Biological membrane in presence of alcohol and anti-asthma drugs: a molecular dynamics study

by

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Declaration

I declare that no material contained in the thesis has been used in any other submission for an academic award and is solely my own work.

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Abstract

This thesis is dealing with drug delivery systems for anti-asthma drugs. We have investigated about the effect of different components in a drug delivery system where liposomes are drug carriers. These components include alcohol, which is used as a solvent in the system and the drug itself. In order to gain a better understanding of the effect of alcohol and anti-asthma drugs on phospholipid bilayer a series of atomistic molecular dynamics (MD) have been carried out. Although alcohol is widely used as a solvent in drug delivery systems, it is known that it can affect a lipid bilayer. The effect of two different alcohol molecules (ethanol and methanol) on lipid bilayer has been compared taking into account different concentrations of ethanol and methanol (0%-30%) in the third chapter of this thesis. In the forth chapter the effect of three hydrophobic anti-asthma drugs including Beclomethasone Dipropionate, Fluticasone Propionate and Prednisone on lipid bilayer is investigated. The behaviour of these three anti-asthma drugs in various positions with respect to the bilayer as well as their preferred position in the bilayer and alcohol role in penetration of drug inside the bilayer is studied in this chapter.

Studying the effect of alcohol on DMPC lipid bilayer has shown that ethanol and methanol have destructive effect on lipid bilayer such as causing lipids expansion (increasing the area per lipid), interdigitation and changing the deuterium order parameter of lipid molecules. It is also shown that ethanol effect on DMPC lipid bilayer is more in comparison with methanol.

The MD studies on three anti-asthma drugs have shown that the drugs are stable inside the lipid bilayer. It is also observed that drug molecules preferable position inside the bilayer is close to the lipid head groups according to the hydrogen bonds and electrostatic interactions between the drug molecule and lipids head groups. It is also shown that presence of ethanol as a solvent, enhance the penetration of drug molecule inside the lipid bilayer.

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Acknowledgement

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Many friends have helped me stay sane through this difficult year. Their support and care helped me overcome setbacks and stay focused on my graduate study. I greatly value their friendship and I deeply appreciate their belief in me.

1. Introduction

1.1. Lipid bilayer

Lipids are amphiphilic molecules. Amphiphiles consist of two parts. One part which is soluble in non-polar solvents such as ethanol and a second part that is soluble in polar solvents such as water. Being amphiphilic molecules, lipids are composed of two main parts, a hydrophobic tail and a hydrophilic head (figure 1.1).

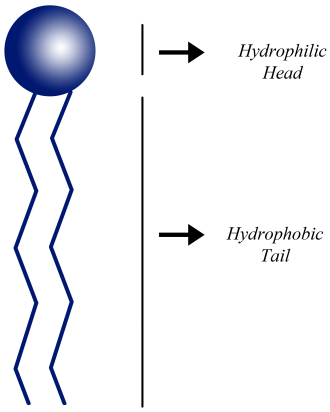


Figure 1.1.: A schematic view of a single lipid molecule. Lipid molecules are mainly made of two parts, a hydrophilic head and hydrophobic tail.

Lipid bilayers are made of two lipid monolayer. In a bilayer structure the non-polar fatty acid tails are in membrane interior to minimise the unfavourable hydrophobic interaction with water molecules, and the polar head groups act as external surface. Liposomes are microscopic vesicles composed of membrane-like lipid bilayers surrounding aqueous compartment.[3]

1.2. Liposomal drug delivery

Liposomes are microscopic vesicles composed of membrane-like lipid bilayers surrounding aqueous compartment.[3] Liposomes were discovered 47 years ago[4] and they have gained interest for a variety of applications including drug delivery.[5]

Having a dual preference to solvent, lipids form closed vesicles spontaneously in aqueous environment, which are like artificial cell membranes.[3] This feature conduct the ability of liposomes to encapsulate both hydrophobic and hydrophilic drugs for drug delivery applications. Liposomes are usually unilamellar and range in diameter from about 50-150nm.[5] A schematic picture of a liposome is shown in figure 1.2.

Liposome mechanical strength as well as its function as a permeability barrier, depends on the packing of the hydrocarbon chains of the lipid molecules.

The exact location of the drug in liposomes will depend on its physio-chemical characteristics and the composition of the lipids.[6] However, as a general rule, the hydrophilic drugs encapsulate in the aqueous space while the hydrophobic and amphiphilic molecules can be retained into the lipid bilayer (figure 1.3).

1.3. Alcohol

Since alcohol is used for different purposes in everyday life, its effect on membranes is widely studied in the literature computationally and experimentally.[7–12] These applications includes alcohol application in medicine industry as a solvent as well as usage of alcohol as beverages and many other applications. Alcohol is used in experiments to solvate anti-asthma drugs in order to entrap them inside liposomal drug delivery systems.[13–15] In the current computational study, the effect of alcohol on bilayer at different concentrations as well as its effect on the behaviour of anti-asthma drugs inside the bilayer is investigated. This computational work also explain the effect of alcohol on drug molecule interaction with the bilayer. Ethyl alcohol, also commonly referred to as ethanol, is a colourless liquid that has many uses. Its formula is CH_3CH_2OH. Apart from

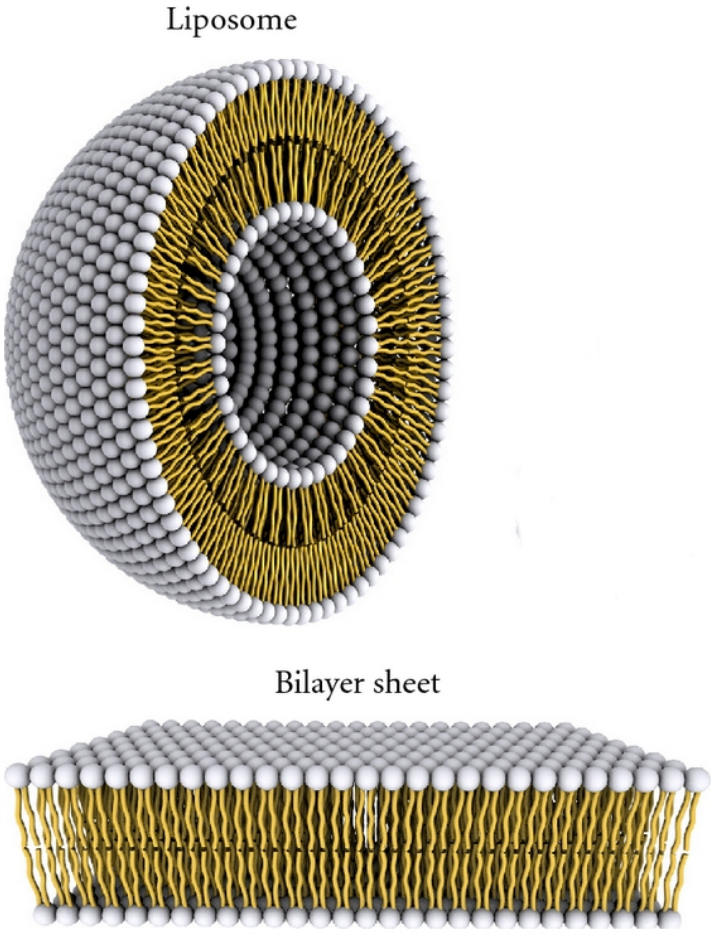


Figure 1.2.: An example of self-assembled structures form by amphiphilic molecules. A bilayer and liposome vesicle are shown. The spheres are the hydrophilic heads and the wavy lines are the fatty acyl chains.[1]

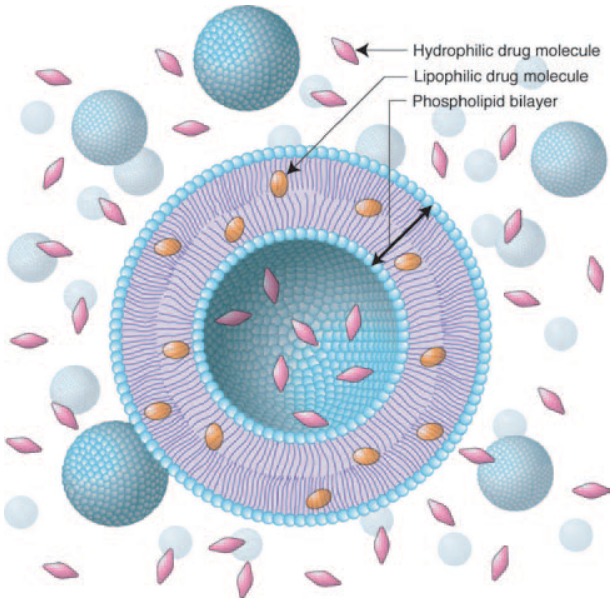


Figure 1.3.: The figure illustrates the position of hydrophobic and lipophilic drug molecules inside a liposome.[2]

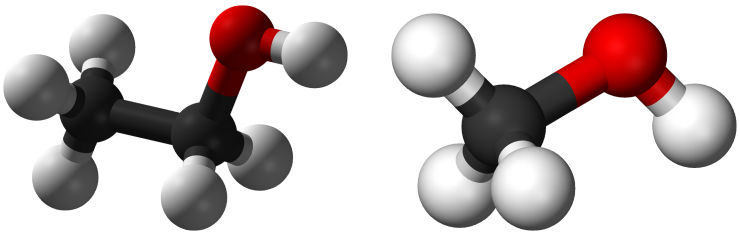


Figure 1.4.: Ethanol molecular structure on the left and methanol molecular structure on the right. The carbon atoms are shown with grey spheres, oxygen with red and hydrogen with white colour.

being the most common type of alcohol found in alcoholic beverages and certain recreational drugs, it is widely used as a solvent. Ethanol is able to solvate many organic molecules, which are not soluble in water.

Methanol is also known as wood alcohol. It has a chemical formula of CH_3OH. It is often used as fuel and also solvent. In the current work its effect on lipid bilayer is investigated.

1.4. DMPC lipid

1,2-dimyristoyl-sn-glycero-3-phosphocholine known as DMPC is a synthesis lipid molecule. Having a molecular formula of $C_36H_72NO_8P$, each DMPC lipid molecule has 128 atoms.

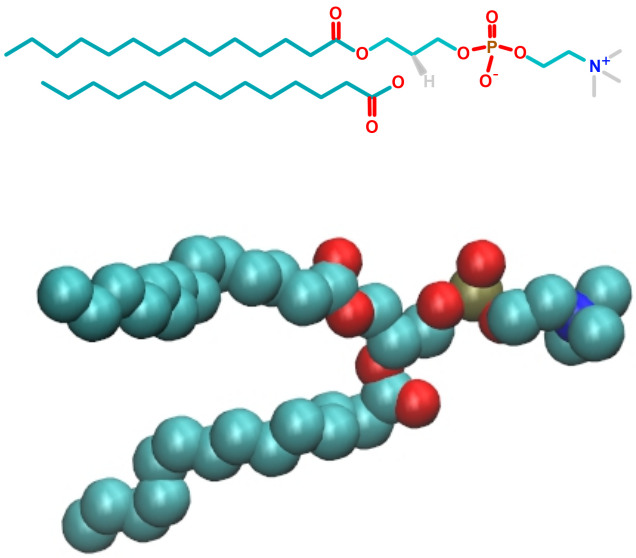


Figure 1.5.: Molecular structure of DMPC lipid molecule. Red atoms are oxygen, light blue refers to carbon atoms, dark blue is the Nitrogen atom and green is the phosphate atom.

This lipid is used experimentally in liposomal form to entrap steroidal drugs because the liposomes it forms, are stable in room temperature.[13] DMPC lipid molecule is also very well parameterised in our chosen force field.[16] Computer simulations are playing a key role in improving our knowledge about the lipid

bilayer behaviour and the interaction of other molecules with them.

1.5. Three anti-asthma drugs

Beclomethasone dipropionate (BDP) is an anti-asthma drug for pulmonary therapy. It can be used in the form of liposomes to be delivered to body organs. BDP is a hydrophobic, partially soluble in water and is able to dissolve completely in ethanol. The molecular structure can be seen below (figure 1.6). This molecule

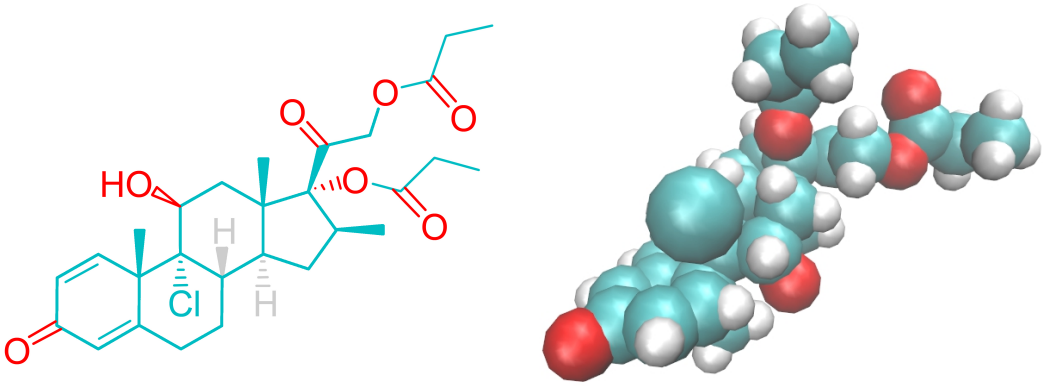


Figure 1.6.: Beclomethasone dipropionate molecular structure. The figure on the right is BDP representation in the current chapter. Light blue spheres are carbon atoms, oxygen atoms are shown with red colour and hydrogens are white. The sphere, which is bigger in size represents chlorine atom.

has 73 atoms in total and its chemical formula is $C_{28}H_{37}ClO_7$. BDP drug has a polar surface area of 106.97Å^2 and the solvent accessible surface area of the molecule is 764.07Å^2. Information about BDP atoms name, charge and mass as well as information about the angles between bonds and so on can be found in topology file.[17] The description about this file is in Appendix B.

Fluticasone Propionate (FPP) is another drug with anti-allergic, anti-asthmatic and anti-inflammatory usage. This drug is hydrophobic and has a high affinity because of existence of fluorine atoms in its structure. The molecular structure of FPP molecule can be seen in figure 1.7. FPP molecule has a chemical structure of $C_{25}H_{31}F_3O_5S$ and 65 atoms. FPP polar surface area is 80.67Å^2 and its solvent accessible surface area is 682.76Å^2.

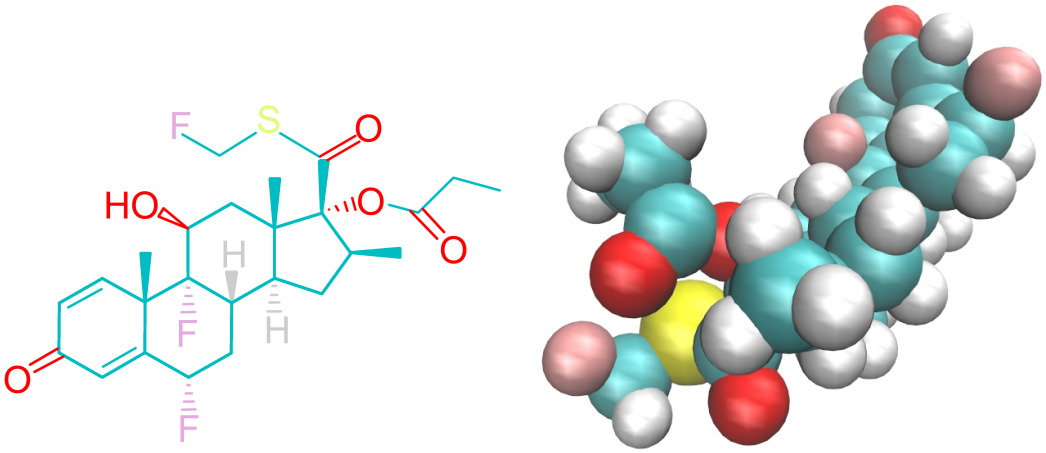


Figure 1.7.: Fluticasone propionate molecular structure. The figure on the right is FPP representation in the current chapter. Colour codes are the same as figure 1.6. In addition pink colour shows fluorine atoms and the yellow sphere represents sulfur atom.

Prednisone is the other anti-asthma drug, which is used in this study. This drug is also an anti-asthma steroidal drug like BDP and FPP, compatible for being used with liposomes. The chemical formula of prednisone molecule is $C_{21}H_{26}O_5$ and it has 52 atoms in total. Figure 1.8 shows the molecule structure. Prednisone

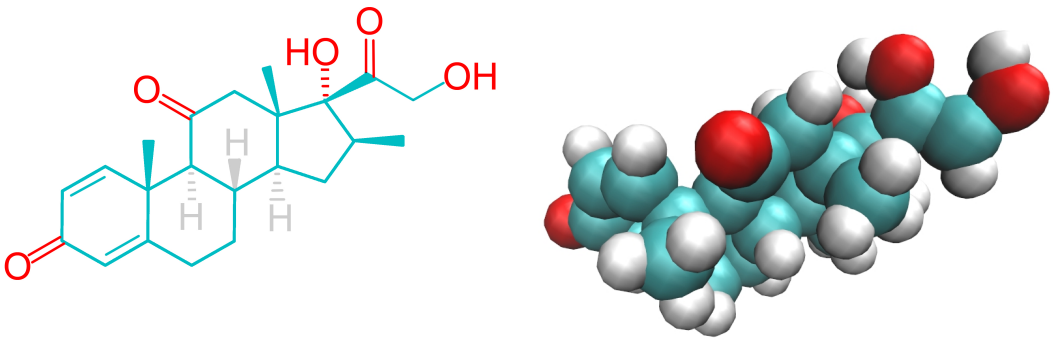


Figure 1.8.: Prednisone molecular structure. The figure on the right is Prednisone representation in the current chapter.

polar surface area is 91.67 $Å^2$ and solvent accessible surface area is 515.07 $Å^2$. Prednisone is the least hydrophobic drug between all three drugs.

1.6. Aim of study

There are many experimental studies done on liposomal drug delivery of anti-asthma drugs and it is very well established in industry as well.[18,19] Since it is not possible to have a look at atomistic level of these complex systems experimentally. Computational studies can help us to understand these systems better in molecular scales. Although many experimental works are done,[13–15] computational studies about systems with anti-asthma drugs and their liposome carriers are lacking. The aim of this study is to gain an atomistic level understanding of liposomal drug delivery system of steroidal anti-asthma drugs. These drugs include beclomethasone dipropionate, fluticasone propionate and prednisone. Finding out the effect of these drugs on lipid bilayer as well as presence of alcohol on drugs behaviour, are the other objectives of the current work. Alcohol has the role of solvent for hydrophobic molecules in the system in this study.

The atomistic level information of these systems can give us information about drugs position inside the bilayer and the effect of these drugs on their carriers.

2. Methodology

2.1. Introduction

Molecular dynamics is a very well known computer simulation method. This technique solves the Newtonian equation numerically with the help of a force field to describe all possible interaction between atoms. Solving the equation of motion for all atoms, it can simulate the motion of atoms and molecules in a desired system. The current chapter introduces Molecular Dynamics(MD) for atomistic simulations.

2.2. Force Fields

Force field is a mathematical function and a set of parameters, which explain the energy of a system regarding to the coordinates of the particles in it. In the classical form of force field there are several terms describing the way atoms are connected together or interacting with each other (equation 2.1).

$$V_{ff} = \sum_{bonds} V^{stretch} + \sum_{angles} V^{bond} + \sum_{angles} V^{tors} + \sum_{pairs} V^{coul} + \sum_{pairs} V^{vdW} \qquad (2.1)$$

Where $V^{stretch}$ is the potential energy of a bond when it is stretched, V^{tors} is the energy of a bond with respect to torsional angles, V^{coul} is the potential energy related to coulombian interactions and V^{vdW} describe the van der Walls potential energy. Many kinds of force fields are available and some of them are

often used for bio-molecular studies such as GROMOS[20,21], AMBER[22], OPLS[23,24] and CHARMM[25,26].

With each force field, two models named all-atom force field and united atom force field are available. In all atom force field all atoms has been modelled while in united atoms the hydrogen atoms are folded with atoms they are bonded to. Both methods have their advantages and disadvantages. While the prior method is more accurate and realistic, the second one is faster and more economical.

2.2.1. Bonded Interaction

The energy of a bond can vary as function of different factors such as bond length, angle and etc. Where two atoms are bonded together with a covalent bond (if the deviation from reference bond length is small) the energy of the system can be expanded using Taylor series (equation 2.2).

$$V(r) = V(r_0) + \frac{\mathrm{d}V}{\mathrm{d}r}|_{r=r_0}(r-r_0) + \frac{1}{2}\frac{\mathrm{d}^2V}{\mathrm{d}r^2}|_{r=r_0}(r-r_0)^2 + \frac{1}{6}\frac{\mathrm{d}^3V}{\mathrm{d}r^3}|_{r=r_0}(r-r_0)^3 \quad (2.2)$$

Assuming $V(r_0) = 0$ and considering that the derivation of energy is equal to zero, when system is at rest ($r = r_0$), Equation(2.2) will become equation 2.3.

$$V(r)_{bonds} = \sum_{bonds} \frac{1}{2} k_b (r - r_0)^2 \quad (2.3)$$

Here k_b is stretching constant representing how stiff the bonds are. The force constant and bond length values are usually achieved from experimental data. The value of this constant can vary as function of the chemical properties of atoms, which are bound together (figure 2.1).

The alterations of a bond from reference angle is also often shown using harmonic potential as it is followed (equation 2.4).

$$V(\theta)_{angles} = \sum_{angles} \frac{1}{2} k_a (\theta - \theta_0)^2 \quad (2.4)$$

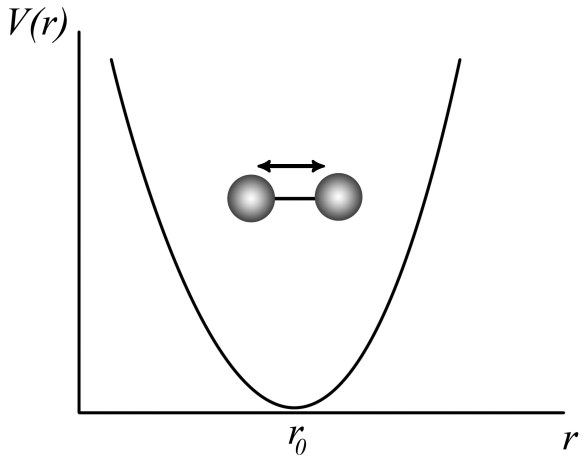


Figure 2.1.: potential energy of a bond in terms of lengths, r_0 is the ideal bond length.

In the equation above θ is the real bond angle, θ_0 is the equilibrium bond angle and k_a is the angle bending force constant.

Much more energy is needed to stretch a bond from equilibrium length compared to the energy needed to change the reference angle of a bond. Therefore, k_a is smaller than the stretching constant. While the constant is bigger in bond stretching potential term, it is more sensitive to changes in length. The accuracy of this term can be improved by considering higher order terms

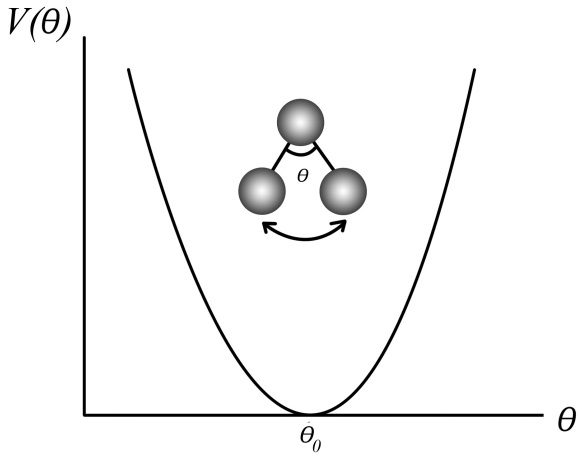


Figure 2.2.: potential energy of a bond in terms of angle between two bonds, θ_0 is the ideal bonds angle.

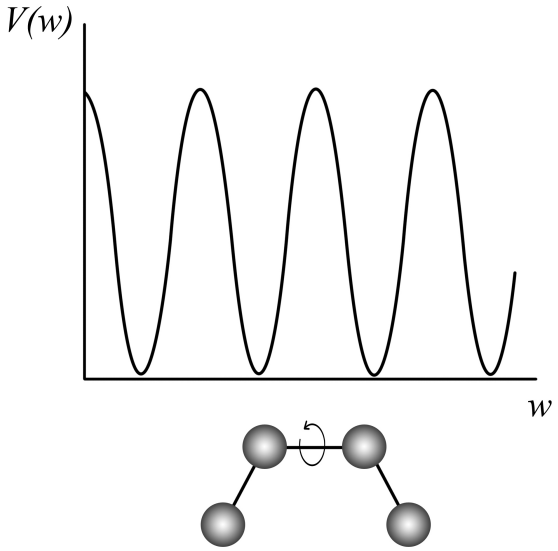
The most common model for describing the torsional interactions is periodic

using Fourier series, often expressed as a cosine function (equation 2.5)

$$V(w)_{torsions} = \sum_{torsions} \frac{1}{2} V_n[1 + cos(nw - \gamma)] \tag{2.5}$$

w is the instantaneous torsion angle, V_n is the height of the barrier to rotation, γ is the angular offset and n is the factor, which shows the number of the oscillations (minima or maxima).

The role of torsional angle rotations in a force field comparing with other terms is of great importance. The reason is that the barriers to these rotations are very low comparing to other interactions. This means that changes in torsional angles can be large. Moreover, the torsional potential is periodic through a 360° rotation.



Therefore, when a bond moves from its equilibrium position either by compressing, stretching or bending, the energy of the bond changes and the bond resist these changes.

2.2.2. Non-bonded interaction

In addition to the interactions explained above, a force field also includes non bonded interactions acting between atoms, which are non-covalently bound together. These interactions may occur between atoms either in the same molecule

or in different molecules. Non-bonded interactions are often defined as electrostatic interactions and van der waals interactions.

The electrostatic interaction happens between atoms when they are permanently charged. Within the force field framework, this uneven distribution of charge can be modelled by placing point charges at each of the atomic sites. Point charges interactions are usually modelled using Coulomb potential (equation 2.6).

$$V(r)_{elec} = \frac{1}{4\pi\epsilon_0}\frac{q_i q_j}{r_{ij}} \tag{2.6}$$

In most of the force fields, van der Waals term is defined, using Lennard-Jones 12-6 potential (equation 2.7).

$$V(r)_{LJ} = 4\epsilon\left(\left(\frac{\sigma}{r}\right)^{12} - \left(\frac{\sigma}{r}\right)^{6}\right) \tag{2.7}$$

Where V_{LJ} is the Lennard-Jones potential for a pair of atoms separated by a distance of r, σ is the finite distance at which the potential of the particle is zero and ϵ is the well depth. The Lennard-Jones potential is also shown in figure 2.3.

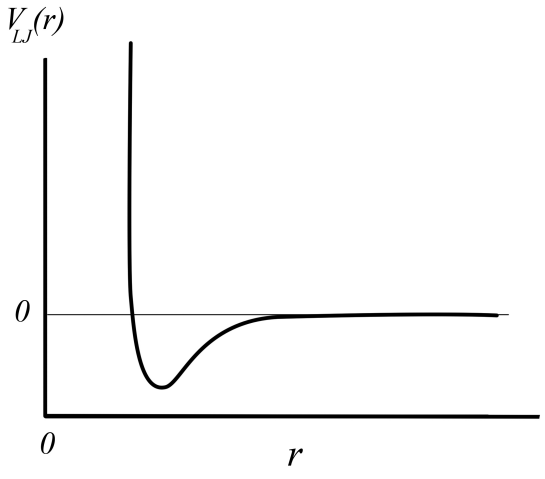


Figure 2.3.: Lennard-Jones potential.

The well depth defines how strong two particles are attracting each other. The parameters in Lennard-Jones equation can be obtained from experimental data or quantum mechanical calculations.

2.3. Molecular dynamics simulation methods

Molecular dynamics is a technique to solve the classical equation of motion for a system of N molecules interacting via a potential. This method was first used to study the interactions between elastic spheres.[27] The equation to describe this interaction can be written in various ways. The most fundamental form is the Lagrangian equation of motion (equation 2.8).

$$\frac{\mathrm{d}}{\mathrm{d}t}(\frac{\partial L}{\partial \dot{q}_k}) - (\frac{\partial L}{\partial q_k}) = 0 \tag{2.8}$$

Where q represent the generalised coordinates and L is the Lagrangian. The Lagrangian function is defined in terms of potential and kinetic energies as shown in equation 2.9.

$$L = H - V \tag{2.9}$$

Considering a simple system of atoms in Cartesian coordinate system and the usual definition of H and V the equation 2.9 becomes equation 2.10.

$$\vec{F_i} = m_i \ddot{\vec{r}_i} \tag{2.10}$$

Where m_i shows the mass of atom i and F_i is the total force applied to that atom.

2.3.1. Numerical integration of equation of motion

The most straight froward method for the solution of equation 2.10 is the finite difference approach. In simple words, having all dynamic information such as positions, velocities at time t we will try to obtain the dynamic information at $t + \delta t$ in which the time interval δt should be significantly smaller than the typical time taken for a molecule to travel its own distance.[28] Where the motion of particles is continuous, the estimation of dynamic parameters at time $t + \delta t$ can be written with Taylor expansion about time t:

$$\mathbf{r}^p\left(t+\delta t\right)=\mathbf{r}(t)+\delta t\mathbf{v}(t)+\frac{1}{2}\delta t^2\mathbf{a}(t)+\frac{1}{6}\delta t^3\mathbf{b}(t)+... \tag{2.11}$$

$$\mathbf{v}^p\left(t+\delta t\right)=\mathbf{v}(t)+\delta t\mathbf{a}(t)+\frac{1}{2}\delta t^2\mathbf{b}(t)+... \tag{2.12}$$

$$\mathbf{a}^p\left(t+\delta t\right)=\mathbf{a}(t)+\delta t\mathbf{b}(t)+... \tag{2.13}$$

$$\mathbf{b}^p\left(t+\delta t\right)=\mathbf{b}(t)+... \tag{2.14}$$

Verlet algorithm is one of the commonly used methods to integrate equations of motion. In this method position of particle has been expanded about time t using Taylor expansion going one time step backward and forward from time t.

Original Verlet algorithm has been developed to other methods such as leapfrog algorithm and velocity Verlet. In the current work we have used leapfrog algorithm for integrating the equation of motion. The reason is that unlike Verlet algorithm the velocities are explicitly included in leapfrog method. The equations of leapfrog algorithm are,

$$\mathbf{r}(t+\delta t)=\mathbf{r}(t)+\delta t\ \mathbf{v}(t+\frac{1}{2}\delta t) \tag{2.15}$$

and

$$\mathbf{v}(t+\frac{1}{2}\delta t)=\mathbf{v}(t-\frac{1}{2}\delta t)+\delta t\ \mathbf{a}(t) \tag{2.16}$$

in which positions and accelerations at time t as well as mid-step velocities are stored.

In this algorithm having equation 2.15, acceleration at time t can be calculated. Afterwards, having velocity half time-step behind from equation 2.16 velocity half time-step in front can be calculated and the positions will be updated using equation 2.15. The full-step velocity may be calculated with the following equation as it will be required to derive the energy of system.

$$\mathbf{v}(t)=\frac{1}{2}(\mathbf{v}(t+\frac{1}{2}\delta t)+\mathbf{v}(t-\frac{1}{2}\delta t)) \tag{2.17}$$

2.3.2. Periodic boundary conditions

Applying periodic boundary conditions is the classical way to minimise the edge effects in a finite system, thus there are no boundaries of the system. In this method, the simulation cell will be repeated in all direction in the space around the original cell, in a way that if any of the molecules leaves the box, its periodic image will enter the box from the opposite face.

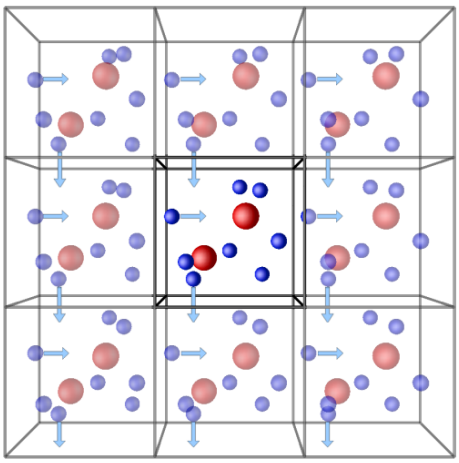


Figure 2.4.: Figure illustrates periodic boundary condition. The main system is in the centre and the periodic images of it are repeated around the system.

2.3.3. Statistical Ensembles

Statistical mechanics explain macroscopic thermodynamic behaviour of a system with microscopic properties and motion of atoms. An ensemble is the assembly of all possible microstates available with given constraints.[29] During an ensemble all variables except those that are fixed, fluctuate about an average value. The system will be thermodynamically in equilibrium state, when the average value becomes constant.[30] All system have been equilibrated through two ensembles in this study. The first ensemble is the canonical ensemble (NVT) in which number of particles (N), volume (V) and temperature (T) are to be kept constant. The second ensemble is isothermal-isobaric ensemble (NPT) in which the number of particle, temperature and pressure (P) remain the same. To start the equilibra-

tion of the system the temperature has been fixed through NVT ensemble to the desired temperature, followed by an NPT ensemble to achieve an equilibrium volume for the system.

2.3.4. The Berendsen thermostat

Different thermostat algorithms are available such as Langevin, Berendsen, velocity re-scaling and Nose-Hoover. One of the popular thermostats, which is also used in this work to control the temperature is Berendsen thermostat. The temperature of any desired system is related to the velocity of the particles in the system as shown followed in equation 2.18:

$$\langle K \rangle_{NVT} = \frac{1}{2} \sum_{i=1}^{N} m_i^2 v_i^2 = \frac{3}{2} N k_B T \tag{2.18}$$

Where m_i is the mass of ith particle, v_i is its velocity, N is the number of particles, k_b is the Boltzmann constant and T is the temperature. Re-arranging equation 2.18, temperature can be written as followed:

$$T(t) = \frac{1}{2} \sum \frac{2}{3} \frac{m_i v_i^2}{N k_b} \tag{2.19}$$

In order to control the temperature the velocity of particles in a system can be re-scaled. Changes in the temperature can thus be determined:

$$\Delta T = \frac{1}{2} \sum_{i=1}^{N} \frac{2}{3} \frac{m_i (\lambda v_i)^2}{N k_b} \; - \; \frac{1}{2} \sum_{i=1}^{N} \frac{2}{3} \frac{m_i (v_i)^2}{N k_b} \tag{2.20}$$

Where λ is the re-scaling factor to change the velocities. Equation 2.20 can be simplified as:

$$\Delta T = (\lambda^2 - 1) T(t) \tag{2.21}$$

The Berendsen thermostat consider a heat bath with a fixed temperature T_{bath}.[31] It scales the velocities in a defined number of steps in order to reach the desired temperature. Berendsen thermostat scales the velocities in a way that the

gradient of the temperature, is proportional to desired and current temperature difference (equation 2.22).

$$\frac{\mathrm{d}T(t)}{\mathrm{d}t} = \frac{1}{\tau}(T_{bath} - T(t)) \tag{2.22}$$

Where τ is the ratio constant(relaxation time), determine the coupling strength. It is also necessary to keep the temperature constant during the isothermal-isobaric ensemble.

2.3.5. The Parrinello Rahman barostat

Parrinello-Rahman barostat is a method to control the pressure. In this method box vectors are represented by matrix and they follow an equation of motion.[32] This means that the box size can change as well as the shape of the box. This feature of Parrinello-Rahman barostat will allow the user to have the control of stress in addition to the pressure. The motion of atoms follows the Nose-Hoover thermostat method in this barostat.

2.4. GROMACS

In this project the 4.5.3 version of Groningen Machine for Chemical Simulations (GROMACS) software has been used to perform molecular dynamics GROMACS is a parallel open source molecular dynamics simulation package[33] available in http://www.gromacs.org. This package is developed by the University of Groningen. Possessing many complicated bonded interactions, as well as being fast in calculating non-bonded interactions, GROMACS is suitable for simulating biological structures such as lipids, proteins and non-biological structures (exp.polymers).

GROMACS is a very popular tool not only because there are a variety of MD integrators, thermostats and barostats available with it in addition to several energy minimisation integrators, but because there are many analysis programs

included in it.

3. DMPC in presence of Alcohol

3.1. Introduction

The effect of short chain alcohols on dimyristoylphosphatidylcholine (DMPC) bilayer is considered in this study in order to gain a better understanding of the ethanol interaction with liposome bilayer when used as a solvent for solvating the hydrophobic drugs.[13–15] DMPC bilayer is also used widely in molecular dynamics simulations as lipid bilayer.[34] Methanol interaction with the same bilayer has been studied to compare the effect of this solvent with ethanol on lipid bilayer. In order to understand the effect of alcohol on DMPC bilayer we have considered the atoms trajectory, bilayer leaflets interdigitation, the average density of different molecules in the system, area per lipid of bilayer and the deuterium order parameter of lipid tails.

The effect of alcoholic solvents on bilayers has crucial bio medical implications. Lipid bilayers are widely used for pharmaceutical purposes such as drug delivery according to formation of liposomes by phospholipids self assembly in aqueous environment. These spherical bilayer structures can be used as pharmaceutical carrier. While many drug molecules are not water-soluble, ethanol and methanol are largely used as co-solutes. The effect of these solutes on liposomes is dependent on their concentration.

Molecular dynamics simulation in recent studies have mentioned the problem of the effect of alcohol on lipid membranes.[7–12] Alcohol is able to create hydrogen bonds with the lipid in the bilayer. These bonds affect the order parameter of lipid hydrocarbon chains and also cause area per lipid and fluidity of membrane to

increase.[9,10] As a result alcohol is able to penetrate easily through the bilayer.[10] It is known that the influence of ethanol on bio-membranes is to cause disorder in lipid hydrocarbon chains[35,36] and increasing the average surface area per lipid leading to membrane expansion.[37] In higher concentrations (more than 12mol%) it has been seen that lipid bilayer can cause formation of micelle-like structures.[11] Moreover, a variety of experimental methods have been used to study alcohol in lipid bilayers such NMR[38,39]and X-ray[40]in which ethanol has been observed to have a disordering effect on lipids.[41] In X-ray studies it has been shown that above the main phase transition temperature of the lipids, ethanol produces structural changes.[40]

Having different chemical structures, methanol and ethanol have contrasting effects on bilayers. Methanol is a smaller molecule, comparing to ethanol. In its structure methanol has one hydrophilic hydroxyl group whereas ethanol possesses an additional hydrophobic carboxyl group.[11] The ethanol molecules have shown to pass through bilayer much more easily than methanol according to hydrogen bond formation between ethanol and bilayer head groups.[11] Because short-chain alcohols are used as solvent for many drugs in preparing liposomes in the process of drug entrapment, It is of great importance to examine how these molecules alter the structure and behaviour of DMPC bilayer. This will improve our insight to many macro-scale phenomena in drug delivery such as entrapped drug leakage from liposomes.[5]

Molecular dynamics simulations are excellent for this study because they illustrate the structural and dynamics of changes of individual lipids in details.[12] In the current study we have investigated the effect of ethanol and methanol on dimyristoylphosphatidylcholine (DMPC) bilayer at different concentrations of alcohol. DMPC is a synthetic bilayer commonly used for creating liposomes.[13,15]

As explained earlier, Many similar studies are done on the effect of alcohol on different bilayers. These studies are done by employing different concentrations and different types of alcohol.[7–12]. The idea of current work is taken from a computational study on ethanol and DPPC bilayer with different concentrations.[10]

In this study by increasing the concentration of ethanol in a system of bilayer, ethanol and water solvent the effect of alcohol on bilayer is investigated. Similar systematical study is done on the behaviour of ethanol and methanol with a different bilayer in the current work.

Although many computational investigation has been done on the effect of short-chain alcohols on different membranes, an atomistic comparison between the effect of ethanol and methanol on DMPC bilayer in different concentrations is lacking.

3.2. Simulation Methods

All molecular dynamics simulations have been performed using GROMACS software. VMD software is used for graphical representation of simulation systems. GROMOS 53A6 force field parameters are used for all components in the system.[21,42] This force field is very well parameterised for lipids. SPC (simple point charge) model has been used to describe water molecules.[43] To set up the simulation box, DMPC bilayer is centred in a box of relevant size and alcohol molecules are placed outside of the bilayer. Next step is to fill all the empty spaces by water molecules. In order to avoid water molecules entering the bilayer as well as overlap between atoms, an artificially large van der Waals radius is created for carbon atoms (0.375 nm). The number of molecules in each system is explained at length in table II. Before to start MD simulation, energy minimisation was conducted by employing Steepest Descent method[28] (please refer to Appendix A for more information). System is also equilibrated through NVT and NPT ensemble respectively. Through NVT ensemble, temperature of the system is coupled to $310°K$ (i.e. $37°C$) for 100 ps with the coupling time of 0.1 ps. This temperature is chosen to be above DMPC bilayer phase transition, which is $297°K$ for DMPC bilayer.[44] Pressure of the system is considered to be constant having a value of 1 bar. NPT ensemble has run for $1ns$. All the simulations have been run for a duration of $100ns$. Particle-Mesh Ewals algorithm were used to maintain long-

range electrostatic interactions.[45] The cut off radios for both van der waals and Coulombic interactions is chosen to be 1.2 *nm*. DMPC parameters for GROMOS 53A6 have been taken from the Automated Topology Builder (ATB) website.[17]

3.3. Initial configuration

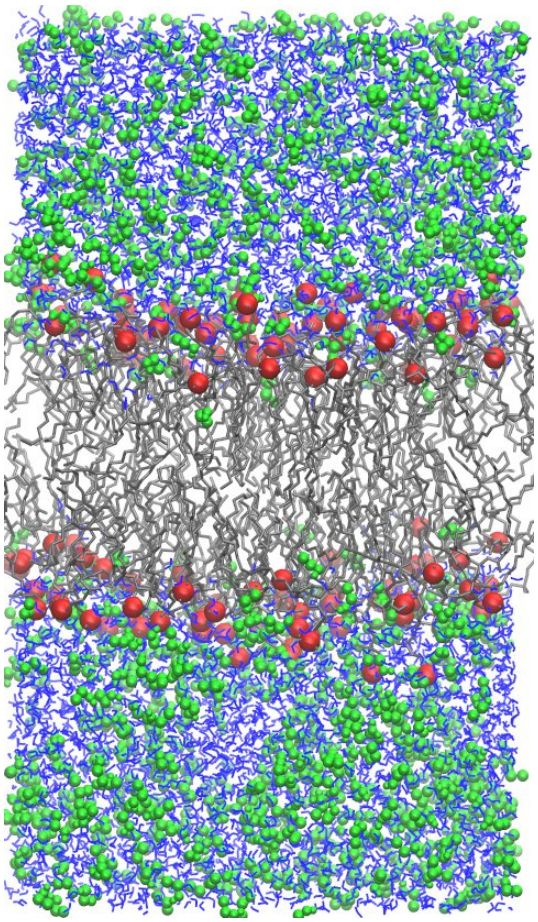


Figure 3.1.: Initial configuration of the system after equilibration for 10 mol% of ethanol. The green dots represent alcohol molecules, the blue lines shows water and the gray part is DMPC bilayer. Red spheres are the Nitrogen atoms in the head groups.

Each studied system includes DMPC bilayer with 128 lipid molecules, water ($\simeq$ 8000 molecules) and ethanol or methanol. Number of ethanol and methanol molecules increase, in respect with changes in concentration of alcohol. The concentration of alcohol was varied systematically from a very low concentration

of 2.5 mol% to a high concentration of 30 mol% (lipid-free basis). This systematic study has been done before by Gurtovenko and Anwar on POPC bilayer and only with ethanol.[10] Table I below describes the molar ratio of alcohol molecules.

System	C_{alc}[mol %]	alcohol/lipid molar ratio
1	0.0	
2	2.5	1.6:1
3	5.0	3.2:1
4	10.0	6.3:1
5	15.0	9.5:1
6	30.0	18.8:1

Table I.: Alcohol concentration of the systems

The simulation box size changes due to the total number of molecules in the system. The box is chosen big enough to accommodate the desired number of alcohol molecules and 8000 water molecules. Total number of molecules in systems varies from 8333 for 2.5 mol% to 11557 for 30 mol%. The number of each group of molecules in systems and the relative simulation box size after equilibrating the pressure can be seen in table II.

System	C_{alc}[mol %]	Alcohol	Water	DMPC	Total	Box Size
1	0.0	0	2538	128	2698	6.16×6.16×5.7
2	2.5	205	8000	128	8333	6.0×6.0×11.42
3	5.0	420	8000	128	5884	5.7×5.7×12.87
4	10.0	886	8000	128	9017	5.7×5.7×14.53
5	15.0	1412	8000	128	9540	5.7×5.7×16.32
6	30.0	3429	8000	128	11557	5.7×5.7×22.62

Table II.: Table illustrates the number of different groups of molecules as well as equilibrated system box size in all systems. All box sizes are in *nm*.

The initial configuration of the systems for 10 mol% of ethanol is shown in the

figure 3.1. As it can be seen from the picture water and alcohol molecules are initially placed outside of the membrane.

3.4. Final configuration

Having a look at final configuration of the system gives a very good understanding of approximate location of molecules in the system.

As it can be seen from the figures (figure 3.3) and later on will be explained in system density terms, ethanol preferably locates in between head groups of bilayer. This is in agreement with previous computational work.[9] As the concentration of ethanol increases the contribution of ethanol inside head groups will increase. At the 10 mol% threshold concentration of ethanol on the right side of the bilayer a pore in which both ethanol and water are present is formed (figure 3.3 (c)). However, this does not happen with the same concentration of methanol. At a concentration of 15 mol% of ethanol as it can be seen in figure 3.2 very large pores are created in the bilayer and not only water and ethanol pass though the pores but the lipids head groups seems to be flowing in the bilayer.

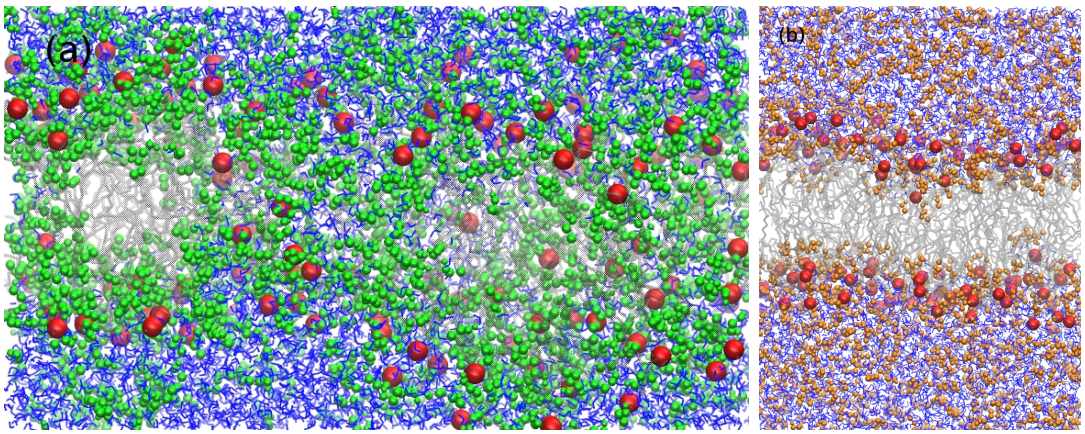


Figure 3.2.: Figure on the left (a) shows system with ethanol and figure on the right (b) illustrate methanol. Final configuration of system with 15 mol% of alcohol. Blue lines are water molecules. Green colour represents ethanol and yellow colour represents methanol. Red spheres are nitrogen atoms and gray lines show lipid acyl hydrocarbon chains.

Comparing two systems with 15 mol% ethanol and 15 mol% of methanol, bi-

layer expansion in system with ethanol is observed to be more. Bilayer thickness also decreases more in the system with 15 mol% of ethanol comparing to the system with the same concentration of methanol.

Hereby in our systematical study we do not consider the systems with alcohol concentration above 10 mol% (15 and 30 mol%) for analysis since the bilayer is destroyed.

3.5. Interdigitation

Interdigitation happens when lipid chains interpenetrating in to the region, which is normally occupied by the opposite leaflet. Changing the bilayer thickness is one of the important effects of interdigitation. Interdigitation effect has been investigated both experimentally[46,47] and through computational works.[10] The previous studies show that the presence of alcohol can increase interdigitation.

Calculation of bilayer leaflets density in each point of the simulation box results to figure 3.4. The areas of leaflets overlap, measures interdigitation. When there are no alcoholic solvent present the interdigitating region thickness is around $1nm$ (black lines). Alcohol enhance further this interdigitation. Changes in interdigitation are concentration-dependent and this value increase to around $2.5nm$ for 10 mol% of ethanol and $2nm$ for 10 mol% of methanol (blue lines). Comparing provided data in the two graphs for methanol and ethanol shows that ethanol cause more interdigitation comparing to methanol.

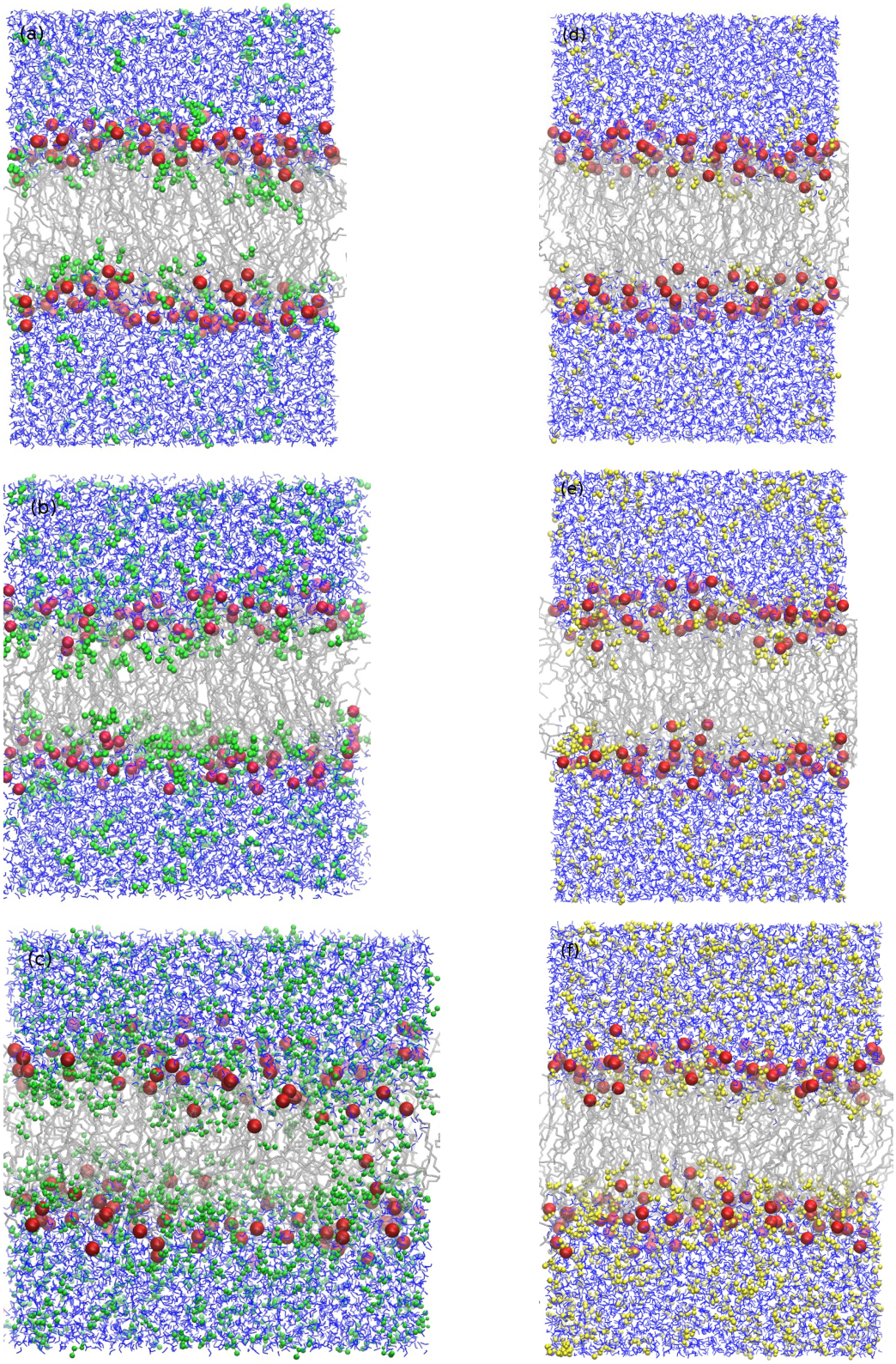


Figure 3.3.: Final configuration of systems after 100 *ns* simulation, figures (a), (b) and (c) are systems with ethanol solvent while figures (d), (e) and (f) present systems with methanol. Figures (a) and (d) are the lowest concentration (2.5 mol%), figures (b) and (e) are 5 mol% and figures (c) and (f) are 10 mol% percent concentration of alcohol. Colour codes are the same as figure 3.2.

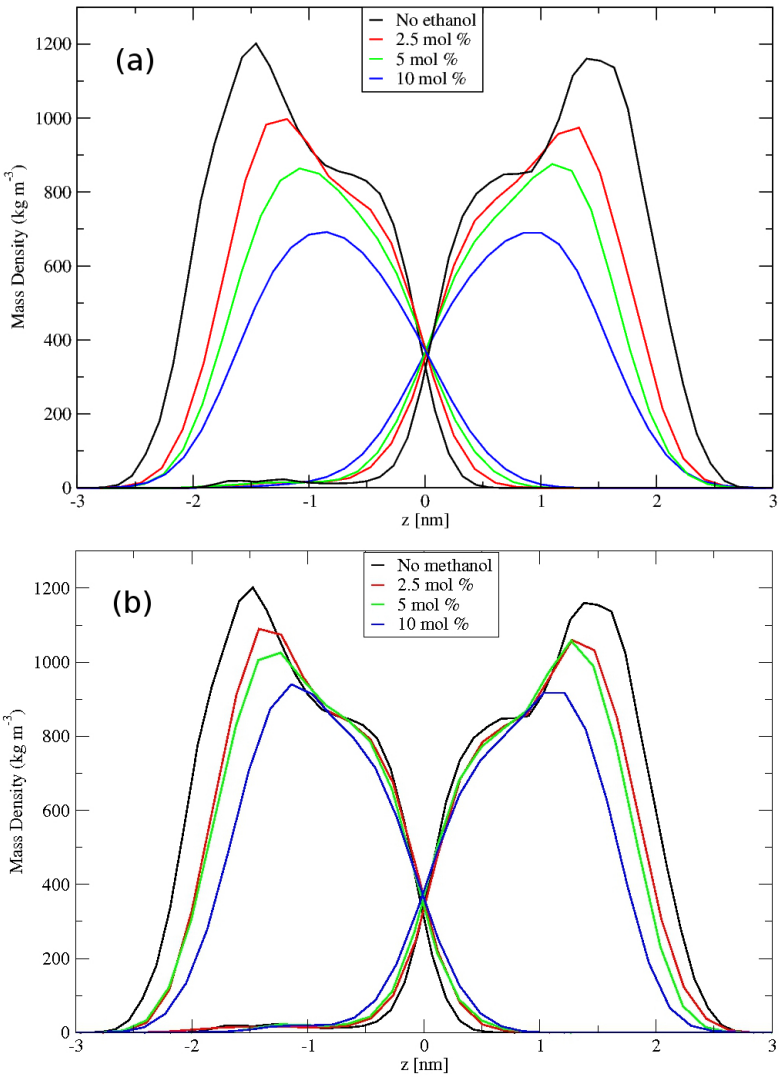


Figure 3.4.: The average density profile of membrane opposite leaflets. Figure (a) shows ethanol and figure (b) shows methanol. Without alcohol (black lines), with 2.5 mol% (red lines), 5 mol% (green lines) and 10 mol% (blue lines) of alcohol.

3.6. System Density Profile

Mass density profile of the bilayer can show the distribution of different groups of atoms in the system. Figure 3.5 is showing how a mass density profile represents a system. In the mass density profile graph each group of atoms is shown with a different line. In the current chapter, each system has four different group of atoms, which includes the bilayer head groups, lipid tail groups, water and alcohol (ethanol or methanol). As the figure and graph illustrate, each line in the graph can represent the average density of one of the groups, which shows the distribution of atoms in the system.

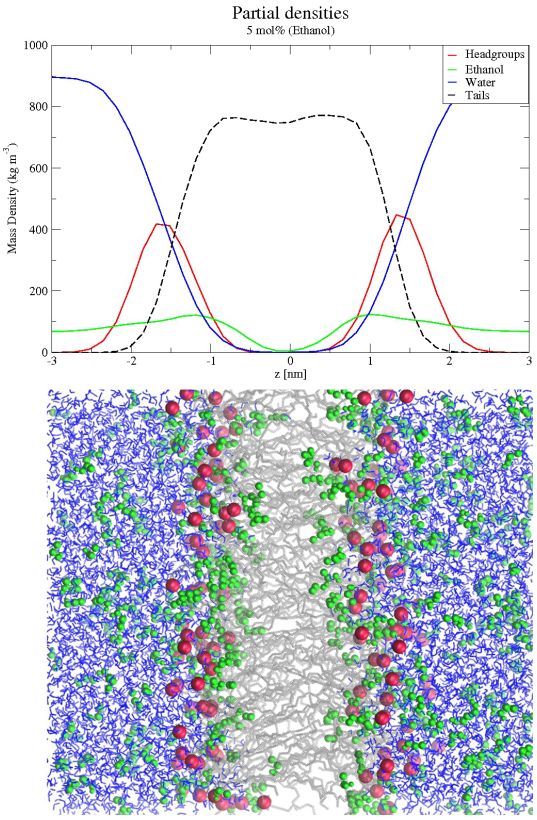


Figure 3.5.: System density profile graph shows the distribution of various atoms in a system. Colour codes are the same as figure 3.2.

Comparing the mass density profile of the system at different concentrations of ethanol and methanol, can give a very good understanding of the destructive behaviour of alcohol on membrane. Ethanol and methanol severely change the shape of the graph. As the concentration increases, the density of head groups decreases while concurrently the density of part of the graph corresponding to tail groups increases. This observation can be explained in interdigitation terms. The density of the hydrocarbon chains in the centre of the bilayer increases because the opposite leaflets interdigitate.

Moreover, the penetration of ethanol and methanol can be compared through these graphs. The more alcohol concentration increase, the more it can penetrate

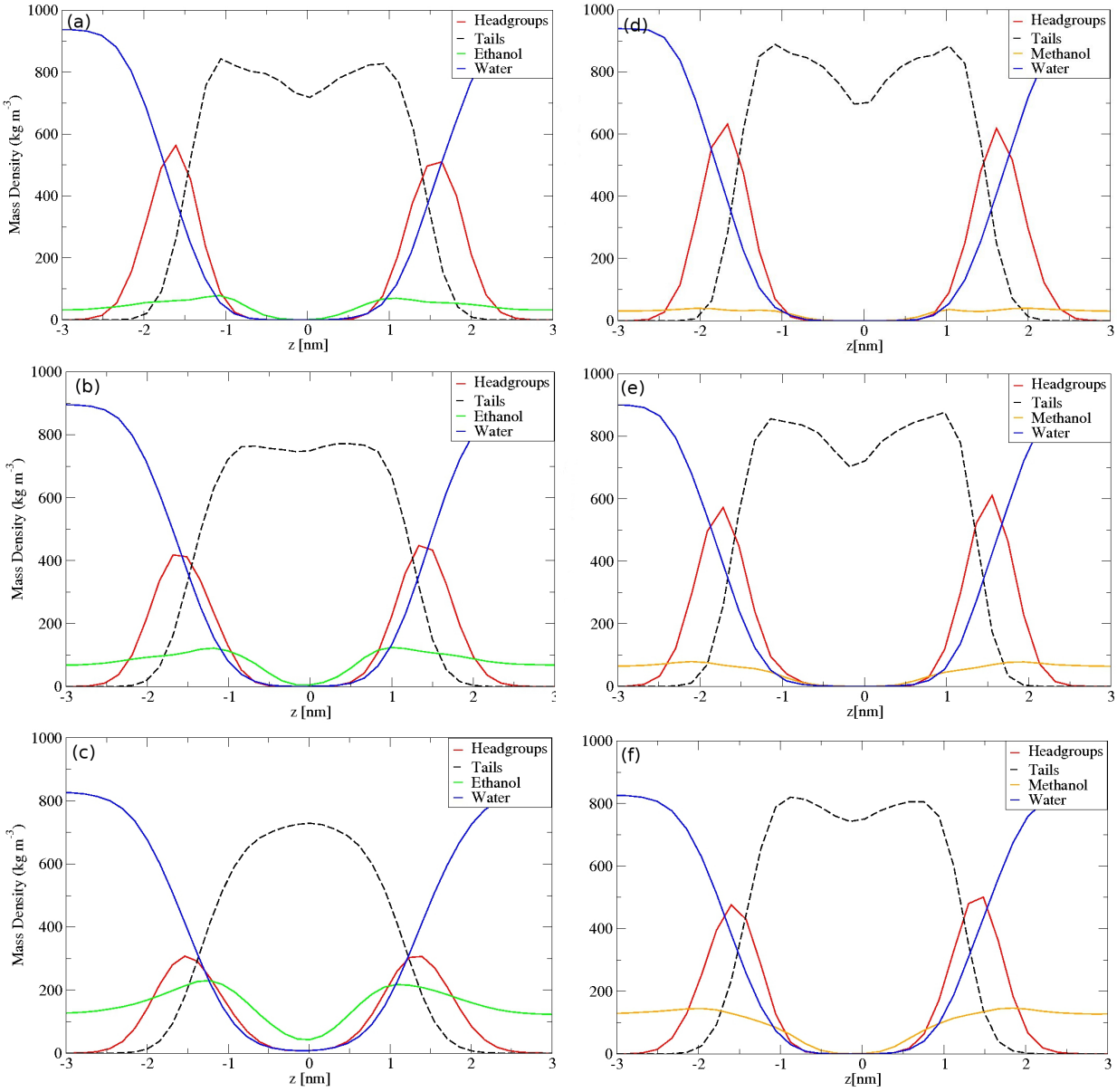


Figure 3.6.: Average mass density of systems after 100ns simulation, figures (a), (b) and (c) are systems with ethanol solvent while figures (d), (e) and (f) present systems with methanol. Figures (a) and (d) are the lowest concentration (2.5 mol%), figures (b) and (e) are 5 mol% and figures (c) and (f) are 10 mol% percent concentration of alcohol

in to the head groups and inside the tails area. For 10 mol% of ethanol pore formation was seen (figure 3.6 (c)). In the lipid bilayer in system with 10 mol% of ethanol, not only ethanol and water molecules are inside the tails area, but nitrogen atoms in the head groups can also be found inside the lipid tails (this can be seen in the right corner of the lipid bilayer). With the same concentration of methanol pore formation is not observed.

3.7. Area Per Lipid

Area per lipid (APL) is one of the important characteristics of bilayers. Area per lipid can be affected by presence of different types of chemical compound. The average area per lipid for a bilayer can be obtained as equation 3.1.

$$\langle A_{lipid} \rangle = \frac{Area\ of\ Simulation\ Box\ in\ xy\ Plane}{Total\ Number\ of\ Lipid\ Molecules\ in\ One\ Leaflet} \tag{3.1}$$

Area per lipid can also be used for equilibrating the bilayer. For Instance DMPC bilayer has been found to have an area per lipid of 60.6$\mathring{A}^2$ experimentally.[44] This value can be used as a reference value in computational work when equilibrating the system. Figure 3.7 shows the time variation for average area per lipid during the simulation for ethanol and methanol respectively.

Comparing the two graphs it can be concluded that ethanol increase the area per lipid of the bilayer more than methanol does. This increase in area per lipid can be explained according to ethanol and methanol tendency to be located between the head groups of the bilayer. More alcohol molecules between the lipids cause the bilayer to expand and as a result have a higher area per lipid.

3.8. Deuterium Order Parameter

Lipids in a bilayer are dynamic. Various movements of lipids are possible to take place and most of these movements influence the deuterium order parameter

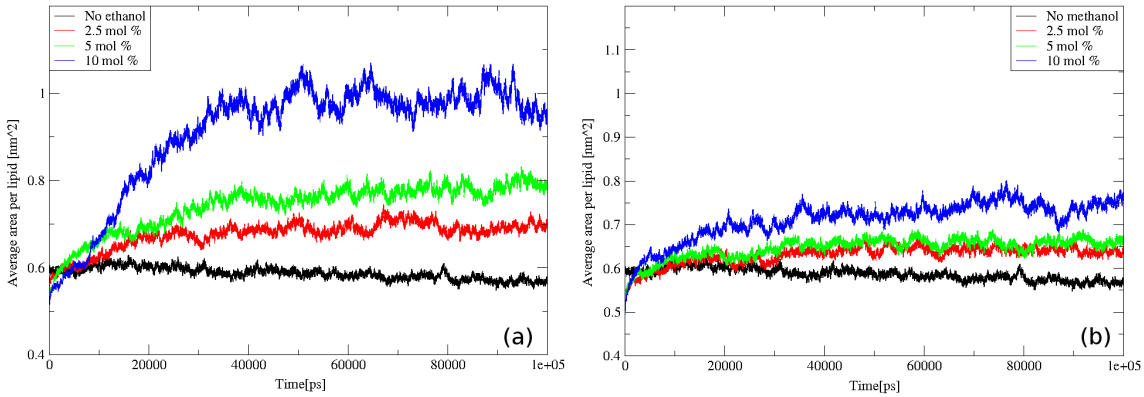


Figure 3.7.: The time evolution of average area per lipid for different concentrations of ethanol. Black line shows the area per lipid of DMPC bilayer in presence of water only while red, green and blue shows the presence of 2.5 mol%, 5 mol% and 10 mol % of alcohol respectively. Figure (a) shows these concentrations for ethanol and figure (b) shows the same concentrations for methanol.

of the acyl chain. Deuterium Order Parameter is measured according to the orientation of the C-D bonds as below

$$S = \left\langle \frac{3\cos^2\theta - 1}{2} \right\rangle \tag{3.2}$$

Where θ is the angle between C-D bond molecular axis and the bilayer normal (z) so It can measure the ordering of lipid tails. There are some examples, which can help us to link the value of $|S_{CD}|$ with lipid tails order. Having a value of one for $|S_{CD}|$ means that the C-D bond vector and the bilayer normal are parallel. A value of -0.5 is when the C-D bond vector is perpendicular to the bilayer normal. $|S_{CD}|$ have a value of zero when random orientation.[9]

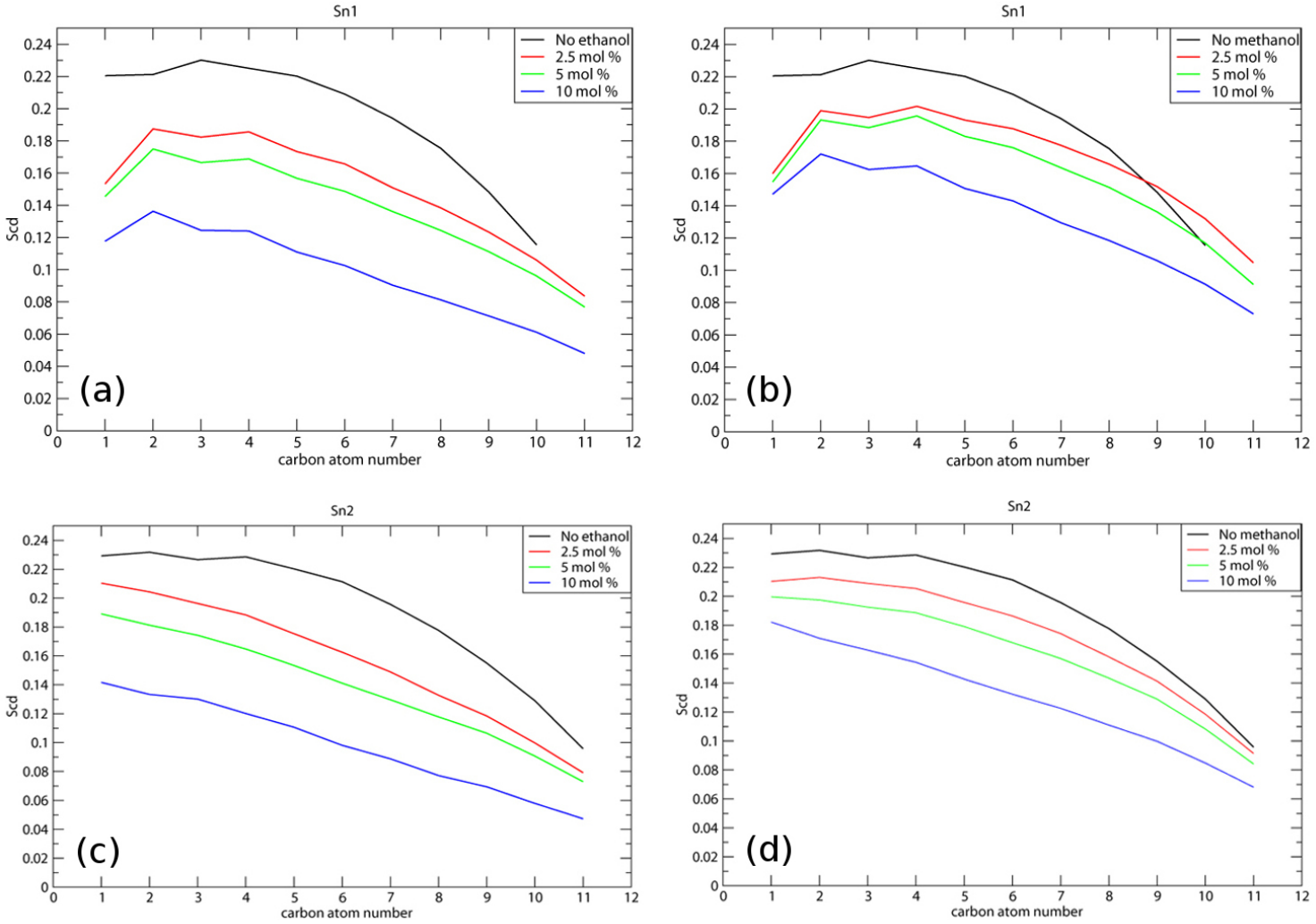


Figure 3.8.: Deuterium Order parameter of $|S_{CD}|$ for Sn1, (a) and (b), and Sn2, (c) and (d), acyl chains of DMPC lipids for different concentrations of ethanol (right column) and methanol (left column). Carbon atoms with lower numbers are closer to the head groups.

As it can be seen from the graphs alcohol generally cause disorder in lipid tails. A decrease in order parameter can be observed from 0 mol% concentration to 10 mol%. Comparing the graphs, it can also be clearly seen that ethanol causes more disorder in lipid hydrocarbon chains comparing to methanol.

The effect of ethanol on different lipid bilayer such as POPC, DOPC and DPPC as well as DMPC is reviewed in previous computational works.[7–12] In the current study, it is shown that alcohol cause interdigitation as well as increase in area per lipid of the bilayer. Ethanol, propanol and butanol are seen to have a similar effect on DPPC bilayer in a computational study done by Dickey and Faller.[12] These effects are shown to be more when the concentration of alcohol increase in the same study. The effect of ethanol and methanol on DPPC and POPC lipid bilayers has been investigated computationally at low concentrations.[11] In this study ethanol molecules are found to penetrate easily in the lipid bilayer since methanol has not penetrated. Comparing the concentration of alcohol in the named study with the current study systems, similar results has been observed in 2.5 mol% of alcohol.

The effect of alcohol on mass density profile in the current study is similar to previous computational studies.[9–11] Effect of ethanol on DMPC bilayer mass density profile in the current study is observed to be more comparing to effect of ethanol at the same percentages on POPC lipid bilayer.[10] Moreover, it is shown in the same work that by increasing the percentage of ethanol, opposite leaflets in POPC bilayer interdigitate. The interdigitation effect on POPC bilayer caused by presence of ethanol this computational work[10] is more than interdigitation in DMPC bilayer at the same percentages of ethanol in the current study. Pore formation in POPC lipid bilayer is observed in this study at a threshold concentration of 12 mol% of ethanol[10], while pore formation has happened at 10 mol% of ethanol in our study. The damaging effect of ethanol observed at 15 mol% of alcohol on both lipid bilayers has been the same. Our findings about the behaviour of DMPC lipid bilayer in presence of alcohol are generally in agreement with previous computational studies, where the effect of alcohol on lipid bilayer

is investigated at different concentrations.[7–12]

3.9. Conclusion

In this chapter performing molecular dynamics simulation for systems including DMPC bilayer and water and different number of alcohol (ethanol and methanol) molecules, we have shown that the effect of alcohol on DMPC bilayer is similar to effect of alcohol on different bilayers such as POPC[10] and DPPC[11] bilayers.

Generally speaking, ethanol affects the bilayer more than methanol according to hydrogen bonding. Ethanol and methanol molecules penetrate inside the membrane head groups. A pore in which ethanol and water molecules as well as nitrogen atoms (of DMPC head groups) are present, is found to be formed at higher concentration of ethanol (10 mol%).

Alcohol causes interdigitation in the bilayer and as a result decreases the bilayer thickness. Furthermore, it affects the deuterium order parameter of the hydrocarbon chains. Presence of alcohol causes disorder in lipid tails. All mentioned effects increase when the concentration of alcohol increases.

4. Effect of anti-asthma drugs on bilayer

4.1. Introduction

Three anti-asthma drugs are the subject of study in the current chapter. Beclomethasone Dipropionate (BDP), Fluticasone Propionate (FPP) and Prednisone are three steroidal anti-asthma drugs. These three drugs are all steroidal drugs and compatible with liposomes. The encapsulation of these drugs in liposomes has been practiced experimentally.[48,49] After being encapsulated in a bilayer these drugs can affect the bilayer in several ways. They can either let the solvent around inside the bilayer and cause pore formation or disorder in lipid bilayer as well as leak from the bilayer. To gain a better understanding of the drugs behaviour within a liposome and its interactions with a bilayer, we consider the atomistic level of some various systems including DMPC bilayer and each steroidal drug. Partitioning of other non-steroidal drugs inside the bilayer such as aspirin and ibuprofen[50,51], naproxen and relafen[34] are examined theoretically with the help of computer simulations but an atomistic level study on steroidal drugs used for the treatment of asthma, and their behaviour inside a drug delivery system is still missing.

4.2. Simulation methods

All the drugs have been placed initially either inside or outside of the membrane (figure 4.1). Moreover, the behaviour of the drug in both positions, either in presence of ethanol or water solvent is examined. In the case of BDP drug more investigations are done. These researches include the drug's behaviour when the concentration of the drug is more as well as if the ethanol molecules are inside the bilayer.

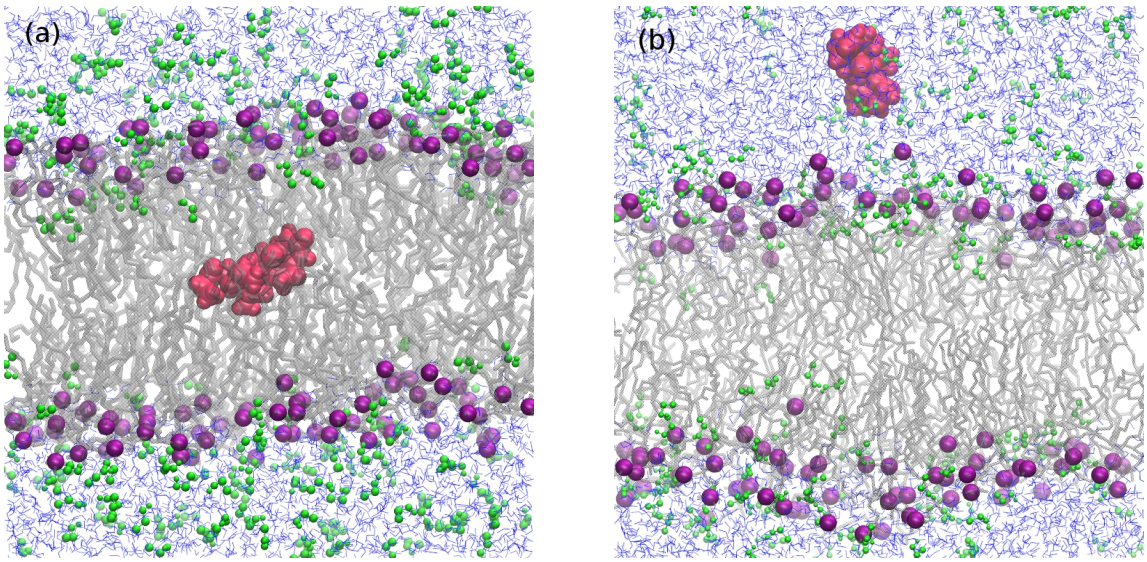


Figure 4.1.: The left hand side figure(a) shows the initial configuration of the system when the drug molecule is inside the bilayer. The right hand side figure (b) shows the initial configuration of the system when the drug is initially outside. Blue lines are water molecules, green spheres are ethanol molecules and purple shows the nitrogen atoms in head groups. Grey lines are lipid tails. The example drug molecule is shown with pink.

Information about creation of the coordinates of the drugs is explained in Appendix B. For every single system, DMPC bilayer and the drug are energy minimised separately, using steepest descent minimisation algorithm.[28] To place the drug inside the bilayer, the bilayer is expanded and the drug is placed between the expanded lipids.[20] After insertion of the drug, lipids are again shrinked in 28 steps until bilayer area per lipid reaches a value close enough to the experimental value for DMPC bilayer area per lipid.[44] Then in order to equilibrate the

bilayer and drug the whole system is subjected to energy minimisation. Having the drug inside the bilayer with correct area per lipid and equilibrated, we insert the ethanol (if considered) and water molecules respectively in the system.

After being energy minimised again, all systems have gone through NVT ensemble at 310 K using V-rescale thermostat (modified Berendsen thermostat).[52] NVT ensemble is run for 100 ps with temperature coupling time of 0.1 ps. Moreover, NPT ensemble is done for $1ns$ for the pressure to reach the value of 1 bar. Through NPT ensemble, the simulation box size will change until the correct density of solvent (water or ethanol and water) is achieved. To place the drug outside the bilayer all mentioned steps are done except expanding and shrinking the bilayer for drug insertion, instead the drug is placed outside of the bilayer and solvent is added afterwards. Periodic boundary condition is applied to the whole simulation box in x, y and z direction.

4.3. Beclomethasone Dipropionate (BDP)

Beclomethasone Dipropionate (BDP) is a hydrophobic steroidal drug. As it has been explained before hydrophobic molecules are not water-soluble and they need a non-polar solvent to be solved in. Thus, ethanol in this chapter is used, to represent the role of solvent in the system. The effect of this alcoholic solvent on encapsulated and non-encapsulated drug is of great importance since it is known that ethanol affect the bilayer and might intensify drug leakage. A thorough investigation is done on BDP interaction with DMPC lipid bilayer. In this section, the drug is considered to be inside and outside of the bilayer in order to consider the encapsulated and non-encapsulated drugs interaction with bilayer. The same simulation are done with water solvent only. The preference of the drug to be either inside or outside of the membrane is also inspected by placing the drug in between the head groups and observing its movements.

	Ethanol	Water	BDP	DMPC	Box size
Alcoholic solvent	205	4140	1	128	6.53×6.53×6.83
Water solvent	0	7913	1	128	6.44×6.44×9.38

Table I.: This table illustrates the information about number of atoms in the two systems. The first system is with BDP inside the bilayer and alcoholic solvent and the second system is with water as solvent.

4.3.1. BDP inside the bilayer

As it has been explained before BDP is a hydrophobic drug and after encapsulation in liposome is going to be placed in between the tail groups of the lipids. In order to gain a better understanding of drug's exact location in the bilayer, BDP is initially placed inside the bilayer. Moreover, to improve our information about the role of ethanol in drug's displacement in the bilayer, behaviour of drug inside the bilayer when there is only water outside is also investigated. This part of the study will help us to have a look at the changes in behaviour of the drug in absence of ethanol. This study also will help in achieving information about drugs effect on bilayer area per lipid and lipid orders.

There are two systems studied in this part. In both systems, one BDP molecule is placed in the middle of the bilayer in a way that the drug's centre of mass and bilayer's centre of mass are coincident. Thus, in the first system the bilayer is surrounded with water and alcohol while in the second system there is only water around the bilayer. Number of different groups of atoms in the system can be found in table I.

For the system with alcoholic solvent the first location of the drug and the final position of the drug after $100ns$ is shown in figure 4.2. As it can be seen from the final snapshot the drugs preferable position in the bilayer is near to the head groups (figure 4.2 (b)). BDP drug is found to be completely in one of the leaflets area and one of the oxygens is close to the head groups all the time during the simulation.

Mass density profile of the system also can be used as a proof of what is shown

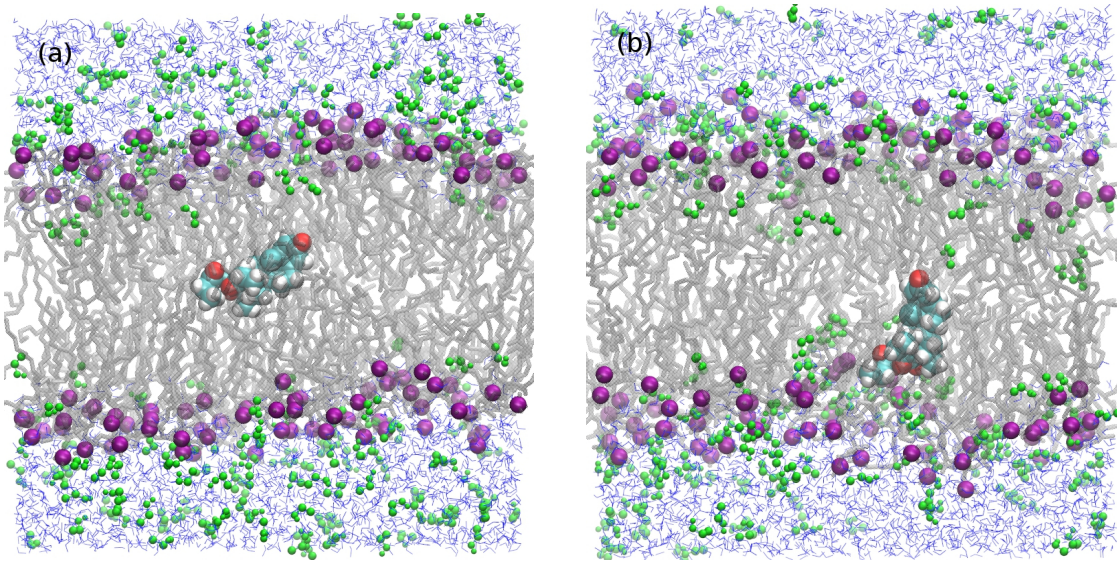


Figure 4.2.: The left hand side figure (a) shows the initial configuration of the system with BDP drug inside the bilayer and ethanol solvent. The right hand side figure (b) shows the final configuration of the same system after 100ns.

in figure 4.2. As it can be seen in the Mass density graph of the system, where the distribution of different group of atoms is shown, the drugs average density (the shortest line) is close to the head groups of the bilayer. The graph also shows penetration of ethanol in the leaflet with BDP is less than the opposite leaflet.

This conclusion is supported by explaining the number of hydrogen bonds the drug forms with different groups of atoms in the system. Between drug molecule and DMPC bilayer molecules no hydrogen bonds are found during the simulation while between the drug and water molecules one or sometimes two hydrogen bonds are found to be formed during the simulation (figure 4.4).

These hydrogen bonds are formed between the oxygen atoms in the drug and water molecules. Therefore, the oxygen molecules in BDP prefer to stay close to the head groups where they are closer to the water molecules.

Figure 4.5 is comparing the area per lipid of the system with 205 number of ethanol when drug is inside the bilayer and the system with 205 number of ethanol when there is no drug in the bilayer. The aim of this comparison is to interpret the effect of drug on lipids expansion in a bilayer.

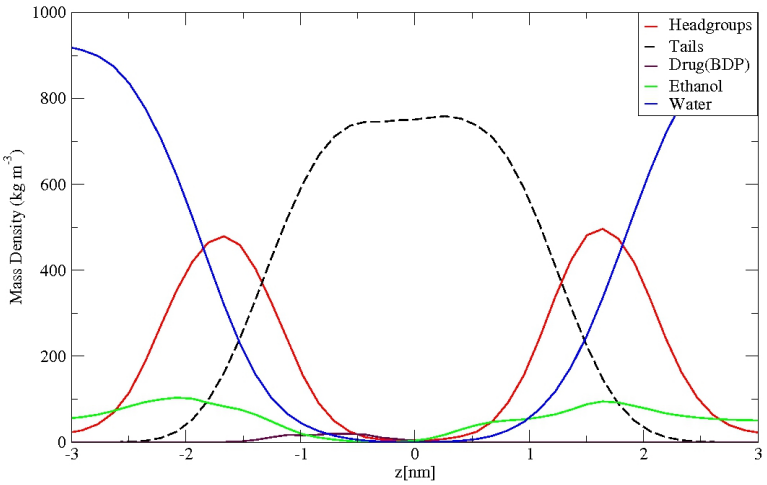


Figure 4.3.: Mass density profile of system with BDP drug inside the bilayer and ethanol solvent.

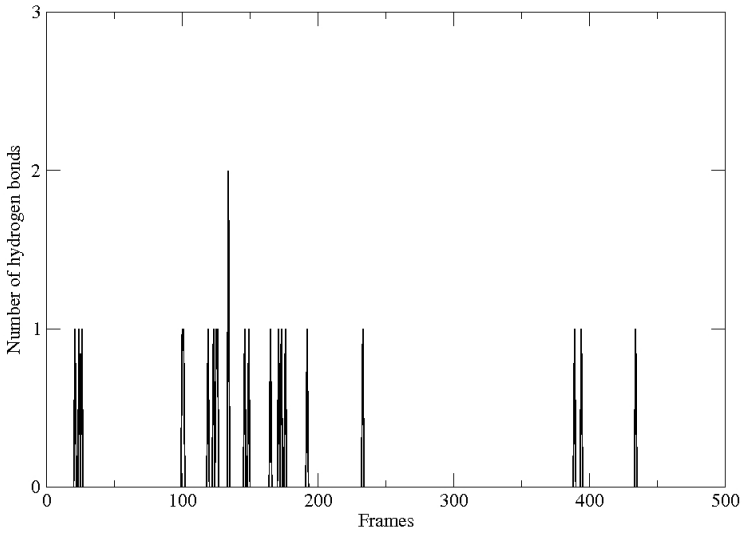


Figure 4.4.: Number of hydrogen bonds between BDP and water molecules during the simulation.

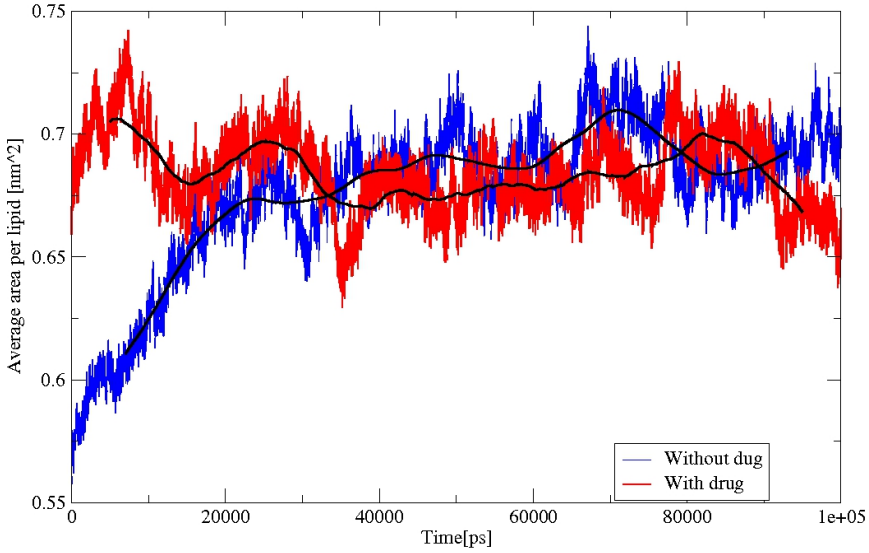


Figure 4.5.: The area per lipid of DMPC bilayer. Red line shows APL for a system without drug while the blue line shows APL of the same system with drug inside the bilayer.

With blue line representing area per lipid of DMPC bilayer in a system without drug and red line representing area per lipid of DMPC in the system with drug it can be understood from the graph that in both systems the bilayer area per lipid changes drastically in the first $30ns$ during which, ethanol molecules penetrate inside the bilayer and affect the area per lipid of the bilayer (this effect is explained at length in chapter 3 of the current thesis). Although systems have started from different area per lipid but in the very short time ($20ns$) they reach almost the same value. After this period, the area per lipid of the system with drug is found to be slightly smaller than the area per lipid of the other system without the drug in it most of the time. This means that the system with the drug in it is less expanded comparing to the system without drug.

4.3.2. Higher concentration of BDP

To consider changes to bilayer properties in presence of higher concentration of BDP, two BDP molecules are initially placed inside DMPC bilayer (figure 4.6). The top view of the bilayer shown in right corner of the picture, illustrates how drugs are placed diagonally in the bilayer. Number of molecules in the system

are illustrated in table II.

	Ethanol	Water	BDP	DMPC	Box size
System	205	5267	2	128	6.44×6.44×9.38

Table II.: This table illustrate the information about number of atoms in the system with higher concentration of BDP drug inside bilayer.

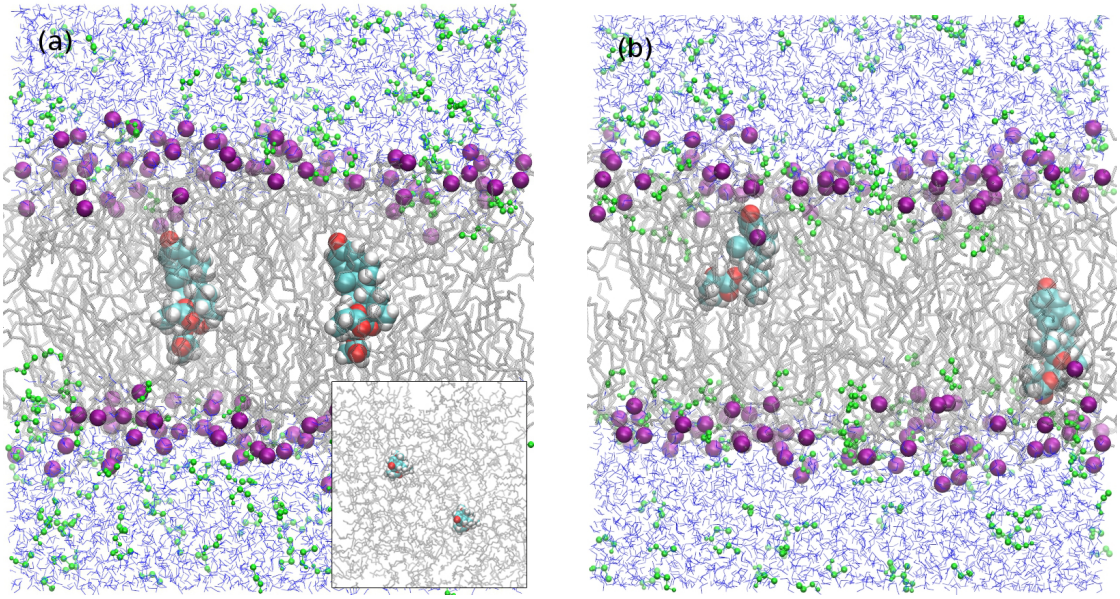


Figure 4.6.: Configuration of system with higher concentration of BDP. left hand side figure (a) is the starting point. In the corner of figure (a) the top view of the bilayer is shown. The right hand side figure (b) is the final system after $100ns$.

From the final configuration of the system it can be seen that the two drugs are anchored to the head groups of the bilayer with one of their oxygens. Once again hydrogen bonds between oxygen atoms in drugs and water molecules can explain this behaviour. As it is shown in the figure 4.7 during the simulation there is most of the time one hydrogen bond and sometimes two hydrogen bonds formed. since there are two number of drug molecules in the system, the total number of the bonds are more than what it can be seen in the figure 4.4. Though, the number of hydrogen bonds is not doubled. This means that there has been more opportunity to form hydrogen bonds comparing to the system with one BDP

molecule, but it is not the same for each single molecule.

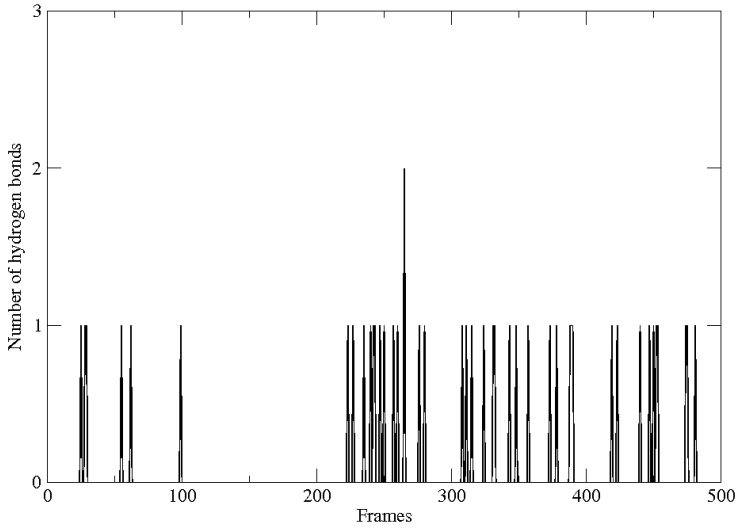


Figure 4.7.: Number of hydrogen bonds between beclomethasone dipropionate and water molecules during the time the simulations.

Mass density profile graph shows the average density of the drugs during the simulation. Interesting information can be found by comparing the mass density profile of the system with one drug (figure 4.3), and the system with two drugs (figure 4.8).

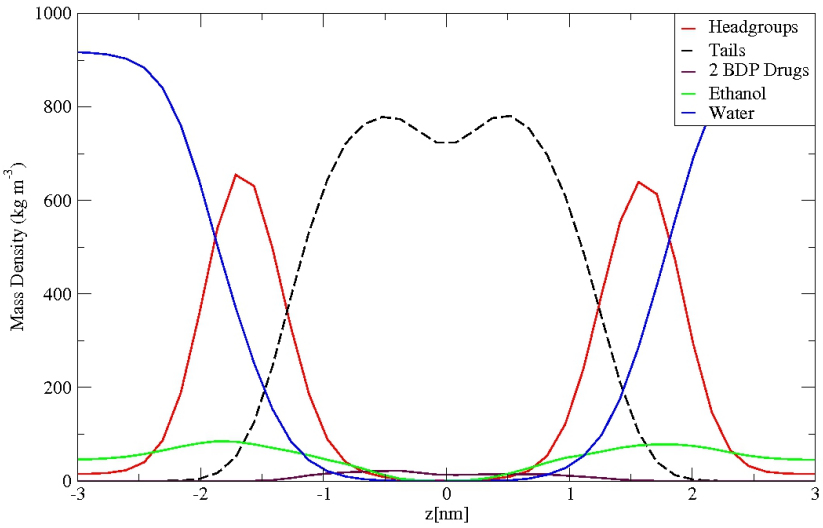


Figure 4.8.: Mass density profile of system with two BDP molecules inside the bilayer (higher concentration).

The first issue attracting ones attention can be the density of the ethanol.

There are less ethanol molecules penetrated inside the bilayer head groups when there are two drug molecules in the system. Thus, the density of the head groups are more in the system with two drugs comparing to the system with one drug molecule.

4.3.3. Ethanol effect on drug inside the bilayer

To understand the effect of ethanol on drug's location in the bilayer, both ethanol and drug molecules are located inside the bilayer initially. This study can show ethanol molecules interaction with the drug and their arrangement around the drug inside bilayer. Details of the simulation system can be found in the table III.

	Ethanol	Water	BDP	DMPC	Box size
System	231	7259	1	128	6.64×6.64×8.84

Table III.: Table shows simulation details for the system with BDP and ethanol molecules inside the bilayer.

The initial configuration shows the ethanol molecules as well as the drug molecules inside the bilayer (figure 4.9 (a)).

As it is expected, ethanol molecules move from the hydrophobic area of the bilayer toward the head groups in few pico seconds after the simulation starts. The effect of ethanol molecules on drug's movements has been checked by looking at the drugs displacements.

The blue line in figure 4.10 shows the drugs centre of mass displacements with respect to the bilayer centre of mass. The green line shows the distance of drug from bilayer centre when ethanol is outside.

As it can be seen from the graph the drug's displacement has more fluctuations when ethanol is inside the bilayer. Therefore, the ethanol molecule can enhance drug's movements and make it to move more freely.

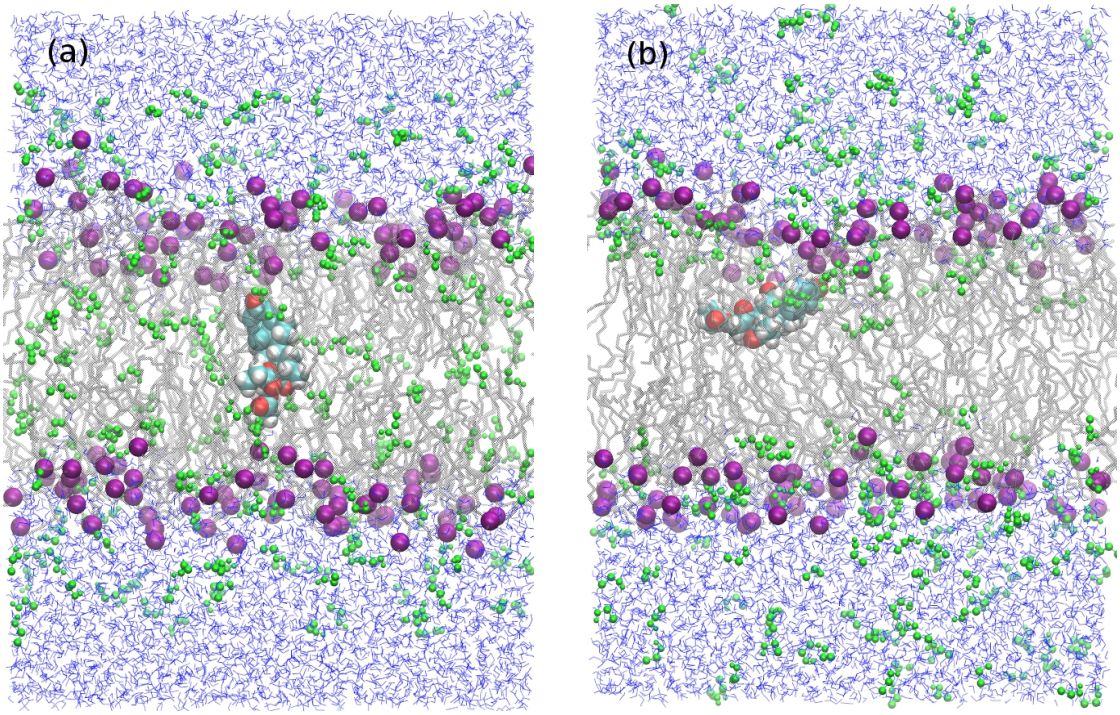


Figure 4.9.: The left hand side figure (a) shows the initial configuration of the system when ethanol is inside the bilayer. The right hand side figure (b) shows the final configuration of the same system.

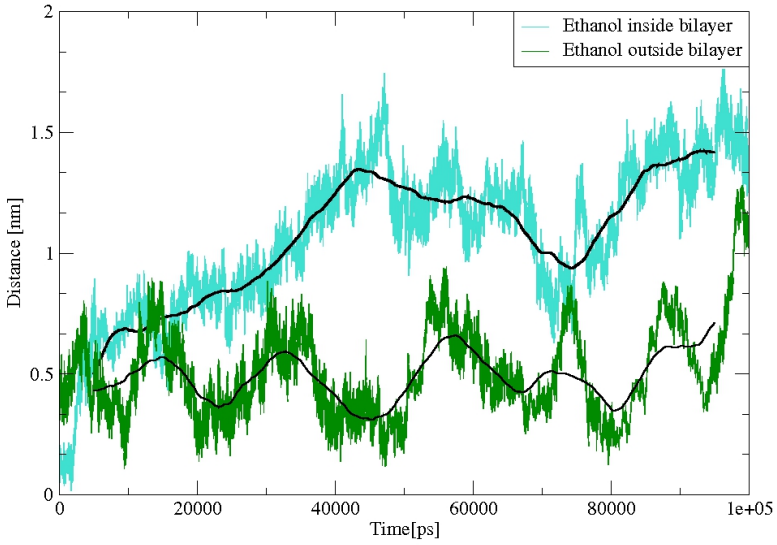


Figure 4.10.: System density profile graph shows the distribution of various atoms in a system.

4.3.4. BDP outside the bilayer

There is always a part of not entrapped drug remained in the solution and these drugs are also able to interact with the bilayer. To consider these interactions two sets of simulations are done. In the first simulation the drug has been placed outside of the bilayer in an aqueous solution (figure 4.12) and in the second simulation the drug is outside of the bilayer where both water and ethanol molecules are present (figure 4.11). Details of the simulations and number of molecules can be found in the table IV.

	Ethanol	Water	BDP	DMPC	Box Size
Alcoholic Solvent	205	5603	1	128	6.58×6.58×7.44
Water Solvent	0	8907	1	128	6.48×6.48×9.67

Table IV.: Table shows simulation details for two systems with the system with BDP molecule outside the bilayer.

Firstly, the system with ethanol solvent is considered. The initial configuration of the system shows that the drug is outside of the DMPC bilayer and is surrounded by water and ethanol (figure 4.11 (a))

From the final configuration we can see BDP position after $100ns$ (figure 4.11 (b)). This snapshot shows that the drug has penetrated inside the bilayer. Although BDP drug penetrates inside the bilayer no hydrogen bonds were found between the BDP and DMPC bilayer during the simulation.

Secondly, the drug is placed outside of the bilayer but this time only water molecules are around the drug (figure 4.12).

The final position of molecules shows that the drug has not moved toward the bilayer. During the simulation BDP molecule flip-flops and move across the bilayer head groups direction.

Thirdly, in another simulation the possibility of drug penetration inside bilayer has been tested by placing the drug initially closer to the bilayer. The result is that if there are no ethanol molecules in the solution the drug will be stuck on

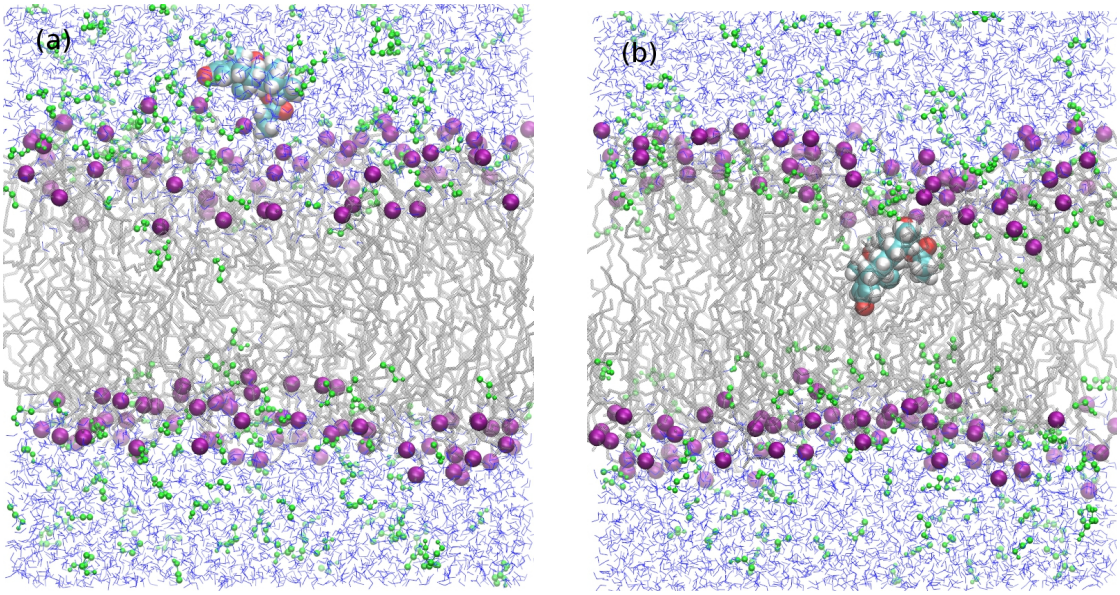


Figure 4.11.: The left hand side figure (a) shows the initial configuration of the system when ethanol is present as a solvent. The right hand side figure (b) shows the final configuration of the same system.

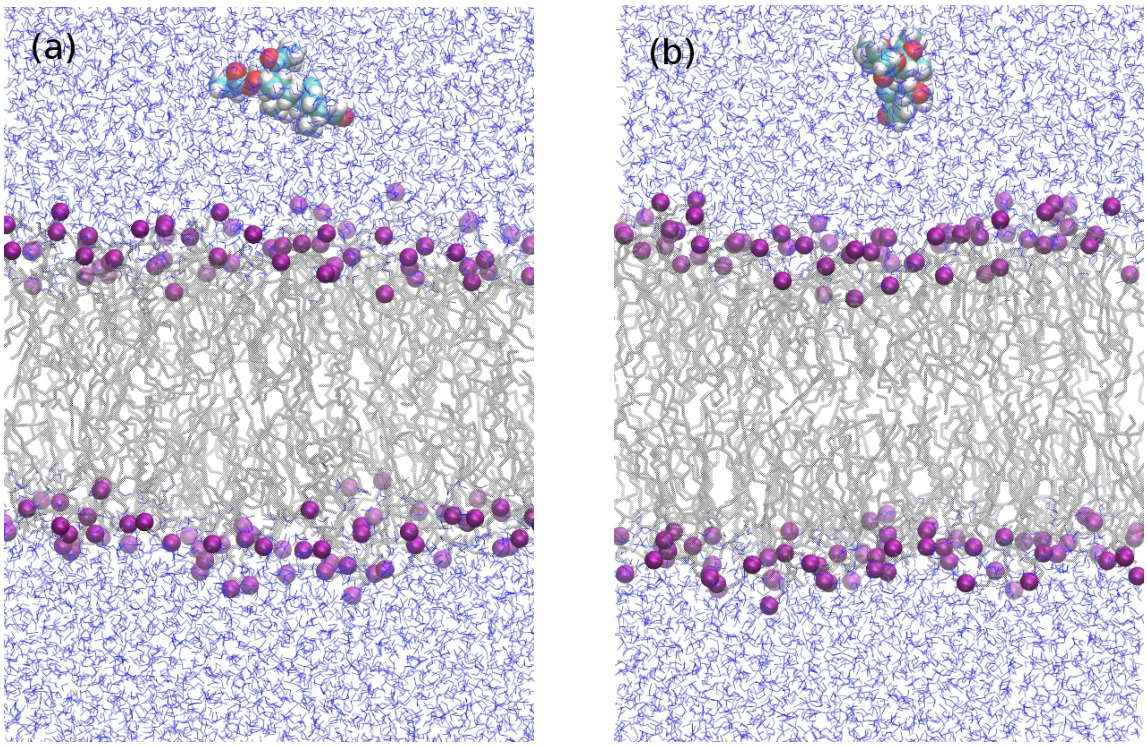


Figure 4.12.: BDP outside of the bilayer in aqueous solution. The left hand side figure (a) is the initial configuration of the system. System after $100ns$ is shown on the right (b).

the head groups and cannot penetrate inside.

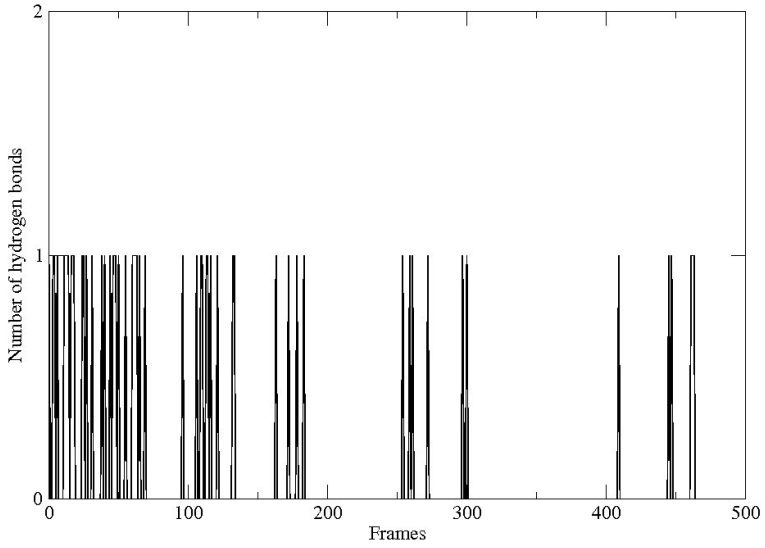


Figure 4.13.: Number of hydrogen bonds formed between water and BDP molecules per each frame during $100ns$ in the system with BDP outside of the bilayer in presence of alcohol and water.

In the system with BDP outside of the bilayer and ethanol and water solvent, the number of hydrogen bonds between ethanol and drug molecule are few (five in the whole simulation). The number of hydrogen bond between water molecules and BDP drug can be seen in figure 4.13. Hydrogen bonding between water molecules and oxygens in drug molecule cause the drug to stay close to the head groups near to water molecules after penetrating inside the bilayer.

The distance between the drugs centre of mass and bilayers centre of mass is shown in figure 4.14 for the system with ethanol and water solvent. The initial starting point of the drug is $2.8nm$ from the bilayer. During the first $20ns$ of the simulation the drug molecule moves toward the membrane when the distance between drug and bilayer decrease to $1nm$. This is when the drug is completely penetrated inside the bilayer. After this time till the end of the simulation, the drug stay close to the bilayer head groups (in the top leaflet) and its distance from centre of mass does not change in normal direction to the bilayer surface.

On the other hand in the system with water solvent, the drug rotates in its place during the simulation and is not attracted to the bilayer.

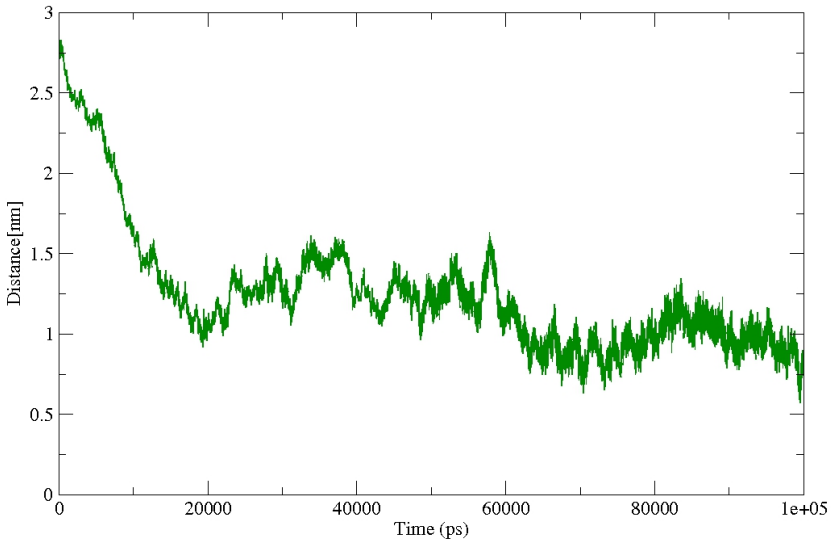


Figure 4.14.: The distance of drug and bilayer during $100ns$ of simulation in the system with BDP outside of the bilayer in presence of alcohol and water.

Further more, to prove the precision of the results the drug has been initially placed in a closer position with respect to the bilayer (very close to the head groups) and inside an aqueous environment. This work is done to see if the drug can penetrate inside the bilayer, in case it is closer to the head groups. The distance of the drug and bilayer centre of mass is $2.6nm$ at the beginning. The results show that even if the drug is closer to the bilayer it cannot go through the head groups and will be stuck on top of the head groups. As it can be understood from the centres of mass distance BDP meets the head groups after $15ns$ and will be on the head groups for the rest of the simulation time. There are no hydrogen bonds between DMPC bilayer and BDP drug found to be formed during this time.

These results can show the effect of alcohol on the energy of the system. The drug molecule to pass through bilayer head groups, has to overcome an energy barrier. The results from system with water solvent shows that when there is no ethanol in the system, the drug cannot overcome this barrier no matter how close to the bilayer it is placed. Furthermore, the results from system with ethanol solvent suggest that presence of ethanol can change this barrier in a way that the

drug can overcome it and penetrate inside the bilayer.

4.3.5. BDP preference between solution and bilayer

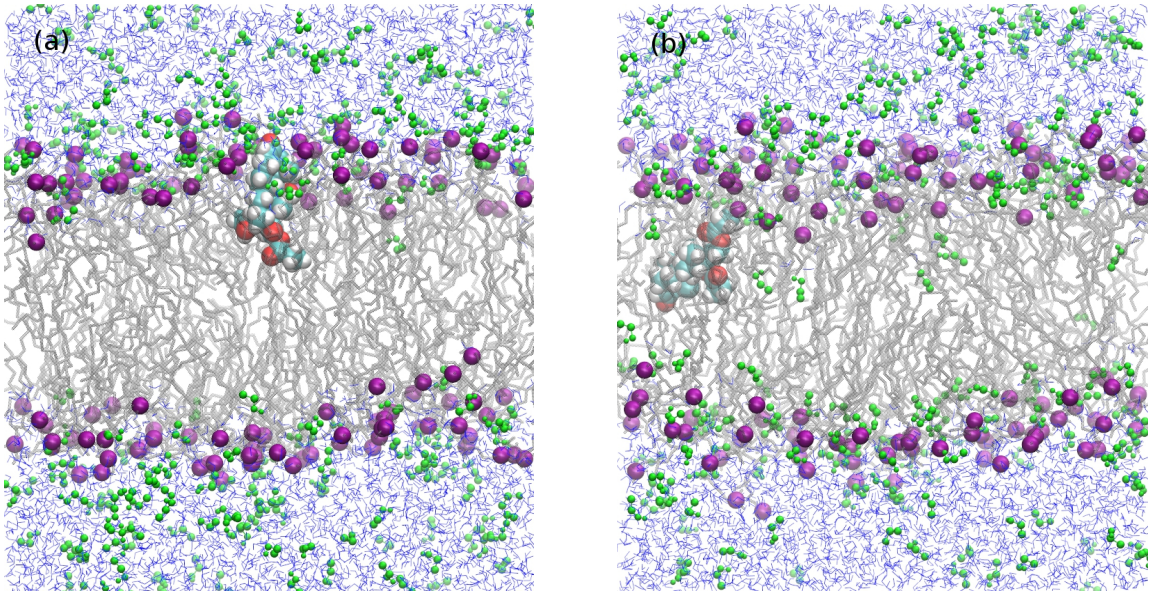


Figure 4.15.: The left hand side figure (a) shows the initial configuration of the system when the drug is in aqueous environment. The right hand side figure (b) shows the final configuration of the same system.

The preference of the drug between being either in the solution or inside the bilayer is examined. This study is done to examine the situation of drugs if in the process of entrapment some of them are entrapped in between the head groups. Though the drug is placed initially between the head groups where it is also touching the bilayer and solvent interface (figure 4.15). The final configuration of the system in figure 4.15 on the left shows that the drug has preferred to penetrate inside the bilayer.

4.4. Fluticasone Propionate

Fluticasone Propionate (FPP) is the other hydrophobic drug, which is used in this chapter. The interactions of this drug with DMPC bilayer is investigated in different ways. Similar to previous section (BDP), FPP is initially placed both

inside and outside the DMPC bilayer. These two simulation are done either when ethanol is present as solvent or not. All four system simulation details can be found in table V.

System	Ethanol	Water	FPP	DMPC	Box Size
FPP inside	205	5257	1	128	6.67×6.67×7.32
FPP inside	0	9010	1	128	6.44×6.44×10.15
FPP outside	205	5654	1	128	6.61×6.61×7.73
FPP outside	0	8917	1	128	6.49×6.49×9.96

Table V.: Table illustrate simulation details for four systems with fluticasone propionate molecule.

In the following sections we will discuss the behaviour of drug in the above mentioned systems. The system in which drug molecule is initially placed inside and outside the bilayer, are discussed in two sections respectively.

4.4.1. FPP inside the bilayer

Firstly FPP molecule is placed inside the bilayer. The starting point of the system is shown in figure 4.16 on the left as well as the final position of molecules in the system on the right.

To start with the drug molecule is placed in the middle of the bilayer and the water and ethanol molecules are outside of the DMPC bilayer. The drugs behaviour is similar to BDP behaviour inside bilayer. FPP prefers to stay close to the head groups of the bilayer and one of the oxygen molecules is anchored to the head groups. Hydrogen bonds between the drug molecule and solvent molecules can explain this matter.

There are no hydrogen bonds found between FPP and the DMPC bilayer. The number of hydrogen bonds between ethanol and FPP as well as bonds between water and FPP are shown in figure 4.17. Comparing figure 4.4 with figure 4.17, it can be seen that the number of hydrogen bonds formed between water molecules

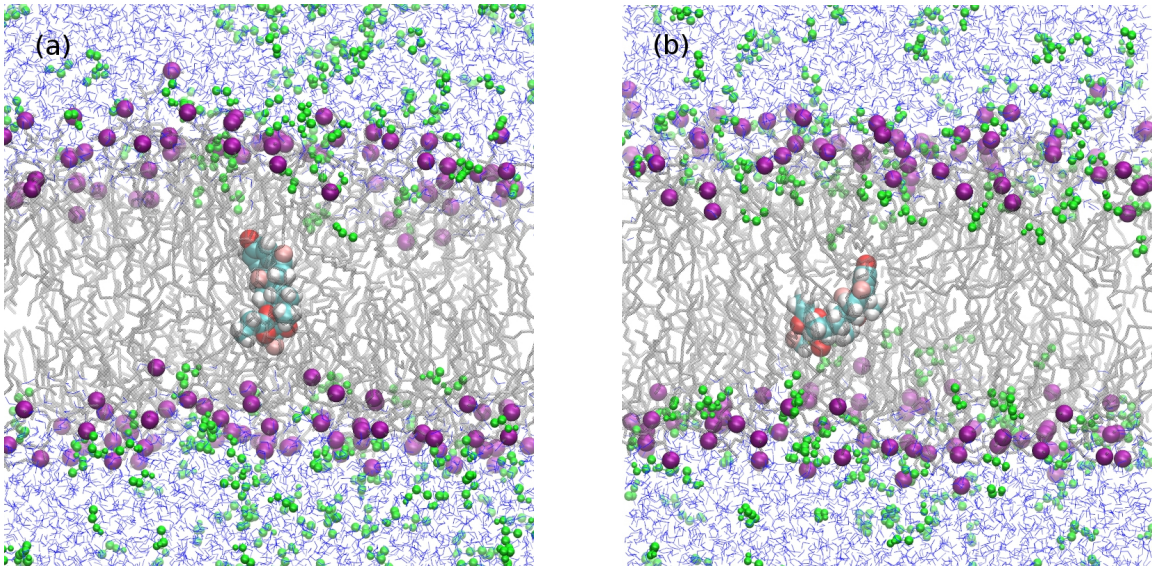


Figure 4.16.: FPP inside DMPC bilayer with ethanol solvent around the bilayer. The left hand side figure (a) shows the initial configuration of the system. The right hand side figure (b) shows the final configuration of the system.

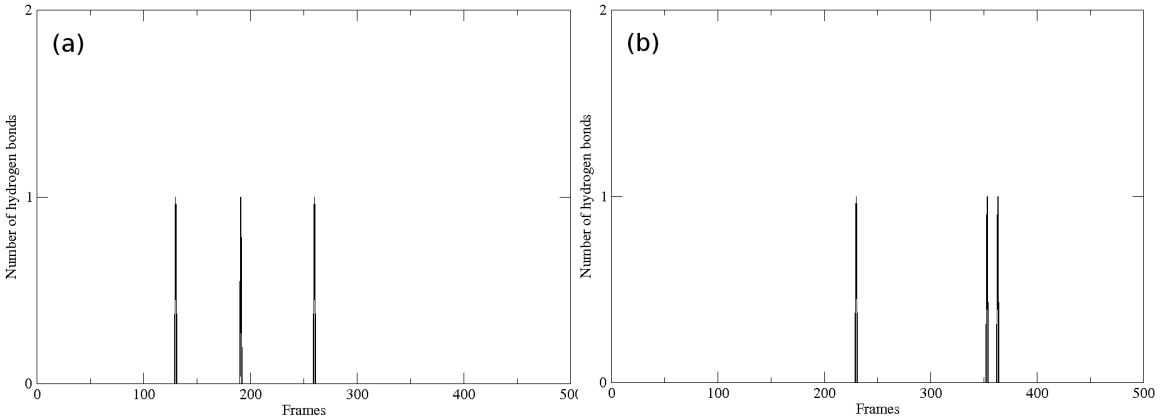


Figure 4.17.: The left hand side figure (a) shows the number of hydrogen bonds between water molecules and drug molecule per frame. The right hand side figure (b) shows the number of hydrogen bonds between ethanol molecules and drug molecule per frame.

and the FPP drug is less than the hydrogen bonds between BDP in the same position. The reason of which can be higher solvent accessible surface area and polar surface area in BDP molecule comparing to FPP molecule. Hydrogen bonds are formed between oxygen atoms of the drug and ethanol as well as water molecules. The graphs also show that the number of hydrogen bonds the drug has formed with either ethanol or water molecules are the same. Thus, the tendency of FPP drug to water and ethanol is the same.

Since the solvent molecules penetrate between bilayer head groups (the mass density profile can support this statement), the hydrogen bonds between FPP drug and solvent molecules can explain the drug's preference to stay close to the head groups in the bilayer.

Moreover, the same study on FPP drug is done with only water solvent. Similar results to the system with BDP and water has been observed. The drug prefers to stay in one of the leaflets close to the head groups where solvent molecules are more accessible. This preference is not as strong as BDP molecule since the number of hydrogen bonds formed by FPP molecules with water during the simulation is less than BDP hydrogen bonds with water.

4.4.2. FPP outside the bilayer

The interaction of drug with bilayer is examined placing the drug outside of the bilayer. Effect of ethanol on this interaction is tested having two different systems where in one of them a low percentage of ethanol is used as solvent and in the other there is only water solvent. The system at initial position is shown in figure 4.18 on the left when ethanol is present. From the final configuration of the system in the same figure on the right it can be seen that the drug has penetrated inside the bilayer.

The distance of drug from DMPC bilayer centre of mass is shown in figure 4.19. From the graph it can be understood that the drug has been initially placed $3.5nm$ away from the bilayer centre of mass, which makes the distance of drugs centre of mass from bilayer head groups about $2nm$. It also shows that immediately after

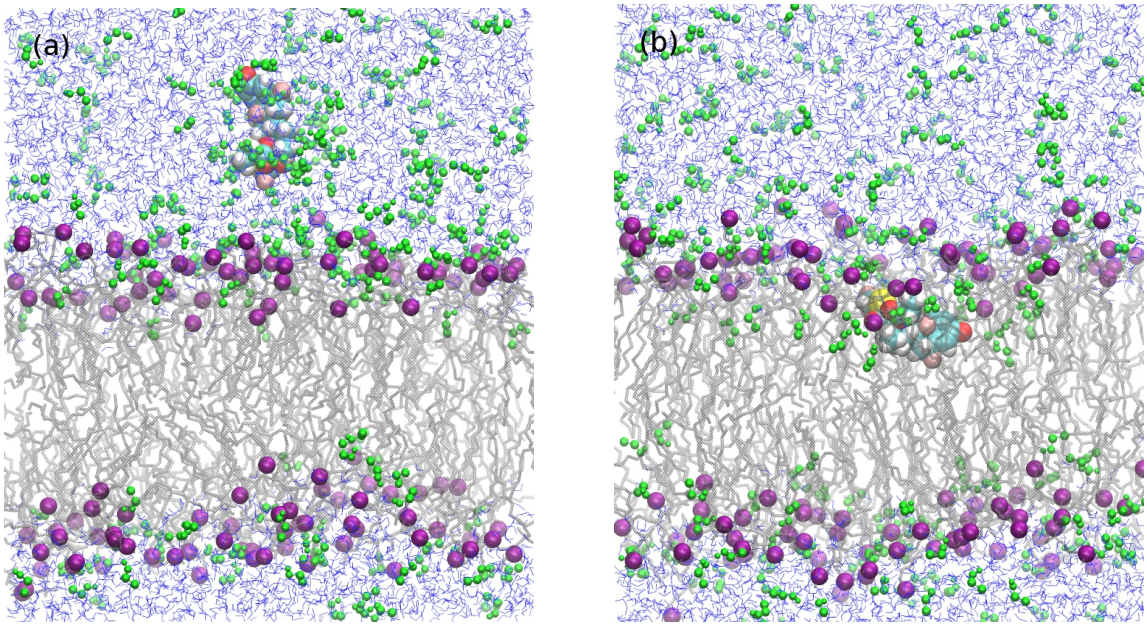


Figure 4.18.: FPP molecule outside of the bilayer in presence of ethanol. Initial and final configuration of the system are shown on the left and right respectively.

the simulation starts the drug starts to move toward the bilayer.

After $20ns$ from beginning the drug's distance from bilayer centre of mass is less than $2nm$, which means the drug is very close the head groups and has started to penetrate. Between $20ns$ and $40ns$ the distance changes from $2nm$ to less than $1.5nm$, which means that the drug is inside the bilayer. After the drug penetrates inside the bilayer, it stays in one of the leaflets close to the head groups of the bilayer like when it was placed initially inside.

The mass density profile of the system supports these results. As it is shown in figure 4.20 the average density of the drug in is inside the bilayer tail groups. Since the drug has been initially placed outside and the average density is inside the lipid tails, the drug has definitely penetrated inside the bilayer.

Furthermore, the drugs is placed outside of the bilayer in an aqueous solution. The aim of this simulation is to understand the role of ethanol in the previous simulation. The initial and final configuration of the system is shown in figure 4.21.

Figure 4.21 (a) illustrates the starting point of FPP molecule in the system.

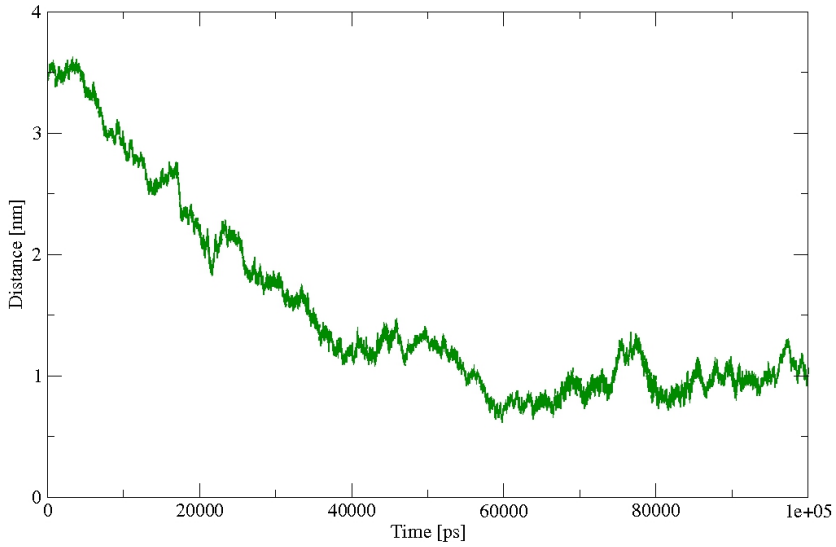


Figure 4.19.: Distance of FPP drug from bilayer centre of mass. The drug is initially outside in ethanol solvent.

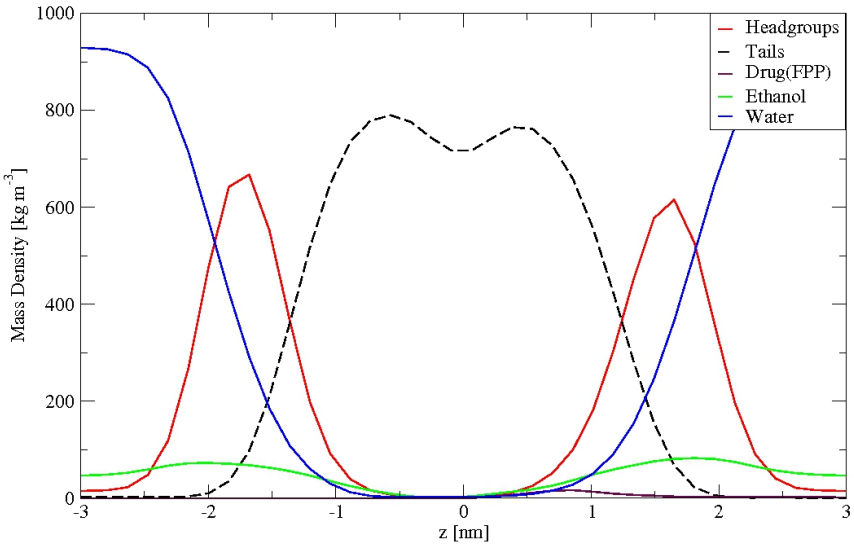


Figure 4.20.: System density profile of system with FPP molecule outside of DMPC bilayer in presence of ethanol.

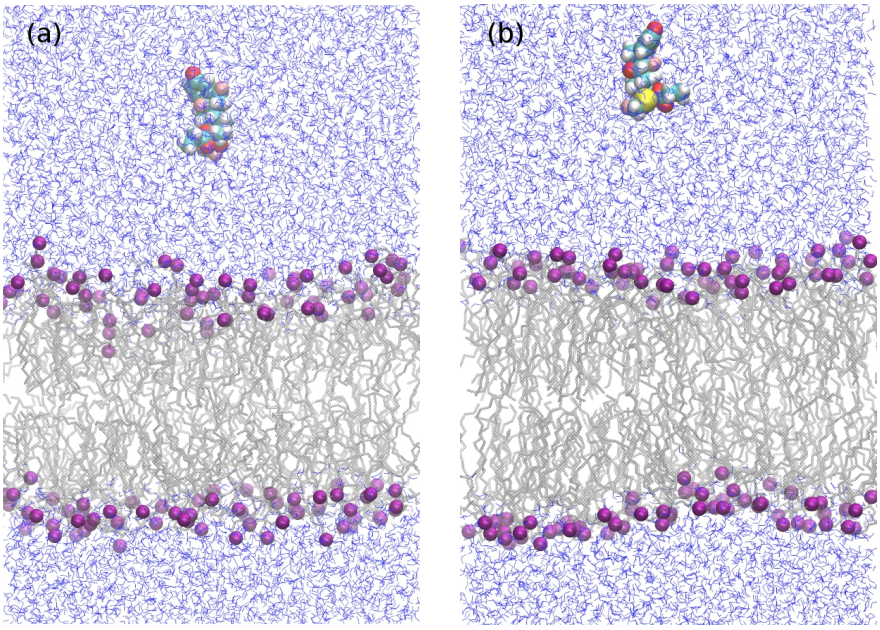


Figure 4.21.: FPP molecule outside of the bilayer in water solution. Figure on the left (a) shows the initial configuration of system. Figure on the right (b) is the final position of molecules in system.

The drug is outside of the bilayer $4.5nm$ away from bilayer's centre of mass and is surrounded by water molecules. This distance makes the drug to be around $3nm$ away from the bilayer head groups. The last snapshot of the system (figure 4.21 (a)) after $100ns$ shows that the drug has remained outside of the bilayer. During the simulation the drug does not move much in z direction and it only moves mostly around in xy plane. These results show that ethanol enhance drug's movement in the solution.

FPP molecule has similar behaviour to BDP molecule in water. As it is shown earlier BDP drug also stays outside the bilayer when there are only water molecules present as solvent. Moreover, BDP and FPP molecules have similar behaviour in ethanol solvent. Both drug molecules move toward the lipid bilayer and penetrate inside the bilayer when ethanol is present as solvent.

4.5. Prednisone

The third anti-asthma drug we study in this chapter is prednisone. Similar to the other two drugs prednisone is a hydrophobic drug. This drug can be entrapped in between the liposome lipid tails. Four different systems are studied in this section. In the first two of the systems drug molecule is placed inside the bilayer to simulate a system in which prednisone is entrapped inside the bilayer. In one of these two systems, solvent around the bilayer is water and in the other it is a low concentration of ethanol. In the other two systems prednisone is initially positioned outside of the bilayer. In one of the systems the drug is surrounded only by water while in the other system ethanol with a low concentration is present outside of the bilayer. The details of these four systems can be seen in the table below

System	Ethanol	Water	Prednisone	DMPC	Box Size
Prednisone inside	205	5265	1	128	6.56×6.56×7.54
Prednisone inside	0	9013	1	128	6.44×6.44×10.17
Prednisone outside	205	5775	1	128	6.48×6.48×8.14
Prednisone outside	0	8929	1	128	6.42×6.42×10.18

Table VI.: Table illustrates simulation details for four systems with prednisone molecule.

4.5.1. Prednisone inside the bilayer

Like the other two drugs prednisone has been initially placed inside DMPC bilayer to investigate its effect on the bilayer when it is entrapped inside liposome. We will firstly discuss the system in which a low concentration of ethanol is present as solvent. Molecules position in simulation box can be seen in figure 4.22 at the beginning and the end of the simulation.

During the simulation prednisone molecule is not found to be as close as the other two drugs to bilayer head groups. Although it sometimes travel to head

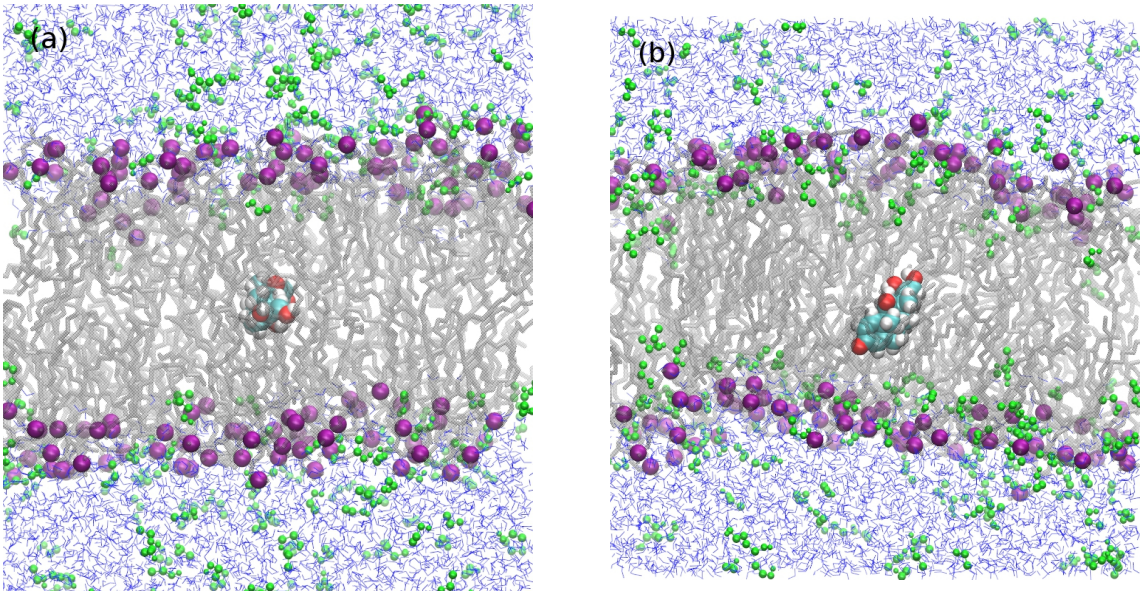


Figure 4.22.: Prednisone molecule inside the bilayer with ethanol solution in system. The left hand side figure (a) is the starting configuration and the left hand side figure (b) is the configuration of system after $100ns$.

groups vicinity, most of the times it prefers to stay in the middle of the bilayer. The average density of the drug in mass density profile of the system can witness this matter (figure 4.23). As it can be seen from the graph the average density of the drug molecule is inside the tail groups very close to the middle in z direction. This means that the drug has spent most of its time close to the centre of the box (in normal direction).

The reason of prednisone behaviour in the bilayer can be explained by having a look at the hydrogen bonds drug molecule forms with other molecules in the system. Unlike the other two drugs, prednisone does not form hydrogen bonds with ethanol molecules. The hydrogen bonds between prednisone and water are shown in left hand side figure 4.24. There are also a considerable number of hydrogen bonds found between prednisone and DMPC bilayer (figure 4.24 (b)). These bonds are formed between the drug and oxygen atoms in lipid head groups. The number of hydrogen bonds between prednisone and the solvent molecules are not as many as the number of bonds BDP and FPP form with solvent molecules.

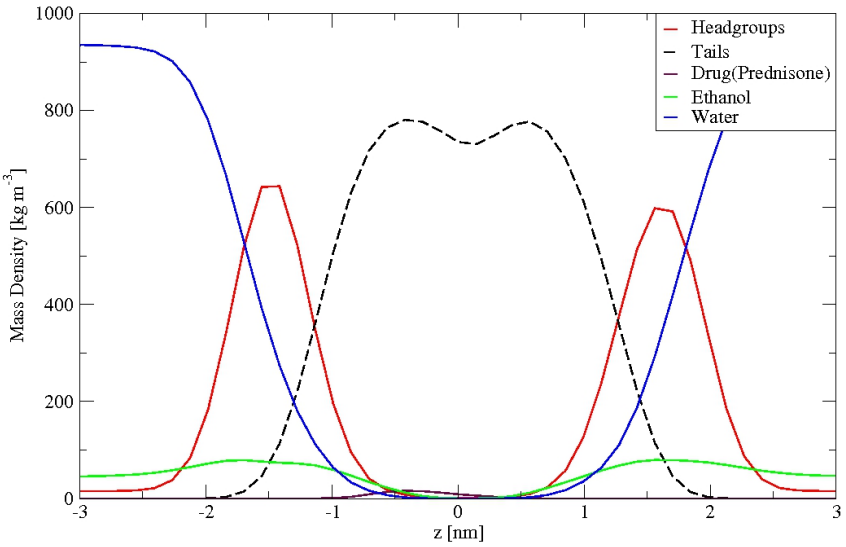


Figure 4.23.: Mass density profile of system with prednisone drug inside DMPC bilayer with ethanol solvent.

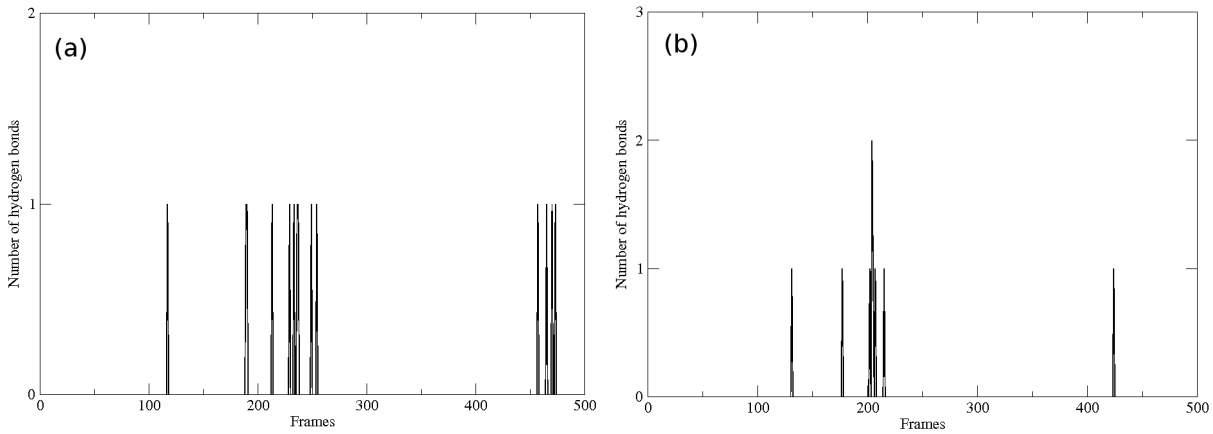


Figure 4.24.: Number of hydrogen bonds per frame. Figure on the left (a) shows the bonds between prednisone and water. Figure on the right (b) shows the bonds between DMPC lipids and prednisone.

Thus, prednisone molecule is not eager to be very close to the area where the solvent molecules are present like the other two drugs. On the other hand prednisone form hydrogen bonds with oxygen atoms bilayer head groups and being in the middle the distance of drug's tail and oxygens in head groups is sufficient to form bonds.

4.5.2. Prednisone outside the bilayer

In the current section prednisone is placed outside of the bilayer in ethanol solvent and water solvent separately for the same purpose explained earlier in previous sections. First of all the drug is placed outside of the bilayer in a solution with a low concentration of ethanol. Initial and final systems are illustrated in figure 4.25. From the final configuration, it can be seen that as it is expected, comparing its behaviour with the other two drugs, prednisone has penetrated inside the bilayer.

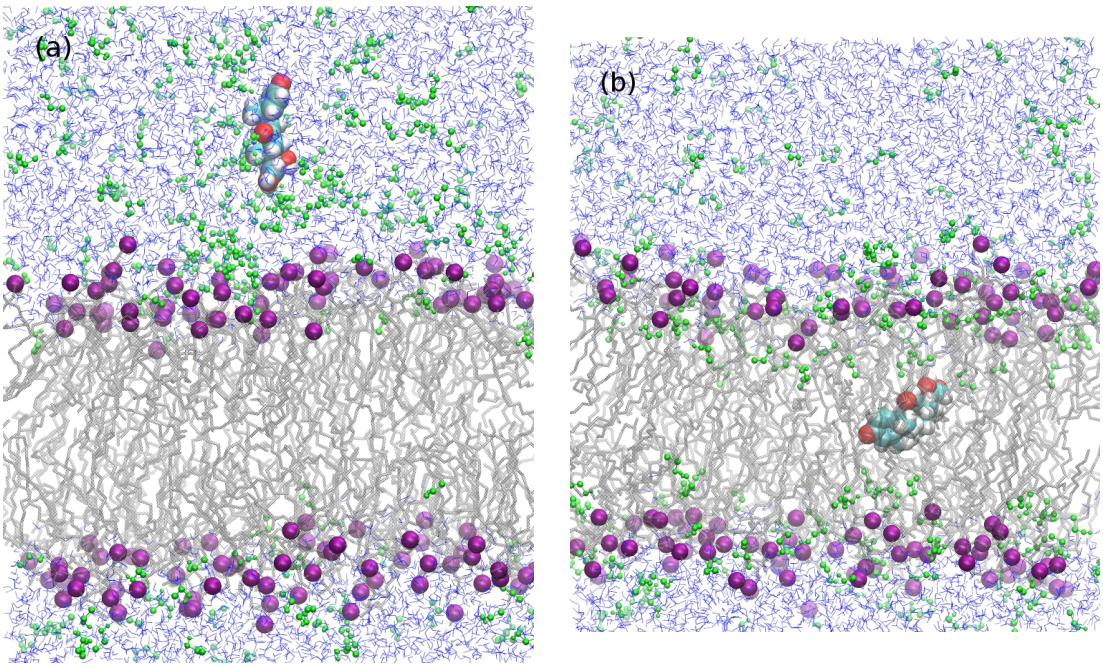


Figure 4.25.: Prednisone molecule outside bilayer in ethanol solution. Figure on the left (a) is the initial position of molecules and figure on the right (b) is the final position of molecules.

The distance of the drug from bilayer centre of mass also support this observation (figure 4.26). Distance between drug and bilayer shows that drug molecule has been initially $3.5nm$ away from bilayer centre of mass, after the simulation begins, the drug immediately start to decrease its distance with the bilayer centre of mass and within $20ns$ the graph reaches a plateau at $1nm$, which means the drug has penetrated inside the bilayer.

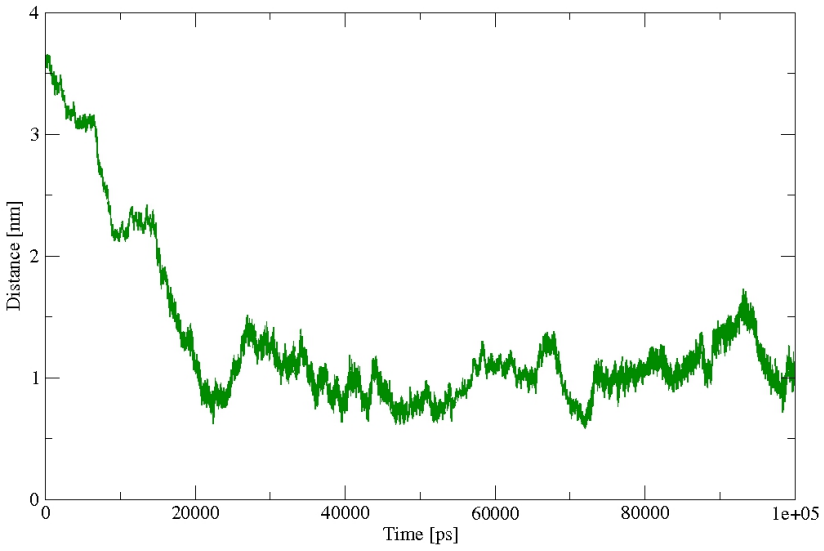


Figure 4.26.: Distance of Prednisone molecule centre of mass, initially outside the bilayer in ethanol solution, from bilayer centre of mass.

Hydrogen bonds between drug and water are shown in figure 4.27. The graph shows that most of these bonds are formed before the drug penetrates inside the bilayer. When drug molecule is inside the bilayer very few numbers of bonds found to be formed with water.

Hydrogen bonds are formed between the drug molecule and DMPC lipid molecules as well as between prednisone and ethanol molecules (figure 4.28). Most of the hydrogen bonds between prednisone and DMPC are formed when the drug has not penetrated inside the bilayer since the oxygen atoms in head groups are more accessible. Few number of bonds are formed with ethanol molecules at the end of simulation time and not any approximately in the first $85ns$. This can be because prednisone molecule solvent accessible surface area is less than the other two drugs.

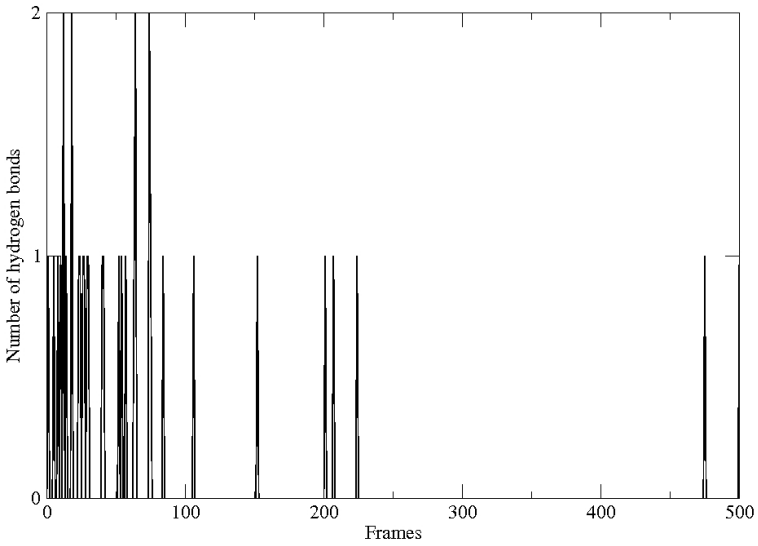


Figure 4.27.: Number of hydrogen bonds between prednisone and water when prednisone is outside of the bilayer in ethanol and water.

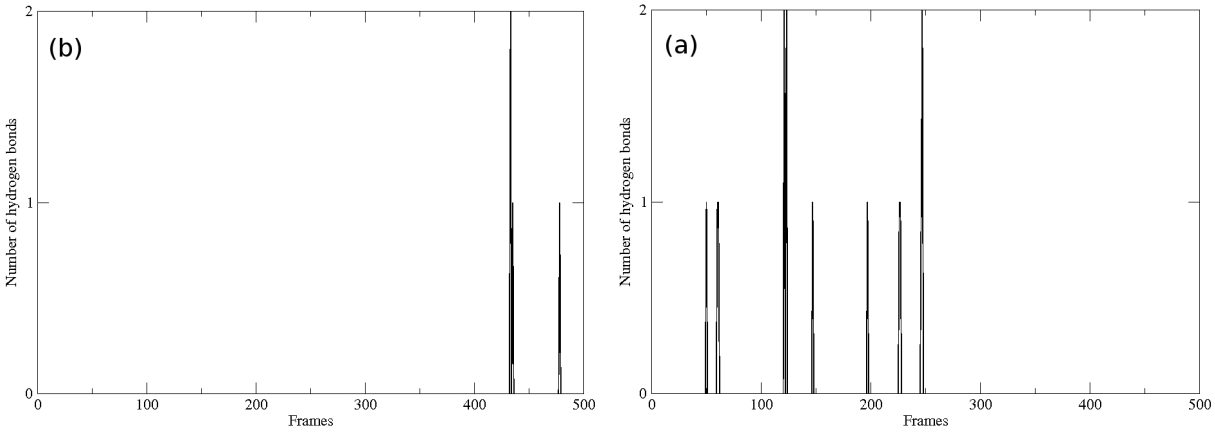


Figure 4.28.: Number of hydrogen bonds per frame. figure on the left (a) shows the bonds between prednisone and water. Figure on the right (b) shows the bonds between DMPC lipids and prednisone.

Secondly the drug is placed in water outside of the bilayer in order to investigate the effect of ethanol on presented results in previous system. Figure 4.29 shows the system at the beginning and the end of simulation. As it can be seen from the figure on the left (the initial configuration) the drug is in aqueous environment outside the membrane.

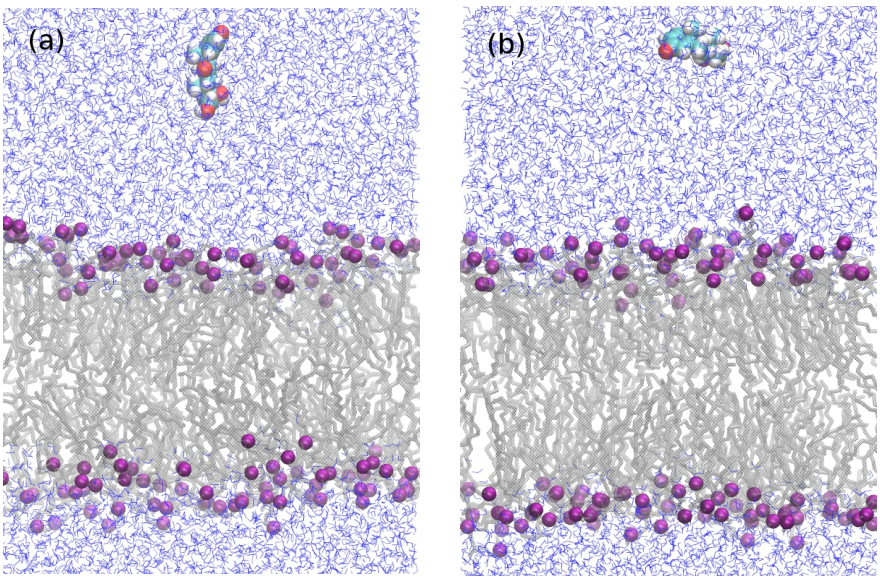


Figure 4.29.: Prednisone molecule in water outside DMPC bilayer. Figure on the left is the initial position of molecules and figure on the right is the final position of molecules.

The right hand side figure shows that the drug is still outside of the membrane after $100ns$. Having a look at the trajectory of the system it can be seen that the drug is not attracted to the bilayer and it either flip flop or move mostly parallel to the bilayer in the box. The distance of drug from bilayer centre is illustrated in figure 4.30. This graph supports the results. The graph shows that the drug molecule is initially $4.7nm$ away from bilayer centre. During the simulation, this distance increase and it does not get closer to the bilayer at any point.

The hydrogen bonds formed between the drug and water molecules are shown in figure 4.31. The number of hydrogen bonds with water molecules are a lot more comparing to when ethanol is present in the solution. Thus, oxygen atoms in the molecule that are hydrogen bond acceptors are always involved in a bond.

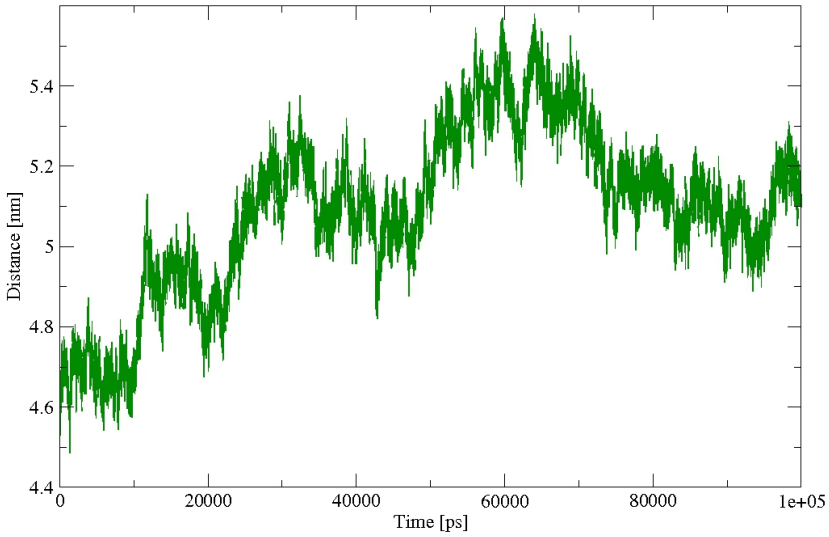


Figure 4.30.: Distance of prednisone from bilayer when drug molecule is outside bilayer in water.

Being surrounded by water molecules and forming hydrogen bonds with them can be a reason why the drug molecule does not move toward the DMPC bilayer.

4.6. Conclusion

Three steroidal hydrophobic drugs where studied in this chapter. The effect of these three drugs on the bilayer as well as the solvent has been the issue of this study. It is shown that all three drugs form hydrogen bonds with water during the simulation. Moreover, the number of bonds is found to depend on the drugs solvent accessible surface area and their polarity.

It is also shown that the drugs presence does not affect the bilayer area per lipid.

When the drugs are initially inside the bilayer, they prefer to stay near the bilayer head groups according to the hydrogen bonds they form with the solvent molecules. Running the simulations for a longer time, due to the bonds formed between the drug and solvent molecules (water and ethanol), the drug might pull the solvent molecules inside the bilayer. This might cause leakage of the drug from bilayer.

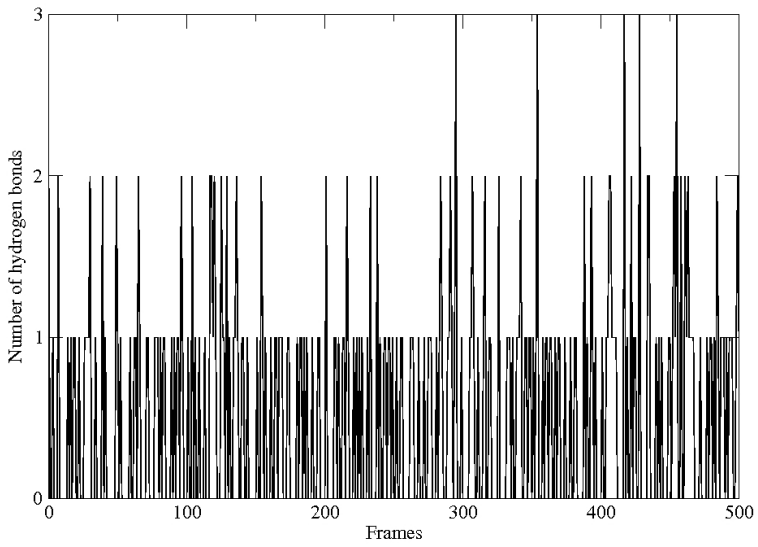


Figure 4.31.: The graph shows the number of hydrogen bonds between prednisone and water. Prednisone is outside bilayer and surrounded by water.

Placing the drug outside of the bilayer, we have shown that if ethanol is present as a solvent all three hydrophobic drugs can penetrate inside the bilayer. On the other hand, if there is no ethanol in the system and water is the solvent, the drug will not be able to retain inside the bilayer. After the drug penetrates inside the bilayer its behaviour will be similar to when, it has been inside from the beginning.

5. Future Work

The current work is done considering the effect of alcohol and anti-asthma drugs on DMPC lipid bilayer. The atomistic level of the systems has been studied using molecular dynamics. Atomic scale systems are well detailed and the amount of information is a lot due to the number of atoms and their interactions. Therefore, it is not possible to run an atomistic level simulation for bigger systems in size as well as for a longer time. The coarse-grained molecular dynamics method provides the opportunity to have less detailed systems, instead larger systems in size and longer simulations in time are possible. As a future work the entrapment of anti-asthma drugs during the liposome formation in aqueous environment can be compared with entrapment of the same drug during the formation of liposome inside ethanol environment.

Furthermore, It is possible to have a look at the free energy profile of the system. This investigation can give us more information about the reason of drug's movement toward the bilayer and its penetration inside the bilayer. The effect of changes in alcohol concentration on energy profile of the system can also be a case of study.

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Appendices

A. Energy Minimisation

Energy minimisation (also known as geometry optimisation) is usually done in computer simulations in order to find a stable configuration of system. There are different methods and algorithm introduced for energy minimisation such as Conjugate Gradient Method and Steepest Descent Method. In most of the methods, energy function of the system with respect to changes in geometry is considered. The gradient of this function at a certain point can be negative or positive and its sign determines where to move to get to the minima. Steepest descent method updates the geometry with the following equation[53]

$$geometry_{(new)} = geometry_{(old)} - AE'(geometry_{(old)}) \tag{A.1}$$

In this equation A is a constant. The updated geometry is shown by $geometry_{(new)}$ and $geometry_{(old)}$ is the previous geometry of the system. The algorithm optimise the geometry firstly in the opposite direction, to which the gradient increases. After finding the first minimum it performs another maximisation starting from the found minimum point.

B. Coordinate and topology Files

The molecular structure of any molecule used in a simulation should be defined in terms of coordinates. The coordinate files in gromacs can be in PDB format. The coordinate files of the drugs are taken from and converted to PDB format. Then the PDB molecules have been submitted in ATB website and the refined coordinate files are used for simulations. In these coordinate files the x,y and z component of an atom coordinate is specified in three columns.

In our simulations, the topology of each component is defined in a separate file with an extension of .itp. The .itp file defines the topology of one molecule of a type. Inside an .itp file all atoms in a molecule are named and the mass and charge of each single atom is provided. This file also includes all information about bonds as well as angles and dihedrals. In introduced work, all drugs force fields were built in ATB website.