Membrane permeation properties of benzo[*a*]phenoxazinium fluorescent probes using molecular modelling techniques

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Abstract: Studies of membrane permeation properties of four benzo[*a*]phenoxazinium fluorescent probes with twenty carbon atoms at the 5- or 9-positions of the tetracyclic ring in 2,3-bis(palmitoyl-oxy)propyl-2-(trimethylammonio)ethyl phosphate, DPPC membranes using molecular modelling techniques were carried out. The molecular mechanism of interaction between the molecular probes and the DPPC lipids is described. It was showed that probes can be preferentially located at the membrane water interface interaction with the lipid polar groups, while others can be cross the membrane in a passive way. This processes is dependent on the tetracyclic substitutions and the alkyl chains present in the fluorescent probe. These findings can provide a detailed rational of the structural properties surrounding the molecular probes that could be correlated with the experimentally measured fluorescence.

Keywords: Molecular modelling, flurescent probes, benzo[*a*]phenoxazinium, DPPC

Introduction

Fluorescent probes are widely used in membrane biophysical studies to investigate the properties of the molecular environment of cell membrane and biochemical processes related to it (Ishii, Shimanouchi, et al. 2010; Bouvrais, Pott et al. 2010). While these probes can provide valuable information of the

membrane molecular properties, a clear structural description, at the molecular scale, of the membrane properties surrounding the fluorescent molecular probe, is essential for the interpretation of the experimental fluorescent signal.

Our recent preliminary photophysical studies in zwitterionic (DPPC) and cationic (*N*,*N*-dimethyl-*N*-octadecyloctadecan-1-aminium bromide, DODAB) vesicles, with benzo[*a*]phenoxazinium derivatives **1a-d** (Figure 1) showed their ability to report the gel to liquid-crystalline lipid phase transition by variations in either the extent of H-aggregation or the acid–base equilibrium (Naik, Alves et al. 2011). As a continuation of this research, the present work describes the evaluation of the membrane permeation properties of these four benzo[*a*]phenoxazinium chlorides in DPPC membranes using molecular modelling techniques. The probes can be preferentially located at the membrane water interface interaction with the lipid polar groups, while others can be cross the membrane in a passive way. The results obtained can provide a detailed rational of the structural properties surrounding the molecular probes, which could be correlated with the experimentally measured fluorescence.

Material and Methods

The structure of the molecular probes were designed with PyMol (DeLano 2008) and modelled with bonded and Lennard-Jones parameters taken from the GROMOS 53A6 (Oostenbrink, Villa et al. 2004). Partial atom charges were calculated using quantum mechanical methods using the following protocol: geometry optimization of the all-atom model of the probes was performed in the gas phase at the Hartree-Fock level, using the 6-31G(d) basis set. Partial atom charges of the united atom models of the probes were obtained using the RESP (Bayly, Cieplak et al. 1993) method using a single step, based on electrostatic potentials calculated with the 6-31G(d) basis set by GAMESS(Schmidt, Baldridge et al. 1993). The probes were modelled using a united-atom description for the methylenic and methyl groups of the aliphatic chains and were inserted in the membrane phase of an equilibrated box of a DPPC membrane. Two lipids were removed to allow the probe insertion. Each system was simulated with molecular dynamics/molecular mechanics (MD/MM) in the isothermal-isobaric

ensemble for 100 ns in order to provide a first equilibration of all system properties at 300K and 1 atm. MD/MM simulations were performed with the GROMACS 4.0.5 (Hess, Kutzner et al. 2008) package. Bond lengths were constrained with LINCS (Hess, Bekker et al. 1997) and the SPC water (Hermans, Berendsen et al. 1984) with SETTLE. Hermans Nonbonded interactions were calculated explicitly up to 0.9 nm and long-range electrostatic interactions were treated with PME (Darden, York et al. 1993; Essmann, Perera et al. 1995) with a grid spacing of 0.12 nm and a fourth-order interpolation. Neighbor searching was done up to 0.9 nm and updated every five steps. An integration time step of 2 fs was used. The pressure control was implemented using the Berendsen barostat (Berendsen, Postma et al. 1984) with a reference pressure of 1 atm, a relaxation time of 2.0 ps, and an isothermal compressibility of 4.5×10^{-5} bar^{-1}. Temperature control was set using the Berendsen thermostat (Berendsen, Postma et al. 1984). Membrane, probes and waters were separated in different heat baths with temperature coupling constants of 0.1 ps. The potential of mean force free energy profile was calculated by pulling the probe towards the interior of the DPPC membrane. For each system, the probe was pulled away to the centre of the membrane along the z-axis over 5000 ps, using a spring constant of 1000 kJ mol^{-1} nm^{-2} and a pull rate of 0.001 nm ps^{-1}. During this process, the head groups of the DPPC lipids were held restrained with a force constant of 100 kJ mol^{-1} nm^{-2}. From these trajectories, 27 snapshots were taken to generate the starting configurations for the umbrella sampling windows. In each window, 5 ns of MD were performed for a total simulation time of 135 ns utilized for umbrella sampling. Analysis of results was performed with the weighted histogram analysis method (WHAM)(Chipot 2002).

Results and Discussion

Four benzo[*a*]phenoxazinium with alkyl chains with twenty carbon atoms at the 5- or 9- positions of the tetracyclic ring (Figure 1) were studied in DPPC membranes using full atomistic molecular dynamics simulations. The four benzo[*a*]phenoxazinium probes (**1a-d**) are amphipathic molecules with a long alkyl chain and a polar group formed by the tetracyclic ring. Compounds **1a-c**

have a single alkyl chain while **1d** holds two alkyl chains (Figure 1). Furthermore, the main difference between **1a**, **1b** and **1c** is in the position where the alkyl chain is attached and the different substitutions at the second amine group. The amphipathic character of these probes clearly suggests their strong affinity towards lipid membranes hence, their use as membrane fluorescent probes in membrane biophysical studies. However, the structural variations in alkyl number and position in the tetracyclic ring as well as amine group derivations, renders different probes with distinctive membrane permeability properties.

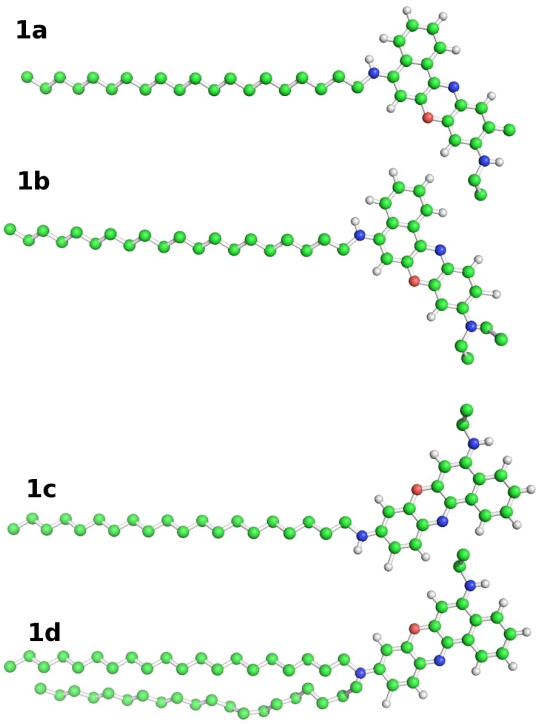

Figure 1. Structure of the benzo[*a*]phenoxazinium molecular probes **1a-d**

The stability of each probe in DPPC was evaluated using a potential of mean force (PMF) approach. The PMF modelling experiments are able to predict the free energy changes of each probe as a function of a reaction coordinate. In our experiments we evaluated the PMF along the z-axis. In this axis the probes initiate their translocation path in the aqueous environment and sample conformations along the membrane environment until they reach the transversal middle plane of the DPPC membrane. In this system the PMF incorporates all relevant effects associated with the translocation phenomena. The free-energy

profiles of membrane permeation for each probe along the membrane z-axis reaction coordinate as shown on Figure 2. The PMF of Figure 2 clearly shows that membrane has different permeability properties towards all the probes under study. The minima of the PMF free energy profile for each probe is less than k_bT, and consequently, this fact indicates that the probes are spontaneously incorporated in the membrane at room temperature. However, we observe that probe **1c** is the one less internalized, while probes **1a**, **1b** and **1d** buried in the membrane environment. Probe **1c** is highly restrained at the surface of the membrane. This fact can be visualized on Figure 3. Here, probe **1c** tetracyclic ring is interacting with the DPPC polar head groups and aqueous media, making difficult the further internalization of this probe. In fact, the positive PMF free energy profile at the middle of the membrane indicates that, it is apparently impossible to spontaneously translocate this molecule across the DPPC membrane at room temperature conditions.

Probes **1a**, **1b** and **1d** have PMF free energy minima at more internalized positions of the membrane z-axis. This clearly suggests that the tetracyclic ring is localized more internally in the membrane environment than the probe **1c**. Figure 3 clearly shows the localization of these molecules in the DPPC membrane. These structures also show that probes **1a**, **1b** and **1d** seem to be in contact with the phospholipid polar head groups from the interior side of the membrane. In addition to these observations, we can also suggest that, the most spontaneous process seems to occur with probe **1b**.

The DPPC also seem to be fully permeable to probe **1b** and **1d**, that is, these molecules seem to be able to completely cross, spontaneously, the lipid bilayer. The reasons for this seem to arise from two distinct molecular properties found in these molecules: 1) the addition of two ethyl groups at the amine position in probe **1b** tetracyclic ring seems to make this probe less polar, hence, with higher preference for the internal membrane environment; 2) on the other hand, in probe **1d**, the tetracyclic ring amine group is single-substituted with an ethyl group, and consequently, with high propensity for this group to act as a hydrogen bond donor in polar environments. However, the inclusion of two alkyl chains makes this molecule highly nonpolar, as a result, it has a high affinity towards the aliphatic phospolipid membranes and, despite the higher polar

character of the tetracyclic ring, it can cross the membrane. Probe **1a** seems to share the same properties as **1b**, however, the presence of an amine group with a hydrogen bond donor group makes impossible for this molecule to spontaneously cross the DPPC membrane.

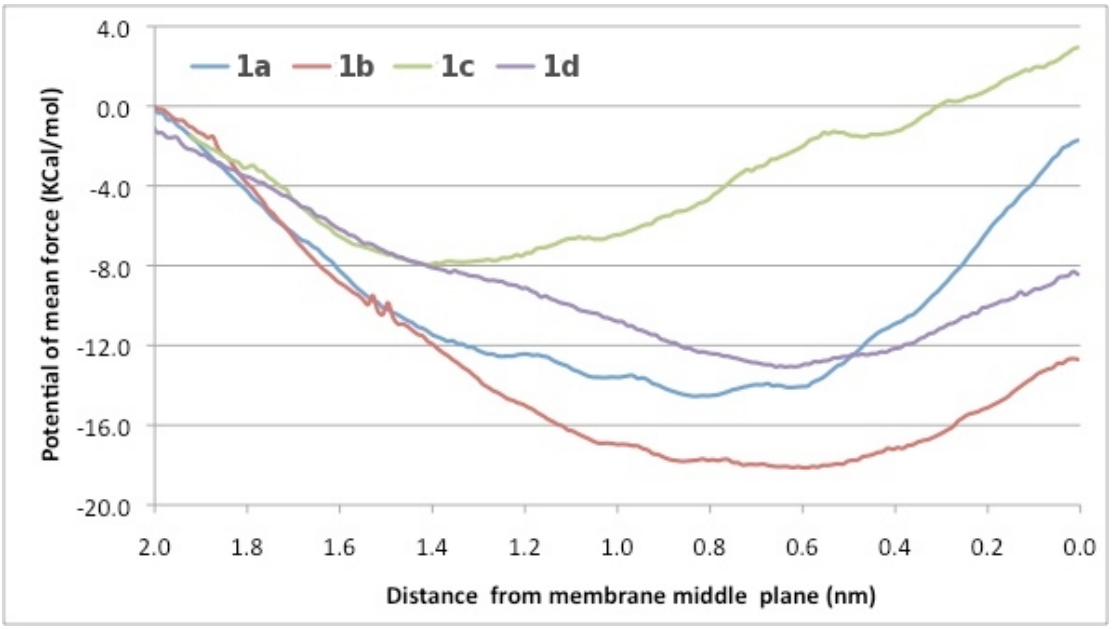

Figure 2. PMF of the molecular probes **1a-d** along the membrane z-axis reaction coordinate. PMF distance was measured using the probe centre of mass.

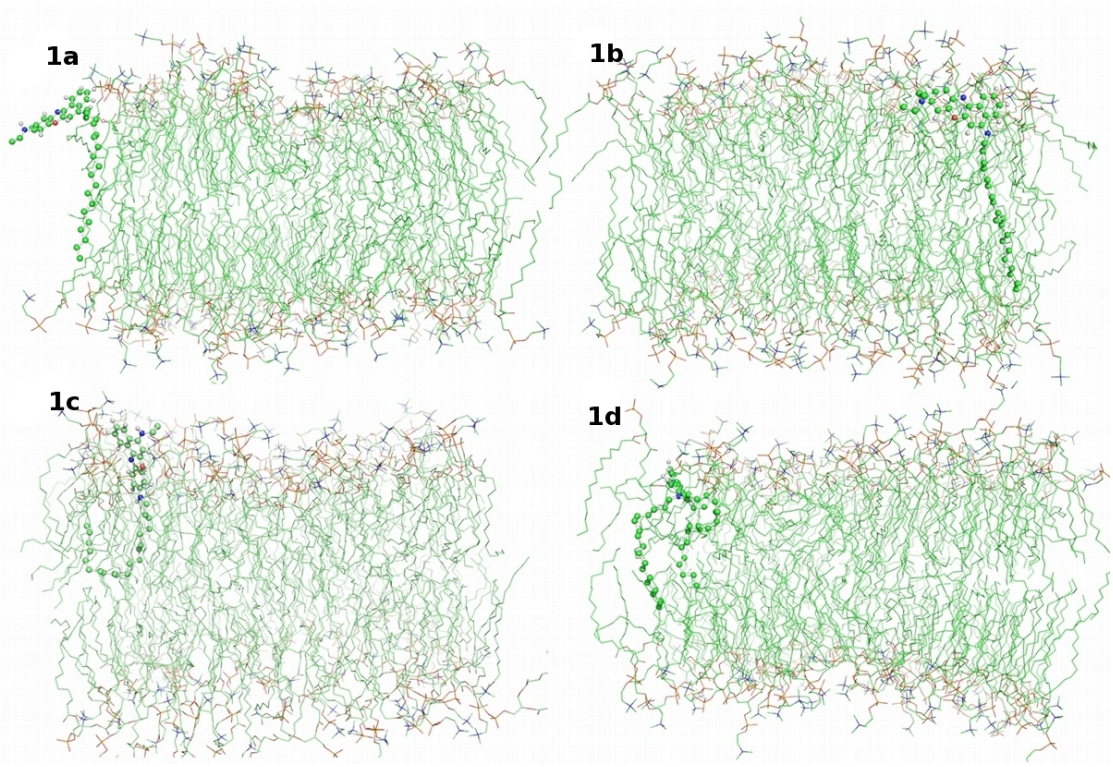

Figure 3. Snapshot of the structure of the DPPC membrane wit probes **1a**-**d**. These structural configurations correspond to the local PMF minima of Figure 2.

Photophysical studies of compounds **1a**, **1c** and **1d** in DPPC liposomes (Naik, Alves et al. 2011) were interpreted by a localization of the tetracycle at the beginning of the interface in a hydrated environment. The simulation results (Figure 3) are compatible with that interpretation with compound **1c** being the most exposed one. It is also interesting to note that H-aggregate formation was only observed for compound **1a**. According to the simulation results shown in figure 3 it seems that compounds **1a** and **1b** have orientations more suitable for aggregation by stacking the tetracycles with the aliphatic chain falling down the menbrane side-by-side.

Conclusions

We studied the permeation properties of a model DPPC membrane towards four different benzo[*a*]phenoxazinium fluorescent probes with different alkyl substituents at 5-, 9-, and 10-positions of the tetracyclic rings. We have shown that, by changing the molecular properties, in particular the hydrogend bond

donor properties of the tetracyclic ring, we can design fluorescent probes with different degrees of membrane internalization. Furthermore, the same effect can be achieved by changing the number of alkyl chains attached to the tetracyclic ring. Probes with amine groups with hydrogen bond donor properties do not cross the DPPC membrane, as shown by probe **1a** and **1c**. However, this impairment can be overcome by adding extra alkyl chains as show by probe **1d**.

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References

Bayly, C. I.; Cieplak, P. et al. (1993). "A well-behaved electrostatic potential based method using charge restraints for deriving atomic charges - the RESP model." J. Phys. Chem. 97(40): 10269-10280.

Berendsen, H. J. C.; Postma, J. P. M. et al. (1984). "Molecular-dynamics with coupling to an external bath." J. Chem. Phys. 81(8): 3684-3690.

Bouvrais, H.; Pott, T. et al. (2010). "Impact of membrane-anchored fluorescent probes on the mechanical properties of lipid bilayers" Biochim. Biophys. Acta 1798(7): 1333–1337.

Chipot, C.; Pearlman, D. A. (2002). "Free energy calculations: the long and winding gilded road." Mol. Simulation 28(1-2): 1-12.

Darden, T.; York, D. et al. (1993). "Particle mesh ewald: An N.Log(N) method for ewald sums in large systems." J. Chem. Phys. 98(12): 10089-10092.

DeLano, W. L. (2008). The PyMOL Molecular Graphics System. Palo Alto, CA, USA, DeLano Scientific LLC.

Essmann, U.; Perera, L. et al. (1995). "A smooth particle mesh ewald method." J. Chem. Phys. 103(19): 8577-8593.

Hermans, J.; Berendsen, H. J. C. et al. (1984). "A consistent empirical potential for water-protein interactions." Biopolymers 23(8): 1513-1518.

Hess, B.; Bekker, H. et al. (1997). "LINCS: A linear constraint solver for molecular simulations." J. Comput. Chem. 18(12): 1463-1472.

Hess, B.; Kutzner, C. et al. (2008). "GROMACS 4: Algorithms for Highly Efficient, Load-Balanced, and Scalable Molecular Simulation." J. Chem. Theory. Comput. 4(3): 435-447.

Ishii, H.; Shimanouchi, T. et al. (2010). "Analysis of the 22-NBD-cholesterol transfer between liposome membranes and its relation to the intermembrane exchange of 25-hydroxycholesterol." Colloids Surf. B. 77(1): 117–121.

Naik, S.; Alves, C.M.A. et al. (2011). "*N*-(Di)icosyl-substituted benzo[*a*]phenoxazinium chlorides: synthesis and evaluation as near-infrared membrane probes." Eur. J. Org. Chem. 2491-2497.

Oostenbrink, C.; Villa, A. et al. (2004). "A biomolecular force field based on the free enthalpy of hydration and solvation: The GROMOS force-field parameter sets 53A5 and 53A6." J. Comput. Chem. 25(13): 1656-1676.

Schmidt, M. W.; Baldridge, K. K. et al. (1993). "General atomic and molecular electronic-structure system." J. Comput. Chem. 14(11): 1347-1363.