most gating-modifiers and MSC-directed peptides are rigid in structure due to the presence of multiple disulfide bonds, pore-forming peptides are often unstructured in solution and only form a more stable secondary structure when in contact with the membrane. For simulations of pore-forming peptides it has also been shown that the coarse-graining of water can affect pore formation [25-26].

Independent of resolution of the force field used, classical (i.e. unbiased) MD simulation are used to address questions including, but not limited to, the spontaneous binding of the peptide to the membrane surface [5, 7, 9-10, 15-16, 43-48] including conformational changes in the peptide, the penetration-depth and orientation of the peptide at the water-lipid interface, the identification of peptide residues that control lipid binding, the effect of peptide binding on the local structure of the membrane, and the effect of lipid composition or mutations in the peptide on these properties. For pore-forming peptides, classical MD simulations are also used to study the structure and stability of pre-formed pores or the first steps in pore formation [46, 49-51].

One of the most valuable properties to obtain from MD simulations is the free energy of binding (ΔG_b), which can be related to the binding affinity of the peptide. The accurate prediction of ΔG_b combined with the structural details obtained from the simulations not only provides valuable mechanistic insight but can be used to predict the effect of mutations in the peptide or lipid composition on membrane binding or pore formation. However, free energy calculations are resource-intensive and the reliable and accurate prediction of ΔG_b for peptide – membrane systems

is far from being routine and still at the limit of current simulation approaches and computer power [52-55].

The most frequently used methods for calculating ΔG_b for peptide – membrane systems is umbrella sampling (for examples, see references [5-6, 13, 15-16, 56-58]). In this method a pathway, referred to as the 'reaction coordinate', connects the two states of interest and the free energy difference between the states along that path is determined. For GMs and MSC-directed peptides this is often a one-dimensional path connecting the peptide in solution to the peptide on the membrane. The reaction coordinate is the distance between the centre of mass of the peptide and that of the membrane. For an accurate calculation of ΔG_b the simulation needs to sufficiently sample the conformations of the peptide in the water and lipid phase, the rotational and translational motion of the peptide on the membrane surface and any changes in the membrane structure caused by the binding of the peptide. A semi-quantitative comparison of relative ΔG_b (i.e. $\Delta\Delta G_b$) for a set of peptides with experimentally known membrane-binding affinities can give a first indication whether the simulation achieves this. For pore-forming peptide the choice of reaction coordinates is often less obvious as the process of membrane disruption is complex and involves multiple steps, each with distinctly different peptide-membrane configurations. In addition, the validation by direct comparison to experimental data is complicated by the fact that simulations are rarely long enough to obtain ΔG_b of peptide-induced pore formation and instead the change in free energy of a specific step in the process is calculated. (e.g the insertion of a single peptide to an intact membrane or the addition of a single peptide to a pre-formed pore). On the other hand, it is difficult to decompose the thermodynamic properties obtained from 'wet-lab' experiments into the contributions from the individual steps. A comparison $\Delta\Delta G_b$ for a set of peptides that are known to have significantly different abilities to form pores might be a start to validate the simulation approach.

2.2. Design and setup of venom peptide-membrane simulations

Given the feasible lengths of MD simulations it is difficult to study the process of membrane binding followed by channel inhibition or pore formation as depicted in Figures 1-3. Thus, most simulations focus on studying part of the process and/or to calculate a set of specific properties or observables. Independent of the question the simulations aims to address, it is important to consider how the setup of the simulation might bias the results. In fact, insufficient simulation time

to overcome or reduce this bias, is one of the problems when comparing results from different simulations studies, even for the same peptide-membrane system.

For GMs and MSC-directed peptides simulations systems usually consist of one or more peptides and a pre-formed (or even pre-equilibrated) lipid bilayer. Peptides are either positioned in solution, a few nm away from the membrane, or on the membrane surface. The starting structure of the peptide is mostly taken from NMR experiments or a homology model based on a structurally-related peptide. The peptide-membrane system is then solvated with explicit water and ions are added to neutralise the charge on the peptide. In some cases, additional ions are added to reach a specific ionic strength. An alternative approach is to start with a random mixture of water, lipids and peptide(s) and allow the lipid bilayer to self-assemble [59-61]. Other simulation studies have used pre-formed micelles to match the experimental conditions of NMR or CD experiments [62-63]. While micelles can mimic some of the essential features of lipid bilayers the effect of the membrane curvature has to be taken into account when interpreting results.

Simulation systems for pore-forming venom peptides show an even wider range of setup and starting structures. For studying surface binding the typical setup of a pre-formed bilayer with one or more peptide is most commonly used. The simulation might be started with the peptide in a random coil or already folded into its membrane-bound conformation. To study the stability and structure of pores, simulations are mostly started from pre-formed pores or pre-arranged, membrane-embedded peptide aggregates. Given the lack of structural detail with respect to the size and shape of these pores choosing a starting structure is not a trivial task. While the formation of transient pores has been reported, the spontaneous formation of stable, long-lived pores is unlikely to be observed in feasible timeframes [51, 64-66] and has not been reported even at very high peptide-lipid ratios [47].

Besides the peptide-membrane configurations used in the setup, the choice of lipids has to be considered. The majority of simulation use membranes composed of one or more type of phospholipid. The specific lipid composition is mostly guided by the biological system of interest, the aim to match conditions of ‘wet-lab’ experiments and the availability of validated force field parameters [67-68]. Until very recently, parameters were only available for a small set of lipids and

simulations of peptide-membrane systems were limited to model membranes consisting mainly of phospholipids. The most frequently used lipids include the zwitterionic lipids POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), POPE (1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine), DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) and DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine). The effect of charged lipids is studied by combining these PC/PE lipids with POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol) or DOPG (1,2-Dimyristoyl-*sn*-glycero-3-phosphorylglycerol). Less common choices are membrane mimetics such as micelles made of SDS (sodium dodecyl sulfate) or DPC (dodecyl phosphocholine), or the use of *n*-alkinethiol self-assembled monolayers (SAMs).

On one hand such simple model membranes are clearly a simplification of bacterial or mammalian membranes which consist of a wide range of phospholipids with different head groups and tails of varying lengths and saturation, as well as many other lipids including sterols, ceramides and sphingomyelines [69]. On the other hand, experiments that characterise the membrane binding of venom peptides are routinely carried using model membranes composed of one or more phospholipid. The use of model membranes in simulations can thus facilitate a more direct comparison to experiment.

It is also important to note that the properties calculated from the simulations can be affected by the choice of force field [43, 70-72]. As pointed out in a recent review on simulations of pore formation in membranes [25], it is important to realise that force fields use different strategies for parameterisation and the "mixing of lipid and protein force field parameters without detailed testing of their compatibility" is not recommended. Other factors that can affect the results are the method used to treat long-range electrostatics and the inclusion or omission of ions. Finally, for MSC-directed peptides or pore-forming peptides the effect of tension or membrane curvature on the binding mode of the peptide might be considered. This can be achieved by applying surface tension [15] or lateral compression [73] to the membrane, introducing asymmetry by using different lipid compositions in the upper and lower leaflets or by using micelles of varying sizes [62, 74].

3. Membrane binding of disulfide-rich peptides targeting voltage-gated or mechanosensitive ion channels

The earliest simulations of GMs were motivated by experiments which showed that many of these peptides partition into membranes. Well-studied examples include VsTx1 [75-76], SGTx1 [77-78], Hanatoxin [78-79], ProTx-II [80-81]. While membrane-binding is not a pre-requisite to act via a gating modifier mechanism [9, 80, 82-83], in some cases altering the membrane-binding affinity of the peptide affects its inhibitory activity on voltage-gated ion channels [10, 37]. Nevertheless, the role of membrane partitioning in the inhibition of voltage-gated ion channels remains an open question. For a review on this topic and a summary of experimental data on GMs, see the recent paper by Agwa et al. [84].

A number of simulations studies have investigated the membrane-binding properties of GMs with a particular focus on defining the location and orientation of the peptide in the membrane as well as identifying the residues that control membrane binding [7, 9-10, 44]. For example, simulations of the spider venom peptides VsTx1, SGTx1, ProTx-I and ProTx-II showed that the preferred location of the peptide is at the water-lipid interface and peptides rarely penetrate into the hydrophobic core of the membrane. This agrees with results from tryptophan fluorescence experiments on the same peptides [84]. The simulations further showed that the peptides bind to membranes in a position such that a cluster of hydrophobic residues on one side of the peptide is orientated towards the hydrocarbon core of the bilayer while the more hydrophilic side of the peptide faces the water. The peptide is further anchored to the water-lipid interface by electrostatic interactions between charged peptide residues and the choline and phosphate groups in the lipids. These charged residues are also likely to play a role in the initial attraction of the peptide to the membrane surface via long-range electrostatics. Simulations also helped identify the residues that form the main peptide-membrane contact surface. Contact residues predicted by simulations of VsTx1 [5], STxG1 [7], ProTx-I [9] and ProTx-II [10] were in good agreement with structural data from NMR experiments and results from studies that compared the membrane-binding properties or inhibitory activities of a series of peptide analogues. For ProTx-II, this information was used to make peptide analogues with improved inhibitory activity on the voltage-gated sodium channel [10].

The challenges of accurately describing the binding free energy, ΔG_b, are clearly demonstrated by the first reports of free energy calculations of GMs and MSC-directed peptides. For example, Wee

et al. [5] aimed to calculate ΔG_b for the binding of VsTx1 to neutral and negatively charged phospholipid bilayers using a CG model and umbrella sampling. As clearly acknowledged by the authors, the binding constants calculated were orders of magnitude larger than experimentally observed data. In a subsequent study by the same authors [6], the free energy profiles for the same system were re-calculated using three different representations; a model in which an all-atom description of the peptide was combined with an implicit membrane-solvent model, a CG model and an all-atom model (both with explicit lipid and water). While the free energy profiles from the three models showed a similar overall shape, the exact location of the energy minima defining the preferred location of the peptide in the membrane were different. Also, the free energy barriers predicted from the three models showed significant differences, and ΔG_b values remained considerably larger than experimental values. Using a similar approach, Chen et al. [13] employed umbrella sampling to calculate ΔG_b for the binding of the GM peptide HpTx2 and the MSC-directed peptide GsMTx4 to a POPC membrane. While the relative binding affinities were in qualitative agreement with experiments, the values were at least 10 kJ/mol larger than experimental values. Similarly, the ΔG_b predicted from simulations of HpTx2 and GsMTx4 with POPC by Nishizawa et al. [16] were much large than experimental values. The authors noted that apart from potential issues with convergence, the high values for ΔG_b might have been the result of restraining the membrane.

A more recent example has demonstrated that longer simulations can significantly improve the accuracy of ΔG_b values. Nishizawa et al. [15] used both all-atom and CG simulations to calculate the free energy for the binding of GsMTx4 and analogues thereof to a POPC membrane. The aim was to investigate the relationship between membrane binding properties of the peptide and activity on the MSC Piezo1 [85]. For this, the simulations studied the effect of K to E mutations on the insertion depths and orientation of the peptide in the membrane and their binding affinities. The free energy profiles from the all-atom and CG simulations for the wild-type peptide were within 6 kJ/mol. The ΔG_b predicted from the CG simulations ranged from -18 kJ/mol to -27 kJ/mol while the corresponding values reported from Trp quenching experiments[85] were -25 kJ/mol to -26 kJ/mol. The $\Delta\Delta G_b$ for the K-E mutants predicted from simulations ranged from -2 kJ/mol to 4 kJ/mol while the $\Delta\Delta G_b$ from experiments was <1 kJ/mol for all mutants. This study demonstrates that it possible to predict ΔG_b that are in acceptable agreement with experiment but accurately predicting small

changes in ΔG_b caused by mutations is still mostly beyond the accuracies than can be achieve with this approach. Nevertheless, realistic ΔG_b values provide more confidence in the structural models obtained from the simulations and the molecular-level insights gained from them. In this study, analysis of the simulations revealed how K to E mutations in different parts of the peptide affected the insertion depths (in agreement with Trp fluorescence data) as well as the tilt angle and orientation of the peptide dipole. The results suggested that the Lys residues fine-tune the depth of penetration and orientation of the peptide and control the balance between a shallow binding mode, which is likely the functionally relevant mode for channel inhibition, and the deep binding mode.

4. Pore-forming venom peptides

Extensive research on the structure, activity and mechanism of AMPs over the past two decades has revealed that pore formation is a complex and highly dynamic, multi-step process [24-25, 27-28, 86]. While the detailed mechanism is peptide specific, the first step of pore formation involves the binding of the peptide to the membrane surface. This is followed by the re-arrangement and assembly of peptides in the membrane to form transient and/or more longer-lived pores that eventually grow into stable pores that disrupt membrane integrity. Because it is difficult to experimentally observe the peptide-induced pore formation at the molecular level, biomolecular simulations have played an important role in elucidating the MOA of pore-forming venom peptides and AMPs in general [24-26]. Yet, many details are still unknown including the exact size and shape for the pore, the number of peptides that line a pore as well as their position, orientation and relative arrangement. Also, the role of aggregation and cooperativity in pore formation is still unclear. While simulations can provide such information, it is precisely the absence of molecular-level structural data from experiments that makes simulations of pore-forming venom peptides so challenging as there often is little data from matching peptide-membrane systems that would allow for direct comparison of simulation and 'wet-lab' data. Also, the structural information obtained from experiments can also depend on the conditions (e.g. micelles *vs* lipid bilayers *vs* supported lipid monolayers) as well as the technique used. This is further complicated by the fact that until very recently, it was not possible to run simulations that are long enough to sufficiently sample the conformations and orientations of the peptide for a quantitative comparison to experimental data. Without such comparison, data from simulations often remains speculative. As pointed out in a

recent paper on the simulations of AMPs by Wang et al [72], "relatively little effort has focused on the validity of such simulations".

Over the years, many simulation studies have focused on modelling the initial absorption of the peptide to the membrane surface. Specific questions include identifying the conformational changes in the peptide induced by membrane binding, the insertion depth and orientation as well as potential aggregation of the peptide at the water-lipid interface. A series of recent studies [43, 45, 47] on the spontaneous binding of the bee venom peptide melittin to neutral phospholipid bilayers have demonstrate the insight that can be gained from atomistic simulations but also clearly highlighted some of the challenges. Andersson et al. [43] reported a 17-μs MD simulation of melittin binding to a DOPC membrane. The insertion depth of the peptide was in good agreement with data from X-ray diffraction measurements [87]. When using the CHARMM22/36 protein/lipid force field the per-residue helicity was in excellent agreement with amide exchange rates from NMR experiments [88]. In contrast, a 2-μs simulation with the OPLS force field failed to reproduce the per-residue helicity. In a similar study, Chen et al. [45] carried out a set of unbiased, ~2-μs long MD simulations to study the folding of melittin on a POPC membrane including the effect of peptide binding on the local structure of the membrane. Simulations were started from a fully extended peptide. The peptide was found to bind to the membrane surface in an unstructured or partially folded conformation, which was followed by rapid folding into a predominantly helical conformation. This is in agreement with data from previous NMR experiments. Furthermore, the simulations suggested that even in a folded and membrane-bound state the peptide remains flexible and samples a large range of conformations. To study the initial steps of membrane disturbance by melittin, Upadhyay et al. [47] carried out multiple unbiased simulations of ~2.5 to 4 μs. Multiple systems with different peptide-lipid (P/L) ratios and varying orientations and positions of peptides on the membranes were used. In general, the peptide remained buried at the water-lipid interface in agreement with previous simulations and experimental data [87]. Similar to the study by Chen et al. the simulations revealed multiple exchanges between different conformations. The findings by Upadhyay et al. and Chen et al. to some extend contradict the findings by Andersson et al, which stated that membrane-bound melittin can be "represented by a narrow distribution of folding states". However, while all three studies used long, unbiased MD simulations there are number of differences that complicate a direct comparison of results. These include, the lipids used (POPC

vs DMPC vs DOPC), the protein force fields (CHARMM22 with CMAP vs CHARMM27 vs CHARMM 36), P/L ratios, and different simulation temperatures. The issues of comparing data from different studies, even for the same peptide, are also demonstrated in a study by Wang et al. [72] in which simulations of four commonly studied AMPs using different force fields showed that results vary significantly between force fields. The combined results from these simulation studies also highlight the slow orientational and conformational fluctuations of peptides that fold at the water-lipid interface and the need for very long simulation times to sufficiently sample them.

Most simulation and 'wet-lab' experiments of melittin suggest that after the initial absorption the pre-dominant conformation of the peptide is one in which the peptide sits more or less parallel to the membrane surface. Thus, a potentially critical step for pore formation is the re-orientation of the peptide from a parallel to a more transmembrane orientation. Irudayam et al. [57] used umbrella sampling simulations to estimate the free energy barrier for this re-orientation of melittin in a POPC membrane for two different P/L ratios (1/128 and 4/128). For the lower ratio, the system contained a single peptide that was initially placed parallel to the membrane surface and the free energy barrier for its re-orientation was calculated. For the higher P/L ratio the system contained four peptides and the free energy barrier for the re-orientation of a single peptide with the other peptides being either all parallel to the membrane or with one already in a transmembrane orientation was calculated. The re-orientation barrier was the largest for a single peptide at a low P/L ratio (13.2 ± 0.8 kcal/mol). The presence of other peptides lowered this barrier to 9.6 ± 1.9 kcal/mol. A nearby peptide that is already in the perpendicular state further lowered this barrier to 5.1 ± 0.8 kcal/mol. This suggests that cooperativity is an important factor in pore-formation of melittin. These findings are consistent with another study by Lyu et al. [58] who used umbrella simulations to quantity the effect of the size of a transmembrane peptide aggregate on the barrier for the addition of another peptide in a DOPC/DOPG membrane. The results showed that the insertion of a single peptide in the absence of any other peptides has the largest free energy barrier (7.2 ± 0.8 kcal/mol). The barrier decreases with each additional peptide already present in a transmembrane orientation and approaches zero when a single peptide is added to an aggregate of six or seven peptides.

As the free energy barrier for the insertion of a single peptide into the membrane is high it has been suggested that the ability of AMPs to recognise local membrane defects or curvature is important for pore-formation [73-74, 89-91]. To gain a more detailed insight into this effect Sun et al. [51] carried out all-atom MD simulations of melittin and a Pro-Ala mutant thereof in the presence of a DPPC membrane. The simulations were started with four helical peptides placed on the membrane surface. Defects were created by pulling three adjacent lipids towards the membrane centre. If a peptide was found to insert into the defect, the external force was removed and the system was allowed to equilibrate for 2 μs. Interestingly, the defects were only short-lived (< 10 ns) and in most simulations the bilayer reformed once the external force was removed, even if a peptide was nearby. Only if the N-terminal of the peptide was close to the defect was the peptide inserted into the membrane. The defect remained open for long enough for other peptides to follow. This occurred on the timescale of hundreds of ns. The simulations further suggested that the Pro-Ala mutation changed the size of the pore consistent with previous data from experimental and simulation studies of melittin. In a similar study [66], the same authors used all-atom umbrella sampling simulations to investigate the free energy cost of pore formation of melittin in DPPC via a lipid flip-flop mechanism. For this, the hydrophilic head of a lipid molecule that is close to a surface-absorbed peptide, was pulled from the outer leaflet away from the peptide to the bilayer centre. As a comparison, the free energy barrier of a lipid flip-flop in a pure DPPC bilayer was calculated. For a pure DPPC bilayer the energy barrier was estimated to be 93 kJ/mol. The barrier was lowered to 64 kJ/mol for lipid flip-flops in the presence of melittin. This suggests that the rate of flip-flop is enhanced by 5 orders of magnitudes in the presence of a single melittin peptide. For pure DPPC the lipid flip-flop caused a water-filled membrane defect but no stable pore. In contrast, when the lipid flip-flop occurred close to a peptide adsorbed on the membrane surface the initial water-filled membrane defect ‘grew’ into a stable pore via the insertion of a peptide. This pore remained stable in a ~1μs-long unrestrained simulation.

Besides surface binding and pore formation, simulations have also been used to study the size, structure and stability of pre-formed pores. Mihajlovic et al. [50] carried out a series of all-atom simulations of pre-formed pores of melittin and five analogues in which 1 or 2 residues were mutated. The simulations suggested that in the case of melittin both charge distribution and imperfect amphipathicity favour the formation of toroidal pores. The stability of toroidal pore has also been reported an another study reporting a 9-μs all-atom simulations of melittin in DMPC [49].

Starting from a closely packed transmembrane tetramer a toroidal pore formed after ~ 1μs and remained stable for the remainder of the simulation despite one or more peptides frequently transitioning between a transmembrane and surface states.

5. Summary and outlook

Characterising the molecular details of how venom peptides interact with membranes will evidently help us to understand their MOA and understand the molecular origins of their biological activities. This will in turn facilitate the more rational design of peptides with specific properties. In this endeavour, biomolecular simulations have become a useful complement to 'wet-lab' experiments. Simulations enable us to study parts of a complex process in isolation or selectively control the environment and parameters that might affect it. Combined with the atomistic-level details of the resulting structural models, simulations can provide a molecular description of membrane binding that is difficult to achieve with 'wet-lab' experiments. Nevertheless, the complexity of venom peptide – membrane systems and the many technical challenges in simulating them makes validation *via* comparison to data from 'wet-lab' experiments critical. Yet until recently, it was not possible to perform simulation that were long enough to sufficiently sample even the seemingly simple process of a peptide binding the surface of a model membrane. As highlighted by the studies in this review, the task of validating simulations is further complicated by the fact that results from simulations of the same peptide-membrane system can depend on the force fields and simulation parameters.

Despite these challenges, the studies described in this review demonstrate that it is becoming increasingly feasible to carry out more realistic and reliable simulations of venom peptide – membrane systems. For GMs and MSC-directed peptides it would be very valuable to develop methods and protocols for the accurate and reliable prediction of relative binding free energies. Most current simulations calculate binding free energies using umbrella sampling with a one-dimensional reaction coordinate. Even very long simulations usually fail to sufficiently sample the rotational and translation motion of the peptide at the water-lipid interface. Combining umbrella sampling with other enhanced sampling methods or the use of different approaches is likely needed to obtain accurate free energies for a wide range of peptides. The development of such methods also requires a strong focus on experimental validation for matching peptide-membrane systems and the use of appropriate controls. Relative binding free energies predicted from simulations can

be compared to data from isothermal calorimetry, surface plasmon resonance or tryptophan quenching experiments. The residues predicted to form the main membrane contact surface can be validated by comparison to data from NMR titration experiments.

For pore-forming membrane peptides the increasing computer power means it will eventually become feasible to study the spontaneous formation of pores and the effect of cooperativity. The ability to perform multiple simulations in the μs range will enable the collection of statistics on the long-scale fluctuations and the complex interplay between peptide conformations, local membrane structure and the shape and size of the pore. To get a more complete picture of the energetic landscape of pore-formation reliable free energy calculation for the insertion and re-orientation of one or more peptides and the growth of pores are required.

An ongoing challenge for all venom peptide – membrane systems is the development of force fields that accurately describe peptide-lipid-water interactions. As pointed out in recent paper by Sadoval-Perez [71] et al. the development of force fields has mostly focused on reproducing properties of water-solvated proteins, pure and mixed membranes and membrane-embedded proteins. Thus, force fields for the accurate description of peptides at the water-lipid interface will require further development and testing. Finally, it would be useful to carry out simulation that systematically study the effect of lipid composition on peptide-membrane interactions and move towards simulations of venom peptides with membranes that are more realistic representations of mammalian and bacterial membranes. This in turn, relies on having force fields for a larger range of lipids and validated simulations of complex membranes. It is promising to see that over the past few years much progress has been made on that front. There are now force field parameters for a wider range of lipids [68, 92] including sterols, ceramides, sphingomyelin and cardiolipins enabling simulations of more complex membranes [41, 93-95]. In particular the presence of cholesterol, which is known to increase the rigidity of membranes, will likely affect the membrane-binding properties of peptides.

Eventually, a detailed understanding of venom peptide – membrane interactions requires a cross-disciplinary approach and simulation studies will always benefit from access to experimental data. Given the diversity of venomous animals in Australia it is not surprising that there are many research groups focusing on different aspects of venom peptides, as highlighted in this special

issue. Combined with our state-of-the-art supercomputing facilities Australia has the potential for a truly integrative and collaborative approach to study venom peptides.

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Figure legends

Figure 1. Schematic illustration of a gating-modifier peptide acting on a voltage-gated ion channel. In many cases, the modulation of the channel involves the binding of the peptide to the membrane surface, followed by binding to the membrane-embedded voltage sensing domain of the channel.

Figure 2. Schematic illustration of a disulfide-rich peptide acting on a mechanosensitive ion channel. In the absence of the peptide, the channel is activated by tension or stress in the surrounding membrane. The peptide inhibits the channel without physical contact by binding to the membrane where it reduces the formation of local stress thus preventing channel activation.

Figure 3. Schematic illustration of pore-forming venom peptides that disrupt cell membranes. The first step in pore formation is the binding of the peptide to the membrane, followed by re-orientation and/or aggregation of multiple peptides in the membrane.

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