J Mol Model (2013) 19:3831–3842
DOI 10.1007/s00894-013-1917-6

ORIGINAL PAPER

Effects of protein binding on a lipid bilayer containing local anesthetic articaine, and the potential of mean force calculation: a molecular dynamics simulation approach

Sepideh Amjad-Iranagh · Abbas Yousefpour · Parto Haghighi · Hamid Modarress

Received: 20 May 2012 / Accepted: 9 June 2013 / Published online: 25 June 2013
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Abstract Articaine, as a local anesthetic drug has been simulated in neutral and charged forms, and its interaction with the dimyristoylphosphatidylcholine (DMPC) lipid bilayer membrane is investigated by molecular dynamics simulation using GROMACS software. In order to obtain the optimum location of the drug molecules, as they penetrate into the membrane, umbrella sampling is applied and the free energy is calculated. The effect of protein binding to DMPC membrane on the process of drug diffusion through the membrane is considered. Five simulation systems are designed and by applying the potential of mean force, the molecular dynamics simulation on the system is performed. In light of the obtained results, the electrostatic potential, variation of lipid bilayer's order parameter and the diffusion coefficient of drug are discussed.

Keywords Articaine · Lipid bilayer · Molecular dynamics · PMF · Transmembrane protein

S. Amjad-Iranagh
Department of Chemistry, Amirkabir University of Technology, Tehran, Iran

A. Yousefpour · H. Modarress
Department of Chemical Engineering, Amirkabir University of Technology, Tehran, Iran

P. Haghighi
Department of Chemical Engineering, University of Kashan, Kashan, Iran

H. Modarress (✉)
424 Hafez Ave, Tehran, Iran 15875-4413
e-mail: hmodares@aut.ac.ir

Introduction

Articaine is a local anesthetic drug commonly utilized in dentistry. Local anesthetics consist of a lipophilic aromatic ring, a hydrophilic amine group and a link which classifies anesthetic drugs into two groups: amide type and ester type [1]. Articaine is an amide type anesthetic that has been synthesized in 1969 [1] and used by general dental practitioners in 1973 [2]. The thiophene ring instead of benzene ring, in articaine structure, causes greater lipid solubility, faster penetration and better function in the cell membranes [3]. Clinical concentration of articaine is in a range of 2–4 % in solution [3] and its half life is less than 20 min, which reduces the effect of toxicity, especially in heart and brain [2]. Interaction of local anesthetics with lipid bilayer is an interesting topic from simulation and experimental viewpoints [4, 5]. Experimental methods have been used to study membrane hydration in the anesthetic presence [6]. Also, the properties of local anesthetics such as diffusion in phospholipid membrane, their effects on the bilayer membrane, melting temperature, perturbation and their pharmacokinetic behavior have been investigated experimentally [2, 7–9].

Molecular simulation can be considered as a preferred method for evaluating predictable factors affecting the anesthetic system [7, 10–15]. For example it has been observed that adding charged and neutral forms of anesthetics have noticeable effects on the obtained simulation results [7, 14]. Presence of membrane protein has influence on the quality of interactions between drug and membrane, and as a result, on the diffusion of drug into the membrane. On the other hand, what has been neglected is whether binding protein in the lipid membrane has advantage or disadvantage on improving these results [16]. However, it has been shown that major facilitator superfamily (MFS) proteins

can transport drugs and other biological substances into the membrane [3, 17–19].

The difficulty in specification of drug properties in various positions in the lipid membrane is a convincing reason to justify the importance of applying molecular simulations to evaluate these properties and free energy calculation is one of the best ways to obtain the desired results [20]. To the best of our knowledge, no published result is available to demonstrate the partitioning of articaine in lipid membranes. Therefore, the aims of this investigation can be classified as follows:

1. Evaluation and comparison of the behavior of articaine in charged and neutral forms in the lipid bilayer.
2. Investigation of optimized location of articaine molecules in the membrane from free energy calculations and by using umbrella sampling.
3. Calculation of lipid membrane characteristics such as diffusion coefficients of the drug, area per lipid and thickness of the membrane in presence of drug molecules.
4. Examining the effect of a drug transporter such as EmrD protein [21] which is a multidrug transporter, and its experimental data such as X-ray pattern are available.

Methodology

Initial structure

Five different simulations were done using GROMACS 4.5 package [22–26]. First simulation was the reference system, containing only dimyristoylphosphatidylcholine (DMPC) lipid bilayer and water molecules. The next two simulation systems were charged and neutral articaine molecules (one drug molecule for each simulation) located near the interface of lipid bilayer and water. One neutral articaine molecule, water and EmrD protein membrane transporter, bind in DMPC, was the fourth simulation system. The fifth simulation system consisted of a neutral articaine molecule and hydrated DMPC whose free energy was calculated by umbrella sampling method. As reported, a well-equilibrated simulation system consists of 128 DMPC lipids, with equal number in each side [27, 28]. The number of species was the same in all simulation systems. The lipid molecular structure is illustrated in Fig. 1. The reason for choosing DMPC was to make the possible improvements on its functional role as a membrane and then compare the results of simulation with those already existing in the literature [7, 14]. The united atom model was used for both articaine and DMPC (except for the polar H atom in the ionized state to save simulation time). The GROMOS force field, modified by Berger [29], as successfully used previously [7, 14, 30–32] was also used in the present simulations. The simple point charge (SPC) model was applied for water molecules. The initial coordinates for both charged and neutral articaine molecules (shown in the Fig. 1) were produced by PRODRG server [33], and partial charges were modified based by Hartree-Fock quantum mechanical calculations [14].

Simulation conditions

Linear constraint solver (LINCS) algorithm was applied to constrain all bonds [34]. A time step of 2 fs and the leap frog algorithm for integrating was used. To obtain a liquid crystalline structure of DMPC, the system temperature was set to 310 K by the Nose-Hoover thermostat [35] with a coupling time constant of 0.5 ps. The pressure was adjusted to 1 bar by Parrinello-Rahman barostat [36] with coupling time constant of 2 ps. The periodic boundary condition was set in xyz directions and the Lennard-Jones cutoff radius was set to 1 nm. Computation of electrostatic interactions was done using a particle-mesh Ewald (PME) sum [37] with a 1 nm cutoff, 0.12 nm fast-Fourier grid spacing, fourth PME order and 1×10^{-5} tolerance. The list of neighbors was updated by searching every 20 fs. In each simulation (except for the reference system) the drug molecules were located outside the membrane; the whole system was solvated with a sufficient amount of water molecules and for the charged drug

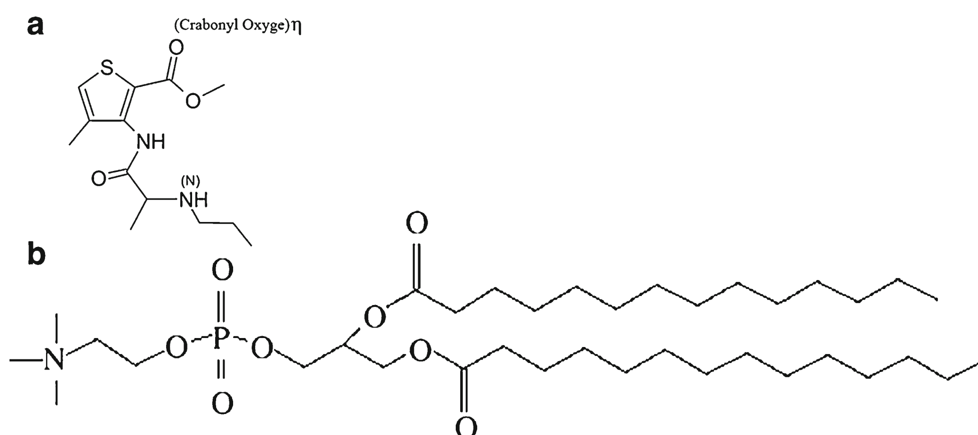

Fig. 1 The structure of articaine (**a**) and DMPC molecule (**b**). Charged articaine has been made by adding one hydrogen molecule to the atom located in (N) position

molecules a Cl^- ion was added to neutralize the system. In the other case (membrane protein), six chlorine (Cl^-) ions were used to maintain the system in the neutral state. To bind the protein in a proper way into the lipid bilayer, inflateGRO methodology was utilized [38]. After energy minimization, the equilibrium step (NVT and NPT) was performed for 10 ns. The MD run step duration for each simulation was 10 ns. All coordinates were saved for the trajectories every 20 ps.

Protein binding

The transmembrane proteins have a significant role in drug delivery processes. To the best of our knowledge, the protein binding in an articaine-lipid bilayer system has not yet been investigated. To show the role of transmembrane protein in a biological system and to evaluate the effects of protein binding on the diffusion properties of the neutral articaine molecule, we used the EmrD, which is a multidrug transporter in our simulation system. The basic structure of this protein with its detailed properties is described in the literature [21], and the protein data bank [39].

This type of protein is known as a major facilitator superfamily (MFS) protein, which can transport the drugs and other biological substances [17–19].

The system was set up by inflating and shrinking the lipid bilayer membrane to insert the protein molecules in the middle of the simulation box (the center of the lipid bilayer membrane). The shrinking procedure had been done until the area per lipid approached the appropriate value for DMPC lipid bilayer membrane. InflateGRO method was used to calculate the area per lipid bound to the protein and the result was compared with the experimental data to reach a reliable area per lipid for DMPC lipid bilayer. Some samples of the system compacting after applying the inflateGRO can be seen in Fig. 2.

Then the drug molecule was inserted into the system, outside of the bilayer membrane and close to the hydrophilic part of the bilayer. As the molecular dynamics simulation begins, the drug molecule changes location to find a position with higher feasibility for diffusion into the membrane. At the end of simulation (after 10 ns) the trajectories of the drug and protein molecules were monitored.

Potential of mean force calculation (umbrella sampling)

The potential of mean force (PMF) is an energy which is gained by integrating the mean force imposed on a certain degree of freedom of the configurations [22–25] and in this way, the free energy of the system is evaluated. Umbrella sampling is a common method in this category. Free energy calculation is an important analysis in molecular dynamics because of its ability to determine the optimized location of the species in the system. Prediction of the macroscopic properties of a material from its microscopic state and energy barriers by determining the energy landscape is the important aim of the umbrella sampling simulation.

To determine the optimum location of the drug molecule in the simulation system, the free energy calculation by umbrella sampling has been applied. This method contains the following steps: Generating a series of configurations

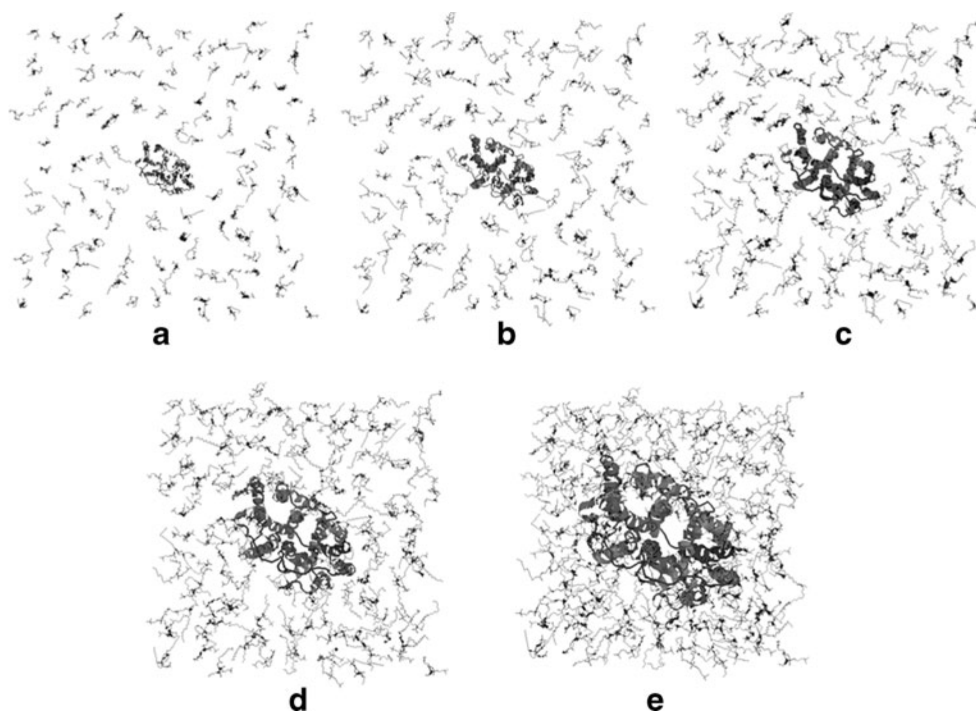

Fig. 2 The procedure of approaching the experimental value of area per lipid for the system bind with the protein

along the Z-coordinate, extracting the frames from the trajectories according to the center of mass spaces, and using the weighted histogram analysis to extract the potential of mean force.

First step was using an external force to make the drug molecule enter the membrane. A force constant of 500-kj.mol^{-1} nm^{-1} and a pull rate of 0.01 nm.ps^{-1} have been applied to pull the drug molecule in the z-direction, from one side of the membrane to the other side. Applying an appropriate pull rate according to the force constant is an important step in calculating the potential of mean force. The utilized pull rate and force constant have been defined by adjusting the force and the drug's position changes intensities along the simulation time.

The calculations contained 50 windows to get a reliable overlap between the windows for the umbrella sampling step. The windows are chosen, ranging from the bulk water to the middle of the bilayer. The PMF is calculated for one monolayer and assumed to apply to the other monolayer, due to the symmetric structure of bilayer between two monolayers. Seven of those windows are illustrated in Fig. 3. The snapshots in Fig. 3 have been taken after the completion of pulling procedure and attainment of equilibration. The location and configuration of the drug in the simulation system and its distance from the membrane are controlled by the tendency to achieve the minimum energy regardless of the biased simulations of several umbrella windows.

The pulling procedure was done in a range of −3–3 nm for the distance from bilayer center, in the z direction. A box length of 16 nm in z direction was used to have enough space in the pulling direction and to allow for a continuous pull without interacting with the periodic images of the system. After making the initial configurations for each window, 5 ns simulation time was given to the equilibration step to reach the pressure of 1 bar and the temperature of 310 K and then 15 ns was given to the MD step.

Results and discussion

PMF and protein binding

The output files provided by harmonic umbrella sampling simulation were analyzed by weighted histogram analysis method (WHAM) [40] and the potential of mean force (PMF) was calculated.

The range of z values where the lipid bilayer exists is shown by arrows in Fig. 4. As is seen from this figure, the maximum value of free energy occurs in the center of the lipid bilayer, which indicates the lack of affinity of the drug molecule to locate itself in the hydrophobic parts of the lipid bilayer. Also, the charged form of articaine does not show any tendency to diffuse into the lipid bilayer membrane and locate in the hydrophobic part of the lipid bilayer [7], and the neutral articaine's behavior in the hydrophobic part is similar to the charged form of the drug due to the defined data from free energy calculations.

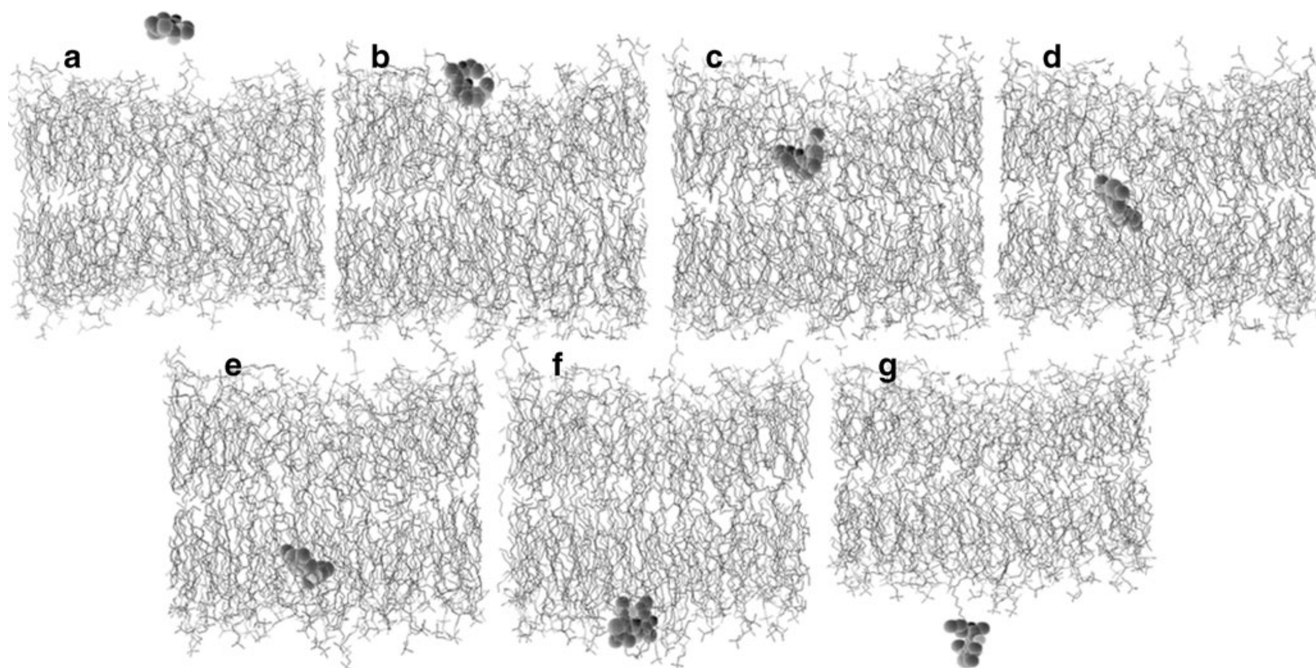

Fig. 3 Pulling procedure: **a–d** the part of pulling from the side of the membrane into the center of the bilayer, **e–g** the part of exiting the drug molecule from the other side of the bilayer

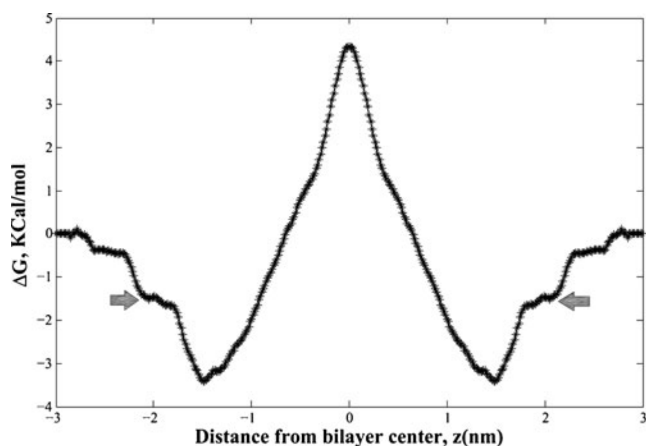

Fig. 4 The free energy of the neutral articaine molecule

On entering the drug molecule in the membrane, a sharp decrease in free energy is observed; this can be due to the lipids head groups packing. The drug-lipid interactions cause a required space for the drug molecule to locate in its preferred location, and subsequently a sudden decrease occurs in the free energy curve.

As is seen in Fig. 4, the minimum free energy is obtained inside the membrane, at a distance of about 1.5 nm from bilayer center. The results are consistent with the previous experimental and simulation studies [6, 7].

To compare two types of drugs with each other, it can be stated that, the defined behavior of the neutral articaine molecule's free energy curve is between the behavior of neutral and charged forms of nonsteroidal antiinflammatory drugs' free energy curve [20]. It can be deduced that, the optimized location of the neutral form of this type of anesthetic drugs is somewhere between the preferred locations of charged and neutral forms of nonsteroidal antiinflammatory drugs, in a lipid bilayer membrane; this can be useful in drug delivery systems to enhance the anti-inflammatory and anesthetizing procedures.

The error analysis of the evaluated free energies was done by using bootstrap technique of the GROMACS software, and this analysis approved the accuracy of the calculated free energies compared with the observed values.

For the system containing the neutral articaine and the protein molecules, the results show that, carbonyl oxygen group and thiophene ring of the drug molecule have strong interactions respectively with VAL232 and TRP289 parts of the protein molecule (illustrated in Fig. 5). The last frames obtained from the equilibration process show the thermodynamic stability of the drug at a distance of 1.72 nm from the lipid bilayer membrane center (shown in Fig. 6). The drug molecule remains in this location, because it is very close to

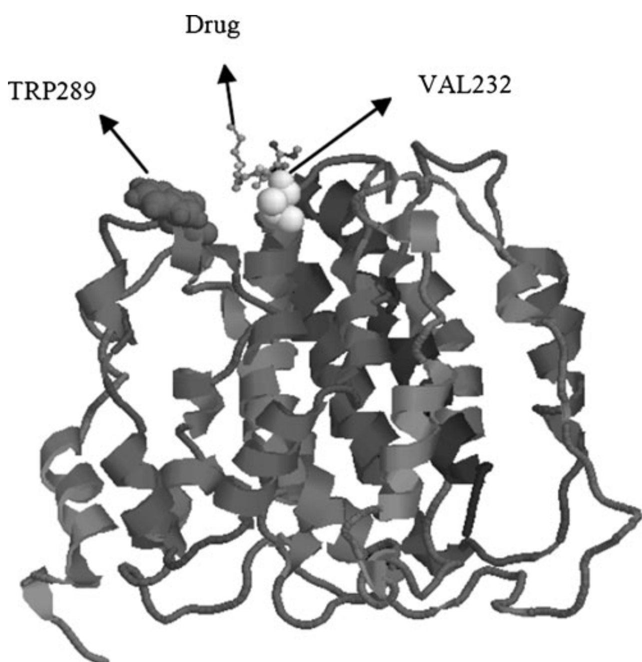

Fig. 5 A schematic of the interactions between the drug and protein molecule

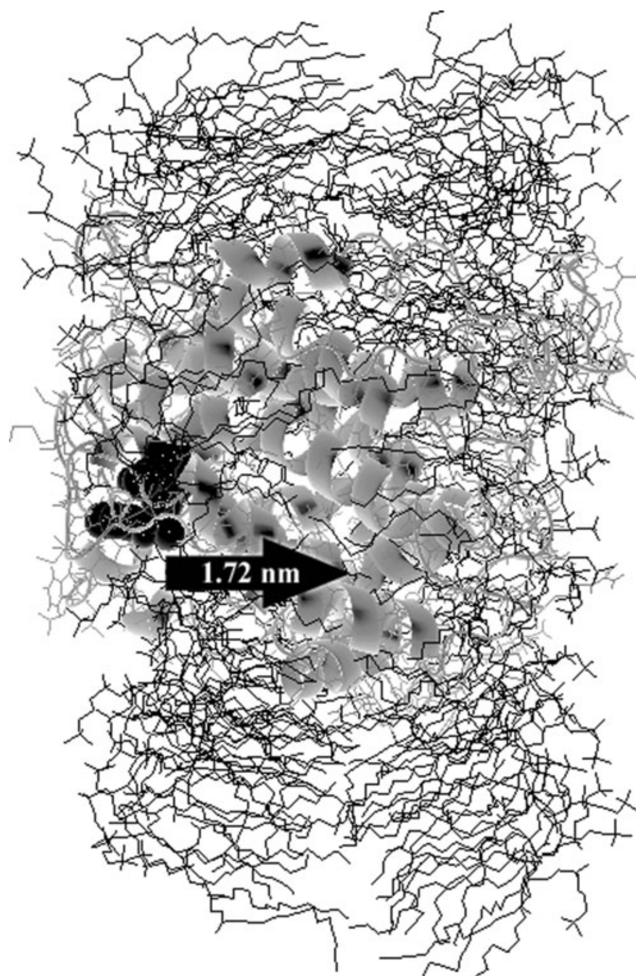

Fig. 6 The drug location after simulation in presence of protein molecules

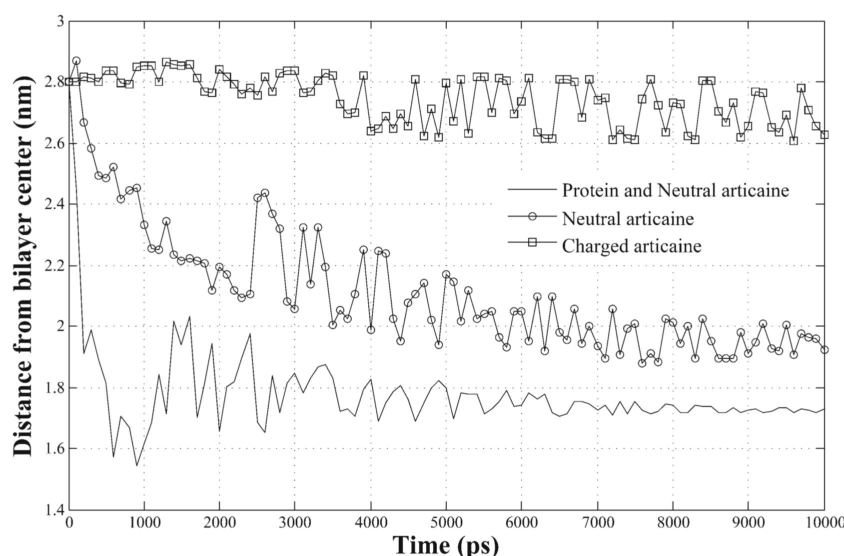

Fig. 7 Distance from the bilayer center to the centers of mass of the drug molecules

the location with the lowest free energy value (1.5 nm from the bilayer center as presented in Fig. 4); therefore, the drug molecule cannot move back in the direction of its initial configuration by increasing the distance from the bilayer center, because this involves an increase in its energy. Furthermore, the drug molecule locates in the cavity of the protein since it has stronger interactions with the protein and has no affinity to interact with the hydrophobic part of the lipid bilayer membrane. This causes the drug molecule to be trapped in the protein's cavity. Figure 7 indicates that, the amplitude of the fluctuations in the distance from the bilayer center decreases after 6 ns for the system containing the protein and neutral articaine molecules, and this confirms the thermodynamic stability of the drug molecule at 1.72 nm distance from the bilayer center. Moreover, the binding sites of EmrD are hydrophobic [21], and facilitate the drug binding into this protein. The drug-EmrD interactions cause the drug binding and change the EmrD initial structure. Therefore, the impossibility of decreasing the drug distance from the lipid bilayer center can be attributed to the binding and compact structure of EmrD which occludes the drug molecule from both sides and prevents its movement [21].

Area per lipid

One of the most common ways to determine the membrane equilibration is to calculate the average area per lipid and compare it with experimental data for X-ray spacing.

The average areas per lipid obtained in our simulations by using GridMat-MD tool [41] are presented in Table 1. The time evolution of the area per lipid for the systems containing charged and neutral forms of articaine molecule is shown in Fig. 8. The insertion of drug molecule in the system increases the area per lipid. Also the area per lipid in presence of the neutral articaine molecules is higher compared with the lower area in presence of the charged molecules. The main reason for this difference is explained by the higher interactions of neutral drug molecules with the membrane which causes a higher affinity of the drug to diffuse into the membrane. On the other hand, as a result of binding of protein molecules to the bilayer membrane, the area per lipid is increased more than in the case of protein absence since the protein molecule occupies the vacant spaces of the lipid membrane and the difference of 13.1 Å^2 for the area per lipid between neutral articaine system and the system with protein is due to this reason.

By checking the z values of the lipid head groups, it is obvious that the tendency for protein binding with molecules causes the lipid molecules to distance from the bilayer center.

By statistical uncertainty analysis, the average error estimates in the area per lipid calculations are as: ±0.50 Å^2 for the systems containing charged and neutral articaine, ±0.65 Å^2 for the reference system, and ±0.40 Å^2 for the system containing protein and neutral articaine.

Order parameter

The movements of the lipids in a fluid bilayer takes place in very short time scale; movements such as rotation around the lipid axis and chemical bonds, fluctuations and some other motions are in a range of picoseconds up to millisecond. We investigated the order parameter as an analysis of the membrane property differences, obtained from simulation and experimental methods.

Table 1 Area per lipid of each simulation system

Simulation system	Charged articaine	Neutral articaine	Protein and neutral articaine	Reference
Area per lipid (Å^2)	63.11	63.19	76.60	63.05

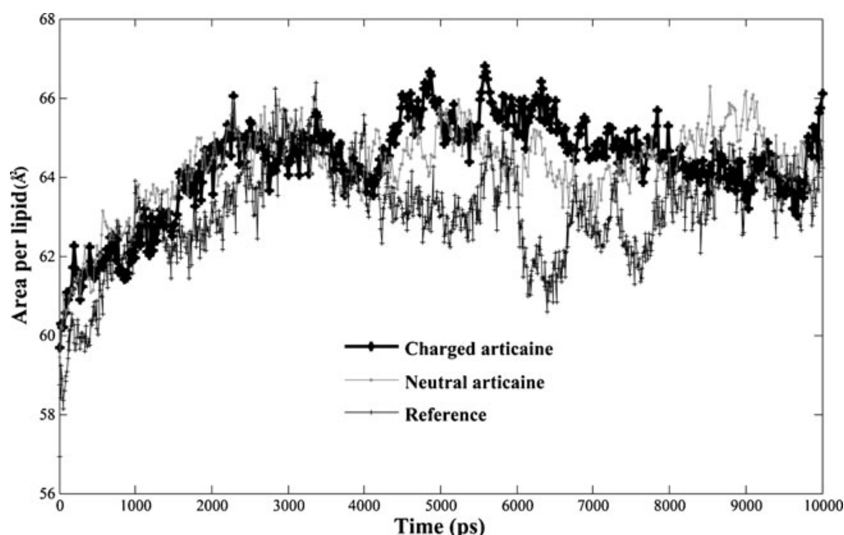

Fig. 8 The time evolution of the area per lipid for the simulated systems

The orientations of the C-H bonds, for defining the lipid order parameters, can be evaluated by the following Eq. 1:

$$S = \langle \frac{3\cos^2\theta - 1}{2} \rangle, \quad (1)$$

where θ is the angle between the normal vector of the lipid chain and the vector which is parallel to the C-H bond. The brackets in the equation show an ensemble time average.

Order parameters can be helpful in verifying whether or not the membrane entered a gel phase during the simulation.

The order parameter data for both chain 1 and 2 of DMPC are illustrated in Fig. 9, where CA, NA, PA and RE indicate the charged articaine, neutral articaine, protein binding and reference systems respectively. As is seen from this figure, the highest disorder is for the system which contains the neutral drug molecule, because its permeation through the bilayer is more than the charged molecule and therefore, has more irregularity. An important result which can be deduced from this figure is that, the order parameter in the head region of lipids has the lowest value in the system with the protein binding, despite the order parameter in the tail region for the protein binding system, which has the highest value. The reason of this high value of the order parameter is due to the perturbation in the order of the lipid chain in the tail region where the protein is located.

Hydrogen bonding

Based on the cutoffs for the angle acceptor-donor-hydrogen and the distance of hydrogen-acceptor, the number of hydrogen bonds can be determined. Functional groups such as NH and OH usually act as donors but O is always an acceptor.

The importance of this analysis is that, both charged and neutral articaine molecules form hydrogen bonds with the lipid and water molecules, therefore, it is questionable which form of articaine molecule (charged and neutral forms) has higher tendency to form a hydrogen bond with the lipid molecules.

As is seen from Fig. 10, the charged articaine molecule has higher tendency to form the hydrogen bond with both water and head groups of the lipid molecules compared with

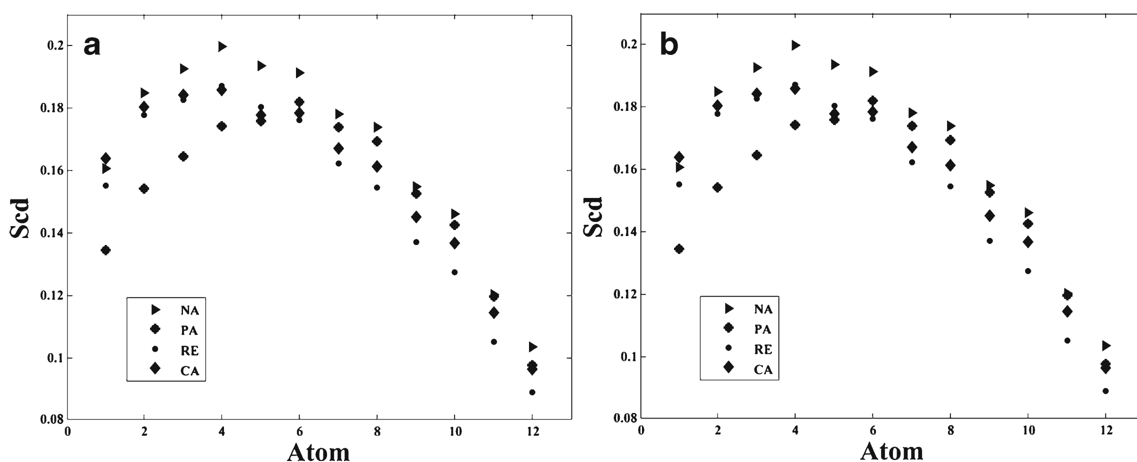

Fig. 9 The order parameters of chain 1 (**a**) and chain 2 (**b**) of the lipid

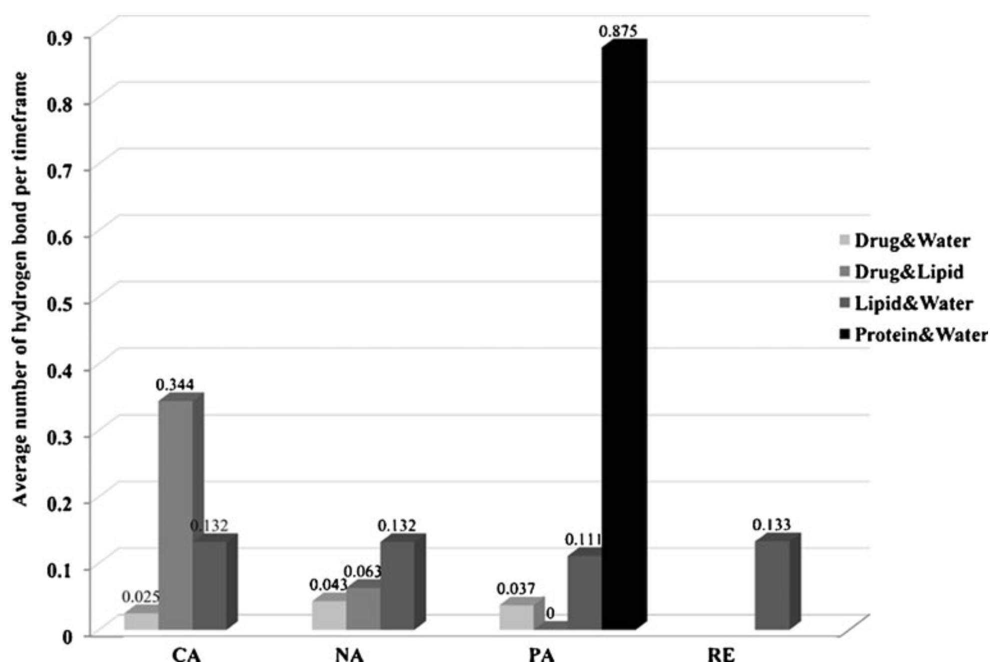

Fig. 10 Average numbers of hydrogen bonds in four simulations

both neutral articaine molecule and protein binding systems. This analysis also indicates that the penetration of neutral drug is due to the existence of a lower number of hydrogen bonds between neutral drug molecule and the lipid head groups as well as the water molecules. In the system which contains the protein molecule as well, the hydrogen bonds with both lipid head groups and water molecules are negligible in comparison with the system containing only neutral drug (0.000 and 0.043 for protein system and neutral drug, respectively).

Mass densities

The mass densities of the lipid and water are shown in Fig. 11. The density curves of charged and neutral forms of the drug are overlapped on each other, due to almost the same results. As it can be seen, the lipid density decreases in the presence of the protein molecule because in the inflating process of the system, a few lipid molecules (seven DMPC, in this study) must be removed from the whole system to conform to the reference box size and also to reach a reliable area per lipid. Therefore, the size of protein is one of the main reasons for decreasing in the lipid densities for PA simulation system, in comparison with the other simulation systems. Moreover, the reason of the differences in water density, in the system with the protein bind is due to allowing the simulation box to deform, by compressing or extending in the z direction, and this causes a difference of solvent density outside of the membrane (in the presence of the drug molecule and some protein terminals). The drug densities in different positions are also illustrated in Fig. 12. The neutral form of the drug shows more of a tendency to have the maximum density inside the lipid bilayer membrane than the charged form of the drug.

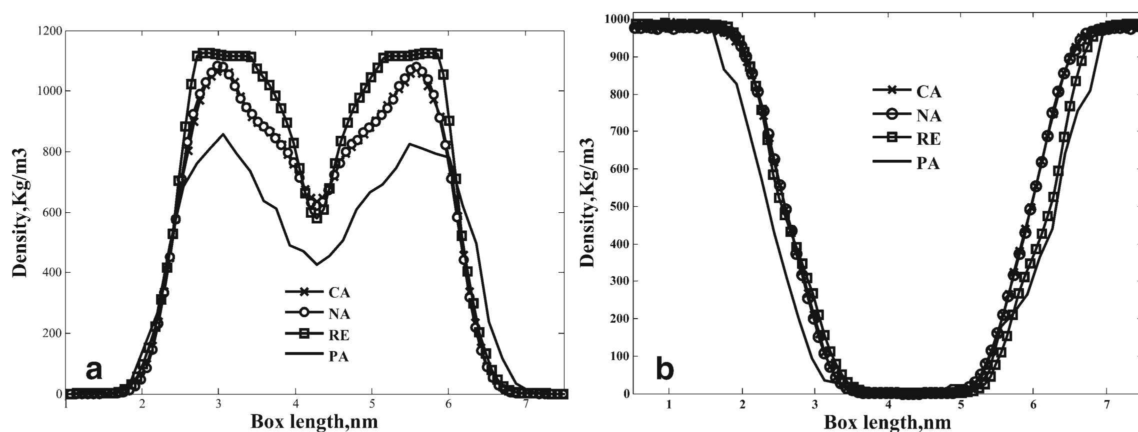

Fig. 11 **a** Mass densities of lipid in the simulations, **b** Mass densities of solvent in the simulations

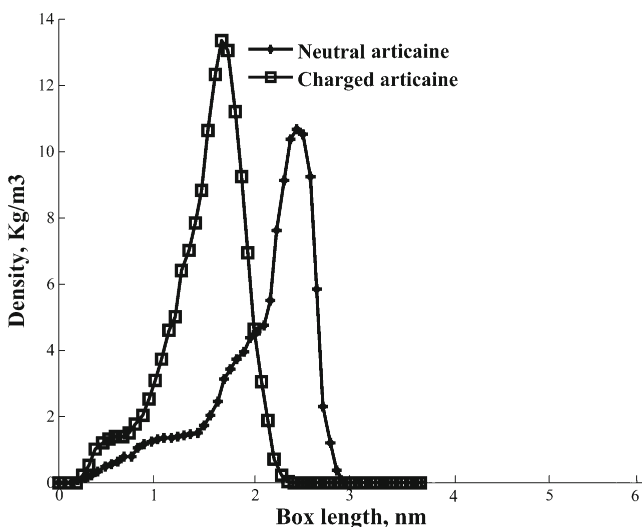

Fig. 12 Mass density of articaine molecules in the simulation

Electrostatic potential

One of the most important properties of biological membranes is their electrostatic potential, which results from charge separation across the bilayer. In 1998, Cafiso [27] mentioned that, addition of anesthetic molecules reduces the amount of the membrane dipole potential, while our simulation results and another report [7] indicate an increase in electrostatic potential for both the charged and neutral system in comparison with the reference system. This inconsistency occurs due to this fact, that to calculate electrostatic potential in the simulations, only the orientation of the lipid head group and water dipoles at the interface are considered, and some other effects have been neglected unintentionally because of the nature of molecular dynamics simulation.

As illustrated in Fig. 13, in our system with the protein binding, we have a reduction in the electrostatic potential at the walls of the simulation box. It is mentioned that, this reduction is consistent with the approach of Cafiso [27]. This means that, the binding protein in the membrane decreases the electrostatic potential of the whole system as is observed in experimental systems [6]. The asymmetric curve in presence of protein is due to the electrostatic potential calculation pathway. This pathway is along Z-axis and passes through protein locations which have different mass densities. The same method of calculating the electrostatic potential is applied to all simulation systems studied in this work, and the decrease in the electrostatic potential of hydrophilic parts of the system in presence of the protein (in comparison with the zero values of electrostatic potential in the other simulation systems) is due to the effects of protein tails on the electrostatic potential of the region containing water molecules.

Diffusion coefficient

The diffusion analysis can lead to a better understanding of protein binding effect on the quality of drug permeability. Calculating the mean square displacement (MSD) of the drug molecule is a way to obtain the diffusion coefficient of the drug molecules. Einstein's equation can be used to calculate the lateral diffusion coefficient [7]:

$$D_{lat} = \lim_{t\to\infty} \frac{1}{4}\frac{d}{dt}\langle[\mathrm{r}(t+t_0)-\mathrm{r}(t_0)]^2\rangle_{t_0}, \tag{2}$$

where $\mathbf{r}$ (t_0) and $\mathbf{r}$ $(t+t_0)$ are respectively the positions of the drug molecules at time t_0 and $t+t_0$ and the angle bracket represents their mean square displacement at t_0. As is seen from Fig. 14, the mean square displacement of the neutral form of articaine has the highest value in comparison with charged articaine and protein binding systems. This fact can justify the reason for high permeation of neutral form in the

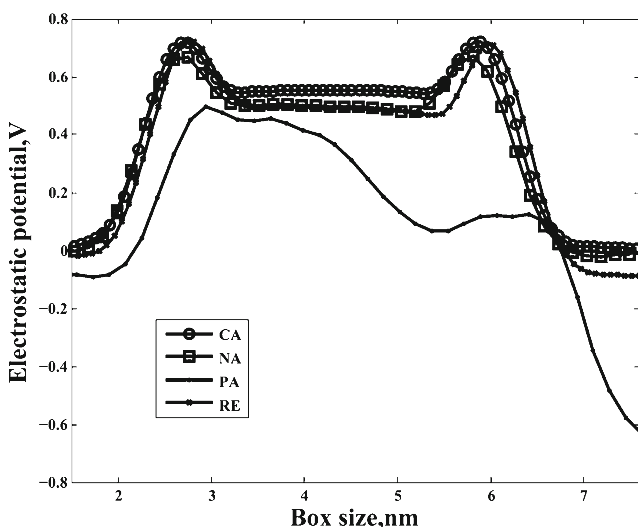

Fig. 13 Electrostatic potential of system in four simulation cases

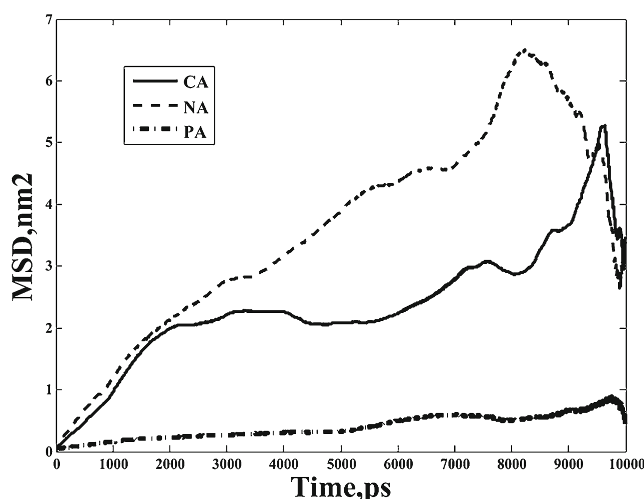

Fig. 14 Lateral mean square displacement of the articaine molecule in three simulation systems

lipid bilayer. In the protein binding system, an unexpected decrease in the diffusion coefficient has occurred. From the reported results in reference [27], the activation state of ion channels occurs at the order of 0.01 V, but in our simulation, only after binding the protein, a meaningful decrease in the electrostatic potential of the whole system is observed. This decrease suggests a way to activate the voltage gate channel of the membrane by enlarging the simulation time. On the other hand, two reasons can justify the decrease in the diffusion coefficient of the binding protein system; the first reason is the potential barrier against the penetration of the drug molecule due to the strong hydrogen bonds between the protein molecule and water molecules, and the second reason is the strong bonds between the drug and protein terminals, as mentioned earlier. This effect, which is due to the binding of the protein molecule, results in a potential barrier effect domination and subsequently a reduction in the diffusion coefficient.

The diffusion coefficient values of 0.0510×10^{-5} cm^2 s^{-1}, 0.1513×10^{-5} cm^2 s^{-1} and 0.0149×10^{-5} cm^2 s^{-1} have been obtained respectively for the charged, neutral and protein binding systems, by fitting the slope of the curves as shown in the Fig. 14 at the time interval of 1000–9000 ps, where the MSD curves have the least fluctuations.

Considering the number of inserted molecules and the used simulation time, the drug molecules in both forms (charged and neutral), have remained in their initial leaflet of the lipid bilayer membrane (the charged form on the membrane surface and the neutral form in the lipid chains' head groups). Furthermore, the neutral form of the drug shows more of a tendency to penetrate into the bilayer; this fact can be seen in Fig. 7, where the drug-bilayer distance is illustrated as a function of simulation time for both neutral and charged drug molecules. The configurations of the drug molecules at the equilibrium position with respect to the lipid bilayer membrane are presented in Fig. 15.

Conclusions

The simulation of anesthetic drug articaine in charged and neutral forms was performed. The effect of the drug on the lipid (DMPC) properties was investigated. The results were compared with the pervious experimental and simulation results [7]. The effect of protein binding on the studied systems was considered, and the free energies were calculated. The penetration of the neutral drug molecule in the lipid bilayer led to determination of protein binding influence on the lipid bilayer membrane behavior and was explained by the force causing the drugs to locate inside the membrane and thereby affects the bilayer from this position. Furthermore, free energy analysis by umbrella sampling determined the optimum position of the drug molecule somewhere inside the membrane. This finding indicates that, the drug molecule penetrates into the membrane and this is consistent with the simulation results, based on mass density calculations, reported in reference [7].

The protein binding in the system has two different effects: First, reducing electrostatic potential, which can be a reliable reason for explaining the higher diffusion of the drug molecule into the bilayer. By applying mean square displacement (MSD) analysis it was found that the penetration was decreased in this state. The second effect is the strong interaction between the drug and some terminals of the protein molecule which can be one of the reasons for the reduction in the diffusion coefficient. Also, the formation of a great

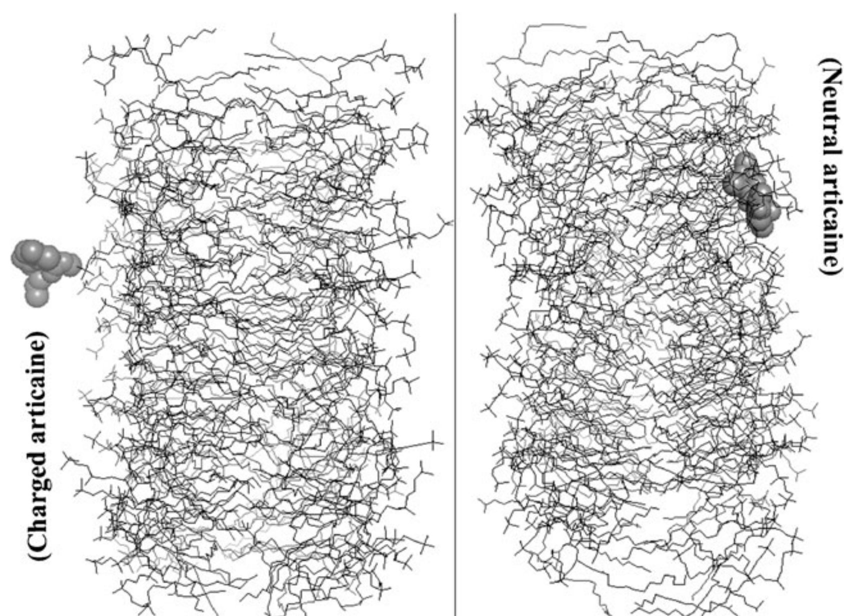

Fig. 15 The configurations of the drug molecules at the equilibrium position

amount of hydrogen bonds between the protein and water molecules makes a potential barrier which causes the drug molecule to be located outside the membrane.

The reduction in the electrostatic potential in the protein binding system suggests that, higher simulation times increase the probability of ion channel activation. In addition, investigating the role of protein binding in the charged articaine system, decreasing the size of the protein binding, applying the effective terminals of the protein as the acceptors in the system and performing the simulation with the different types of lipid bilayer and different doses of the drug are our suggestions for further studies.

Acknowledgments The authors express their thanks and gratitude to the High Performance Computing Research Center (HPCRC) of Amirkabir University of Technology (Tehran Polytechnic) for providing computer facilities.

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