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Cell penetrating peptide modulation of membrane biomechanics by Molecular dynamics

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ABSTRACT

The efficacy of a pharmaceutical treatment is often countered by the inadequate membrane permeability, that prevents drugs from reaching their specific intracellular targets. Cell penetrating peptides (CPPs) are able to route across cells' membrane various types of cargo, including drugs and nanoparticles. However, CPPs internalization mechanisms are not yet fully understood and depend on a wide variety of aspects. In this contest, the entry of a CPP into the lipid bilayer might induce molecular conformational changes, including marked variations on membrane's mechanical properties. Understanding how the CPP does influence the mechanical properties of cells membrane is crucial to design, engineer and improve new and existing penetrating peptides. Here, all atom Molecular Dynamics (MD) simulations were used to investigate the interaction between different types of CPPs embedded in a lipid bilayer of dioleoyl phosphatidylcholine (DOPC). In a greater detail, we systematically highlighted how CPP properties are responsible for modulating the membrane bending modulus. Our findings highlighted the CPP hydropathy strongly correlated with penetration of water molecules in the lipid bilayer, thus supporting the hypothesis that the amount of water each CPP can route inside the membrane is modulated by the hydrophobic and hydrophilic character of the peptide. Water penetration promoted by CPPs leads to a local decrease of the lipid order, which emerges macroscopically as a reduction of the membrane bending modulus.

1. Introduction

Cell membrane acts as a filter regulating the flux of species between intra- and extracellular environments, which is at the basis of almost all the processes at cell level. The main mechanisms of translocation through the plasma membrane are endocytosis, diffusion mediated by a small perturbation of the membrane (Schmidt et al., 2010) and protein-assisted mechanism such as passive or active protein channels. However, several hydrophilic molecules, and drug candidates among them, are unable to target the intracellular environment because they cannot spontaneously pass through the lipid bilayer (Vivès et al., 2008). This membrane barrier effect has stimulated the scientific research toward effective methods able to facilitate the transport of compounds within cell membranes. In this regard, in the last decade it has been discovered that short peptides derived from protein-transduction domains, called *Cell-Penetrating Peptides* (CPP), can elicit the transportation inside living cells of a variety of covalently or non-covalently linked cargoes (Lindgren et al., 2000; Schwarze et al., 2000). Taking advantage of this mechanism, a large number of cargoes, such as antigenic peptides (Shibagaki and Udey, 2002), peptide nucleic acids (Pooga et al., 1998), antisense oligonucleotides (Astriab-Fisher et al., 2002), full-length proteins (Nagahara et al., 1998), nanoparticles (Weissleder et al., 2000) or even liposomes (Torchilin et al., 2001) have been internalized. As a main common feature, penetrating peptides are all amphipathic, net positively charged, non-toxic, and rich of arginine and lysine (Regberg et al., 2012). In 1988, the first CPP discovery by two independent research groups was based on the observation that that the trans-activator of transcription (TAT), a protein involved in HIV-1, is able to enter the nucleus crossing the cell membrane (Vives et al., 1997). In recent years, the CPP capability to penetrate cells has been largely investigated (Derossi and Joliot, 1994; Deshayes et al., 2005; Lindgren et al., 2000; Zorko and Langel, 2005). However, CPP internalization mechanisms are not yet fully

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understood, mainly because of their complex dependence on a wide variety of aspects such as the nature and size of the CPP and its cargo, the concentration and the working temperature (Ben-Dov and Korenstein, 2015; Brock, 2014; Heitz et al., 2009). Technically, there are two pathways of CPPs internalization: endocytosis and direct permeation of the membrane. With regards to direct translocation, different mechanisms may take place, such as the inverse micelle model (Derossi et al., 1996), the carpet model, the membrane-thinning model (Lee et al., 2005) and the pore formation (Allende et al., 2005). The CPPs-induced water pore formation has been experimentally observed by applying, e.g., X-ray with neutron-reflection (Choi et al., 2012), conductance measurements (Herce et al., 2009) and solid-state nuclear magnetic resonance spectroscopy (NMR) (Li et al., 2010).

The peptide penetration into the hydrophobic lipid core may also result in alterations of membrane thickness, area per lipid (Cseh and Benz, 1999; Dunkin et al., 2011), and membrane mechanical performance (Agrawal et al., 2016; Zemel et al., 2008). The interest of the scientific community in investigating the effects on membrane mechanical properties of embedded peptides and proteins is underlined by a large body of recent literature (Bouvrais et al., 2010, 2008; Dimova, 2014; Häckl et al., 1997; Mishra et al., 2011; Pabst et al., 2007; Pan et al., 2009; Pott et al., 2015; Ratanabanangkoon et al., 2003; Shchelokovskyy et al., 2011; Tristram-Nagle Stephanie et al., 2010; Vitkova et al., 2006). Computational modelling has been widely used to support the rationalization of experimental findings concerning biological and biomimetic complex systems (De Napoli et al., 2014; Havelka et al., 2014; Massai et al., 2013; Pennella et al., 2012). In this context, Molecular Dynamics (MD), able to link microscale phenomena to macroscopic thermodynamic properties (Deriu et al., 2014; Paciello et al., 2011; Soncini et al., 2007), has been successfully employed to investigate properties of phospholipid bilayers. In particular, all atom and coarse grained MD simulations have been employed to investigate how membrane properties are affected by environmental factors (Ding et al., 2017; Koshiyama et al., 2008) and composition (Akabori et al., 2014; Ding et al., 2015; Kandasamy and Larson, 2006; Orsi et al., 2010; Orsi and Essex, 2013). Particular attention has been paid to the CPP internalization process (Li et al., 2012, 2013; MacCallum et al., 2011; Yesylevskyy et al., 2009) and to membrane defects created by the presence of CPP (Herce and Garcia, 2007; Huang and García, 2013; Jean-François et al., 2008). In this regard, a comprehensive study has been performed on the Alamethicin peptide, and a decrease in bending modulus as a function of the concentration of the peptide has been observed (Pan et al., 2009). Furthermore, Coarse Grained models and Umbrella Sampling have been proposed to investigate the free energy cost of pore formation in a system made of a nona-arginine peptide interacting with 18 different lipid types (Hu et al., 2015), and a correlation between free energy and the conformational properties of lipid bilayers, including membrane thickness and membrane bending rigidity has been reported.

Despite the high CPPs' efficiency in translocation via biological membranes, the mechanism is still unclear. Aimed at providing novel insight to better elucidate how different types of CPPs affect the membrane's mechanical properties, here, a systematic investigation is proposed based on computational modelling with atomic resolution. In a greater detail, MD simulations were employed to investigate how embedded CPPs may modulate the mechanical performance of the dioleoyl phosphatidylcholine (DOPC) bilayer. Technically, the effect of six different peptides (pAntp, Tat, $(ARG)_9$, Cady, Pep and MAP) was investigated.

Findings of the present study support the hypothesis that the presence of the cell penetrating peptide increases locally lipid disorder, which directly relates to the membrane stiffness. In particular, the observed local lipid disorder is markedly caused by the amount of water molecules conveyed within the lipid bilayer by the peptide, due to its biophysical character.

2. Materials and methods

2.1. Cell penetrating peptides and lipid membrane models

The six cell penetrating peptides considered in this study are listed below.

- The pAntp $_{43}$(RQIKIWFQNRRMKWKK)$_{58}$ is the Antennapedia homeodomain fragment responsible for the penetration characterized by seven positively charged residues and it is the one of the most studied CPPs. A study has demonstrated the importance of residual W_{14} by mutation it and showing that the uptake is abolished (Prochiantz, 1996).
- The Tat $_{47}$(YGRKKRRQRRR)$_{57}$ is the minimal sequence of HIV-TAT required for cellular uptake and the translocation into the nucleus. Recently non-covalent strategies for protein and oligonucleotide delivery with Tat-CPP have been studied (Meade and Dowdy, 2007).
- The ARG_9 is a polyarginine that has been shown to be very efficient in translocation (Mitchell et al., 2000).
- Cady (GLWRALWRLLRSLWRLLWRA) consists of 20 residues, including aromatic tryptophan and cationic arginine residues, recently used to siRNA delivery (Crombez et al., 2009).
- The Pep (KETWWETWWTEWSQPKKKRKV) is composed by 21 residues, divided into three domain: a N-terminal hydrophobic motif, the interval KKKRKV responsible for interactions with nucleic acids and the interval WSQP which is a flexible linker (Morris et al., 2008).
- The amphipathic peptide, i.e., MAP, (KLALKLALKALKAALKLA) is a fully artificial peptide capable of transporting oligonucleotides and peptides (Saar et al., 2005).

Sequences of pAntp, Tat, $(ARG)_9$, Cady, Pep and MAP were considered. The pAntp and TAT were extracted from the Protein Data Bank (PDB ID: 1OMQ (Lindberg et al., 2003), and 1TAC respectively). The remaining peptides, $(ARG)_9$, Cady, Pep and MAP, were built by the PEP-FOLD 3 server (Shen et al., 2014).

In the present study, a model of DOPC bilayer constituted by 128 lipids was built with the CHARMM-Builder (Lee et al., 2016), choosing 3 nm of water thickness.

2.2. System configuration and force field

Based on models described above, seven systems were built, six consisting of one CPP embedded in a DOPC lipid bilayer (128 lipids), and one consisting of a pure DOPC bilayer (128 lipids). The membrane length was about 7 nm for all considered systems.

Main features of investigated systems are also reported in Table S1 and Table S2 in Supporting Information.

It is worth mentioning that the peptide density used in this work (~1%), computed as the peptide/DOPC molar ratio, is lower than the one at which the phospholipids undergo a phase transition (12.5%) (Herce and Garcia, 2007). Moreover the selected peptide density is in line with previous experimental studies evaluating the membrane effects caused by peptide insertion in a molar ratio range of 1–10% (Chen et al., 2017).

The CPPs were embedded in the bilayer center and grown by the *g_membed tool* (Wolf et al., 2010); the initial orientation was obtained using the OPM server (Lomize et al., 2012). To describe protein, membrane and water topology, the CHARMM36 force-field (Huang and MacKerell, 2013) and TIP3p model were adopted. Ions were added to neutralize the system charge. Each DOPC-CPP

complex was placed in an *xyz* Cartesian coordinate reference system, with the membrane normal aligned with the z-axis. Non-bonded interactions were cut off at 1.2 nm. The Particle-Mesh Ewald method was used to handle electrostatic interactions (Essmann et al., 1995). Periodic boundary conditions were considered in all directions.

2.3. Minimization and molecular dynamics

All the simulated systems were first minimized using the steepest-descent algorithm and then equilibrated in an NVT ensemble for 100 ps at 1 atm and 300 K, by means of the V-rescale algorithm (1 fs as time step; time constant $\tau = 1.0$ ps) (Bussi et al., 2007). NPT equilibration of 200 ps was then carried out, adopting the Berendsen semi-isotropic pressure coupling scheme (1 fs as time step; barostat $\tau = 1.0$ ps) (Berendsen et al., 1984), at a reference pressure of 1 atm. Finally a 200 ns long production MD was performed (2 fs as time step) in the NPT ensemble, using the V-rescale thermostat (T = 300 K; $\tau = 1.0$ ps) together with the semi-isotropic Parrinello-Rahman barostat (P = 1 atm, $\tau = 2.0$ ps) (Parrinello and Rahman, 1981) as previously done in literature (Apicella et al., 2013; Deriu et al., 2014; Grasso et al., 2018a,b,2017, 2015).

All MD simulations were performed by using the GROMACS 5 package (Abraham et al., 2015). The Visual Molecular Dynamics (VMD) software was used to monitor and visually inspect all simulation trajectories (Humphrey et al., 1996). More information on DOPC-CPP system and starting configuration is reported in Supporting Information S1.

2.4. Lipid order and membrane modulus

Lipid order and mechanical parameters have been evaluated in last 50 ns of each MD simulation.

A useful parameter for characterizing the chain mobility in the lipid bilayer is the deuterium NMR order parameter (ρ), that can be defined for every CH_2 group in the chains as:

$$\rho = \frac{1}{2}(3\langle cos^2\theta_{CD}\rangle - 1) \quad (1)$$

where θ_{CD} of C_n (carbon of the CH_2 *n*th group) is the angle between the vector connecting C_{n-1} to C_{n+1} and the membrane normal (z-axis in this case). The order parameter, ρ, is the average along the observation time interval and over the whole lipid chain. The order parameter, ρ, varies between 1 (i.e., full order along the interface normal), and $-1/2$ (i.e., full order along the perpendicular to the interface normal). When $\rho = 0$ an isotropic orientation along the normal is considered.

The membrane bending mudulus was estimated by applying a recently proposed approach (Khelashvili et al., 2013) for the case of small membranes made of lipid mixes, as explained below. In a greater detail, the bending rigidity is associated with the ability of the lipid membrane components to change their orientation with respect to each other (Khelashvili et al., 2013; Khelashvili and Harries, 2013a,b). The above mentioned lipid ability is quantified as the so called splay angle (α, ranging in 0–90°) and directly related to the splay modulus χ_{12} as follows. By monitoring the variation of splay angles on both monolayers it is possible to obtain a normalized probability distribution used to calculate the potential of mean force (PMF) as:

$$PMF(\alpha) = -k_BT\ ln\frac{P(\alpha)}{P_0(\alpha)} \quad (2)$$

where $P_0(\alpha) = sin(\alpha)$ is the probability distribution of a hypothetical non-interacting particle system (Khelashvili et al., 2010b), k_B the factor of Boltzmann and T the temperature of the system. Other details are reported in Supporting Information S2.

In the investigated systems, the splay mudulus χ_{12}, can be extracted by a quadratic fit of the PMF data (Fošnarič et al., 2006; Kozlovsky and Kozlov, 2002; Watson et al., 2011) and is representative of the membrane monolayer bending modulus, K_m.

Following a heuristic approximation (Khelashvili et al., 2013) the membrane bending modulus, K_c, can be obtained as.

$$K_c = 2K_m = 2\chi_{12} \quad (3)$$

Results will be mainly shown in terms of monolayer bending modulus, K_m.

3. Results

In the last 50 ns of the MD simulation, the structural stability of CPP was reasonably proven, in all systems under investigation, by a cluster analysis. The structural similarity of each CPP at the trajectory equilibrium was assessed by computing the Root Mean Square Deviation (RMSD) of the C-alpha atomic positions after optimal superposition, as previously done in literature (Grasso et al., 2018a,b,2016). Snapshots taken from the last 50 ns of the MD trajectory (i.e., time range 150–200 ns) were considered for the calculation. Each peptide snapshot was considered as part of a cluster when its distance to another element of the cluster was less than a critical threshold of 0.15 nm. Two clustering tests have been performed following single-linkage (Abraham et al., 2015) and gromos (Daura et al., 1999). In every performed test, one single cluster containing the 100% of explored conformations was detected. It can be noticed that the COM_{CPP} equilibrium position within DOPC membrane was different for each considered peptide. Notably, it was observed that: MAP equilibrium position was characterized by the helix axis parallel to the P-layer (Fig. 1) with a COM_{CPP} distance from the P-layer of about 0.5 nm (Fig. 2B); ARG_9 was positioned perpendicular to the P-layer (Fig. 1); Cady, Pep and Tat were stably positioned with their COM_{CPP} in the center of the lipid bilayer, at an average distance between 1.6 and 1.8 nm from the P-layer; pAntp oriented its structured polar part parallel to the P-layer, with the unstructured region buried in the DOPC hydrophobic region.

The orientation of phospholipids surrounding CPPs was differently influenced by the CPP presence, as detected analyzing the lipid order parameter reported as function of the distance from the COM_{CPP} in Fig. 3A. Overall, it can be observed that the CPP within the membrane can markedly affect both order and structural rearrangement of the surrounding phospholipids. As expected, the order for lipids closer to the CPP is more disturbed by its presence. Nevertheless, the lipid rearrangement-induced-by-CPP is still non-negligible at a distance of about 3.5 nm, in presence of Pep, Tat, and Arg9, and Cady. Interestingly, MAP does not act as disturbing element of lipid ordering.

This observed lipid rearrangement is reflected on membrane mechanical properties, in terms of bending modulus (Fig. 3B).

Here we evaluated the monolayer bending mudulus (χ_{12}) for pure DOPC equal to 9.5 k_BT, in close agreement with recent studies (Liu and Nagle, 2004; Pan et al., 2008). MAP, that was observed to poorly affect lipids order (Fig. 3A), is responsible for a very moderate variation of bending stiffness. Cady, similarly to MAP, did not markedly affect DOPC bending modulus. ARG_9, Tat and Pep, identified as the most disturbing elements of lipid order (Fig. 3A), markedly affect the membrane bending modulus. In particular, Pep drastically reduces the elastic modulus of the DOPC membrane as shown by Fig. 3B. Information concerning splay angle distributions and associated PMF (Eq. (2)) profiles is detailed in (Supporting Information S2).

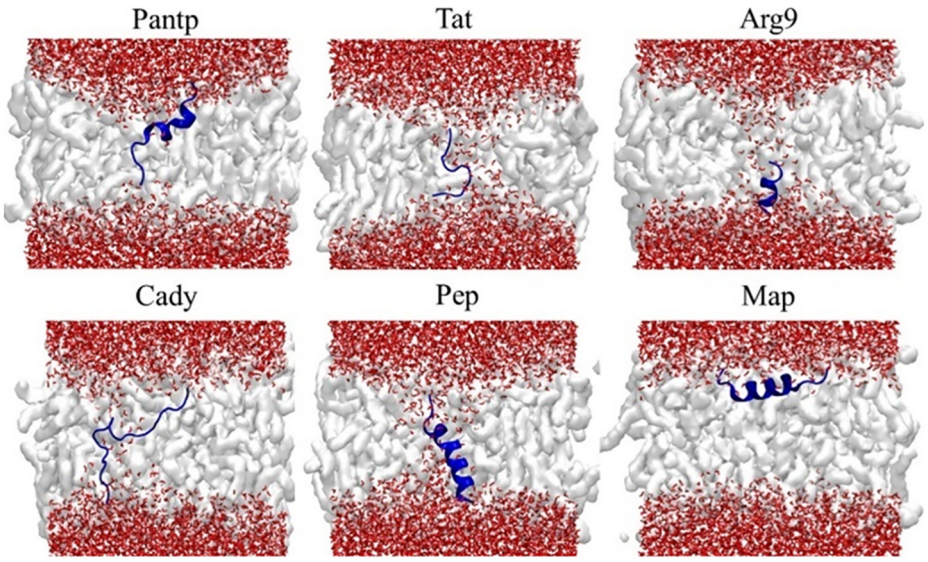

Fig. 1. Visual inspection of DOPC-CPP system after a 200 ns MD. Color code is: CPP-blue, water-red, DOPC-grey. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

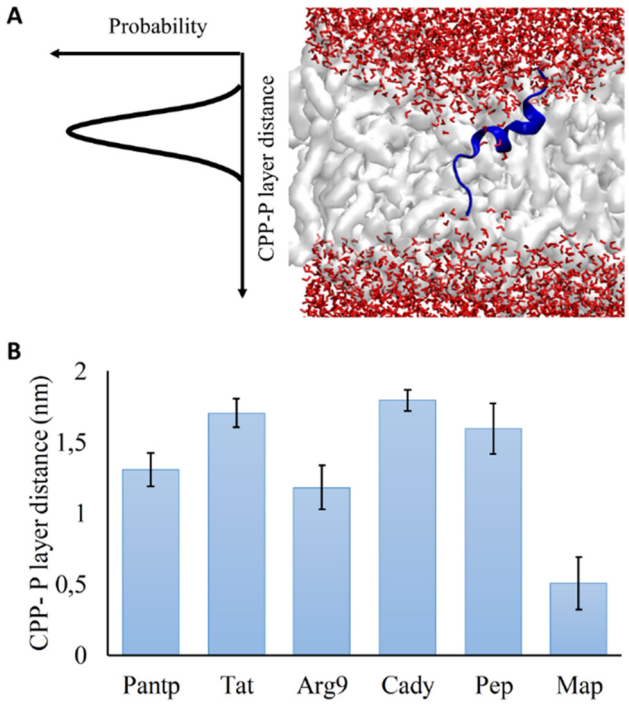

Fig. 2. (A) schematic representation of the normalized probability of distance between the CPP's center of mass and the hydrophilic membrane layer (identified by the position of phosphorous atoms). (B) average and standard deviation of the above mentioned probability distribution for each DOPC-CPP system. The probability distribution had, in all cases, a Gaussian shape.

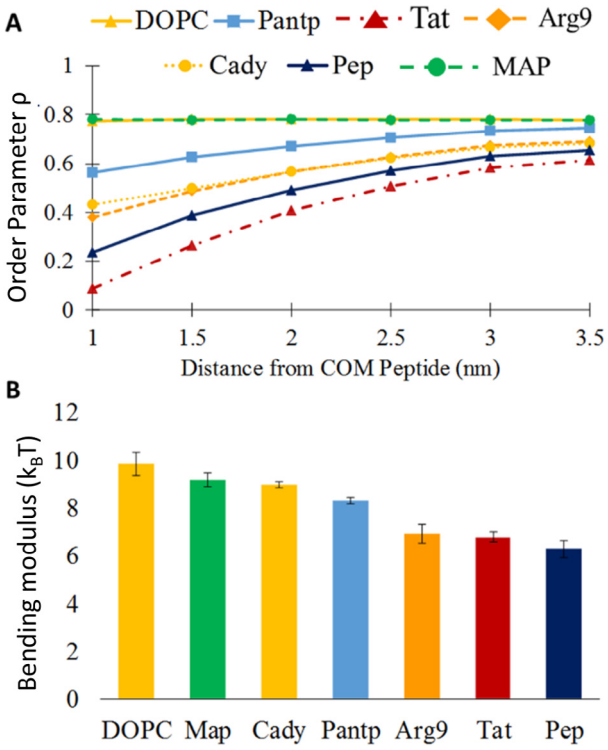

Fig. 3. (A) Lipid order parameter along the membrane as a function of the distance from the CPP center of mass. Only the average value is shown since associated standard error is always lower than 0.1% of the average value (B) Monolayer bending modulus calculated for each simulated system.

All the above mentioned findings clearly suggest that membrane mechanics is differently influenced by the presence of CPPs with different features. It is then intuitive to consider that, the CPP ability to convey water molecules into the lipid bilayer may be responsible for affecting locally the membrane bending modulus.

In this connection, the results summarized in Fig. 4 clearly show that a relationships does exist between the number of average water molecules surrounding the peptide at the equilibrium and: (i) the DOPC bending mudulus (Fig. 4A); (ii) the hydropathy of the peptide (Fig. 4B) . Technically, each CPP hydropathy coefficient (Kyte and Doolittle, 1982) was obtained as an average over all the CPP amino-acids hydropathy coefficients, each varying in the range −4.5 (highly hydrophilic), 4.5 (highly hydrophobic). Interestingly, a negative linear trend between the bending modulus and the number of water molecules surrounding the peptide (Fig. 4A) was observed. Moreover, the amount of water molecules conveyed within the membrane was found to be closely related to the peptide hydropathy coefficient. As an example, MAP is the peptide

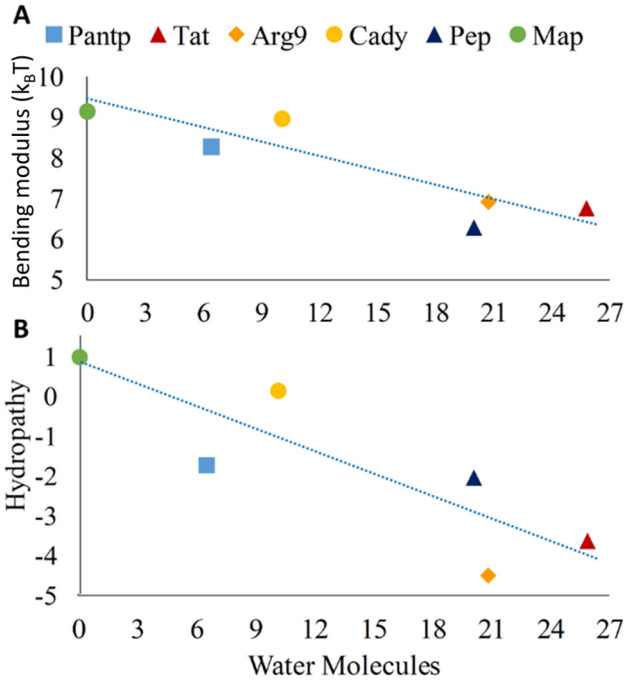

Fig. 4. (A) Monolayer bending mudulus and (B) hydropathy coefficient as a function of the number of water molecules embedded together with the considered CPP. A linear fit is shown in plot A and B (regression coefficients $R_A = -0.89$ and $R_B = -0.84$).

characterized by the highest hydropathy coefficient and the one attracting less water molecules within the membrane (Fig. 4B).

4. Discussion

Biological membranes are composed by amphiphilic phospholipids with hydrophilic heads exposed to the aqueous solvent and interacting with each other due to weak van der Waals interactions. In this context, the lipid bilayer can be considered as a fluid surface characterized by mechanical performance dependent on both the lipid components and the presence of foreign bodies or local defects (Bouvrais et al., 2010, 2008; Dimova, 2014; Häckl et al., 1997; Pabst et al., 2007; Pan et al., 2009; Pott et al., 2015; Ratanabanangkoon et al., 2003; Shchelokovskyy et al., 2011; Tristram-Nagle Stephanie et al., 2010; Vitkova et al., 2006). In recent years, much attention has been given to CPPs that, being able to cross the lipid membrane by conveying different types of cargoes (Astriab-Fisher et al., 2002; Nagahara et al., 1998; Pooga et al., 1998; Shibagaki and Udey, 2002; Torchilin et al., 2001; Weissleder et al., 2000), are candidates of election as tools supporting localized pharmaceutical treatments. In this connection, the ability of CPPs to locally modify the membrane stiffness may be a crucial parameter to design strategies for cargo transportation. However, the interplay between lipids and peptides has remained an elusive problem in membrane studies for a long time and there are still many outstanding questions to be resolved. In this context, understanding how a CPP may locally affect mechanical properties of lipid membranes is timely. Our investigation employed atomistic molecular modelling to characterize the dynamics of six different CPP-DOPC lipid bilayer systems. The findings of this study may contribute to enrich knowledge of those complex mechanisms by means of which CPPs can modulate membrane mechanics.

In detail, our findings clearly highlighted that the presence of a charged peptide inside the DOPC membrane could markedly impact on lipids organization and on lipid membrane mechanical properties as well. As noted by the lipid order parameter quantified in Fig. 3, the influence of charged peptides is remarkable. This influence is also peptide-distance dependent. In particular, phospholipids closest to the CPP were more disturbed and disordered, whereas the disturbing effect is expected to vanish for distances larger than 3.5 nm. The observed local lipid reorganization may be justified as an attempt to minimize the hydrophobic mismatch between the lipid chains and the embedded CPP. A similar influence has been recently observed as dependent on the type of membrane component (Pourmousa and Karttunen, 2013). Our simulations kept into account that the peptide presence in membrane is accompanied by a certain penetration of surrounding water (Fig. 1) coordinated by the CPP polar and hydrophilic residues (Khelashvili et al., 2010a; Sankararamakrishnan and Weinstein, 2002). This is a well-known aspect of CPPs, as it emerges from experimental studies on the HIV Tat peptide, where water penetration coordinated by arginine and lysine residues was reported (Herce and Garcia, 2007).

In this work we systematically highlighted how CPP structural and biophysical properties are responsible for modulating the membrane bending modulus. In detail, CPP hydropathy has shown to be directly correlated with penetration of water molecules in the lipid bilayer. This can lead to the assumption that, the amount of water conveyed by a CPP is modulated by the hydrophobic and hydrophilic character of the specific peptide. The overall effect is a local decrease of lipid order, emerging as a reduction of the membrane bending stiffness (Fig. 4).

A decrease in stiffness is documented in all systems with a peptide inside the DOPC membrane. In detail, among the peptides here investigated, MAP is the one that affects less the membrane properties, whereas Pep is the one that reduces most the stiffness of the lipid layer. Interestingly, peptides and proteins might be intuitively regarded as rigid inclusions in the soft membrane suggesting a stiffening of the latter (Andersen and Koeppe, 2007). Instead, outcome of the present study reminds that the insertion of CPPs inside the lipid membrane drives toward a local membrane softening, as a consequence of the induced lipid reorganization, at least for investigated peptides. A similar result had been obtained for HIV-1 fusion peptide and assuming that each protein has a unique mechanical signature, parametrized by its specific interfacial coupling to the surrounding membrane (Agrawal et al., 2016). Several experiments on HIV-1 fusion indicated a stiffness decrease as a function of peptide concentration (Agrawal et al., 2016; Shchelokovskyy et al., 2011; Tristram-Nagle and Nagle, 2007; Tristram-Nagle Stephanie et al., 2010). Moreover, a similar result was found for Alamethicin (Pabst et al., 2007; Pan et al., 2009) and for the melittin peptide (Pott et al., 2015).

5. Conclusions

In the present work we have characterized the impact that several CPPs embedded in a DOPC bilayer have on membrane mechanical properties. Particular attention has been given to the relationship between biophysical properties of the CPP and the variation of membrane bending modulus. The findings of this study highlight that: (1) CPP hydropathy highly correlated with the amount of water the peptide can attract within the membrane; (2) the presence of water molecules coordinated by the CPP locally destabilize the lipid order softening the membrane. Although other complex combinations of several parameters might play an important role in modulating membrane mechanics, such as steric hindrance and secondary structure, the CPP hydropathy is here highlighted as a crucial property for peptide design strategies.

Competing interests

The authors declare no competing interests.

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Author Contributions

GG, and MAD conceived the research.
GG, MR, SM, did the molecular dynamics simulations.
UM, AD, GG, AA and MAD analyzed and rationalized the data.
All authors wrote the paper and critically commented to the manuscript for important intellectual content.
All authors read and approved the final manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jbiomech.2018.03.036.

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