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# Interaction of PEGylated anti-hypertensive drugs, amlodipine, atenolol and lisinopril with lipid bilayer membrane: A molecular dynamics simulation study

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ABSTRACT

The interaction of PEGylated anti-hypertensive drugs, amlodipine, atenolol and lisinopril with lipid bilayer membrane dimyristoylphosphatidylcholine (DMPC) has been studied in nine different simulation systems consisting of 128 lipid molecules and appropriate number of water molecules by molecular dynamics method and by utilizing GROMACS software. The influences of PEGylation on the mentioned drugs and the differences in application of two types of spacer molecules on the performance of drugs and DMPC membrane have been evaluated and mass density of the components in the simulation box, mean square displacement (MSD), electrostatic potential, hydrogen bonding, radial distribution function (RDF), area per lipid, order parameter, and angle distribution of the component molecules including drug, DMPC and PEG has been investigated. Furthermore, umbrella sampling analysis indicated that, PEGylation of the drugs made amlodipine to behave more hydrophilic, whereas in case of lisinopril and atenolol, PEGylation made these drugs to behave more hydrophobic. In almost all of the simulated systems, PEGylation increased the diffusion coefficient of the drugs.



## 1. Introduction

In kidney and cardiovascular diseases hypertension is a risk factor [1]. Hypertension is treated by regulating blood pressure and electrolyte balance [1]. Amlodipine, atenolol, lisinopril and captopril are some common drugs that are prescribed for high blood pressure treatment [1–3]. The classification of hypertension into mild, moderate and severe requires specific treatment for each level of suffering from the disease [4].

Amlodipine (shown in Fig. 1a) ((RS)-3-ethyl 5-methyl 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate) is a dihydropyridine $Ca^{2+}$ channel blocker and is widely used as a hypertension treatment drug. Moreover, amlodipine known as Norvasc is useful in cancer treatment and can be synthesized by crystallization from organic solvents [5,6]. 1,4-Dihydropyridine-based drug molecules such as amlodipine are used to treat cardiovascular disorders [7,8].

Atenolol (shown in Fig. 1b) (2-(4-{2-hydroxy-3-[(propan-2-yl) amino] propoxy} phenyl) acetamide) is a frequently used beta-blocker in the treatment of hypertension and cardiovascular diseases due to its anti-hypertensic and anti-arrhythmic characteristics [9–12].

Lisinopril (shown in Fig. 1c) ((2S)-1-[(2S)-6-amino-2-{[(1S)-1-carboxy-3-phenylpropyl] amino}hexanoyl] pyrrolidine-2-carboxylic acid), as an ACE (Angiotensin I-Converting Enzyme) inhibitor is widely used in the treatment of hypertension [13–16]. Lisinopril is marketed with the trade names of Zestril and Prinivil [17]. One of the benefits of using Lisinopril is the ability of the drug to maintain its properties, when applied with other compounds such as nano carriers [18–20].

In drug delivery systems, the main goals are targeting and protecting of the drug molecules. Nanomedicine has simplified the way to reach the best drug design mechanism. Nanoparticles, dendrimers, liposomes are applied as vehicles for safe targeting delivery of the drugs and are considered as the novel areas for research. Poly(ethylene-glycol) (PEG) (shown in Fig. 1d) is one of the most commonly used coating materials for nanoparticles [21,22], since PEG is soluble in both polar and nonpolar solvents which is due to the presence of polar oxygen atom and nonpolar $(CH_2)_2$ group in ethylene glycol. The procedure in which the drug molecule conjugates to PEG is called PEGylation and the PEGylated hydrophobic drug is protected from immune system of the human body. Furthermore, the structure of PEG and its interactions with the drug molecules in the physiological environment enhance the circulation of blood [22]. Therefore, PEG and monomethoxy PEG (mPEG) play a main role in polymer-based drug delivery systems [22]. A PEGylated prodrug consists of three molecules: drug, spacer and PEG. An important point in conjugation of drug molecules with PEG is the presence of functional groups (such as —COOH, —OH, —SH and

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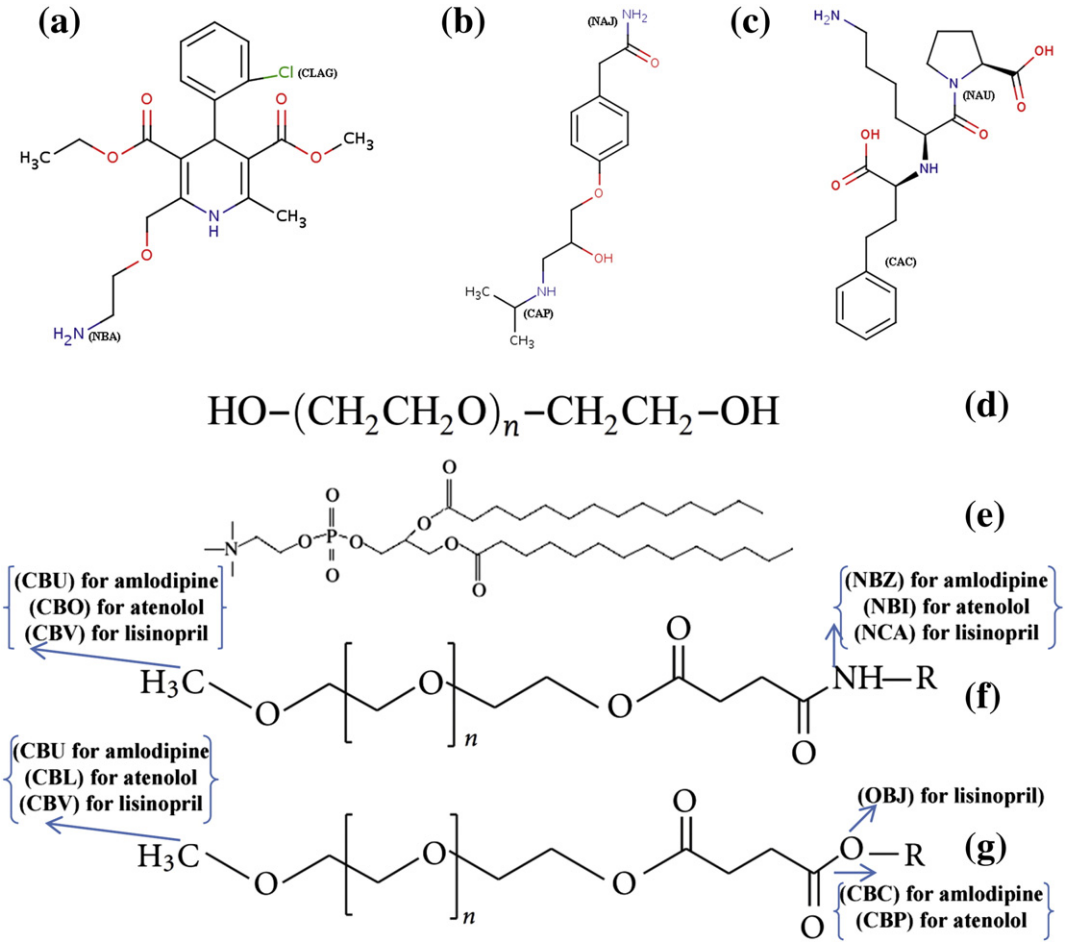


**Fig. 1.** The structure of (a) amlodipine, (b) atenolol, (c) lisinopril, (d) PEG, (e) DMPC, (f) mPEG with amide spacer and (g) mPEG with ester spacer with their atom names referred in the text.

$NH_2$) in both drug and spacer molecules. Covalent bonds in prodrug such as ester or amide which are formed between drug-spacer-PEG are the determining factors in the stability of the prodrug [23–30]. The characteristics gained by the drugs in conjugation with PEG have led the food and drug administration (FDA) to approve their pharmaceutical applications [31]. On the other hand, side-reactions, drug functionalities and availability of the reactive groups must be taken into account to reduce the disadvantages of this process. PEGylation has been applied to nano drugs which are prescribed as anti-tumor, antiviral, anti-inflammatory, and anti-microbial [31]. In the case of nano drugs, not only the PEGylation sites and PEG chain length are important [32], but the interactions between the drug molecules (PEGylated or not) and lipid bilayers are also determining factors which should be considered to understand the behavior of drug molecules when they come into contact with the phospholipid cell membranes. The phosphatidycholines, which are the chain branchings in phospholipid molecular structure, are the most abundant components in the plasma membrane of vascular systems [3]. The drug interactions with phospholipid DMPC (dimyristoylphosphatidylcholine) membrane (Fig. 1e) have been investigated by many researchers [33–53]. Therefore, in this work the drugs amlodipine, lisinopril and atenolol have been PEGylated and then their interaction with DMPC membrane has been studied to elucidate the drug and membrane behavior.

Molecular dynamics (MD) simulations as an effective tool have been utilized to achieve the purpose of this research. The almost entirely unknown mechanism of altering drug behavior after PEGylation and its interactions with DMPC membrane is one of the fundamental reasons to use molecular simulation to obtain interpretable results which are not attainable by experimental procedures. MD simulation is a reliable method which has been applied to study the interactions of drug molecules and model phospholipid membranes [49–55].

In this study, we focused on the role of PEGylation in hydrophilic drug transport through the lipid bilayer membrane. The effect of PEGylation on changing and interfering with the drugs' functionality and also using a membrane with a receptor (as used in [56]) are important topics for our future studies; therefore, the aims of this study are outlined as follows:

1) Comparing the drug diffusion into the DMPC membrane in both ordinary and PEGylated forms.
2) Studying the behaviors of three types of anti-hypertensive drugs (amlodipine, atenolol, and lisinopril) with three functionalities such as calcium channel blockers, beta-blockers and ACE inhibitors.
3) Evaluating the orientation, location and agglomeration of the drug molecules in the studied system.
4) Considering the membrane perturbation and configuration in the presence of different types of drug molecules.
5) Determining the influence of different types of spacers on the PEGylated drug behavior.

## 2. Simulation methods

Nine different molecular dynamics simulations in the simulation boxes of $6 * 6 * 10$ nm were performed by using GROMACS (Groningen machine for chemical simulations) 4.5 package [57–61]. The first three simulations were done on three types of the anti-hypertensive drugs (amlodipine, atenolol and lisinopril), PEGylated with tetraethylene

glycol by a spacer molecule containing an amide functional group (as shown in Fig. 1f) in the presence of appropriate number of water molecules and a well-equilibrated DMPC lipid bilayer membrane with 64 lipid molecules per leaflet [62,63]. The next three simulations were performed containing the drug molecules in their ordinary form (not PEGylated), and the last three simulations were done on the drugs PEGylated by ester form spacers (as shown in Fig. 1g). The components and their amounts in each simulation system are presented in Table 1; also, the schematic presentations of three of the simulation systems, as examples, are provided in Fig. 2 (and for other systems, the schematic presentations are given in the supplementary data as Figs. S1 to S6). After equilibrating the bilayer membrane for a 20 ns MD simulation, four drug molecules were inserted in the simulation box (all in the solvent bulk and on one side of the lipid bilayer membrane). The SPC (single point charge) approximation for water molecules was then utilized to solvate the whole system. The united atom model, for reducing the computation time, was used for the DMPC and the drug molecules [64]. The reliable GROMOS force field [65–69], modified by Berger [70] was applied. The initial structures of the drug molecules were obtained by PRODRG server [71]. Moreover, the atomic charges prepared by PRODRG were modified by Hartree–Fock quantum mechanical calculation, using Spartan software (Wavefunction, Irvine, CA) [67]. After neutralizing the simulation systems with the appropriate number of ions (Na and Cl), each system was energy minimized and equilibrated in NVT (for 10 ns) and NPT (for 10 ns) ensembles. After setting the temperature of the simulation systems at 310 K (to have a liquid crystalline structure of DMPC) by Nose–Hoover [39] thermostat (with a coupling time constant of 0.5 ps) and the pressure at 1 bar by Parrinello–Rahman barostat [72] (with a coupling time constant of 2 ps), the 100 ns production run was set by using the following simulation conditions: the linear constraint solver (LINCS) algorithm [73] was applied to constraint all bonds. The leap frog algorithm was utilized for integration with a time step of 2 fs. Periodic boundary condition was used in x, y and z directions. A Lennard–Jones cutoff radius of 1 nm, a particle-mesh Ewald (PME) sum [74] with a 1 nm cutoff, fourth PME order, 0.12 nm fast-Fourier grid spacing and $10^{-5}$ tolerance were also applied and every 10 fs the neighbor list was updated.

Also, potential of mean force (PMF) has been calculated using umbrella sampling with a force bias perpendicular to the membrane to investigate the partitioning of the drugs and their permeation into the membrane by considering the obtained free energy profiles. The drug molecules (PEGylated and non-PEGylated) have been pulled from their first location (bulk water) into the lipid bilayer along the z-direction using a pulling rate of 0.01 nm/ps and a force constant of 1000 kJ/(mol·nm$^2$) for a harmonic spring. For each simulation system (three systems were used for the PEGylated forms of the drugs, and another three systems for the non-PEGylated forms), 15 frames with a distance changes of about 0.2 nm between the center of mass of the drug and the lipid bilayer have been extracted. After constraining the z distance between the center of mass of the drug and the bilayer using a biasing harmonic potential, each frame (window) has been equilibrated for 5 ns and a production run of 15 ns has been applied. The weighted histogram analysis method (WHAM) has been used to calculate the potential of mean force (PMF) across a monolayer of the membrane [49]. Due to the bilayer structural symmetry, the obtained PMF profile for a monolayer has been applied to the other monolayer of the DMPC bilayer membrane. Furthermore, the bootstrap technique has been used to approve the accuracy of the obtained results. It should be noted that, the two spacers used in the drug–PEG conjugation did not indicate a significant difference on the energy profiles; therefore, the simulation results, presented in Fig. 8 are only for the amide bonded spacer molecule.

**Table 1**
The components of the simulation systems.

| Simulation system name | Components |
|---|---|
| Amlodipine | DMPC lipid bilayer (128 lipid molecules) + 4 amlodipine molecules + water (about 3300 molecules) + 4 Cl ions |
| Atenolol | DMPC lipid bilayer (128 lipid molecules) + 4 atenolol molecules + water (about 3400 molecules) + 4 Cl ions |
| Lisinopril | DMPC lipid bilayer (128 lipid molecules) + 4 lisinopril molecules + water (about 3300 molecules) |
| Amidamlodipine | DMPC lipid bilayer (128 lipid molecules) + 4 amlodipine molecules, PEGylated by amide spacers + water (about 3300 molecules) |
| Amidatenolol | DMPC lipid bilayer (128 lipid molecules) + 4 atenolol molecules, PEGylated by amide spacers + water (about 3300 molecules) + 4 Cl ions |
| Amidlisinopril | DMPC lipid bilayer (128 lipid molecules) + 4 lisinopril molecules, PEGylated by amide spacers + water (about 3300 molecules) + 4 Na ions |
| Esteramlodipine | DMPC lipid bilayer (128 lipid molecules) + 4 amlodipine molecules, PEGylated by ester spacers + water (about 3300 molecules) |
| Esteratenolol | DMPC lipid bilayer (128 lipid molecules) + 4 atenolol molecules, PEGylated by ester spacers + water (about 3300 molecules) + 4 Cl ions |
| Esterlisinopril | DMPC lipid bilayer (128 lipid molecules) + 4 lisinopril molecules, PEGylated by ester spacers + water (about 3300 molecules) + 4 Na ions |

## 3. Results and discussion

### 3.1. Mass densities

Mass density analysis has been performed to evaluate the behavior of the simulated systems in the presence of the different types of the drug molecules. Fig. 3 illustrates the mass density distribution of drugs amlodipine, atenolol, lisinopril in the ordinary and PEGylated forms, DMPC lipid bilayer membrane and solvent molecules. The asymmetry in the membrane localization of the drugs is due to the drug insertion from one side of the membrane. Fig. 3a indicates that, the PEGylation caused amlodipine molecules to be located in the solvent bulk and at a distance from the lipid bilayer membrane, whereas in the ordinary form there is a tendency of the drugs to penetrate into the lipid bilayer and locate itself somewhere between the hydrophobic and hydrophilic region of the membrane. Moreover, the sharp curve obtained from the simulation of amlodipine molecules PEGylated by the spacers containing ester and amide groups can be due to the accumulation of the drug molecules (as shown in Fig. 2). This accumulation in some cases can be useful for drug effects on the target, as well as for PEGylated mitosome, which accumulates more in tumor tissues by an enhanced permeability [5]. The mentioned behavior for the PEGylated atenolol is not as sharp as amlodipine. However, a smooth shift in the density profile of the PEGylated atenolols to the hydrophobic region is seen in Fig. 3b. As shown in Fig. 3c, PEGylation's influence on the density distribution of lisinopril molecules indicates a slight increased tendency for the PEGylated drugs to locate themselves closer to the hydrophobic regions of the lipid bilayer membrane.

Fig. 3d indicates that, the PEG molecules have their hydrophilic behavior except in the system containing PEGylated atenolol with an amide bond. However, (even very small) the density of PEG in the hydrophilic region confirms the amphiphilic nature of PEG. In Fig. 3e the density distributions of lipid and water molecules are presented.

It can be deduced that, PEGylation has the highest effect on the amlodipine molecules location in the membrane, compared with the other types of the simulated drugs in this work. However, this is not a desired effect, since it prevents the diffusion of the drug molecules into the lipid bilayer membrane. Furthermore, the type of spacer used in the PEGylated drugs has a negligible effect on the drug behavior.

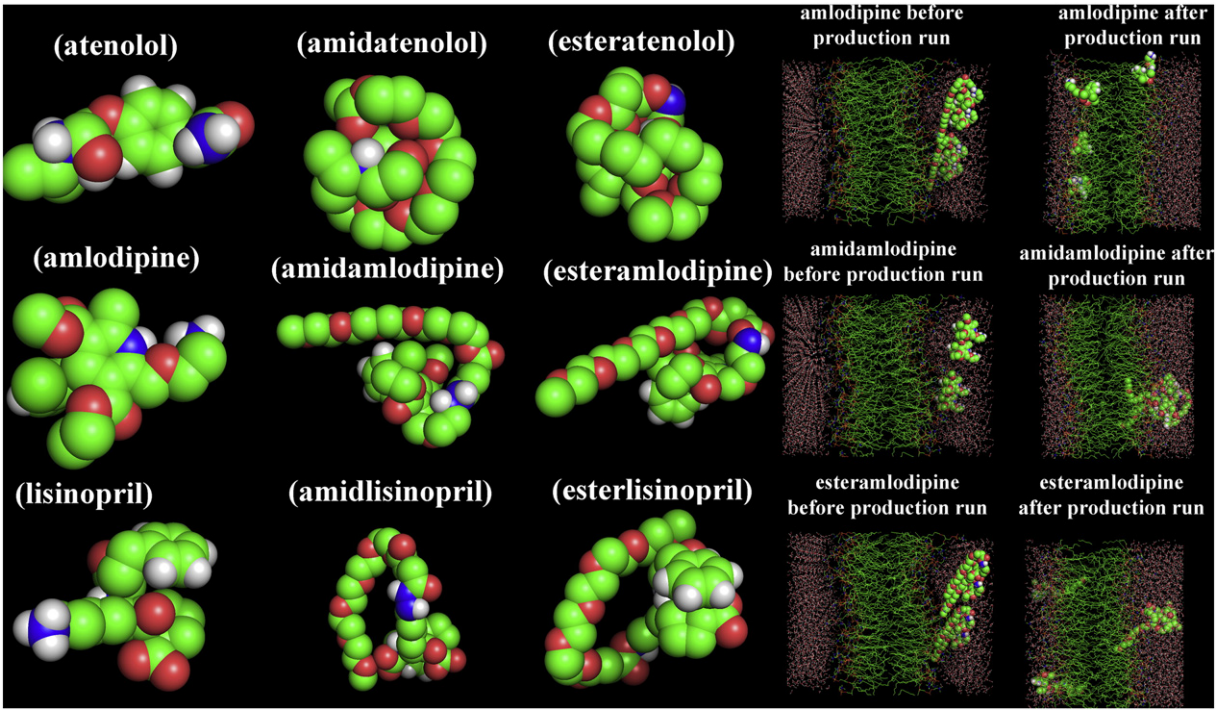


**Fig. 2.** The drug molecules' shapes after equilibration and the snapshots of the simulation systems containing amlodipine (PEGylated and ordinary forms).

### 3.2. Mean square displacement

Mean square displacement (MSD) calculation can be helpful in evaluating the drug's movement in the simulated systems. Diffusion coefficient also can be determined from MSD calculation using Einstein's equation [49]:

$$D = \lim_{t\to\infty} \frac{1}{4}\frac{d}{dt}\left\langle [r(t+t_0)-r(t_0)]^2 \right\rangle_{t_0} \quad (1)$$

where $\boldsymbol{r}(t_0)$ and $\boldsymbol{r}(t+t_0)$ are the position vectors of the simulated molecules at time $t_0$ and $t+t_0$ respectively, and the angle brackets show their mean square deviation at time $t_0$. Table 2 represents the diffusion coefficients of the drug, lipid and PEG molecules in different simulation systems. The results are obtained from the slope of the curves that are shown in supplementary data as Figs. S7–S13 at the time interval of $10^4$–$9\times10^4$ ps, where the least fluctuations are observed in the MSD curves. The highest values of the diffusion coefficients correspond to the drugs PEGylated by the ester spacer. For the systems containing lisinopril, a meaningful increase in diffusion coefficient can be seen by an ester PEGylation in comparison with the ordinary and PEGylated forms (by a spacer containing amide group). On the other hand, the ordinary form of atenolol represents lower values of the diffusion coefficient in comparison with the PEGylated (ester and amide) forms. In both systems, containing lisinopril and atenolol, the PEGylated forms of the drugs represents more movements compared with the ordinary form; whereas, the ordinary form of amlodipine has the diffusion coefficient and mean movement values, which are in between the values of the two forms of PEGylated drugs (shown in Figs. S7–S9). Fig. S10 illustrates the MSD curves of lipid molecules in the systems containing different forms of amlodipine. It can be deduced from this figure that, the differences between the lipid movements in the simulated systems are negligible Also, Figs. S11 and S12 represent the same behavior for the lipid molecules in the presence of atenolol and lisinopril. The behavior of PEG molecules are also similar to the drug molecules, which means the higher values of diffusion coefficients and molecular movements are obtained for the systems containing PEGylated drugs by ester bonded spacer compared with the ordinary and amide bonded systems (see Fig. S13).

As a result, almost in all of the simulated systems, the PEGylation increases the diffusion coefficient and the ester bonded spacers indicate a much more effect on the molecular movements.

### 3.3. Electrostatic potential

Drug insertion into the system containing lipid bilayer membrane and water molecules can change the electrostatic potential due to orientation of water dipoles and lipid head group dipoles at the water–membrane interface; moreover, in some of the simulated systems the ions added to neutralize the whole system may cause an electrostatic potential. Poisson equation relates the charge density distribution along Z-axis $\rho(z)$ to the electrostatic potential $\psi(z)$ by [49]:

$$\frac{d^2\Psi(z)}{dz^2} = -\frac{\rho(z)}{\varepsilon_0} \quad (2)$$

where $\varepsilon_0$ is the vacuum permittivity (the relative permittivity is set to 1 in atomistic simulations). Integrating Eq. (2) twice and utilizing the boundary conditions lead us to evaluate the electrostatic potential. The charge distribution is calculated from the partial atom charges set in the force field. The total electrostatic potentials for the simulation systems are illustrated in Fig. 4.

The electrostatic potentials as evaluated for all of the simulation systems containing the anti-hypertensive drug molecules show an increase compared with the system without the drug molecules (as shown in our previous studies [49–51]). It can be seen in Fig. 4 that, the highest and the lowest values for the electrostatic potential respectively have occurred in the systems consisting of amlodipine and lisinopril molecules (in their ordinary forms, without PEGylation). Whereas, PEGylation (with both ester and amide spacers) increases the electrostatic potential in the systems containing atenolol and lisinopril molecules.

According to Fig. 4, applying both types of spacer molecules has the same effect on increasing (for lisinopril and atenolol) and on decreasing (for amlodipine) the electrostatic potential compared with the values obtained in the systems containing the ordinary forms of the mentioned

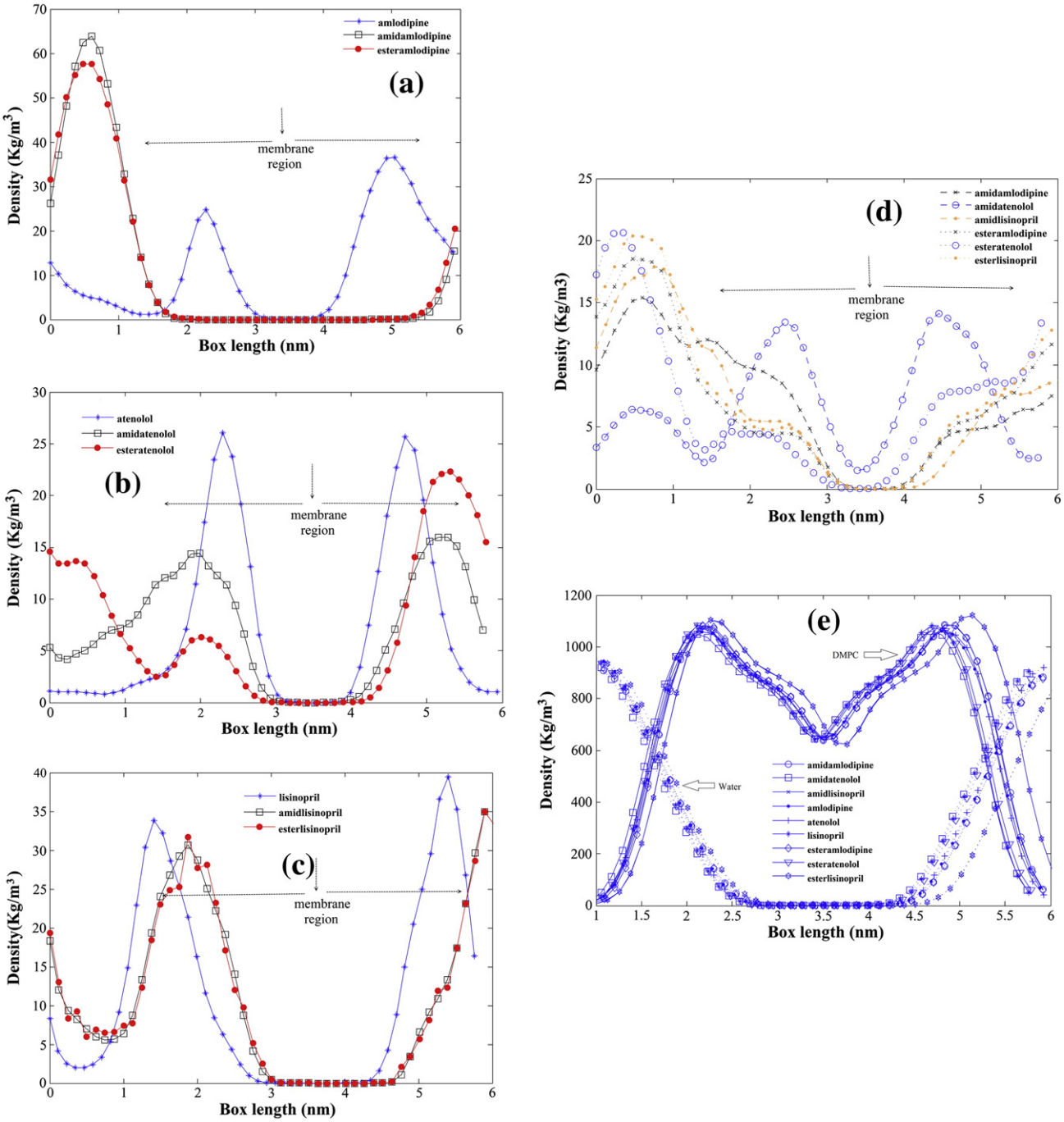


**Fig. 3.** Mass densities of (a) amlodipine, (b) atenolol, (c) lisinopril, (d) PEG, and (e) water (down) and lipid (up) molecules in different systems.

**Table 2**
Diffusion coefficients ($\times 10^7$ cm$^2$ s$^{-1}$) of different components in different systems.

| Simulation system | Drug | DMPC | PEG |
|---|---|---|---|
| Amlodipine | 3.67 | 0.43 | – |
| Atenolol | 0.81 | 0.84 | – |
| Lisinopril | 0.58 | 0.54 | – |
| Amidamlodipine | 1.38 | 0.43 | 2.58 |
| Amidatenolol | 1.41 | 0.36 | 1.90 |
| Amidlisinopril | 0.93 | 0.44 | 1.84 |
| Esteramlodipine | 3.91 | 0.43 | 2.58 |
| Esteratenolol | 2.64 | 0.89 | 2.28 |
| Esterlisinopril | 1.43 | 0.59 | 2.20 |

drugs; also, the amount of the electrostatic potential differences between PEGylated and ordinary forms is independent of the spacer type (amide or ester bonded). The occurred changes in electrostatic potential can be due to the flexibility of PEGylated drugs in altering the orientation of the lipid chains [30].

It can be concluded from this analysis that, the insertion of the drug molecules in the studied simulation systems increases the electrostatic potential, but this increase varies for different types of antihypertensive drugs (both in ordinary and PEGylated forms). This increase can be compared with the electrostatic interaction blockings caused by PEGylation, as reported in the other studies [27]. The meaningful difference between the electrostatic potential in the presence of

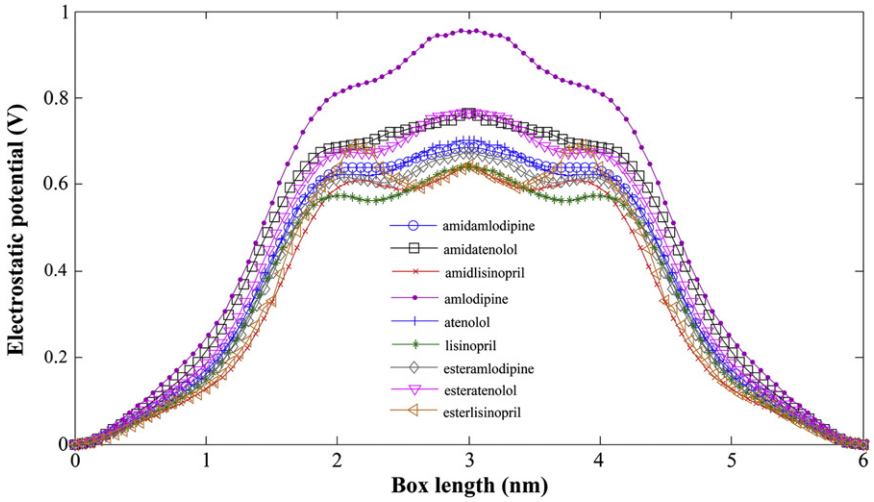


**Fig. 4.** Electrostatic potential of the simulated systems.

amlodipine in the ordinary form and the other types of the drugs can be due to the Cl atom in the structure of amlodipine; therefore, PEGylation of this drug reduces the Cl effect on increasing the electrostatic potential, since PEGylation changes the orientation of the lipid head groups against the drug molecules.

### 3.4. Hydrogen bonding

Hydrogen bonding strongly affects the distances between electron donor hydrogen and electron acceptor atoms (such as oxygen), and also the acceptor–donor bond angle, where hydrogen bonds are formed between the functional groups OH and NH. The bond distance of less than 2.5 Å and simultaneously the bond angle of acceptor–hydrogen-donor more than 90° are considered as the evidences of hydrogen bond formation [49].

Table 3 illustrates the average number of hydrogen bonds formed between drug–drug, drug–DMPC, drug–water, drug–PEG, and DMPC–water in different simulation systems. The highest number of hydrogen bonds is formed between drug–drug for the system containing PEGylated atenolols by ester bonded spacer, and the strong hydrogen bonds between drug–drug cause their agglomeration. Moreover, the lowest numbers of hydrogen bonds in this analysis correspond to the systems consisting of atenolol and amlodipine in their ordinary form. As can be seen in Fig. 3a and b, the flat mass density distribution for the mentioned systems confirms this point. On the other hand, almost the same mass density distribution for the three simulation systems containing lisinopril molecules, as shown in Fig. 3c, is consistent with the number of hydrogen bonds formed between these molecules. It can be deduced from the results in Table 3 that, PEGylation of the three simulated drugs leads effectively to drug–drug hydrogen bonds formation, compared with their ordinary forms, and this can be attributed to the presence of more donor groups in the PEGylated systems. Therefore, formation of higher number of hydrogen bonds in the PEGylated drugs acts as a strong driving force for drug diffusion. As can be seen in Table 3, the systems containing PEGylated atenolols (both with ester and amide spacers) have the highest number of drug–drug hydrogen bonds which can be due to the presence of more donor groups in these systems; whereas, for the system containing the ordinary form of atenolol, the number of hydrogen bonds is lower. Therefore, the steric altering of the drug molecule (atenolol) causes more hydrogen bond formation.

**Table 3**
Average number of hydrogen bonds between the components in the simulation systems per time frame.

| System | Drug and drug | Drug and DMPC | Drug and water | Drug and PEG | DMPC and water |
|---|---|---|---|---|---|
| Amlodipine | 0.008 | 5.662 | 17.251 | – | 801.116 |
| Atenolol | 0 | 7.279 | 6.157 | – | 794.84 |
| Lisinopril | 0.062 | 2.566 | 58.38 | – | 726.35 |
| Amidamlodipine | 0.348 | 0.015 | 29.089 | 0.209 | 801.421 |
| Amidatenolol | 0.49 | 2.93 | 39.91 | 10.28 | 808.237 |
| Amidlisinopril | 0.072 | 2.581 | 35.84 | 0.013 | 725.10 |
| Esteramlodipine | 0.093 | 0.025 | 21.794 | 0.172 | 800.266 |
| Esteratenolol | 0.870 | 1.628 | 7.420 | 0.222 | 800.072 |
| Esterlisinopril | 0.065 | 2.573 | 43.72 | 0.008 | 725.033 |

The systems containing ordinary forms of atenolol or amlodipine molecules have lower number of drug–drug hydrogen bonds and higher number of drug–DMPC hydrogen bonds, but the number of hydrogen bonds is the lowest for the systems containing PEGylated amlodipines (both ester and amide). The higher number of drug–DMPC hydrogen bonds brings the drug molecules closer to the DMPC membrane.

The highest number of hydrogen bonds is formed between drug–PEG in the systems containing the PEGylated forms of atenolols, in both ester and amide forms, but for the amide form it is higher than the ester form. Hydrogen bond formation changes the PEGylated drugs structure from linear to a semi-spherical structure. This is seen in the snapshots of the drug molecules which are taken after the production run (Fig. 2).

The system containing lisinopril molecules has the highest number of hydrogen bonds between drug–water molecules; formation of these bonds decreases the diffusion coefficient of the drug and retards the drug movements in the simulation system (this can be seen in Table 2).

The number of hydrogen bonding between water–DMPC molecules is in the lowest for the systems containing lisinopril (PEGylated or not). This is due to the fact that, while in this system the lisinopril molecules find a pathway to move between the water molecules toward the DMPC lipid bilayer membrane, in the systems containing amlodipine and atenolol molecules, the water molecules are pushed toward the bilayer and as a result they will be located closer to the membrane These phenomena cause formation of lower (for the systems containing lisinopril) and higher (for the systems containing atenolol or amlodipine) number of hydrogen bonds between water–DMPC molecules compared with the system in the absence of the drug molecules [49].

It can be concluded from this analysis that, PEGylation has almost the same influence on amlodipine and atenolol; while, PEGylated lisinopril shows more tendency to behave as a hydrophobic drug.

### 3.5. Radial distribution function (RDF)

Fig. 5 illustrates the radial distribution function (RDF) of the simulated drugs. By this analysis, the probability of water molecules around the drug molecules, and their hydration can be evaluated. The first maximum point in each curve determines the first layer of water molecules around the drugs. This figure indicates that, the drug affinity to accept water molecules for the ordinary forms of amlodipine and atenolol is less than that of their PEGylated forms. On the other hand, an inverse behavior is observed for lisinopril molecules, which indicates that, the RDF for ordinary form is higher than its PEGylated form. It can be concluded from Fig. 5 that, PEGylation affects the RDF curves for the systems containing amlodipine or atenolol much more than the system containing lisinopril. These results are consistent with the results obtained for hydrogen bond formation (see Table 3). The higher tendency of the drugs to be surrounded by water molecules is consistent with their strong affinity to form hydrogen bonds with the water molecules.

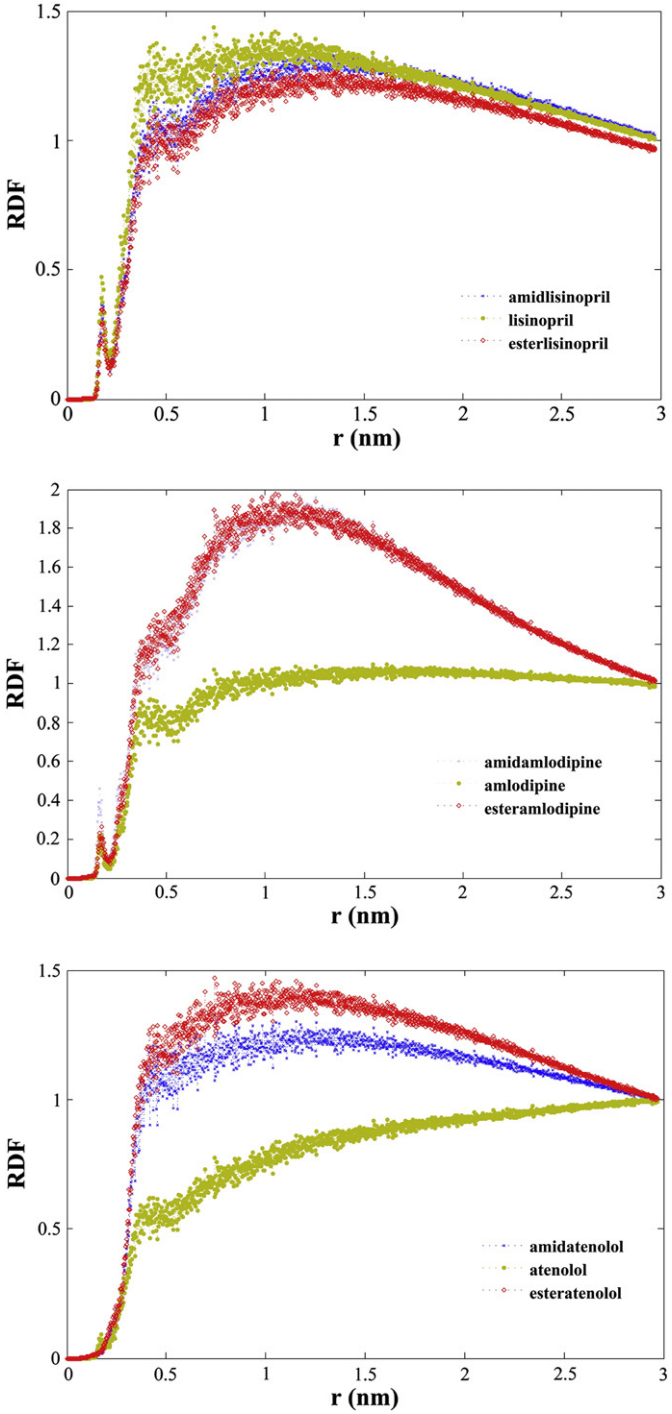


**Fig. 5.** Radial distribution function for several simulation systems.

Hydration number also can be calculated by integrating Eq. (4) up to ($r$) the first minimum in the RDF curves [49]:

$$N = \int_{r=0}^{r} N(r) = \int_{r=0}^{r} 4\pi r^2 \rho g(r) dr \tag{3}$$

where $N(r)$ is the number of water molecules in the first shell with a thickness of $dr$ at a distance $r$ from the center of mass of the drug molecule and ρ is the number density of water molecules. The hydration numbers for the studied systems are presented in Table 4.

For all of the simulated systems (in Fig. 5), the first minimum, in the RDF curves, occurs at a distance of 0.218 nm; therefore, the probability of the water molecules surrounding the drug molecules (both in ordinary and PEGylated forms) are almost the same. The calculated hydration numbers given in Table 4 indicate that, the maximum and the minimum probabilities of water molecules surrounding the drug molecules are respectively for the systems containing ordinary lisinopril and PEGylated atenolol by an amide bonded spacer. The differences between the number of hydrogen bonds and the RDF for the system containing PEGylated atenolol by amide bonded spacer are due to the stronger affinity of the PEGylated drug to form hydrogen bonds even with a low number of water molecules. Therefore, a direct relationship between hydration number and number of hydrogen bonds cannot be always true.

### 3.6. Area per lipid

By multiplying the two dimensions ($xy$) of the simulation box and dividing the result by the number of lipid molecules in one leaflet of the lipid bilayer membrane (64 molecules in this study), an important parameter called area per lipid can be evaluated. The lipid membrane equilibration and its distance from the phase transition condition can be studied by area per lipid analysis. The average area per lipid and the time evolution of the area per lipid (DMPC) for the simulated systems are presented in Fig. 6 (the time evolutions of the area per lipid for all of the simulation systems are provided separately in the Supplementary data, Figs. S14–S22). It is evident that, all the systems remained in the liquid crystalline phase [49].

The maximum area per lipid corresponds to the system containing amlodipine molecules in their ordinary form. For all of the simulated systems a meaningful reduction has been obtained in comparison with a system in the absence of the drug molecules [49]. These calculated results show that, the lipid molecules have tendency to approach each other. As can be seen in Fig. 6, the system containing the PEGylated lisinoprils (with a spacer containing amide bond), has the least value of area per lipid.

Insertion of lisinopril molecules (PEGylated or not) in the simulation systems has the highest influence on the lipid (DMPC) chains to change

**Table 4**
Average hydration numbers of the simulated drugs.

| System | Hydration number |
|---|---|
| Amlodipine | 0.0981 |
| Atenolol | 0.0516 |
| Lisinopril | 0.2330 |
| Amidamlodipine | 0.1840 |
| Amidatenolol | 0.0492 |
| Amidlisinopril | 0.1940 |
| Esteramlodipine | 0.1340 |
| Esteratenolol | 0.0599 |
| Esterlisinopril | 0.1880 |

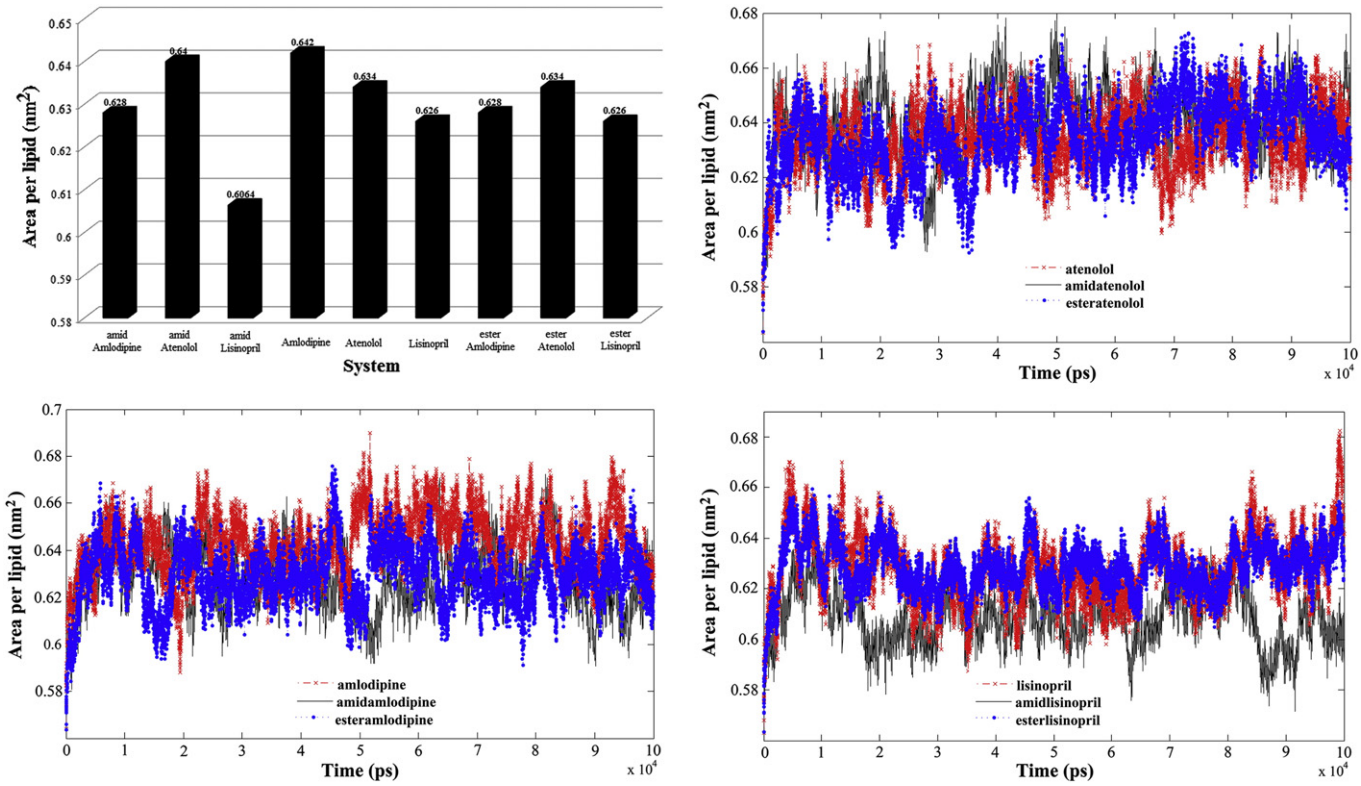


**Fig. 6.** The area per lipid and the time evolution of the area per lipid for the simulated systems.

their locations and causes the highest fluctuations in the calculated area per lipid (as shown in Fig. 6). One of the obvious reasons of this phenomenon is the low number of hydrogen bonds formed between DMPC–water molecules. More interactions between the lipid head groups, due to the lack of water molecules in their vicinity, bring the DMPC molecules closer to each other and would make a compact structure for the DMPC molecules compared with in the other simulation systems.

### 3.7. Order parameter

By order parameter analysis, the movements for lipid (DMPC) chains and the fluctuations that occur in a very short period of time can be taken into account. Also, lipid chain disorders in the membrane can be evaluated by this analysis. The order parameter is expressed as [49]:

$$S = \left\langle \frac{3\cos^2\theta - 1}{2} \right\rangle \tag{4}$$

where $\theta$ is the angle between the molecular vector and the vector which is parallel to the bilayer normal (z). The angle brackets in this equation correspond to the time averaged value.

Fig. 7 illustrates the order parameter data for both chains 1 (Fig. 7a) and 2 (Fig. 7b) of DMPC lipid bilayer membrane. As can be seen in this figure, the highest and the lowest values of the disorders are respectively for the systems containing amide form of the PEGylated lisinoprils and the ordinary form of amlodipines. The results are consistent with those of Fig. 6 for the area per lipid in different simulation systems. The maximum variation in the lipid chain locations occurs in the system containing amide form of the PEGylated lisinopril; therefore, the maximum value of order parameter should be expected for this simulation system. These results indicate the influences of PEGylation, in amide form of lisinopril molecules, on the lipid chain disorders.

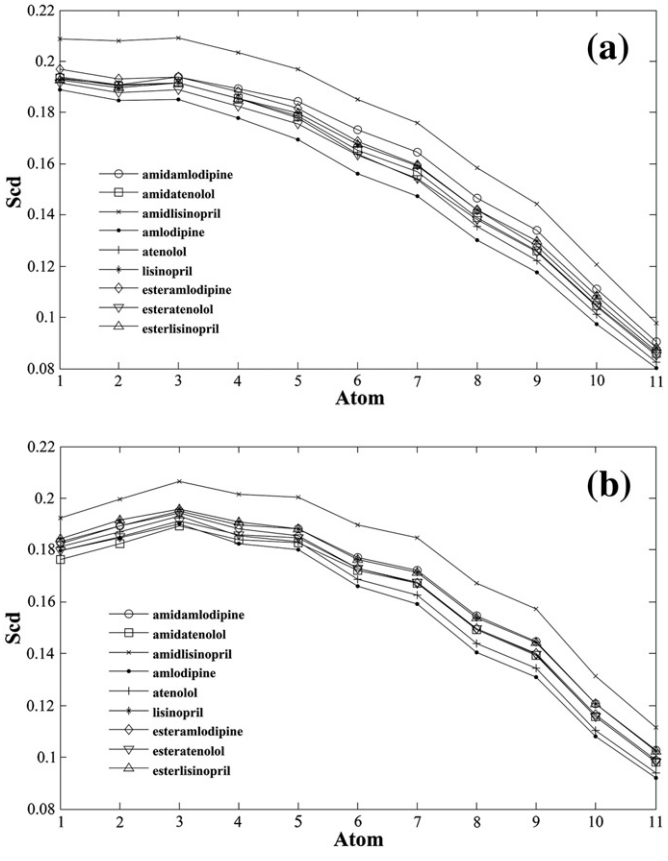


**Fig. 7.** The order parameters of chain 1 (a) and chain 2 (b) of the lipid.

### 3.8. Angle distribution

To evaluate the system behavior during the simulation time, the angle distribution analysis has been performed. The drug and lipid chain orientations, as the important parameters in the molecular simulation, can be defined by appropriate vectors for both drug and lipid molecules. In this research, the vectors formed by the two specified atoms in the drug and lipid molecules are considered as represented in Fig. 1 and in Table 5.

The angle between the mentioned vectors and the normal vector of the lipid bilayer membrane, which is parallel to the z-axis, has been analyzed by GROMACS angle distribution calculators during the 100 ns time of MD simulation. The zero angle is for the direction out of the (or perpendicular to the) lipid bilayer membrane surface. Table 6 shows the average and the highest probable values (in degrees) for the calculated angles (for the drug molecules). The data obtained for the lipid chains are for the angles between the normal vector of the bilayer and the vector formed by the atoms P and N as shown in Fig. 1.

Figs. S23, S24 and S25 (in the supplementary data) show the angle distribution during the 100 ns production run. The angle distribution of PEG molecules in the simulation systems is provided as the Supplementary data (Figs. S26–S28).

As is shown in the mentioned figures and tables, amlodipine molecules (PEGylated or ordinary) prefer to be oriented perpendicular to the lipid chains. Almost the same average angles for the amlodipine molecules in the PEGylated and in the ordinary forms justify the fact that, PEGylation does not have significant effect on the angle distribution. The highest effect of PEGylation on the angle distribution on the amlodipine molecules appears as a 7° decrease in the orientation of the drug molecules which makes the angle more compatible for the drug to diffuse into the lipid bilayer membrane. The maximum effect of PEGylation on the angle distribution corresponds to the system containing atenolol molecule PEGylated by an ester spacer. Atenolol molecules in their ordinary form prefer to be oriented perpendicular to the lipid bilayer membrane, but the PEGylation reduces the angle between the drug vector and the lipid bilayer normal vector (the differences are about 30° and 70° for the PEGylated drugs respectively for amide and ester spacers). The drug molecules diffuse into the lipid bilayer membrane and the drug–drug interactions make changes in the drug orientation to be located in an optimized orientation in respect to the lipid chains. PEGylation by both types of ester and amide spacers resulted in an angle change of about 60° for the systems containing lisinopril molecules. These significant differences in angle distribution justify the importance of PEGylation in making the drug molecules to achieve an optimized orientation. The other fact deduced from the angle distribution analysis of lisinopril molecules is the negligible effect of the spacer type (amide or ester) on the drug orientation where both of the spacers resulted in almost the same change in the drug orientations (but in different directions in respect to the normal vector of the lipid bilayer).

The PEG angle orientations are reported in Table 6 and also are provided as the supplementary data, which show how the long chains of PEG are oriented during the simulation time. The sharp PEG angle distribution curves for the PEGylation by amide spacers (for the three studied drugs), are compared with those of ester spacers, which demonstrate higher stability for the PEG molecules orientation in the PEGylation by amide spacer. Furthermore, whenever the PEGylated drug orientation in respect to the membrane is studied in a simulation system, the PEG stability should also be taken into account.

**Table 5**
Specified atoms in different systems to form vectors.

| Simulation system | Drug vector (atom 1–atom 2) | PEG vector (atom 1–atom 2) | |
|---|---|---|---|
| | | Amide spacer | Ester spacer |
| Amlodipine | CLAG-NBA | NBZ-CBU | CBC-CBU |
| Atenolol | NAJ-CAP | NBI-CBO | CBL-CBP |
| Lisinopril | NAU-CAC | CBV-NCA | CBV-OBJ |

**Table 6**
Drug, lipid and PEG molecules orientation, average angle (AA) and most probable angle (MPA).

| Simulation system | Component | | | | | |
|---|---|---|---|---|---|---|
| | Drug | | Lipid | | PEG | |
| | AA | MPA | AA | MPA | AA | MPA |
| Amidamlodipine | 90.5 | 91.4 | 26.2 | 37.3 | 129.1 | 156.3 |
| Amidatenolol | 95.8 | 61.4 | 23.2 | 19.7 | 75.6 | 62.2 |
| Amidlisinopril | 48.0 | 161.5 | 85.7 | 84.4 | 101.3 | 164.3 |
| Amlodipine | 83.2 | 84.3 | 31.5 | 31.6 | – | – |
| Atenolol | 92.0 | 95.1 | 23.5 | 24.2 | – | – |
| Lisinopril | 81.1 | 102.4 | 93.4 | 94.3 | – | – |
| Esteramlodipine | 90.0 | 91.5 | 30.8 | 37.2 | 105.4 | 95.7 |
| Esteratenolol | 59.0 | 20.1 | 28.4 | 23.5 | 87.9 | 120.3 |
| Esterlisinopril | 61.7 | 21.3 | 88.3 | 94.3 | 89.5 | 143.2 |

As can be seen in Figs. S23(b), S24(b), S25(b) and in Table 6, a difference of about 23° to 30° occurs in the lipid chains to become parallel to the normal vector of the lipid bilayer membrane, in all of the simulation systems, except in the systems containing lisinopril (PEGylated or not) molecules. These changes in the lipid chain orientations in the presence of lisinopril molecules are consistent with the results obtained in the order parameter and area per lipid analyses in this study. It is clear that, lisinopril (both in PEGylated and ordinary forms) insertion in the simulation systems has a considerable effect on the lipid chain orientations, compared with the other two types of simulated drugs (atenolol and amlodipine). The different effects of PEGylation on the drugs are consistent with the results presented by Yu et al. [75], where PEGylation of lysozyme altered the preferred orientation of lysozyme at the silica surface.

The negligible effect of PEGylation on the orientation of the amlodipine molecules, the same effects on the angle distribution by both of the spacers and changing the directions of atenolol and lisinopril molecules toward the bilayer membrane, are the most import results of this analysis.

### 3.9. Free energy analysis

The obtained free energy profiles, by integrating the mean force imposed on the z-direction, for amlodipine, atenolol and lisinopril (PEGylated and non-PEGylated) are presented in Fig. 8. As can be seen in Fig. 8, the maximum point of the profiles in the hydrophobic region shows the hydrophilic behavior of the simulated drugs, but as it is obvious in this figure, the effect of PEGylation on the drugs is different in the simulated systems. For atenolol and lisinopril, the meaningful decrease at the maximum point of these profiles verifies that, the lipophilic effect of PEG on the drugs changes their behavior and facilitates the diffusion into the bilayer membrane. On the other hand, amlodipine shows a higher hydrophilic behavior after PEGylation which is due to the dominant hydrophilic effect of the PEG as an amphiphilic molecule. The results of free energy calculations for the simulated drugs are consistent with the mass density, hydrogen bonding, RDf obtained in this work. From the results presented in Fig. 8, it can be concluded that, PEGylation reduces the energy barrier against the penetration of the drugs into the lipid bilayer for lisinopril and atenolol, but in contrast for amlodipine, an undesired increase in the energy barrier is observed after PEGylation.

## 4. Conclusion

Three types of anti-hypertensive drugs, amlodipine, atenolol and lisinopril, were studied in their ordinary and PEGylated forms (by two types of amide and ester bonded molecules). All of the simulations were performed in the presence of DMPC and appropriate number of water molecules. The behavior of the drug molecules and the lipid

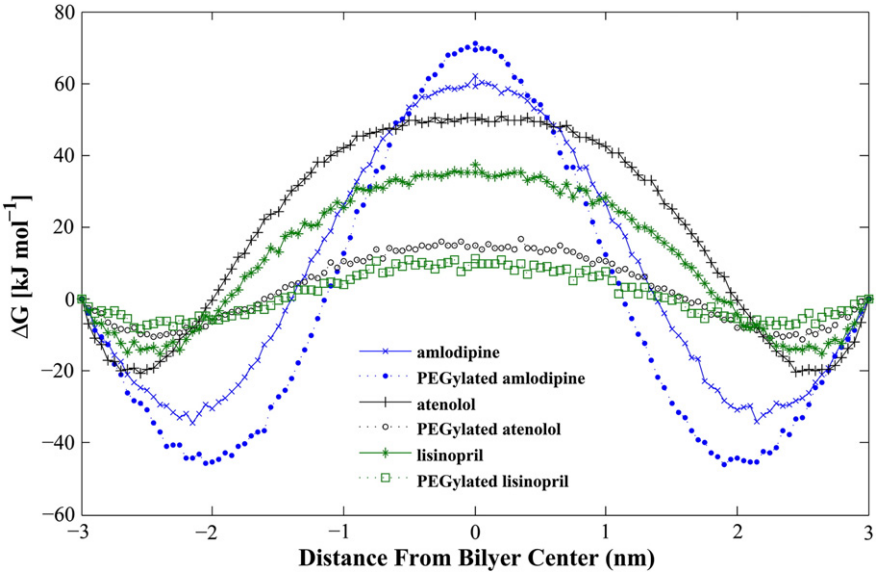


**Fig. 8.** Free energy profiles of the simulated drugs across the DMPC lipid bilayer membrane.

bilayer membrane were considered in the simulated systems. The mass density analysis shows that while the ordinary form of amlodipine molecules shows slight tendencies to diffuse into the lipid bilayer membrane, the PEGylated forms of the drug (both with ester and amide spacers) indicate the hydrophilic behavior. Moreover, the influence of drug PEGylation on the location of lisinopril molecules appears as a shift toward the lipid bilayer membrane, and the highest effect appears for the system containing amlodipine molecules. The sharp curve obtained from the mass density analysis of the systems containing PEGylated amlodipine corresponds to accumulation of the drugs. From the MSD analysis, it is concluded that, the ester form spacer has the highest effect on the drug movement for all the simulated drugs. On the other hand, the presence of the drug molecules increases the electrostatic potential of systems (for all of the simulated systems which include water, DMPC and drugs) compared with the absence of the drug molecules (only DMPC and water). This increase in the electrostatic potential does not depend on the type of the used spacer molecule. One of the interesting results obtained in this analysis is that, the PEGylation reduces the electrostatic potential by the insertion of amlodipine molecules in the simulation systems. The results indicate that, the drug characteristics can influence the electrostatic potential and the PEGylation decreases this influence. The other structural effect on the results is due to the hydrogen bond formation, where the PEGylated atenolols have the highest number of drug–drug hydrogen bonds due to their higher number of electron donor and acceptor atoms. Furthermore, the steric effect of the drug molecules, results in formation of higher number of hydrogen bonds. An interesting effect of hydrogen bonding is seen in the PEGylated atenolol molecules, where the higher number of drug–PEG hydrogen bonds causes the drug molecule to form a spherical structure. The low value of diffusion coefficient resulted for the system containing lisinopril molecules is consistent with the number of hydrogen bonds between the drug–water molecules that hinder the drug molecules movements. The analysis of the order parameter and area per lipid shows that, lisinopril molecules have the highest influence on the lipid chain deformation. The results obtained for angle distribution analysis indicate the presence of an extreme perturbation in the lipid orientations, in the systems containing the lisinopril molecules (PEGylated or not).

The main results of this study can be summarized as follows:

1. PEGylation increases the tendency of lisinopril and atenolol molecules to locate themselves inside the lipid bilayer membrane (with different extents, as reported by Han and Lee [27]), but the PEGylated amlodipine molecules become more hydrophilic than their ordinary forms (free energy analysis justifies these results in Fig. 8).
2. PEGylation increases the diffusion coefficients of the simulated drugs, but other factors such as the hydrophilic nature of the drugs and the energy barrier in the simulation system, as well as the dislocations of lipid molecules should be considered to justify the diffusion process.
3. PEGylation makes atenolol and lisinopril take up an orientation toward the bilayer which facilitates the diffusion into the membrane, but PEGylated amlodipine molecules have the same orientations as the ordinary forms of this drug.
4. PEGylation increases the electrostatic potential of atenolol and lisinopril which can be due to their instability in the transition region between hydrophilic and hydrophobic states, but the electrostatic potential of amlodipine molecules is less than that of the ordinary form of the drug due to the presence of more stable level of hydrophilicity after PEGylation which is the same behavior as reported for the stability of PEGylated insulin [25].
5. Type of the spacer molecule (amide or ester bonded) does not have a structural effect on the drug's behavior; however, the ester bonded form (for all the three drugs) increases the diffusion coefficient and the amide bonded form of PEGylated lisinopril has higher effect on the DMPC orders.

The results indicated that, decreasing the hydrophilic behavior of the drugs increases their tendency to diffuse into the lipid bilayer membrane for the systems containing lisinopril and atenolol molecules, but an inverse effect is observed for the systems containing amlodipine molecules.

In addition, in future research we intend to study the drug dosage, novel multiarm PEGs, anti-hypertensive drug mixtures, the effectiveness of the conjugated drugs, lipid bilayer membrane with a receptor and the other types of the anti-hypertensive drugs.

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## Transparency document

The Transparency document associated with this article can be found, in the online version.

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