UNDERSTANDING CELL PENETRATING PEPTIDE MECHANISMS USING COMPUTATIONAL ELECTROPHYSIOLOGY SIMULATIONS

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ABSTRACT

Cell-penetrating peptides (CPPs) can enter cells without inducing cytotoxicity and can be coupled with cargo molecules to be used to deliver drugs, DNA, or nanoparticles into cells. The peptide-membrane interactions driving the internalization mechanism are not completely understood. In this study, we introduce Computational Electrophysiology (CompEL) as a tool for the computational investigation of CPP and membrane interaction leading to internalization, focusing on cationic CPPs such as Arg9, MAP, TP10, and TP2. CompEL induces membrane stress through ion imbalance, prompting the membrane to alleviate this stress via pore formation. Using double bilayer molecular dynamics (MD) simulations with one or eight peptides, we show that CPPs can use these pores to translocate, whereas non-CPP nonaleucine peptide fails to cross the membrane and instead contribute to pore stabilization. In the eight-peptide systems we observe that some peptides can cooperate to reach translocation or to foster pore stabilization. This study introduces CompEL as a powerful tool for CPP research, shedding light into the molecular peptide-membrane interactions governing CPP translocation, and offering valuable insights for the design of next-generation delivery systems.

Keywords: cell-penetrating peptides, molecular dynamics, computational electrophysiology, pore dynamics, peptide cooperativity.

1. INTRODUCTION

Cell-penetrating peptides (CPPs) represent a diverse class of molecules renowned for their ability to traverse biological membranes and facilitate the intracellular delivery of various cargoes without inducing cytotoxicity[1–3]. CPPs can internalize into cells coupled with various cargoes, such as proteins, DNA, RNA, nanoparticles or low molecular weight drugs[4–9]. Moreover, CPPs are currently being used for therapeutic purposes, such as cancer treatment, Alzheimer's disease, and

immunotherapy[10–14]. Nevertheless, the molecular mechanism of CPP translocation and internalization requires further investigation[15], for controlled rational design of these biomedically relevant molecules.

Many molecular mechanisms have been proposed, which can be classified into energy-dependent and energy-independent methods[16]. Energetic mechanisms entail endocytosis[17,18], whereas energy-independent mechanisms include pore formation[19], the carpet-like model (through membrane destabilization)[20], the membrane thinning model[21], and inverted micelle[22]. More recently, CPPs have been discovered to translocate across both the endosomal and plasma membranes by a vesicle budding and collapse (VBC) mechanism[23,24]. In fact, the diverse possible translocation methods lead to talk about a *landscape* of different internalization mechanisms[25]. In addition to these mechanisms, other variables, such as peptide concentration, can also affect the internalization dynamics[26].

CPPs exhibit a wide range of structural and biochemical characteristics, often classified based on their predominant physicochemical properties. Among these classifications, peptides can be categorized as cationic, hydrophobic or amphipathic[27]. Cationic peptides, such as Arg9[28] are characterized by an abundance of positively charged residues, such as arginine or lysine, which promote electrostatic interactions with negatively charged cell membranes. Hydrophobic peptides, such as TP2[29], and K-FGF[30] possess a high proportion of hydrophobic residues, facilitating interactions with lipid bilayers. Amphipathic peptides, such as TP10[31] or MAP[1,32], can be further divided into primary amphipathic (featuring distinct hydrophobic and hydrophilic regions within their sequences), secondary amphipathic (which adopt amphipathic conformations upon interaction with lipid membranes), proline-rich, and histidine-rich[27,33]. Understanding the

classification of CPPs based on these physicochemical properties is essential for elucidating their mechanisms of cell penetration and optimizing their utility in biomedical applications[34].

Molecular dynamics (MD) simulations have been extensively used to investigate the internalization mechanisms, since it can provide meaningful insights of peptide-membrane interaction at atomic level[35–37]. In this study, we seek to study the insertion or translocation abilities of CPPs. Nonetheless, the translocation process takes from seconds to minutes[38,39], and is too computationally demanding to be observed in a conventional Molecular Dynamics (cMD) simulation. In this regard, enhanced sampling techniques have been employed to explore a larger conformational space, such as Umbrella Sampling (US)[40,41], Replica Exchange (RE)[42,43], adaptive Steered Molecular Dynamics (aSMD) in combination with cMD[44], and Weighted Ensemble (WE)[45]. Besides, coarse graining techniques, such as MARTINI, have also been used to study the thermodynamics of CPP translocation[46] (interested readers are redirected to the exhaustive review by Ouyang and colleagues [47]). However, these methods have inherent limitations. For instance, US is primarily used to calculate free energy of translocation, aSMD requires powerful computational resources, and they are strongly biased methods requiring reaction coordinates, whereas coarse-grained simulation have lower resolution. Therefore, we repurpose computational electrophysiology (CompEL)[39] to elucidate the key steps involved in CPP cell penetration. CompEL has been previously used to study membrane proteins, mainly ion channels (see the CompEL review in Ref.[48]), but, to the best of our knowledge, this is the first study to employ CompEL for the study of CPPs and their mechanism of action.

Simulations at high temperatures (up to 500 K) have been performed to study membrane disruption of small molecules[49], following the high kinetics rationale to enhance sampling [50]. Following a parallel reasoning, in CompEL, we generate a difference in potential through ion imbalance using

a double membrane configuration[39], allowing for enhanced sampling and easier CPP-mediated membrane disruption. Through this approach, we seek to identify critical molecular events and kinetic barriers that dictate the efficiency of CPP internalization. We aim to provide a new possibility to unravel the interactions between CPPs and lipid membranes, shedding light on the processes governing their cellular uptake.

In this regard, we chose four representative CPPs (Arg9, MAP, TP10, and TP2, to compare them with our previous study[44]) and a negative control (Leu9, non-CPP) to conduct the study. First, we ran simulations with one peptide at different potentials to decide the appropriate transmembrane potential for this system setup. Then, we analyzed the simulations at the chosen transmembrane potential. Last, we simulated the systems with eight peptides to study and analyze CPP cooperation and aggregation.

In this study, we aim to expand the computational toolkit for investigating membrane active peptides[51,52], with a particular emphasis on CPPs. To this end, we employ CompEL –an entry-level, rapid, and reproducible computational technique– to explore the molecular mechanisms underlying CPP internalization. Our objectives are to differentiate between CPP-like and non-CPP peptides, and to compare single- to multiple-peptide simulations. Our findings hold promise for guiding the rational design of CPP-based delivery systems and advancing targeted therapeutic interventions in biomedical research.

2. MODELS AND METHODS

2.1. Systems preparation

Peptides were initially modelled with ColabFold notebook[53], which uses AlphaFold[54] monomer prediction to model the peptides. Each peptide was put in a 7.5x7.5x7.5 nm box solvated with TIP3 waters. The systems were minimized for 5000 steps, equilibrated in the NVT ensemble for 125 000 steps, and the production was run for 250 ns. The forcefield chosen was CHARMM36m, which has been maintained throughout the study. GROMACS[55–62] software was used to run the simulations. The temperature of all the simulations was maintained at 350 K, to use the same temperature that is going to be used in CompEL. Periodic boundary conditions (PBC) were applied.

The simulations were analyzed using Jupyter Notebook integrated development environment (IDE)[63] and GROMACS. The trajectory of the peptide was clustered using MDAnalysis package in Python[64,65], and the centroid structure was used as input for the CompEL simulations run using GROMACS.

The membrane was composed of 128 POPC (1-palmitoyl-2-oleoylphosphatidylcholine) molecules per leaflet (256 POPC each bilayer and 512 in total in the system) and solvated with TIP3P water. The system was neutralized with KCl to a final concentration of 150 mM. The systems were built using CHARMM-GUI[66–68] web server.

2.2. Computational electrophysiology simulations

In CompEL, a transmembrane potential triggered by charge imbalance (ΔQ) is generated between one side and the other of the membrane. But, since PBC are applied, a double membrane configuration system must be designed to achieve the desired transmembrane potential. Simulations were run at 350 K. To prepare the system, we followed the methodology described in

previous studies[39,48,69]. In this regard, we used GROMACS built-in *gmx* utilities: first, we duplicated the system, then, rotated the second system, doubled the box size, and concatenated both files into a single box with a double membrane configuration. After that, the desired transmembrane potential was achieved by ion imbalance between both membranes: a positive charge (K^+ ions) was added to the inner water compartment, and a negative charge (Cl^- ions) was added to the outer water compartment. Thus, water molecules were swapped with ions into the corresponding water compartment, done using *gmx insert-molecules*. In Table 1, we present the configurations for all the systems simulated in this study. When a peptide was added to the system –in the inner water compartment–, the corresponding counterions were inserted –in the same water compartment– following the same procedure as before. Thereafter, the system was minimized for 5 000 steps and equilibrated for approximately 2 ns (the different steps were of 125, 125, 125, 500, 500, 500 ps) while gradually lowering the positional restraints (1 000, 400, 400, 200, 40, 0 $kJ \cdot mol^{-1} \cdot nm^{-2}$, respectively). Last, a 250 ns simulation was run for each system. The ion number was kept constant in each water compartment through the whole duration of the simulation using computational electrophysiology protocol in GROMACS, which controls ion/water position exchanges (production files can be found at the GitHub repository). A summary of the number of simulations run can be found at Table 2 (see next section for explanation of simulation number and/or difference in simulation length), accounting for 31.5 μs of simulation time (ca. 3μs for membrane only systems, ca. 14 μs for systems with one peptide, and 15 μs for systems with eight peptides). The simulations were run using a workstation with a GPU RTX3080Ti, at approximately 80 ns per day.

2.3. Data analysis

MD simulation analysis was executed in a Jupyter Notebook IDE[63], used along with the MDAnalysis package in Python, and *gmx* utilities[56,59,61,62,64,65]. PyLipID was used to analyze lipid occupancy, which is a measure of the simulation time that a protein residue is in contact with the lipids[70]. Visual inspection and molecular graphics were performed in VMD[71]. Secondary structure was analyzed in VMD using STRIDE program[72]. An in-house Python script was utilized to calculate the radius pore size, employing Scipy[73]. Basically, the script computes the maximum distance between water molecules in each membrane z-stack (2 Å thick) and outputs the minimum radius among all z-stacks, repeated for each simulation frame. Lipid order parameter (S_{CD}) computes the orientation of the lipids with respect to the membrane normal[74]. S_{CD} is calculated using Equation 1:

$$S_{CD} = \left\langle \frac{3 \cdot (\cos^2 \theta_{CD}) - 1}{2} \right\rangle \quad (1)$$

Where θ_{CD} stands for the angle between the carbon-deuterium bond and a reference axis, and the angular brackets indicate an ensemble average over time and lipid molecules. S_{CD} values close to 1 indicate perfect alignment with the bilayer normal, and values close to 0 indicate complete disorder. Matplotlib[75] was used for plotting Figures 1-5.

2.4. Data availability

System, files simulation inputs, and code to reproduce the analyses presented can be found at:

https://github.com/APMlab-memb/CompEL_CPPs.git

Due to file size limitations, the simulation trajectory file will be shared upon request.

3. RESULTS & DISCUSSION

3.1. ΔQ benchmarking

We decided to use 4 canonical CPPs: Arg9, MAP, TP10 and TP2 (Table 3). Besides, we chose Leu9 as a negative control, a peptide not expected to present CPP-like capacities due to its high hydrophobicity[76]. In parallel, we ran a simulation without a peptide, as a membrane only control. We chose POPC as membrane lipid, since it is extensively used owing to its biological relevance, reliability and stability in MD simulations, and relevance in physiological systems[77]. The starting configuration of the peptides and the membranes are presented in Figure 1, where the total number of membrane lipids and water residues are also displayed. Besides, the electron density plot is included, indicating membrane, water, and ions densities.

In order to conduct the CompEL simulations, we created a series of systems with increasing ΔQ, starting from 0 (0 net charge in any of the two compartments) to 24, where the charge imbalance was obtained by placing ΔQ/2 K+ ions in the inner water compartment and the other half by placing ΔQ/2 Cl- ions in the outer water compartment. We initially ran 4 sets of 250 ns simulations: at ΔQ 0, 8, 16, and 24, with 2 replicas for each simulation. However, given the sudden change in simulation outcomes between ΔQ 8 and 16 (see below), we decided to add a new set of simulations, at ΔQ 12, to try to better describe the phenomena occurring.

In Figure 2, we show the number of occurrences of each behavior in every transmembrane potential simulation (Figure 2A). In ΔQ 16 is where the most different behaviors can be seen and, moreover, a similar occurrence ratio of each behavior is observed, allowing to differentiate between peptides. Thus, we decided to carry on the analysis mainly with the simulation of ΔQ 16 (Figure 2B). Here, we differentiate between three possible behaviors. First, inner leaflet adsorption or partitioning: the peptide stays in the inner water compartment and interacts with the inner part of the membrane.

Second, insertion and pore (Figure 2C: TP2, Leu9): the peptide gets in contact with the polar heads in the outer part of the membrane, but it is still in contact with the inner part of the membrane. Moreover, there is a flow of water molecules, defining a water pore, with a toroidal-like nature[78,79]. Third, outer leaflet stabilization or translocation (Figure 2C: Arg9, MAP, TP10): the peptide is able to cross the membrane and interact with the outer part of the membrane and the outer water compartment.

The potential and field distributions were calculated using GROMACS built-in *gmx potential* tool (Figure S1). We see how the potential and field grow as the transmembrane charge imbalance increases, reaching to a voltage of approximately 2 V in CompEL ΔQ 16 simulations (the ones chosen to be analyzed). Besides, the calculated field in one membrane averages to ca. 0.3 V/nm (considering both membranes, the field adds up to 0). Therefore, potential and field values are both in the normal range used in other biological studies[80–82].

3.2. CompEL simulations at ΔQ 16

Peptide analyses

To increase the simulation set and achieve better sampling of the phenomenon with greater certainty, we ran a third replica of simulations in ΔQ 16. The results of all the simulations, divided by peptide, can be seen in Figure 2B (and Table S1). We see how the translocation is the most common result for TP10, whereas Arg9 and MAP partition in most cases, but they also have the ability to translocate the membrane. On the other hand, TP2 and Leu9 are the only peptides that do not show translocation capabilities and get inserted in the membrane in most cases.

These behaviors can be spotted in Figure 3, which shows the occupancy of the peptide residues by lipid molecules. Occupancy, analyzed with PyLipID[70], measures the simulation time in which a residue is in contact with a lipid. In short, Figure 3 shows how do peptides interact with POPC membranes. We differentiate between the inner and the outer part of the membrane, and between the interaction with the polar head and the hydrophobic tail of the lipids. In this regard, TP10, the peptide that translocates in most cases, shows a higher occupancy in the outer part of the membrane, demonstrating the translocation behavior. On the other hand, in the case of the other four peptides, we see a higher occupancy in the inner part of the membrane. Besides, TP2 and Leu9, the peptides that do not translocate in any replica, also show interaction with the outer leaflet, indicating that the insertion mechanism entails interaction with both leaflets.

Observing the residues that show a higher occupancy, Arg9 and Leu9 show a homogeneous behavior, with a similar occupancy for all residues, given that they are polychains of the same amino acid. Nonetheless, we see a clear difference in the occupancy of these two peptides: Arg9 preferably interacts with POPC polar heads, whereas Leu9 shows a higher interaction with the lipid tails. These behaviors are explained by the cationic polar nature of arginine, preferably interacting with the negative charge in the phosphate group, and the hydrophobicity of leucine, rather interacting with the hydrophobic lipid tails, respectively[83,84]. In parallel, in the cases of MAP, TP10 and TP2 we see how, in average, cationic (K, R) or -partly- polar (Y, N, Q) amino acids show a higher occupancy with the polar heads[83] and, conversely, hydrophobic amino acids (L, I, W) preferably interact with the lipid tails. Interestingly, we see how W12 has a high occupancy with the lipid tails in TP2, which proves that this residue, together with L7-L8, are stabilizing the peptide in the hydrophobic core of the membrane and, therefore, hampering its

translocation. In fact, tryptophan was found to strongly interact with the hydrophobic part in the interfacial region of the membrane[85].

In parallel, peptide secondary structure was analyzed (Figure 4). We observe that Arg9 and MAP are mostly unstructured during the simulations, as seen in previous studies[86,87], TP2 and TP10 remain at a stable structure, and Leu9 is the only peptide where we see a drastic change during the simulation, reaching more than a 50% of α-helix structure when it gets inserted in the membrane, phenomena previously described in other studies[88–90]. TP2 is highly structured in the N-terminal, whereas TP10 shows higher α-helix percentage in the C-terminal part[91]. Furthermore, the hydrogen bonds analysis (Figure S2) shows that Arg9 prefers to interact with lipids, predominantly polar heads as discussed before, which correlates with the lack of secondary structure. On the other hand, Leu9 or TP2 have the highest number of intrapeptide hydrogen bonds, owing to their higher hydrophobicity. Leu9 and TP2, together with TP10, show the highest percentage of secondary structure, again, correlated with higher number of intrapeptide H bonds[92].

Membrane disruption analysis

An interesting comparison is the pore that is formed in the membranes during the simulations. Transient, small pores are spontaneously formed in biological membranes[93]. In CompEL, owing to membrane stress[94] caused by the ion imbalance, pores are also generated without the presence of a peptide (membrane control, Table 4)[95,96], highlighting that peptides are not responsible for pore formation. Still, some of these peptides are able to translocate using the pre-formed pores, as seen in previous studies[97–100].

Lipid order parameter (S_{CD}) analyses have been performed to test the organization and orientation of lipids in the membrane. S_{CD} calculates the angle between the carbon-hydrogen bond in the acyl chains of lipids. Here, we focused in the palmitoyl segment of POPC, the sn-1 tail (Figure S3). We see a similar results to the ones observed by Ferreira *et al.*[101], and thus conclude that the lipids are well-oriented in the membrane.

3.3. CompEL simulations at higher Peptide:Lipid ratio

Peptide analysis

Simulations with only one peptide are useful to test the method, but since CPPs do not cross a membrane as individual monomers[102,103], we simulated the same POPC CompEL system with 8 peptides, and 256 lipids (per bilayer), corresponding to a 1:32 protein:lipid (P:L) ratio, as in previous studies[104]. This P:L ratio showed full peptide binding in previous studies[26], it was shown enough to create stable pore channels for melittin [105], and is close to the ratio used by Herce and Garcia[106] in the study with Tat peptides (1:18). Besides, more peptides would require larger water compartments, and we wanted to keep the same system conditions as for one peptide.

Two replicas of CompEL 250 ns simulations were run for ΔQ 0, 8, 12, and 16. ΔQ 24 was discarded in order to reduce the electric field applied to the system. As in the CompEL simulations with one peptide, the ΔQ 16 simulations presented the possibility of observing peptide translocation and were, thus, used for simulation analysis (Figure 5). The global results seen for ΔQ 0, 8, and 12 can be seen in Figure S4 and Table S2.

System stabilization was not achieved with 250 ns (as shown by the RMSD analysis, Figure S5), thus the ΔQ 16 simulations were extended to 500 ns. Besides, a third replica was run to improve

statistical power. As shown in Figure 5A and Table S2, the results reveal different translocation and insertion behaviors among the peptides. From a total of 8 peptides, 1 Arg9 peptide is able to translocate in each replica, achieving a total of 3 translocation events in total in the 3 replicas. MAP shows 1 translocation and 1 insertion in each replica, with a total of 3 translocations and 3 insertions among the 3 replicas. Thus, Arg9 and MAP were the peptides with the highest translocation ratio. For TP10, only 1 TP10 translocates in one replica, whereas in the other two replicas, 2 and 3 TP10 peptides get inserted in the membrane. For TP2, which showed no translocation capacity in simulations with one peptide, 1 TP2 peptide is now able to translocate in 2 replicas, with a total of 2 translocation and 7 insertion events in the 3 replicas. Last, as in simulations with one peptide, Leu9 does not show translocation capacity, but it does show insertion behavior, with 22 peptides getting inserted across the 3 replicas. In short, ΔQ 16 simulations demonstrate the CPP behavior of the four CPPs used in the study, whereas Leu9, the negative control without CPP characteristics, can get inserted but has no translocation capacity.

At the molecular level (Figure 5 B-F), Arg9 peptides show two possible behaviors: partitioning, where they get adsorbed to the inner leaflet and interact with the polar heads, or translocation, where they cross the membrane and interact with the polar heads in the outer leaflet. In both cases, Arg9 interacts with POPC polar heads, owing to the positive charges in Arg9 structure and negative charges in the polar heads, as discussed previously. The opposite behavior is seen for Leu9 peptides, which mostly get inserted in the membrane, preferring the interaction with the hydrophobic lipid tails. The other 3 CPPs (MAP, TP10, TP2) have hydrophobic, polar, and charged amino acids in their structure and can, therefore, interact with the polar heads in the partition and translocation behaviors, or get inserted and interact with the aliphatic lipid chains.

Regarding the secondary structure (Figure S6), we see similar results to the ones discussed for simulations with one peptide (Figure 4), with Arg9 mainly unstructured, most MAP peptides unstructured, even though some showed β-sheet structure, TP10 with a stable α-helix structure in the C-terminal, and Leu9 gaining secondary structure owing to the membrane insertion. Only TP2 shows considerable differences, since it changes from β-sheet to α-helix, with most peptides unstructured during the simulation, which can be related to a higher partitioning or translocation behaviors and, therefore, lower insertion ratio, typically associated with α-helix formation, as seen for Leu9.

Comparing the results to the simulation with one peptide, we see now more translocation or insertion events, demonstrating that peptide-peptide interactions are relevant for translocation capacity[15,87,100]. However, the high numbers affect differently for each peptide. As seen in Figure 5 B, Arg9 peptides do not show strong interaction among them[86,99,107,108] and, instead, Arg9 peptides primarily interact and form H bonds with lipids (Figure S7). Besides, Arg9 peptides form most of the protein-protein bonds within the same peptide (80 % intra-protein H bonds) (Table 5), indicating that Arg9 peptides are mostly present in the monomeric form. Conversely, the other four peptides do show cooperativity[91], supported by a higher inter-peptide H bond ratio (Table 5). Specially, Leu9 and TP2 show the highest self-assembly capacity to get inserted in the membrane[109]. Leu9 shows the highest self-assembly capacity, preferably interacting among them or with lipids tails. A closer look at the pore is seen in Figure S8, where we see how Leu9 peptides can stabilize the pore. In order to do so, Leu9 peptides present the polar atoms (N, O) oriented to the water molecules, whereas C atoms point to the aliphatic lipidic acyl chains.

Membrane disruption analysis

Table 6 shows the pore analyses conducted for ΔQ 16 simulations with 8 peptides. Arg9 and MAP do not contribute to the pore persistence, but TP10, TP2, and, specially, Leu9 are able to stabilize it. This behavior is depicted in Figure S8, where we see how one MAP peptide is inserted in the bilayer, but does not allow water flow, opposite to TP10, TP2, and Leu9, which cooperate and are able to stabilize the pore through the formation of a barrel-stave-like pore[79], with mostly peptides stabilizing the pore. The pore nature is different from the one seen for simulations with one peptide, where the polar heads played a higher part in pore formation.

These simulations have also allowed us to observe lipid flip flop from upper to lower leaflet (Figure 5 B-F). Lipid flip-flop occurs at the same time as the peptide is translocating across the water channel[15,86,104,110,111], from the peptide-enriched to the peptide-free bilayers[104].

In Figure 6, snapshots of the translocation (A-G) and insertion (H-M) processes are shown. The phases of pore creation and annihilation are compared to those described by Levine & Vernier[96]. Taking the Arg9 simulations, we observe a 4-step process. First, pore formation starts with a water defect, resulting in the interaction of water molecules from both water compartments, defined as pore initiation (Figure 6B). As seen in the previous section, peptides are not responsible for this process. Second, pore construction (Figure 6C) takes place, where polar heads from both bilayers enter in contact, which is followed by pore maturation (Figure 6D), characterized by a large number of waters and polar heads in the pore. In this step we can see how peptides get attracted to these pores. Third, one Arg9 peptide gets inserted into the membrane through the pore (Figure 6E). The peptide remains stable in the pore for approximately 300 ns, until it crosses to the lower leaflet and starts the process of pore annihilation (Figure 6F). Pore closure involves i) pore degradation, when water molecules and polar heads start to migrate out of the bilayer and the pore starts to thin,

and ii) pore deconstruction, when there are no polar heads involved in the pore formation. Fourth and last, the Arg9 peptide has crossed the bilayer and stabilized in the lower leaflet, triggering pore dissolution (Figure 6G) and completing pore annihilation, when all water molecules are expulsed from the membrane. A similar process is followed in the first steps of Leu9 peptides insertion. First, a water defect is created, and the pore initiation starts, with one Leu9 peptide already close to the formed pore (Figure 6I). Second, pore construction and peptide insertion happen concomitantly (Figure 6J), followed by pore maturation (Figure 6K). Third, more peptides get attracted to the pore and get inserted in the membrane (Figure 6L), until all peptides are stabilizing the pore (Figure 6M), structure stable during the rest of the 500 ns simulation. In Figure 6, we can also see how peptides are parallel to the membrane when they are partitioning, rotate and are perpendicularly oriented to the bilayer when they achieve insertion, and they finally adopt an orientation parallel to the bilayer when they complete the translocation[104].

3.4. CompEL analysis

In the CompEL simulations, we have seen how CPPs are able to use the pores formed due to ion imbalance to internalize[98,108]. Moreover, when a CPP translocates, it is able to trigger pore closure[41,100,108] (Figure 6D), with rapid water expulsion from the membrane[96], as seen for Arg9 and MAP, which have the least average pore sizes (still, this may be related to the underlying charge imbalance in CompEL, and further studies need to be conducted to confirm this statement). TP10 only translocates in the second replica (Table S2), precisely the one in which pore closure occurs (Table 6), confirming the relationship between these two events. TP2 also shows translocation in two of the replicas, but pore closure is not seen, which can be related to the TP2 lower net charge (+2), possibly not being enough to reduce the transmembrane potential under the

pore-forming threshold. Still, we hypothesize that in longer timescales, more TP2 peptides are able to translocate, ultimately inducing pore closure (we extended the TP2 simulations to 1 μs, but no significative changes were observed, so larger timescales may be needed). In short, the four CPPs have demonstrated translocation capabilities, as opposed to our previous CPP study[44], where only Arg9 (TP10 was not studied) was able to achieve translocation. In this sense, we have seen how CPP translocation can occur through pore formation, leading to pore closure and potentially explaining the lack of cell toxicity of CPP translocation[112]. On the other hand, Leu9 causes larger pore sizes, marking the difference compared to peptides with CPP characteristics, since Leu9 peptides are not able to translocate and they are indeed able to stabilize the pore, a behavior correlated with other bioactive peptides such as antimicrobial peptides (AMPs)[104].

Charge seems to be an important factor in deciding translocation, so simulations containing K-FGF[113], a neutral CPP, and Dynorphin A[114,115], a positively charged peptide that did not show internalization behavior in our last study [116] (Table 3), have been run. Both systems containing 8 peptides and ΔQ 16, showed no translocation behaviors, with only insertion or partitioning in both cases (Figure S9). Therefore, we can conclude that the charge is not the sole determinant of the translocation process in CompEL.

In conclusion, CompEL simulations are valuable for enhancing the dynamics of the system by inducing pore generation thanks to the ion imbalance. According to the CPP translocation process[106,117], peptides adsorb or partition to the upper leaflet. They then destabilize the membrane while attempting to reach the polar heads in the lower leaflet, ultimately causing pore formation and allowing peptide translocation. However, it is challenging to observe pore formation

in cMD, owing to the energetic cost of the process[93]. Precisely, CompEL allows for pore formation, thus enabling the observation of unbiased translocation of CPPs.

4. CONCLUSIONS

In this study, we have performed CompEL simulations to study CPP behavior under applied potential in model lipid membranes. The first step in CPP translocation through pore formation is the peptide adsorption to the membrane, destabilizing the membrane and allowing pore formation, which the CPPs will use to translocate. However, to observe the complete translocation in cMD simulations is cumbersome, owing to the energetic cost of pore formation. Precisely, CompEL enhances pore formation via ion imbalance in the membrane, allowing the computational study of CPP translocation.

In short, in ΔQ 16 CompEL simulations with one peptide, TP10 showed the highest translocation capacity, followed by Arg9 and MAP, with TP2 and Leu9 not showing internalization behavior. More translocation events were seen in ΔQ 16 CompEL simulations with eight peptides, where Arg9 and MAP showed the highest translocation capacity, followed by TP2 and TP10. Moreover, CompEL simulations have also revealed that once pores spontaneously form in the cell membrane under charge imbalance conditions, peptides can stabilize them (such as Leu9) or translocate through them (such as Arg9, MAP, TP10, or TP2).

We present CompEL, a method with significantly lower computational requirements compared to US, where up to 4 μs[41] are needed. Besides, CompEL is entry-level, easier to parallelize, and less GPU-intensive than aSMD[44]. In this study, simulations at several ΔQ values were required to perform method calibration, but in subsequent studies, simulations at only one ΔQ (e.g. ΔQ16)

need to be run, considerably decreasing the overall computational cost. Additionally, CompEL increases the feasibility of performing replicas and allows simulations with higher number of peptides, being able to analyze peptide cooperativity or aggregation. Moreover, CompEL is an enhanced molecular dynamics technique, but, contrary to aSMD or US, in CompEL the peptide is not forced to cross the membrane, rather it provides an unbiased exploration of the peptide-membrane interaction. Thus, CompEL can be used to describe at the molecular level the peptide interaction with the membrane, and with higher resolution than coarse-graining methods. Simulations using a potential difference and a single membrane can be conducted, but peptides may translocate to the opposite membrane via the PBC, which is prevented in CompEL, enabling the encapsulation of peptides between two membranes, which also simplifies analysis and enhances control over the system.

We believe that this study can be a first step in the use of CompEL for CPPs computational research. However, CompEL simulations only account for CPP translocation via pore formation, whereas CPPs can internalize through additional mechanisms, as discussed in the introduction. Besides, this study only conducted simulations with neutral, zwitterionic POPC bilayers; future work should explore negatively charged bilayers (e.g., containing POPS or POPG) and more physiologically relevant compositions (e.g., asymmetric containing POPE or cholesterol) in order to perfect this technique. Lastly, CompEL requires the application of a high voltage, which necessitates cautious interpretation of the results. Additional simulations may be needed to further increase the sampling of the systems, possibly combining CompEL with elevated temperatures to increase molecular mobility. Future studies should also investigate different P:L ratios to assess the influence of P:L ratio in CPP internalization.

In conclusion, we propose the use of CompEL to computationally study CPP insertion or translocation at a molecular level, which is challenging to achieve with other techniques. Furthermore, CompEL can be expanded to study the interaction of other types of membrane active peptides, such as AMPs, antiviral, or anticancer peptides.

DISCLOSURE STATEMENT

The authors declare nor conflict of interest or competing interest.

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FIGURE LEGENDS

Figure 1. Graphical representation of CompEL initial configuration. Initial structure of Arg9, MAP, TP10, TP2 and Leu9, as well as the membrane used are shown. The number of lipids and waters are indicated, and the electron density ratio plot of the system is shown. The circle and the peptide colored in orange indicate the peptide starting position in the computational electrophysiology (CompEL) simulation. Peptides are represented as Van der Waals spheres, and colored as: Arg9 in cornflower blue, MAP in green, TP10 in orange, TP2 in purple, Leu9 in red. The polar heads (phosphate and choline groups) of the phospholipids are represented as surface, and the lipid tails are represented as licorice. The inner and outer membrane are colored in dark and light gray, respectively. Waters are colored in cyan and represented as licorice. Hydrogens are hidden for clarity. These colors are maintained throughout the study.

Figure 2. Results in CompEL simulations with one peptide. (A) Illustrative summary of behaviors seen throughout the CompEL simulations in each transmembrane potential (ΔQ). We differentiate between peptide partitioning, insertion, and translocation. The results represent the ratio of behaviors in the two (ΔQ 0, 8, 12, 24) and three (ΔQ 16) replicas conducted. (B) Summary of the outcomes in the simulations of ΔQ 16 divided by peptide. (C) Molecular representation of the final snapshot in the ΔQ 16 CompEL simulations: top pose (upper image) and side pose (lower image). Two behaviors are observed: translocation of Arg9, MAP, and TP10, and insertion with pore formation of TP2 and Leu9. Peptides are colored in its own color, inner membrane in white, outer membrane in gray and waters in cyan. Peptides are depicted as spheres, membrane and waters as licorice. Only waters pertaining to the pore are shown in the top pose. Hydrogens are omitted

for clarity. A scale bar is added for size clarity. The black box indicates the peptide starting position.

Figure 3. Lipid occupancy. Occupancies are differentiated by each peptide residue, divided by inner and outer membrane contacts, and by interaction with the polar heads or lipid tails. Residue occupancy is defined as the ratio of simulation time that a residue is in contact with the lipid. The average values for the three replicas are represented.

Figure 4. Secondary structure analysis of the five peptides. The analysis has been done through a tcl script that employed VMD Secondary Structure tool[71]. The arrow indicates the time in which the peptide got inserted in the membrane.

Figure 5. Results of CompEL ΔQ 16 simulations with 8 peptides. (A) Number of behaviors observed. Only relevant (i.e., translocation and insertion) results are shown. A complete depiction of the results can be seen in Figure S4. The values include the results in the three replicas. (B, C, D, E, F) Molecular representation of the system at the end of the 500 ns of simulation. The peptides represented are Arg9, MAP, TP10, TP2, and Leu9, respectively. Peptides are shown as Van der Waals spheres (left) or cartoon (right). Surface of the polar heads is shown, differentiating between inner (white) and outer (grey) leaflets. Water molecules are shown as licorice, representing in bigger size the water residues in the pore. Hydrogens are omitted for clarity. A scale bar is added for size clarity.

Figure 6. 1. Arg9 translocation procedure in CompEL ΔQ 16 simulation with 8 peptides. Representative snapshots illustrate the structural organization of the membrane during the translocation process, portraying the key steps: (A) simulation start, some peptides adsorb to the

bilayer surface, (B) pore initiation, with water molecules entering in contact, (C) pore construction, the polar heads from both bilayers interact, (D) pore maturation, with polar heads connected, resulting in a larger pore; a peptide gets attracted to the pore and starts the insertion, (E) one peptide gets inserted into the pore, (F) the peptide reaches the lower leaflet, causing pore deconstruction, with no more interactions between polar heads, and (G) the peptide finalizes the translocation and stabilizes into the outer leaflet, leading to pore dissolution. **2. Leu9 insertion procedure in CompEL ΔQ 16 simulation with 8 peptides.** (H) Start of the simulation, (I) Pore initiation, and peptide insertion, (J) Pore construction, (K) Pore maturation, (L) More peptides insertion, and (M) Eight peptides insertion. Arg9 and Leu9 peptides are colored in cornflower blue and red respectively, and represented as Cartoon, while showing the sidechain atoms (C in cornflower blue or red, respectively, N in darker blue, O in white). The polar heads surface is shown and colored based on the bilayer: inner in white, outer in gray. Water molecules are shown as transparent cyan surface. Lipid tails are omitted for clarity.

TABLES

Table 1. System configurations in the computational electrophysiology (CompEL) method. The composition is indicated for the system without peptides (membrane control). When a peptide is added to the system (in the outer water compartment), the corresponding counterions were added (in the same water compartment).

Transmembrane potential (ΔQ)	# K+ in outer water compartment	# K+ in inner water compartment	# Cl- in outer water compartment	# Cl- in inner water compartment	# water residues
0	28	28	28	28	22850
8	30	26	26	30	22820
12	31	25	25	31	22831
16	32	24	24	32	22839
24	34	22	22	34	22832

Table 2. Summary of the simulations and replicas run in this study. The columns differentiate the transmembrane potential (ΔQ), and the rows indicate the number of peptides. If not indicated otherwise, the simulations have been run for 250 ns.

Number of peptides	0 ΔQ	8 ΔQ	12 ΔQ	16 ΔQ	20 ΔQ

1	2 replicas	2 replicas	2 replicas	3 replicas	2 replicas
8	2 replicas	2 replicas	2 replicas	3 replicas (500 ns)	-

926

927 **Table 3.** Sequence and characteristics of the peptides used in this study. GRAVY score is
928 calculated using the Kyte-Doolittle scale[118].

Peptide	Length	Sequence	Type	Net charge	GRAVY score
Arg9	9	RRRRRRRRR	Cationic	+9	-4.5
MAP	18	KLALKLALKALKAALKLA	Amphipathic	+5	0.99
TP10	21	AGYLLGKINLKALAALAKKIL	Amphipathic	+4	0.93
Dynorphin A	17	YGGFLRRIRPKLKWDNQ	Amphipathic	4	-1.26
TP2	13	PLIYLRLLRGQWC	Hydrophobic	+2	0.42
Leu9	9	LLLLLLLLL	Hydrophobic	0	3.8
K-FGF	17	AAVALLPAVLLALLAP	Hydrophobic	0	2.42

929

930 **Table 4.** Average pore radius (Å) during the 250 ns of CompEL ΔQ 16 simulation. Standard
931 deviation (SD) values are shown. The pore radius is averaged over all frames in the simulation.

# Replica	Control	Arg9	MAP	TP10	TP2
1	0	31.8 ± 6.7	0.6 ± 0.3	11.5 ± 2.8	4.7 ± 1.9

2	24.8 ± 5.5	6.3 ± 2.1	8.2 ± 2.8	3.7 ± 0.6	10.6 ± 2.6
3	21.2 ± 5.4	0	0.4 ± 0.2	9.3 ± 1.8	16.7 ± 3.4

932

933 **Table 5.** Average ratio of intra-peptide H bonds formed during the 3 replicas of 500 ns of CompEL
934 ΔQ 16 simulations with 8 peptides. Standard error of the mean (SEM) values are shown.

H bonds	**Arg9**	**MAP**	**TP10**	**TP2**	**Leu9**
Average	82.5 ± 0.7	60.7 ± 3.5	57.8 ± 4.2	51.7 ± 4.9	51.8 ± 1.9

935

936 **Table 6.** Average pore radius (Å) during the 500 ns of CompEL ΔQ 16 simulation with 8 peptides.
937 SD values are shown. The pore radius is averaged over all frames in the simulation. The second
938 line indicates the number of peptides involved in pore formation in each replica.

# Replica	**Arg9**	**MAP**	**TP10**	**TP2**	**Leu9**
1	6.2 ± 1.5 1 peptide	6.2 ± 1.6 1 peptide	11.5 ± 2.2 3 peptides	4.7 ± 1.2 2 peptides	15.5 ± 2.3 7 peptides
2	5.3 ± 1.4 1 peptide	5.5 ± 1.4 1 peptide	5.7 ± 1.6 1 peptide	10.6 ± 2.1 3 peptides	20.1 ± 2.7 8 peptides
3	6.0 ± 1.5 1 peptide	5.7 ± 1.5 1 peptide	9.3 ± 1.8 2 peptides	16.7 ± 2.3 3 peptides	24.7 ± 3.8 7 peptides
Average	**5.3 ± 0.5**	**5.8 ± 0.5**	**8.8 ± 0.9**	**10.7 ± 0.8**	**20.1 ± 1.6**

939