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Teaser This review discusses the simulation techniques currently being used to study cell-penetrating peptides, along with the mechanistic insights gained and challenges associated with such studies.

The role of molecular simulations in understanding the mechanisms of cell-penetrating peptides

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Cell-penetrating peptides (CPPs) offer an exciting approach to tackle the pharmacokinetic challenges associated with the delivery of large, polar molecules to intracellular targets. Since the discovery of the first CPPs in the early 1990s, vast amounts of research have been undertaken to characterise their cellular uptake mechanisms. Despite this, the precise mechanisms of cellular internalisation of many CPPs remain elusive owing to inconsistent experimental results. Molecular dynamics (MD) simulations provide an approach to probe specific aspects of the internalisation process and many published CPP studies have incorporated simulation data. This review provides a critical evaluation of the current approaches that are being used to simulate CPPs interacting with artificial lipid bilayers.

Introduction

Cell-penetrating peptides (CPPs) are short, often cationic and/or amphipathic, peptides that can enter cells with minimal disruption to the cell membrane [1,2]. This is a desirable property for intracellular drug delivery systems, which are required to pull therapeutic cargo (such as proteins and siRNA) into the cell to reach their targets. The need for intracellular drug delivery is becoming increasingly evident because there is growing interest in using biopharmaceuticals and biomimetics to treat intracellular diseases (such as stapled peptides for the inhibition of protein–protein interactions) [3]. CPPs have been a topic of interest in this field since the discovery of two proteins that can enter cells *in vitro*: Tat, an HIV-1 transactivator protein, discovered in 1988 [4]; and Antennapedia, a homeoprotein of *Drosophila melanogaster*, discovered in 1991 [5]. Sequence research identified the short peptide sequences within the proteins that were responsible for their cellular uptake, leading to the discovery of the Tat peptide and penetratin [6,7] (Table 1). Since then, many other CPPs have been derived from proteins, synthesised or produced chimerically, and have been dubbed 'Trojan horse' molecules, owing to their ability to pull large, polar cargo

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TABLE 1

Examples of cell-penetrating peptides (CPPs)

Name	Origin classification	Physicochemical classification	Amino acid sequence	Net charge	Refs
Tat Peptide	Protein-derived	Cationic	YGRKKRRQRRR	+8	[6]
Penetratin	Protein-derived	Cationic/ secondary amphipathic	RQIKIWFQNRRMKWKK	+7	[7]
pVEC	Chimeric	Primary amphipathic	LLIILRRRIRKQAHAHSK	+8	[99]
Pep1	Chimeric	Primary amphipathic	KETWWETWWTEWSQPKKKRKV	+3	[100]
M918	Chimeric	Primary amphipathic	MVTVLFRRLRIRRACGPPRVRV	+7	[101]
TP	Synthetic	Secondary amphipathic	GWTLNSAGYLLGKINLKALAALAKKIL	+4	[102]
TP10	Synthetic	Secondary amphipathic	AGYLLGKINLKALAALAKKIL	+4	[103]
MAP	Synthetic	Secondary amphipathic	KLALKLALKALKAALKLA	+5	[104]
Cady	Synthetic	Secondary amphipathic	GLWRALWRLLRSLWRLLWRA	+5	[105]
Arg_9	Synthetic	Cationic	RRRRRRRRR	+9	[106]
Arg_{12}	Synthetic	Cationic	RRRRRRRRRRRR	+12	[106]
RW9 (R6/W3)	Synthetic	Cationic	RRWWRRWRR	+6	[107,108]
TP2	Synthetic	Hydrophobic	PLIYLRLLRGQF	+2	[21]
CC12	Synthetic	Proline-rich amphipathic	EMFTPPSMIERL	−1	[27]

into the cell [2] (Table 1). A comprehensive collection of CPPs that have been identified and studied can be found in the CPPsite2.0 database [8].

To design new CPPs for intracellular drug delivery, it is important to understand how they enter cells. Many experimental studies have aimed to characterise the cellular uptake of CPPs but, owing to differences in laboratory protocols, experimental conditions or assay types, conflicting results have emerged, and the topic remains controversial [9–12]. It is now widely believed that CPPs are internalised via numerous mechanisms and that a single peptide can participate in a number of pathways; the internalisation process can be modulated by altering the physicochemical properties of the peptide, peptide concentration, lipid composition or pH [11,13].

The ability to gain detailed mechanistic insights into these pathways has benefited from developments in the applications of computational methods such as molecular dynamics (MD) simulations. The development of MD methods (Box 1) and biomolecular force fields (Box 1) has provided the means to probe biological mechanisms at the atomistic level [14]. What is more, the ability to control or alter simulation conditions, such as temperature, ion concentration and pH, has the potential to allow researchers to monitor the effects of these parameters closely and disentangle the controversies surrounding experimental results. However, simulations directed at understanding the interactions of CPPs with models of cellular membranes (CPP–lipid simulations) come with their own set of challenges, including the limited accuracy of biomolecular force fields, the difficulties associated with sampling the full or relevant parts of the underlying energy landscape and the simplified models of cell membranes [15–17]. To obtain valid and useful results from CPP–lipid simulations, these issues must be addressed. As a result, several CPP simulation studies have been published that implement a variety of intelligent techniques to probe CPP translocation across models of membranes. Each technique comes with its own advantages and challenges. This review will provide a brief description of CPP properties and their experimentally deduced uptake routes and applications, followed by an in-depth discussion of the various simulation techniques that are being used to probe their membrane penetration mechanisms. Specific attention will be paid to how the aforementioned simulation challenges are being tackled.

Experimental knowledge of CPPs

Physicochemical properties

CPPs can be classified based on their physicochemical properties, with categories including cationic, amphipathic and hydrophobic peptides (Fig. 1, Table 1) [18]. Cationic CPPs contain a stretch of positive charges essential for cellular uptake and are generally unstructured [18]. Amphipathic CPPs can be divided into four subcategories:

i Primary amphipathic peptides, which have defined hydrophobic and cationic domains.
ii Secondary amphipathic peptides, which form α-helices with one hydrophobic face and one hydrophilic face.
iii Amphipathic β-sheet peptides, which have a hydrophobic stretch and a hydrophilic stretch.
iv Proline-rich amphipathic peptides, which form polyproline II (PPII) structures.

The cell-penetrating properties of amphipathic peptides can be attributed to their ability to interact with the polar head and lipid chain regions of cell membranes [19]. The final class of CPPs are the hydrophobic peptides, which contain mainly hydrophobic residues or have a hydrophobic stretch essential for cellular uptake [18]. The uptake mechanisms of hydrophobic peptides have been less well studied; however, it is proposed that they are able to undergo energy-independent translocation across cell membranes owing to their favourable interactions with the hydrophobic membrane interior [20,21]. Although the majority of the CPPs documented in the literature are positively charged, a small set of anionic peptides has also been reported [18].

Applications

There are a vast range of potential applications of CPPs and many of these have been studied and reviewed in the literature [22]. An important application is the intracellular delivery of therapeutic agents in the treatment of cancer. For example, CPPs have been effectively conjugated (covalently and noncovalently) to siRNAs, increasing the ability of the siRNA to enter the cell and modulate

BOX 1

Simulation methodology descriptions

Molecular dynamics (MD): a simulation technique used to generate the dynamic evolution of atoms in a molecular system through time [93]. MD codes implement iterative algorithms that calculate atomic positions, velocities and accelerations, using Newton's laws of motion.

Forcefield: the underlying equation and parameters that describe the energy of the system based on the interactions felt by each atom [93]. The equation includes terms that model all the bonded and nonbonded interactions; for example, bond stretching, angle bending, electrostatic interactions, among others. The atomic energies provided by the force field are used to derive the forces, which are consequently used in the MD simulation.

Collective variable (CV): a parameter of the system that describes the process of interest, such as the distance between two groups. The purpose of an MD simulation is to sample data across a range of values of the CV; enhanced sampling methods might be required to achieve this (see below).

Metadynamics: a technique used to increase system sampling along a defined CV by applying a biasing force that drives the simulation away from previously visited areas of the energy landscape [94]. The biasing force is in the form of energy Gaussians (or hills), which are deposited throughout the simulation to fill the over-explored energy minima and push the system over energy barriers into different areas of the CV space. As a result, the deposited hills provide a negative imprint of the energy landscape with respect to the CV, much like sand deposited in a sand pit [94].

Umbrella sampling (US): another technique that increases sampling along a defined CV. The approach involves running multiple simulations with the system restrained at given values of the CV, to collect data at the different values [95]. These data are then reweighted to remove the restraining bias, using methods such as the weighted-histogram analysis method (WHAM) [96], and combined to produce a potential of mean force (PMF) profile, providing the free energy surface associated with different locations along the CV.

Replica exchange methods: a series of enhanced sampling techniques that increase conformational sampling. The simplest example is temperature replica exchange MD (T-REMD), where multiple replicas of the system are run in parallel: a base replica at the temperature of interest, and a series of replicas at higher temperatures that can access more areas of the conformational landscape [97]. Coordinate swaps between adjacent replicas are attempted frequently throughout the simulation based on a Metropolis acceptance criterion (a criterion that assesses the thermodynamic probability of the move), such that the base replica is able to 'jump' over energy barriers and access different energy minima [97]. Other replica exchange methods define the replicas in different ways; for example, replica exchange with solute tempering (REST) scales the interaction parameters to create an artificially 'hot' solute in a 'cold' solvent (the hot solute has more conformational flexibility) [77].

Coarse-grained (CG) models: molecular systems described using interaction beads that represent groups of atoms [98]. CG reduces the number of particles in the system and allows larger MD time steps to be used, as the lower resolution removes the high frequency motions. As a result, computational efficiency is increased and bigger systems and longer timescales can be accessed. CG models are parameterised to reproduce experimental and/or atomistic MD observations.

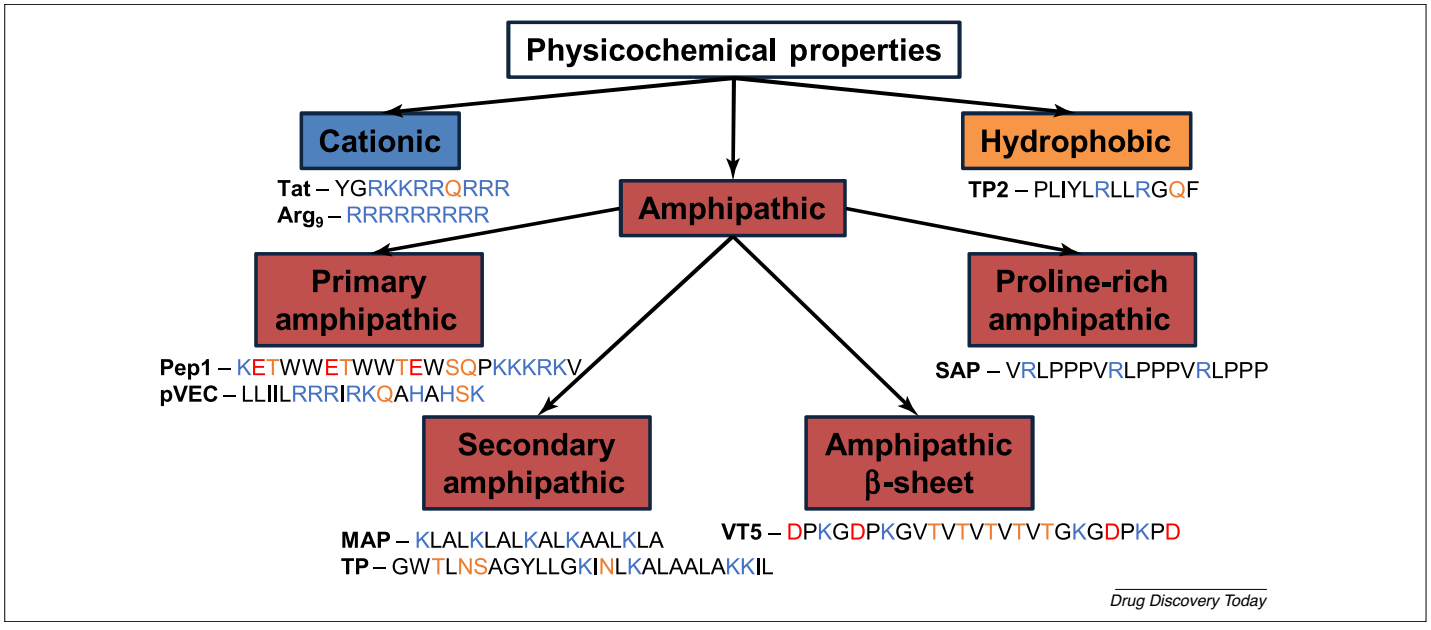

FIGURE 1

Classification of cell-penetrating peptides (CPPs) based on their physicochemical properties, including examples. Blue residues are cationic, red are anionic and orange are polar.

function [22,23]. Hyun *et al.* recently developed moderate RNA-binding peptides that can also act as CPPs, meaning they are able to enter the cell and release siRNA into the cytosol [24]. The library of peptides was lysine- and leucine-rich and incorporated hydrocarbon staples, and the peptides were demonstrated to chaperone the siRNA into cells [24]. CPPs can transport many other classes of molecules with anticancer properties into cells, such as proteins, plasmid DNA and oligonucleotides, and have often demonstrated

selectivity for cancer cells over healthy cells [23,25]. This selectivity can also be utilised to deliver imaging agents to tumour cells. For example, Du *et al.* found that the oligoarginine R11 can deliver a fluorescent probe to bladder tumours, producing a more intense signal than that seen in normal bladder tissue [26]. More-extensive reviews of the applications of CPPs as anticancer therapies and diagnostics can be found in Refs. [22,23].

CPPs also have applications in other disease areas. Chen *et al.* demonstrated the ability of the CC12 peptide (Table 1) to deliver antiangiogenesis peptides to the posterior segments of the eye for the treatment of neovascular fundus diseases via topical application [27]. CC12 forms an amphipathic, PPII helix that can permeate through the multiple barriers of the eye before reaching the retina. Furthermore, CPPs are being developed as potential vehicles to allow oral delivery of insulin. Fukuoka *et al.* studied the effects of co-administering insulin with oligoarginines loaded into hydrogels [28]. They demonstrated that the combination results in improved intestinal absorption of insulin in rats via a noncovalent CPP uptake strategy. More-general applications of CPPs can be found in Ref. [22].

Finally, it is possible that certain peptides can possess cell-penetrating and therapeutic properties. Chang *et al.* published a study that optimised stapled α-helical peptides to work as dual inhibitors of the MDM2 and MDMX p53 regulatory proteins [29]. The lead peptide, ATSP-7041, was a hydrophobic stapled peptide and showed proficiency at entering living cancer cells and inhibiting MDM2 and MDMX. The combination of favourable pharmacokinetics, cell penetration and target binding resulted in suppression of tumour growth *in vivo*.

Cellular uptake mechanisms

Cellular uptake mechanisms of a CPP can vary depending on the experimental protocol and conditions. In fact, it is common to observe energy-dependent and energy-independent internalisation of the same CPP. The former involves endocytosis and subsequent release from the endosome, whereas the latter involves direct translocation, pore formation or physical endocytosis (Fig. 2). These processes can occur via numerous mechanisms, which will be described here.

To probe endocytotic uptake of CPPs, studies have utilised various endocytic markers, fluorescent probes or have been performed in conditions that prohibit endocytosis [10,30]. Using specific endocytic markers, Lundin *et al.* demonstrated the presence of three distinct endocytosis pathways for CPPs: macropinocytosis, caveolae endocytosis or clathrin-mediated endocytosis (CME) [10]. Macropinocytosis is caused by the rearrangement of cytoskeleton leading to ruffling of the cell membrane to form sheet-like extensions that fold around and

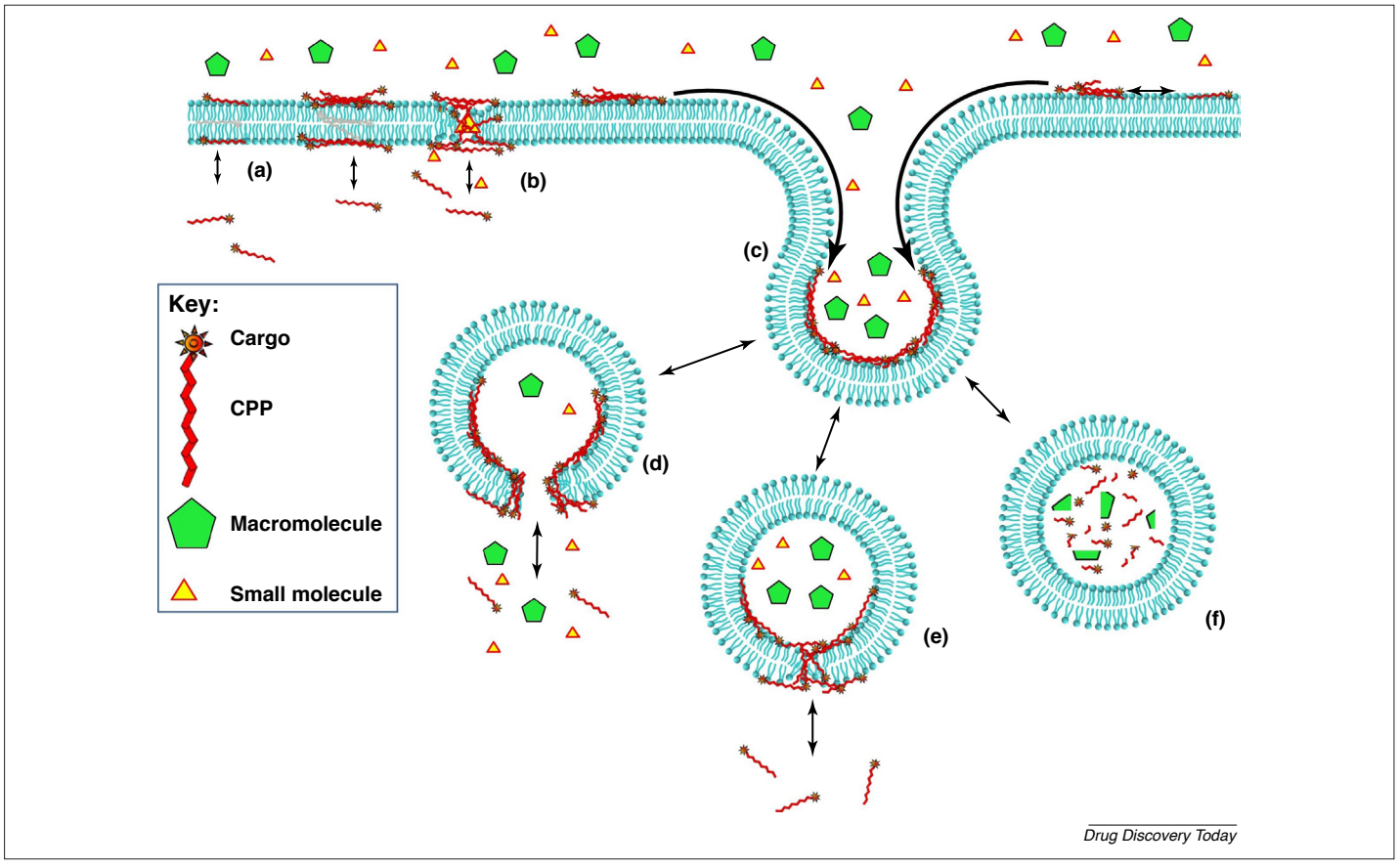

FIGURE 2

Internalisation pathways for cell-penetrating peptides (CPPs). **(a)** Spontaneous membrane translocation. **(b)** Peptide aggregation leading to direct penetration or pore formation. **(c)** Endocytosis. **(d)** Endosomal membrane lysis. **(e)** Translocation across endosomal membrane. **(f)** Degradation and/or recycling of endosomal CPP. Image reprinted from Trends in Biochemical Sciences, 50, B. Kauffman *et al.*, Mechanism matters: a taxonomy of cell penetrating peptides, 749-764, Copyright (2015), with permission from Elsevier [11].

non-selectively entrap extracellular fluid and particles in macropinosomes [31]. Lundin *et al.* and Duchardt *et al.* showed that cationic CPPs can be internalised via this mechanism [10,32]. Caveolae endocytosis involves the clustering of caveolin-1 proteins and the formation of flask-shaped invaginations in lipid raft regions of the cell membrane. Phosphorylation of caveolin-1 then leads to internalisation of the vesicle [33,34]. Caveolae endocytosis was observed by Fittipaldi *et al.* and Duchardt *et al.* for cationic peptides, such as Tat and Arg9 (Table 1) [32,33]. Finally, CME involves recognition of internalisation cargo by membrane-bound receptors, resulting in membrane curvature and the formation of an endosome [35]. Lundin *et al.* observed CME as the main cellular uptake pathway for amphipathic CPPs [10]. For CPPs to reach the cell cytosol, it is important that the peptide is released from the endosome; this can involve endosomal membrane disruption or direct translocation across the endosomal membrane (Fig. 2d,e) [11].

Energy-independent mechanisms (Fig. 2a,b) have also been observed using experiments that utilise artificial lipid membranes or that inhibit endocytosis [13,30]. These mechanisms include direct penetration (with very little disruption to the membrane), pore formation (with varying degrees of membrane disruption) or physical endocytosis (with vast membrane deformations) [11]. Direct penetration is an attractive route for the design of intracellular delivery vectors, owing to the low likelihood of causing cell leakage or cytotoxicity [36]. Swiecicki *et al.* used large unilamellar vesicles (LUVs) with a combination of anionic and zwitterionic lipids to observe direct translocation of Tat, Arg9 and R6/W3 (Table 1) [12]. This occurred via aggregation of the peptides on the membrane surface, leading to lipid flip-flop, where the anionic lipids chaperone the positive charges in the peptide across the hydrophobic membrane interior. Maniti *et al.* observed spontaneous translocation of penetratin, Arg9 and RW9 (Table 1) in plasma membrane spheres (PMSs) but also observed endosome-like invaginations in the membrane, along with external budding and tubulation [37,38]. The authors suggested a physical endocytosis mechanism, which involves membrane manipulation via the formation of large peptide aggregates on the membrane surface, leading to electrostatic and H-bonding interactions that induce negative or positive curvature [37–39]. Marks *et al.* also identified a series of peptides, including TP2 (Table 1), observed to spontaneously translocate across artificial and cell membranes [21]. The spontaneous membrane-translocating peptides (SMTPs) are shown to internalise inside cells via nonendocytic mechanisms and with no concentration dependence, suggesting that uptake happens via direct translocation as monomers [21,30]. What is more, the SMTPs were shown to efficiently pull large, polar cargo inside the cell, meaning they have ideal properties as intracellular drug delivery systems.

Finally, CPPs are also hypothesised to enter cells via pore formation (Fig. 2b), specifically via toroidal pores [13]. Although the structures of the pores are similar to those proposed for antimicrobial peptides (AMPs) [40], CPPs cause less cell leakage and cytotoxicity, because the pores are smaller and shorter lived [41]. Di Pisa *et al.* also proposed a carpet model or barrel-stave model resulting in CPP-induced pores; however, these models are more commonly associated with AMPs [13]. Islam *et al.* observed translocation of TP10 (Table 1), followed by TP10-induced pore formation in 80/20 DOPG/DOPC giant unilamellar vesicles (GUVs), using a fluorescent probe and confocal microscopy [42].

In summary, numerous energy-dependent and energy-independent cellular uptake mechanisms have been determined for CPPs. However, the experimental techniques used to characterise these mechanisms do not provide atomistic information and the molecular interactions that occur between CPPs and lipid bilayers remain uncharacterised. MD simulations provide the means to gain insight into these atomistic interactions and probe the various energy-independent mechanisms. Although endocytosis is not currently accessible to simulations, owing to the energy-dependence and large scales, MD simulations provide a way to study the interactions of the peptide with the membrane that could be crucial to allow the peptide to escape from the endosome. The next section will discuss the simulation techniques that are currently being used to study CPP translocation. Box 1 describes the various simulation methodologies discussed throughout this review, whereas Table 2 summarises the example usages, advantages and disadvantages and software implementations of each technique.

Techniques for CPP simulation

Force fields

To simulate CPP–lipid systems, biomolecular force fields (Box 1) have been developed at varying simulation scales; examples inlcude the atomistic Amber and CHARMM series [43,44], the united atom GROMOS series [45] and the coarse-grained (CG) MARTINI and SIRAH series [46,47]. The CG simulation method is described in Box 1 and discussed in more detail below. The choice of simulation scale depends on the system size, timescales and resolution needed to observe the biological phenomena of interest [48]. The parameterisation of atomistic force fields involves fitting to *ab initio* and experimental data, whereas CG force fields can be parameterised using 'bottom-up' (fitting to atomistic simulation data) or 'top-down' (fitting to experimental data) approaches. More extensive discussions of the development of biomolecular force fields can be found in several excellent references [17,48].

The ability of a force field to recreate experimental results can greatly depend on the fitting procedure and data utilised, meaning different force fields can often result in different simulation outcomes. Various studies have compared the different protein–lipid force fields and often conclude that the best choice depends on the system and the nature of the calculated property [15,16]. Piggot *et al.* performed a comprehensive comparison of five lipid force fields from the GROMOS [45], CHARMM [44] and Berger [49] series by simulating DPPC and POPC bilayers and comparing various calculated and experimental physical properties [15]. The study concluded that the calculated physical properties of lipid membranes depends substantially on the choice of force field and, although a 'best' force field was not determined, certain ones are better or worse for certain measurements. Sandoval-Perez *et al.* also published a comparative study of common protein–lipid force fields, specifically calculating protein interactions with lipid bilayers [16]. In this study, GROMOS 54A7 [50], CHARMM36 [51], Amber14SB/Slipids [52] and Amber14SB/Lipid14 [43,53] force fields were used to compare membrane protein secondary structure conservation, transmembrane (TM) peptide positioning and free energy of amino acid adsorption. The study showed that Amber14SB/Slipids, Amber14SB/Lipid14 and CHARMM36 performed

TABLE 2

A summary of the molecular dynamics (MD) methods for cell-penetrating peptide (CPP)–lipid simulations discussed in this review, including their features, example usages, advantages, disadvantages, software implementations and references

Method	Features	Example usages	Advantages	Disadvantages	Software	Refs
Atomistic MD	Unbiased time evolution of atoms in a molecular system	• To study peptides interacting interfacially with lipid bilayers • If *a priori* knowledge is available, use to corroborate results, for example place a peptide inside a membrane or pore using experimentally derived conformations or configurations and monitor the behaviour	• Samples configurations based on the force fields and starting coordinates • Easy to analyse	• Computationally expensive • Convergence issues • Unlikely to sample full CPP translocation event • Systems likely to get trapped in local energy minima	• GROMACS • Amber • CHARMM • NAMD • LAMMPS	[109] [110] [111] [112] [113]
HT-MD	Perform simulation at elevated temperature	• To study known thermostable TM helices	• Speeds up the kinetics of peptide insertion into the membrane • The peptide is able to flip between thermodynamic minima multiple times, enabling an extrapolation of the Arrhenius equation to calculate energy barriers at relevant temperatures	• Unrealistic dynamics of peptide in water • Force fields were not parameterised or tested at high temperatures; artefacts can occur • Need to prove peptide thermostability in membrane using experimental approaches to be sure that the simulation samples relevant states	• GROMACS • Amber • CHARMM • NAMD • LAMMPS	[70]
Steered atomistic MD	Apply a harmonic force between two groups and pull together	To provide starting configurations for umbrella sampling of: • a peptide passing through a membrane • peptides coming together in an aggregation event Atomistic or coarse-grained models can be used	• Can force the peptide through the lipid bilayer • Speeds up translocation event	• Non-equilibrium dynamics • The slow changes in peptide conformation as it interacts with the membrane will be missed • Might not sample the most relevant free energy path; important minima could be missed	• GROMACS • Amber • CHARMM • NAMD • PLUMED (MD engine patch) • LAMMPS	[114]
Umbrella sampling	Collect data in windows along a chosen CV; provides PMF	• To sample a peptide moving through a bilayer • To sample peptides coming together in an aggregation event • Atomistic or coarse-grained models can be used	• Increases sampling along the reaction coordinate • Forces the system to sample unfavourable areas of the energy landscape • Allows the construction of a PMF along the CV	• Difficult to choose a CV that sufficiently describes the reaction path, for example sampling the centre of mass (COM) distance between a peptide and a bilayer might neglect peptide folding and aggregation events that occur in the process • Often many umbrella sampling (US) windows are required, resulting in great computational expense	• GROMACS • Amber • CHARMM • NAMD • PLUMED (MD engine patch) • LAMMPS	[95]
Metadynamics	Force the system to sample unvisited areas of the energy landscape by adding a history-dependent bias	• To sample a peptide moving through a bilayer • To sample peptide folding events • To sample peptides coming together in an aggregation event • Atomistic or coarse-grained models can be used	• Increases sampling along a reaction coordinate • Forces the system to sample unfavourable areas of the energy landscape • Allows the construction of a PMF along the CV • Less expensive than US as the CV is sampled in one simulation • Multiple CVs can be sampled (either in one simulation or using replicas)	• Difficult to choose a CV that sufficiently describes the reaction path • Difficult to choose other parameters, for example the height or width of the energy bias and the pace at which the bias is added • Difficult to know when all areas of the energy landscape have been explored	• GROMACS (patched with plumed) • Amber [adaptively biased molecular dynamics (ABMD)] • CHARMM [adaptively biased path optimisation (ABPO)] • NAMD • LAMMPS	[94]

TABLE 2 (*Continued*)

Method	Features	Example usages	Advantages	Disadvantages	Software	Refs
Replica exchange methods	Simulate replicas of the system in parallel, with properties (e.g., temperature or interaction energies) of higher order replicas scaled to allow more sampling. Perform exchanges between replicas	• To sample peptide folding events • To sample peptide aggregation events	• Increases conformational sampling in an unbiased way • No user-defined CV needed	• The use of multiple replicas results in increased computational expense	• GROMACS • Amber • CHARMM • NAMD • PLUMED (MD engine patch) • LAMMPS	[97] [77]
Coarse-grained MD	Model groups of atoms as interaction beads	• To study large peptide–lipid systems at longer timescales	• Computational expense reduced • Can study large systems and long timescales	• Lower resolution means atomistic information is missed • The energy landscape is smoothened so the kinetics are scaled in an unpredictable way • Parameterisation and unrealistic atom grouping may lead to artefacts	• GROMACS • CHARMM • NAMD • LAMMPS	[98]
Implicit membrane	Model the solvent and bilayer as a continuous medium	• To study the peptide behaviour as it interacts with media of different dielectric constants	• Computational expense greatly reduced • Atomistic peptide conformational sampling is increased	• Major over-simplification of the membrane • Important atomistic interactions are neglected	• CHARMM	[89]

well in secondary structure conservation and were able to predict TM peptide orientation and insertion depth, whereas GROMOS 54A7 performed poorly. Furthermore, CHARMM36 was best able to predict adsorption free energies of Wimley White peptide amino acids [54], closely followed by Amber14SB/Lipid14, whereas Amber14SB/Slipids and GROMOS 54A7 each performed significantly worse [16]. In summary, it is advisable for researchers to use multiple force fields to compare simulation and experimental observations to choose the force field most suited to describe their system.

Atomistic MD

The first atomistic MD simulations (Box 1, Table 2) of spontaneous CPP translocation events were published by Herce and Garcia [55]. Multiple Tat peptides were placed on one side of a DOPC bilayer and atomistic MD was performed using the Berger lipid force field [49]. The peptides were observed to insert under the phospholipid head groups, making favourable H-bonding and electrostatic interactions with the phosphates. As additional peptides were added to the system, they accumulated under the phospholipid head groups and the increased positive charge attracted the phosphates from the opposite leaflet of the bilayer, resulting in membrane thinning. Eventually, an arginine side chain was able to reach across the hydrophobic membrane interior and pull a phosphate head group from the opposite leaflet up into the lipid chain region. This resulted in the formation of a toroidal pore, with the peptides at the centre, after only 400 ns of simulation [41,55]. It should be noted, however, that these simulations were performed in the absence of neutralising counterions, which has been shown to cause membrane thinning and destabilisation owing to the charge imbalance across the bilayer [56]. This method is useful as an approach to increase the likelihood of peptide-induced pore formation, allowing the study of pore structure and peptide insertion into the pore, but the observations should be treated with caution owing to the artificial charge imbalance.

Self-assembly approach

Atomistic MD has also been used as an *a priori* approach to predict the position and orientation of peptides in membranes. For example, Farrotti *et al.* studied the designed peptide LAH4 (KKALLALALHH-LAHLALHLALALKKA-NH2), which is known to possess AMP or CPP activity based on pH [in acidic conditions the histidines are cationic (LAH4-c) and the peptide acts as an AMP, whereas in basic conditions the histidines are neutral (LAH4-n) and the peptide acts as a CPP] [57]. The authors comment on the sampling difficulties associated with peptide insertion into a preformed bilayer and instead utilise an atomistic self-assembly approach. This involved randomly placing the helical peptide, POPC lipid molecules, water and neutralising ions in the system and observing self-assembly of a POPC bilayer with the peptide embedded. An annealing approach, where the system was heated from 300 K to 375 K and then cooled back to 300 K multiple times, was needed to observe bilayer formation in the presence of the peptides. This approach was successful in identifying the different observed experimental behaviours of LAH4-n and LAH4-c, with the neutral histidine species forming a TM helix resulting in little bilayer disruption, and the cationic histidine species sitting approximately parallel to the membrane surface and causing more bilayer disruption [57]. The study also involved other simulation methods, namely umbrella sampling (US) and CG MD that are discussed in subsequent sections, to corroborate the results.

Atomistic MD with initial configurations selected using a priori knowledge

Atomistic simulations carried out with the CPPs placed near the membrane usually result in the peptide engaging in occasional binding interactions with the phospholipid head groups, yet spontaneous pore formation or translocation is unlikely to occur within the timescales currently accessed by MD simulations [56]. However, there have been a number of CPP simulations reported in the literature that probe the membrane translocation mechanism by selecting the initial configuration based on *a priori* knowledge [58–62]. This requires knowledge derived from experiments or previous simulations.

One method for investigating the ability of CPPs to stabilise membrane pores is to place the peptide inside a pre-formed pore and monitor the rate at which the pore closes. This is a useful technique if the translocation mechanism has been shown to involve pore formation experimentally. Akhunzada *et al.* used this approach to study the effects of Tat monomers, Tat-TAMRA monomers and Tat-TAMRA dimers on the stabilisation of DOPC pores [58]. First, the configuration of the Tat dimers was taken from a previous study by Macchi *et al.* that had shown that dimerisation is the main form of Tat aggregation using experimental and computational approaches [63]. In this aggregation study, UV-vis and NMR were used to prove the existence of Tat dimers, whereas metadynamics (Box 1, Table 2) was used to predict the dimer configurations. The inter-peptide contact number was set as the collective variable (CV) (Box 1) for the metadynamics, effectively pulling the monomers together. High-contact structures were extracted and used to seed MD simulations to assess the stability of the dimers [63]. The stable dimers identified in this study were pulled to the centre of a pre-equilibrated 30 Å DOPC pore, which was formed by inducing surface tension in the membrane and then restraining the pore while simulating the membrane back to equilibrated conditions [58]. Next, unconstrained MD was performed to observe the effect of the peptides on the pore. Monomers were shown to translocate out of the pore after 450 ns, stabilising the pore for much longer than the membrane-only simulation, in which the pore closed after 30 ns. The dimer had even stronger interactions with the lipids and stabilised the pore across 1 μs [58]. These simulation studies proved useful in generating Tat dimer configurations and testing the stabilisation of DOPC pores by Tat monomers and dimers and are a good example of how MD simulations can be used in conjunction with experiments. Nevertheless, it might be interesting to investigate the dependence of dimer configuration prediction on the choice of CV, specifically whether the inclusion of different peptide-folding CVs could cause the peptides to fold and dimerise in different ways.

Other simulation studies have also used this approach to investigate the stabilisation of pores by CPPs. For example, Sun *et al.* simulated Arg8 at the centre of a DPPC bilayer pore by removing the restraint from the last frame of a US window (US is discussed in more detail in a later section and in Box 1) [59]. They found that the interactions between Arg8 and the lipids in the pore stabilised the system throughout a 200 ns simulation. This is in contrast to the pore closure observed after 5 ns in a system with no peptides. Sun *et al.* used this approach again to study the differences in the abilities of Arg8 and Lys8 to stabilise pores in DOPE/DOPS bilayers [60]. They found that a pore with no peptide closed after 30 ns but a pore with an Arg8 peptide at the centre remained open for a full 800 ns simulation. A pore with Lys8 at the centre closed after only 10 ns, consistent with experimental evidence that Lys8 is a much less efficient CPP than Arg8 [64]. This can be attributed to the fact that the bidentate guanidinium side chain of arginine is able to form stronger interactions with the phosphates in the lipid head groups than the amino side chain of lysine [65].

Experimental structures of CPPs, when available, are used as initial starting configurations for atomistic MD. For example, Bera *et al.* derived the 3D structure of the penetratin-derived peptide DK17 (DRQIKIWFQNRRMKWKK) in the presence of different artificial lipid membranes using NMR, and then used these structures as initial conformations in atomistic simulations [61]. They also used the Orientation of Proteins in Membranes (OPM) server to predict the orientation of the derived peptide structure in the membrane, giving the initial simulation configurations [66]. Briefly, the OPM server allows users to access a database of predicted TM protein spatial arrangements in membranes [66]. The database was built by minimising the transfer free energies from water to a hydrophobic slab of all the TM proteins in the protein data bank (PDB) [67]. Bera *et al.* performed 100 ns of atomistic simulation and monitored the stability of the secondary structure of the peptide in the membrane, revealing that the peptide had different preferential structures in different membrane compositions [61].

Grasso *et al.* also recently used experimental NMR structures of the Tat peptide and penetratin, along with the OPM server, to generate initial peptide-embedded DOPC configurations for atomistic simulations [62]. They also studied Arg9, Cady, Pep and MAP (Table 1) embedded in DOPC with initial conformations predicted by the PEP-FOLD 3 server [68]. The CPP-DOPC systems were each simulated without restraints for 200 ns and peptide and membrane properties were monitored, revealing different residing depths and orientations for each CPP in the membrane. They also linked the physicochemical properties of the CPPs to their effects on the membrane-bending modulus, finding that more-hydrophilic peptides encouraged more water molecules to enter or remain inside the membrane, leading to increased membrane destabilisation [62].

The studies discussed above are examples of how MD simulations can validate experimental results and provide additional insights. However, it is important to note that observations taken from atomistic peptide–lipid MD simulations are likely to depend on the initial conformation/configuration and force field used. It is advisable for atomistic simulation studies to include repeat simulations starting from independent starting configurations and different force fields, to statistically validate the conclusions drawn. Furthermore, to derive valid thermodynamic and kinetic information about a process, multiple, reversible events must occur within the simulation; for example, pore formation and closure must happen several times to ensure statistical convergence. It is very unlikely that atomistic MD can achieve the timescales needed to observe this with the computational resources currently available. However, advancements in computer technology, such as the purpose-built Anton supercomputer for MD simulations, promise longer timescales becoming accessible [69].

High temperature MD (HT-MD)

Ulmshneider *et al.* published a series of studies that utilise HT-MD (Table 2) to investigate membrane-active peptides (MAPs), such as TM helices and AMPs, and have suggested that the technique could be

extended to study CPPs [70–72]. The principle behind the technique is to simulate peptide–lipid systems at an increased temperature to speed up the kinetics involved with peptide folding on the membrane surface and insertion into the bilayer. TM helices are known to be unstable in aqueous solution but are stabilised by the membrane, as a result of the increased resistance to break favourable backbone H-bonds in a hydrophobic environment [71,72]. Simulations and circular dichroism (CD) experiments of TM helices at varying temperatures have shown that their structures are thermostable in the membrane, suggesting that increased simulation temperatures can be used without altering the preferred structure of the embedded peptide [71,72]. Wang *et al.* used HT-MD in conjunction with various experiments to study the effect of the AMP maculatin (GLFGVLAKVAAHVV-PAIAEHF-NH_2) on lipid bilayers [72]. Using simulation temperatures of up to 150 °C, the simulations in this study were initiated with multiple surface-bound helices on a range of phosphatidylcholine lipid membranes and were able to capture transmembrane insertion and multiple pore formation events over 10–20 μs. The simulations revealed a variety of oligomer and pore structures and numerous pore-forming mechanisms (Fig. 3) [72]. Given that most force fields were not parameterised at such high temperatures, more studies may be needed to establish the accuracy of their performance. Also, although it is shown that the secondary structures of TM peptides are thermostable in the membrane, the nature of the thermodynamic landscape at high temperatures needs more investigation.

Steered MD

Steered MD or pulling simulations (Table 2) provide a way to force the peptide across the membrane. This often involves harmonically restraining a part, or the centre of mass (COM), of the peptide to the COM of the lipid bilayer and applying a force that pulls the two groups closer. Alaybeyoglu *et al.* performed steered MD of pVEC (Table 1) across a POPE bilayer [73]. The peptide was placed above the membrane in a random coil position, with a β-turn at residues Arg8 to Lys11, and a force was applied to the N terminus C_α atom to pull the peptide to the centre of the membrane. The force needed to pull the peptide through the membrane and the presence of water molecules in the bilayer were monitored. From this study, Alaybeyoglu *et al.* suggested a translocation mechanism by which pVEC initially binds to the negative phospholipid head groups, via electrostatic interactions of its cationic stretch with the negative phosphates, followed by the formation of a water defect in the membrane that shields the cationic residues from the hydrophobic core [73]. However, the dynamics observed from steered MD do not represent equilibrium dynamics and it is possible that mechanistic details taken from these simulations are artefacts of the force applied to the N terminus. The study did not generate a potential of mean force (PMF) via US and just deduced mechanistic detail by assessing the pulling force needed to keep the peptide moving at a constant rate. In addition, the energy landscape associated with peptide folding and membrane translocation is complex and it is likely that longer simulations will be needed to capture the peptide conformational changes that could occur as it interacts with the membrane. We would like to note that the authors of the steered MD study discussed here also released a later study in which they more thoroughly sampled pVEC passing through a POPE bilayer by using a US approach [74]. US is discussed in more detail in the following section.

Atomistic US

A more stringent approach to investigate the mechanism and energy associated with peptide translocation is to use snapshots from pulling simulations to perform atomistic US (Box 1, Table 2), as has been shown by several studies [56,59,60,75]. The general approach is to set the US CV as the distance, z, between the COM of a peptide and the COM of a lipid bilayer. Initial configurations along z are generated by pulling the peptide to the centre of the bilayer or by growing the peptide into the membrane at different z values. These initial configurations are then used to seed US simulations, as described in Box 1. The PMFs produced by performing US of CPPs passing through a bilayer often show similar characteristics: an energy minimum is observed when the peptide binds to the membrane surface, followed by a steep increase in energy as the peptide is introduced into the hydrophobic membrane interior and a kink in the PMF is observed if a water pore is formed (Fig. 4).

In 2013, Huang and Garcia used US to investigate the movement of cyclic Arg9 across a DOPC bilayer [75]. The study used two different approaches to pull the peptide into the membrane:

i The Cz atom of one arginine residue was restrained to the COM of the bilayer.

ii The Cz atom of one arginine residue was restrained to the centre of weighted mass (COWM) of the lower bilayer

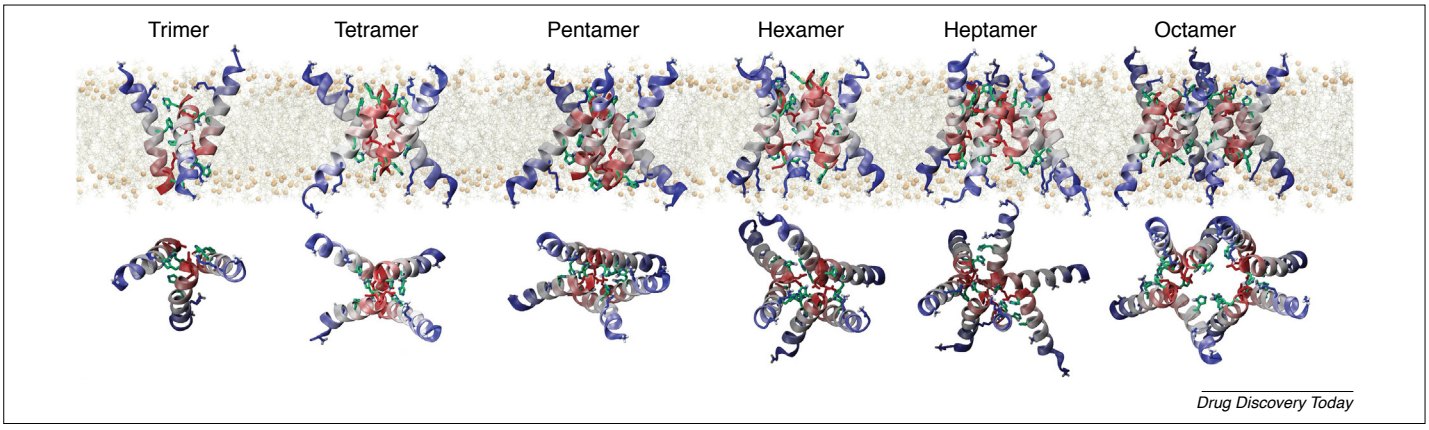

FIGURE 3

Snapshots of the most populated transmembrane oligomeric structures of maculatin in phosphatidylcholine bilayers, formed in a high temperature molecular dynamics (HT-MD) simulation. Image modified, with permission, from Ref. [72] (licence available at https://creativecommons.org/licenses/by/4.0/).

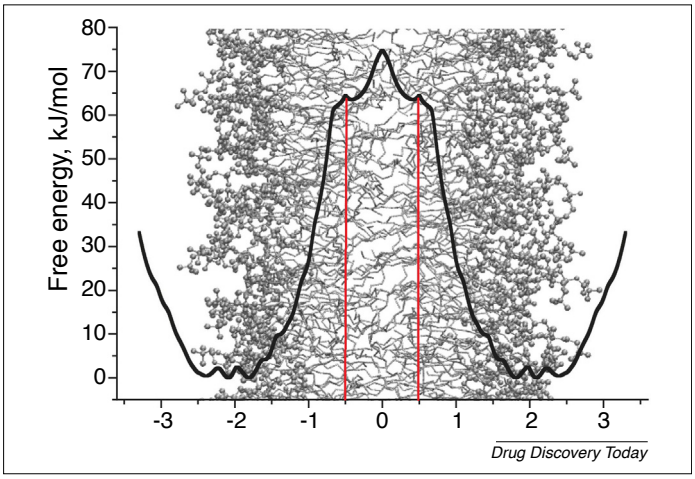

FIGURE 4

An example of a potential of mean force (PMF) produced from atomistic umbrella sampling (US) of penetratin at different z distances through a DPPC bilayer. The kinks in the PMF associated with pore formation in the membrane are indicated by the red lines. Image reprinted from Biophysical Journal, 97, S.Yesylevskyy *et al.*, Alternative mechanisms for the interaction of the cell-penetrating peptides penetratin and the TAT peptide with lipid bilayers, 40-49, Copyright (2009), with permission from Elsevier [56].

phosphates that fell within a cylinder with diameter comparable to the guanidinium group.

They found that using different CVs resulted in different translocation mechanisms. The first pulling approach did not result in pore formation and the associated PMF showed an increase in energy all the way to the bilayer centre, with a huge energy barrier of 200 kJ mol^{-1}. The second approach pulled the lower leaflet phospholipids closer to the peptide, resulting in the formation of a pore, with a much smaller activation energy of 120 kJ mol^{-1} [75]. The authors comment that the pore formation mechanism is more likely because of the smaller activation energy; however, this could have resulted from the artifical force that was applied to the lower phosphates thus forcing them into the bilayer centre, and not necessarily through interactions felt with the peptide alone. Surprisingly, adding more unrestrained peptides to the system increased the energy barrier, which is not consistent with experimental evidence that suggests the arginine-rich CPPs form pores by aggregating on the membrane surface. This further emphasises the importance of choosing the right reaction coordinate in US calculations.

Sun *et al.* also performed pulling and US simulations of Arg8 into the centre of a DPPC bilayer and compared the resulting PMF with that produced from pulling a phospholipid head group into the centre (lipid flip-flop) [59]. They found that both pulling simulations resulted in pore formation, giving characteristic kinks in the PMFs. The energy barrier for Arg8 pore formation was 87.5 kJ mol^{-1}, slightly higher than the energy barrier measured for lipid flip-flop, which was 76 kJ mol^{-1}. As with Huang and Garcia's US simulations, they found that adding more unrestrained peptides resulted in a higher energy barrier for Arg8-induced pore formation and reduced the binding interactions between the peptide and the membrane. Although neither study produced results in agreement with experimental data, i.e. adding more peptide should increase permeation, it is interesting to note the similarities in their results. Furthermore, Sun *et al.* did observe a slightly smaller energy barrier to lipid-flip-flop-induced pore formation with the addition of unrestrained peptides (probably resulting from the increased lipid disorder induced by peptide binding), which could suggest that Arg8 enters the cell by increasing the likelihood of spontaneous lipid flip-flop events [59].

The challenge associated with atomistic pulling and US simulations to study CPP penetration into lipid bilayers is that the resulting PMF is likely to depend on the initial peptide conformation and orientation relative to the membrane. As previously described, the energy landscape associated with peptide conformational changes and membrane translocation is complex and US simulations can only sample one possible energy 'route' into the membrane. It is possible that the peptide secondary structure changes as it moves from bulk water to the membrane surface and then again as it enters the hydrophobic interior of the membrane. The simulations described above are of arginine-rich peptides that are found to have random coil structures in water; US simulations might not sample all the possible random coil conformations that the peptides adopt during translocation. When using pulling and US simulations to study peptide insertion into a bilayer, it might be advisable to create repeats with independent initial configurations to assess the dependence on the resulting PMF.

Replica exchange methods

To better understand the conformations accessible to peptides in the presence of membranes, replica exchange approaches can be applied to increase conformational sampling (Box 1, Table 2). Nymeyer *et al.* performed temperature replica exchange MD (T-REMD) (Box 1) on WALP-16 (AWWLALALALALAWWA), a model TM α-helical peptide, in the presence of a DPPC bilayer [76]. An unstructured peptide was placed above the membrane surface and simulated in NVT conditions with the area per lipid big enough to be comparable to that of a previous DPPC/WALP simulation (i.e., greater than that of pure DPPC). The study used 38 replicas exponentially spaced between 350 and 505.8 K, simulated for 1 ns, followed by 64 replicas exponentially spaced between 350 and 800 K, simulated for 1.6 ns. A planar restraint was added to the C2 atoms of the lipids to prevent the bilayer from disintegrating. The simulation resulted in the peptide inserting into the DPPC bilayer before folding into an α-helix, providing insight into the insertion mechanism. However, the results should be interpreted with caution owing to the fact that the DPPC bilayer was simulated with an artificially high area per lipid, meaning that the peptide had more room to insert in between the phospholipid head groups [76]. It is also possible for the peptide to fold into a helix upon binding to the membrane, allowing the peptide to interact with the lipids in a way that pushes the head groups apart and facilitates insertion. Also, although T-REMD was applied to enhance the peptide conformational sampling, longer simulations will be needed to interrogate convergence of the peptide secondary structure and depth in the membrane.

Smith *et al.* used replica exchange with solute tempering (REST) (Box 1) to enhance the conformational sampling of the Aβ10-40 amyloidogenic peptide in the presence of a DMPC bilayer [77,78]. They set the peptide and counter-ions as the 'hot' solute, whereas the remaining water, ions and lipids were set as

the 'cold' solvent, resulting in an increase in the peptide conformational sampling. In the initial configuration, the peptide was bound to the DMPC bilayer through four or five residues and, throughout the course of the simulation, hydrophobic regions were able to insert under the phospholipid heads. The simulations allowed useful comparisons to be made between the secondary and tertiary structures of the peptide in bulk water and on the membrane. Additionally, important binding interactions between the peptide and the lipids were identified. This method was used again by Parikh and Klimov to investigate the effect of including lipopeptides in a DMPC bilayer on the binding and secondary structure propensity of the Aβ10-40 peptide [79]. Both studies highlighted the usefulness of the REST method in identifying peptide–lipid interactions and exploring the conformational space accessible to the peptide while in contact with the membrane; this method could be applied to CPP–lipid systems to investigate the mechanistic role of peptide conformations in membrane translocation.

Coarse-grained simulations

Another way to enhance sampling is to use CG simulations (Box 1, Table 2, Fig. 5). Although this approach decreases the resolution of the model, it vastly increases the computational efficiency and allows for larger systems to be simulated for longer timescales. There have been numerous examples of CG simulations of CPP–lipid systems described in the literature [80–84].

CG US was used by Hu and Patel and Zhang *et al.* to study the translocation of the SMTP TP2, and compare it with negative controls [81,83]. Both studies used the Martini CG force field [46] to describe the peptide and a POPC (or POPC/POPG) bilayer, and performed US to assess the free energy change associated with the peptides penetrating to the centre of the bilayer. Hu and Patel used a growing approach, where they gradually switched on the interactions of the peptide at insertion depths, separated by 0.1 nm, in the membrane [81]. They then restrained the COM of the peptide at the insertion depths and performed a 300 ns production simulation at each depth. Zhang *et al.*, however, built an initial system with the peptide at the centre of the bilayer and then pulled the peptide into bulk solvent [83]. They also used snapshots every 0.1 nm and performed a 250 ns production simulation for each snapshot. Both studies were consistent in predicting a higher energy barrier for penetration of the negative control: ONEG (PLGRPQLRRGQF), compared with TP2 and observed a stronger and deeper binding interaction of TP2. The studies revealed that TP2 induces less membrane deformation than ONEG, even though it resides deeper in the membrane; this is consistent with experimental evidence showing that TP2 does not cause cell leakage [85]. Hu and Patel also compared their findings with other SMTPs and control peptides, showing that the SMTPs had lower energy barriers for translocation (all SMTPs had barriers of $<$96.2 kJ mol^{-1}, whereas all the control peptides had barriers of at least 125.5 kJ mol^{-1}). Zhang *et al.* took the lowest energy structures (when the peptides were bound to the membrane surface) and converted them into atomistic models, using the CHARMM36 force field. This allowed a comparison of the membrane-bound TP2 and ONEG structures at the atomistic level, confirming that TP2 indeed resides deeper in the membrane, probably because of its high hydrophobicity.

Via *et al.* used the Martini CG force field to study the effects of transmembrane potentials on the ability of Arg9 and R8W3 to translocate across a DOPC bilayer [84]. The simulations involved using well-tempered metadynamics with the CV set as the z distance between the COM of the peptide and the COM of the membrane; this allowed the construction of a PMF from the metadynamics hills. Simulations were run for 6–8 μs, providing a less expensive way of constructing a PMF than from US simulations, which requires multiple simulations to sample different values of the CV. The authors observed pore formation when the peptide reached the centre of the membrane in all the simulations and were able to relate CPP activity to the presence of a transmembrane potential (applied by adding more ions on one side of the membrane and restraining them from crossing the periodic boundary), showing that CPPs are able to translocate with a lower energy cost in the presence of a charge imbalance. However, it should be noted that this approach may not sample configurations along the bilayer as completely as US and hence the lowest energy translocation path might not be sufficiently sampled.

An advantage of using CG models is that larger and more realistic cell membranes can be simulated [86], as opposed to the simplistic phospholipid bilayer models accessible to atomistic MD. In reality, eukaryotic cell membranes are complex and contain many different phospholipids, cholesterol, sphingolipids and glycolipids. Also, an asymmetrical distribution of these components means that the upper and lower membrane leaflets have different overall properties, such as charge and curvature, which could affect CPP activity. Marrink *et al.* recently published a review that discusses the development of more-realistic cell membrane models [17]. The next steps in the study of CPPs could involve utilising these complex membrane models to understand the influence of the different membrane components on cell-penetrating activity.

Although the use of CG models vastly increases the ability of the simulation to sample longer timescales and larger systems, they

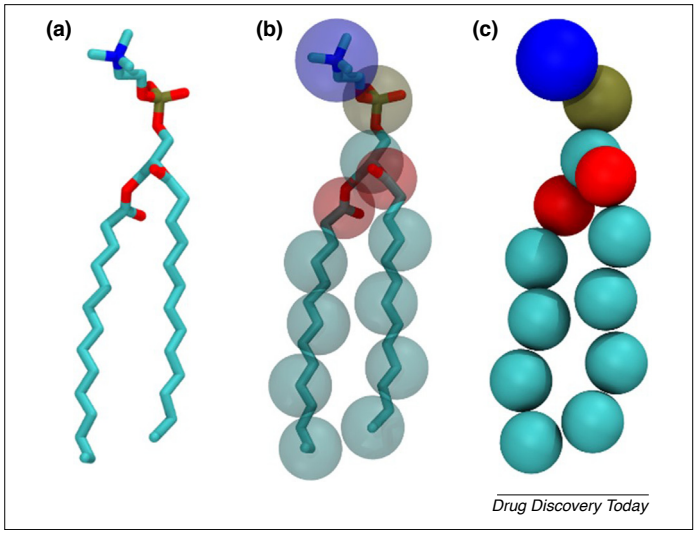

FIGURE 5

Mapping of united atom DMPC **(a)** to SIRAH CG beads [47] **(c)** with overlaid structures shown in **(b)**. Conventional CPK colouring used for united atom representation. CG bead colouring as follows: dark blue = choline, orange = phosphate, red = ester, light blue = hydrocarbon chain.

also lose atomistic detail, which could lead to parameterisation artefacts. For example, the 4–1 mapping of heavy atoms to Martini interaction beads means that lipids with 12 or 16 carbon atoms per acyl chain are split into three or four interaction beads, respectively. However, lipids with 14 carbon atoms cannot be split perfectly, so their length is either over- or under-estimated [87]. Further, CG simulations speed up sampling by smoothening the energy landscape; the kinetics are therefore modified in an unpredictable way and are no longer comparable with real-time experiments [87]. In addition, it is not possible to sample peptide conformational transitions, because the description of the backbone is simplified. If the peptide secondary structure is known *a priori*, restraints can be applied in the Martini force field to retain the structure throughout the simulation, however this will result in the neglect of any conformational changes associated with the translocation process [87].

Implicit membranes

Implicit membrane force fields describe the bilayer as a continuous medium, where the hydrophobic membrane interior is modelled as a low dielectric slab with a high dielectric slab on either side, representing the water and polar lipid head groups [88]. This vastly decreases the computational expense, which increases the simulation time accessible to the system, meaning an atomistic peptide can sample a wider conformational space. However, simplification of the membrane comes with the problem that the membrane thickness is fixed and simulating water defects is challenging, which may not be able to capture important events associated with CPP translocation [89].

To study the structure of TP2 and ONEG in the presence of a membrane, Lazaridis *et al.* used a modified version of the IMM1 implicit membrane model that takes into account changes in membrane thickness and water defects [89,90]. Membrane deformations were accounted for by simulating the system at multiple membrane thicknesses, T, and estimating the free energy of membrane deformation, ΔG^{def}, as a function of T. The optimal T was then chosen as the value that minimised ΔG^{def}. Water defects were accounted for by altering the free-energy profile according to all-atom simulations. The study initiated atomistic simulations of a single TP2 peptide, constrained to different secondary structures, near the implicit membrane surface and measured binding. The study also investigated the transition state configurations of helical-constrained TP2 and ONEG passing through the membrane. These simulations involved restraining the peptides at grid values of membrane depth (z) and tilt (θ), to sample data and construct a 2D free-energy surface. This produced results consistent with the previously described CG US simulations of TP2 and ONEG [81,83]; TP2 binds more strongly to lipid bilayers than ONEG and has a smaller free-energy barrier to translocation through the bilayer (104.6 kJ mol^{-1} as opposed to 184.1 kJ mol^{-1} reported for ONEG) [89].

Although the use of implicit membranes with atomistic peptides decreases the computational cost and increases the sampling efficiency of the peptide (and Lazaridis *et al.* address the issues of membrane thinning and water defects with their modified model [89]) it loses the atomistic detail of the membrane; for example, the specific role that arginine could have in binding to the phosphates.

Concluding remarks

There is much interest in the use of CPPs as intracellular drug delivery systems because they are shown to pull large, polar cargo into cells [2]. The experimental literature reveals various energy-dependent and energy-independent cellular uptake mechanisms for the same peptides, which have been discussed here. It is now widely believed that many CPPs enter cells via endocytosis at low concentrations but via energy-independent mechanisms at high concentrations [11]. While the endocytosis mechanisms remain inaccessible to MD simulations owing to their energy-dependence and large scales, energy-independent mechanisms can be studied using a variety of simulation techniques and might prove helpful in understanding how CPPs are able to escape endosomes. Examples of simulation studies that have provided useful atomistic insights into CPP translocation have been discussed in this review, along with associated advantages and limitations.

It is generally accepted that conventional MD is unable to reach the timescales needed to observe CPP translocation, which might involve multiple peptide folding, aggregation and insertion events. However, many studies were able to utilise atomistic MD simulations by selecting initial configurations based on knowledge gained from experiments [58–62]. For example, simulations can probe the ability of a peptide to stabilise pores by placing it in a preformed pore and monitoring its closure [58]. This is a useful method if mechanistic detail is already known; however, caution must be exercised since biasing simulations in this way could result in incorrect assumptions being made if experimental data are not available. In this review, the inclusion of experimental data in CPP–lipid simulations was discussed in the context of choosing appropriate initial coordinates. In addition, there have been methods reported that directly incorporate experimental data into the simulation by reweighting or biasing the structural ensemble [91]. To the best of our knowledge, these methods have not yet been applied to CPP–lipid simulations but could offer an exciting new approach for the future.

Ulmshneider *et al.* also demonstrate the usefulness of using elevated simulation temperatures in studying TM peptides, showing that TM helices are thermostable in the membrane and that high temperatures can speed up the kinetics [70]. Given that the biomolecular force fields were not parameterised at high temperatures, additional studies are needed to establish their applicability. Atomistic steered MD and US are commonly used to study CPPs, where the peptide is pulled across the membrane and US is used to produce a PMF [56,59,60,75]. This yields the free-energy changes associated with the peptide translocating across the lipid bilayer. However, the free-energy barriers calculated for CPPs are often still very large and inconsistent with the fast translocation rates observed experimentally. This could result from missing the lowest free energy translocation path by choosing an incorrect CV or under-sampling the conformational changes adopted by the peptide in contact with the membrane. The result of the simulation is therefore very likely to depend on the initial conformation and orientation of the peptide. It could also be a result of the over-simplification of the membrane; in reality, membranes are likely to consist of multiple lipid types, cholesterol and many membrane proteins. A possible resolution of the conformational sampling problem is to use a replica exchange method on the peptide in the presence of the membrane [78,79]. This could identify important

conformational transitions undertaken by the peptide in the translocation process. Finally, CG or implicit membrane simulations can be used to decrease the computational cost associated with sampling large systems and long timescales. Although there are CG US simulations described in the literature that produce PMFs with relative energy barriers consistent with experiments [81,83], it is important to recognise that the over-simplification of the system may not capture important binding interactions and conformational changes. Similarly, implicit membrane models will not capture important details of the membrane.

Owing to the complex nature of the energy landscapes associated with peptide folding, aggregation and penetration into the membrane, the choice of initial peptide conformation and configuration relative to the membrane is important for all simulation techniques. For atomistic MD, it is likely that peptide–lipid simulations will sample only local minima, so it is vital to seed the simulation from experimentally significant conformations. For example, NMR or crystal structures, prediction software to determine membrane insertion depth and/or orientation or experimental assays could be used to determine whether peptide aggregation and/or membrane pore formation is involved. This is also important when performing atomistic pulling and US because the CV might not describe the folding or orientation of the peptide in the membrane. In theory, replica exchange approaches can sample from an equilibrated ensemble of structures, so the initial peptide conformation is less important; however, multiple simulations should be performed to check convergence. Additionally, CG and implicit membrane simulations enable longer timescales to be accessed, meaning convergence is more likely to be achieved, but it is again advisable to repeat simulations to validate results.

In conclusion, MD simulations provide a useful approach to complement experiments and to validate experimental results. However, researchers should be aware of the limitations, some of which are discussed in this review. In particular, simulations should be repeated with independent starting conditions and multiple force fields, and simulation convergence must be carefully assessed. Future efforts in CPP simulations will undoubtedly tackle the sampling issues discussed above, including the development of new enhanced sampling approaches and more-descriptive CVs for CPP translocation; in parallel, development and refinement of more-accurate force fields are in progress. Furthermore, complex membrane systems and longer simulation times, using CG and mesoscale models [92], will likely be applied to CPP studies in the future. It is also important to use experimental knowledge to guide simulation.

Conflicts of interest

C.S.V. is the co-founder of Sinopsee Therapeutics; there is no clash with the current work.

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