A molecular dynamics study of NBD-cholesterol in phase separating ternary lipid mixtures.

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Summary The aim of this project is to investigate the partitioning of NBD-cholesterol in phase separating bilayers with the aid of molecular dynamics simulations using the Martini 3.0 force field. Lipid domains in membranes are likely to play an important role in drug delivery and the understanding of many cell processes. However, investigating lipid domains in vivo is challenging. Fluorescent probes such as NBD-cholesterol allow imaging at very high resolution, but it cannot be assumed that these probes behave exactly the same as the untagged lipid. Therefore it is not certain that NBD-cholesterol partitions preferentially into the liquid ordered phase, as cholesterol is known to do. For simulations with experimental data, a coarse grained model of NBD with a four carbon chain, NBD-C4, was developed and compared to an atomistic mapped model. The Martini 3.0 force field is currently being modified in order to represent aromatic molecules better. The first model of NBD-C4, model A, was not very flat and the carbon chain was flipped between above and below the aromatic plane of the molecule. The design was not without flaws since the 2-1 mapping for tiny beads was not observed, and after the introduction of more constraints the model became numerically unstable. Therefore a new model, model B was created. This model kept the aromatic rings of NBD-C4 flatter, and the angle and bond distributions compared well to the mapped atomistic reference. Based on solvent accessible surface area (SASA) analysis and PMF measurements using umbrella sampling, a version of model B was chosen for use in further simulations. This model was attached to cholesterol and subsequently placed in a ternary lipid mixture, which was known to phase separate at temperatures of 320 and 330 K. Visual analysis and measuring of the contact fractions indicated that NBD-cholesterol does not behave in the same way as cholesterol. In the studied system, cholesterol partitioned into the liquid ordered phase, whilst NBD-cholesterol showed a small preference for the liquid disordered phase. More analysis regarding the orientation and exact position of NBD-cholesterol would be beneficial to understanding how and why NBD-cholesterol prefers the liquid disordered phase and for designing better probes. The results found in this study suggest that NBD-cholesterol is not a good fluorescent probe to use for studied lipid domains. However, this must be viewed with some reservation, since there are ways in which the testing system was not ideal. For example, it has been shown that NBD-cholesterol is sensitive to the polarity of the environment, so it is possible that the system that was created does not resemble an experimental system enough.

Introduction

Importance of understanding membranes Membranes are fundamental components of cells. Their major function is to define a boundary between the inner and outer cell environments, which is essential for maintaining a steady state in the inner cell environment. [1] Furthermore, it has been shown recently by [2],[3] that biological membranes are involved in many processes, including energy production, protein synthesis and secretion, signal transduction and cell–cell interactions. The membrane can also play a role in drug delivery. For some diseases there are drugs available, but a challenge is to bring the drugs to the malfunctioning tissue through complex membrane structures, and this is especially a problem in diseases of the central nervous system. Currently knowledge of how drugs cross this barrier is limited. [4] A better understanding of the structure, components and mechanisms of cell membranes might help to treat many people.
Lipid rafts A view of the membrane composed of gel and liquid crystalline phases has been replaced by descriptions of the membrane as a complex system. This system is a combination of so-called phase separated liquid ordered (Lo) and disordered (Ld) domains. The liquid disordered domain is mainly composed of unsaturated lipids, whilst the liquid ordered domain is composed of saturated lipids and cholesterol. [5] These domains are represented in figure 1 showing a lipid raft. It is thought that lipid rafts play a significant role in regulating signal transduction, cellular transport, and lipid sorting [6–13]. Membrane proteins were shown to partition preferentially to one specific domain or to the interface between domains. These proteins can only perform their function correctly when in the appropriate environment [14]. Changes in membrane lateral organization have been identified in cases of Alzheimer disease [15]. Alterations in lipid domains have also been linked to the mechanism of action of general anaesthetics [16, 17].

Characterizing lipid domains Understanding lipid rafts in vivo will contribute a lot to biomedical sciences. Unfortunately lipid domains are very difficult to characterize because the domains are typically smaller than is visible at optical resolution. [5] Employing fluorescence microscopy or spectroscopy to study these lipid structures and their dynamics in vivo is very challenging. This is a result of the limitations of imaging techniques combined with the scarcity of suitable probes. [18] Over the past decades, optical microscopy has undergone dramatic improvements regarding sensitivity and resolution. With contrast agents, or fluorescent probes, optical microscopy currently allows imaging at a single molecule level at tens of nanometres resolution.

Since biological systems have insufficient intrinsic fluorescent species, this technique requires the introduction of fluorescent probes into the system. [19] In figure 2 a depiction of fluorescent sampling is shown. Often an artificial chromophore such as NBD, BODIPY, anthracene, pyrene or diphenylhexatriene is attached to one of the fatty acid side chains of a lipid. The majority of these probes were introduced before the relevance of lipid phase separation was acknowledged. As a result, despite the huge number of available probes, most probes do not provide a good contrast of the lipid rafts in both model and cell plasma membranes. [18] For example, it was shown recently using fluorescence quenching assays that attaching a fluorophore to a raft-associated lipid can alter the lipids’ ability to preferentially partition to a domain. [20–22] The structure of a raft probe should fit perfectly into the tightly packed Lo phase, or the raft probe should clearly prefer the Ld phase. This required selectivity makes the design of these probes very challenging. Furthermore, their partitioning often depends on the lipid composition of the phases, so that makes it difficult to predict a probes’ behaviour in live cells. [19]
NBD-cholesterol Because of the importance of cholesterol (Chol) in membrane structure and function, fluorescent sterols are an important class of membrane probes. [23] A cholesterol labeled with the nitrobenzoxadiazole (NBD) group, 22-(7-nitrobenz-2-oxa-1,3-diazol-4-yl-amino)-23,24-bisnor-5-colen-3β-ol (22-NBD-cholesterol) is used in membrane biophysics. The structure is shown in figure 3. [24] Some properties of NBD include a high fluorescence quantum yield, sensitivity to the polarity of the environment, and suitability for Forster resonance energy transfer (FRET) experiments. [1] However, although it is often assumed that labeled lipids show very similar behaviour to their untagged analogues, this is not always the case. It has been shown by Scheidt et al. and Xu et al. that a minor change in the structure of cholesterol can substantially decrease its partitioning into the Lo phase.[25, 26] Furthermore, several studies utilizing various experimental approaches have implied that 22-NBD-Chol does not mimic Chol correctly. [26-30] For example, it was shown with time-resolved fluorescence that NBD cholesterol preferentially partitions into the Ld phase, rather than the cholesterol rich Lo phase. [28] In Ld/Lo phase mixtures of giant unilamellar vesicles (GUVs), NBD-chol partitions preferentially into Ld phases. [31] One hypothesis is that because NBD is rather polar it prefers to sit at the membrane surface. Since cholesterol's hydroxyl functionality also sits by the membrane headgroups the result is a loop toward the membrane surface. This looping probably disturbs the proper cholesterol insertion into the Lo phase. This theory is supported by molecular dynamics studies of NBD-labeled cholesterol in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayers. [24] Another study suggested that domain perturbation can be influenced by certain properties. Aromatic compounds were shown to stabilize phase separation by partitioning into Ld domains and as such excluding cholesterol from the disordered domains into the Lo domains. [5]

Molecular dynamics simulations Simulations can give a detailed insight into processes that cannot be observed at experimental resolution. Much of our current knowledge about the physical world is generated by doing experiments. As these experiments have limitations for the time scale and size of observations, the extent of our knowledge is impaired. However, with increasing computational power and better algorithms it has become possible to do molecular simulations which can give insight into processes occurring at atomistic scale and femtosecond timescale. [32] As it is impossible to do measurements at the time scale of individual conformational changes, experimental methods will only reveal an average of conformations. A computer simulation might reveal structures that are too short-lived to observe experimentally. In this project, molecular dynamics simulations are used to investigate the influence of tagging cholesterol with the NBD chromophore on the partitioning behaviour in phase-separating lipid bilayers. The Martini 3 force field is used, which is currently under development to improve modelling of aromatic rings. First a coarse-grained model of NBD-C4 is made and tested against experimental partitioning data. We use NBD with a chain of four carbons attached to the amine for experimental verification because partitioning data is available for this molecule. Once a satisfactory model was made of this molecule, NBD was attached to a cholesterol model which was developed separately. The partitioning of cholesterol was then compared to the partitioning of nbd-cholesterol in ternary bilayers.
Theory

Simulations The software package GROMACS [33-41] was used for this project. GROMACS is able to perform molecular dynamics simulations and provides a set of analysis tools. It uses four different ingredients as input; the building blocks of the molecule, the energy of interactions, the motion of the building blocks and the system in which the building blocks preside, which include boundary conditions, temperature and pressure. This information is stored for the GROMACS software to read in .top, .itp, .gro and .mdp files, respectively.

The building blocks of the molecule can be defined with different amounts of detail. In this study we use the ‘coarse-grained’ (cg) MARTINI force field [42], which treats a group of approximately 4 heavy atoms, excluding hydrogens, as a normal bead. In the MARTINI 3 force field [43], which is currently being developed, tiny beads are introduced. The tiny beads map two heavy atoms to onemartini bead. These beads are introduced to improve the modelling of aromatic rings. The approximations made with MARTINI are made to reduce the computational power and memory necessary to perform simulations, which allows for much longer simulations.

An atomistic model of NBD-C4 is created to serve as a reference of angle and bond distributions, and solvent accessible surface area (SASA) analysis for the coarse grained (cg) model. For the atomistic simulations the GROMOS 54A7 force field is used. [44] All atoms are treated separately, which makes this model much more precise and thus suitable to serve as a reference. For comparison with the cg model, the atomistic trajectory is mapped onto the cg topology. This is described in more detail in the methods section.

The energy of the system depends on bonded interactions and non-bonded interactions. The bonded energies are described by harmonic potential, angle vibrations and torsional motion. Due to the different characteristics of the building blocks, the non-bonded interactions between these particles are not all equal in energy. The interactions are determined by Coulomb’s law for charged particles and the Lennard-Jones potential for neutral particles. The potentials are used to calculate the motion of the particles in the system. For this only Newtonian mechanics are used, which means that chemical bonding cannot be simulated. The assumption is made that the force is constant during every time step, so in the simulation the positions of all the particles change only after every time step.

A force field contains the parameter set to calculate the potential energy of the system. Energetic penalties are given for deviation of bonds and angles away from the reference defined in the force field. The trajectory of particles in a simulation is determined by using the equations of motion derived by Newton to find the forces on the particles. [45] The forces are the negative derivative of a potential function. The output is a list for all particles with coordinates vs time. This is called a trajectory. A force field ignores any electronic motions in the system. It takes only the nuclear positions of particles into account. Legitimacy of this simplification comes from the Born-Oppenheimer approximation: Motion of the nucleus and electrons can be separated. [46]

Theory umbrella sampling The potential of mean force (PMF) is the free energy that is associated with moving a molecule from one environment to another. In this study the PMF is calculated with the aid of umbrella sampling, a method that is explained in the partitioning techniques tutorial [47]. Umbrella sampling moves the molecule along an axis from a reference position to another position on the axis. This method is very useful for the situation when a molecule is not likely to visit a certain phase. In this study NBD-C4 is expected to preferentially sit in the membrane rather than the water phase. In order to determine the free energy related to the different environments umbrella sampling can be used to force
the molecule to visit the water phase. In the umbrella method the potential function of the system is adjusted to equally sample all states along an axis. First a set of initial configurations along the chosen axis is generated. For this system the axis that lies along the normal of the membrane is chosen. Subsequently, simulations are run at each initial configuration whilst the molecule is restrained with a harmonic potential to the centre of the window. Finally, the weighted histogram analysis method (WHAM)\[48\] is used to calculate the PMF. From the PMF we can obtain the difference in Gibbs free energy (\( \Delta G \)) associated with being in the membrane and being in the water phase. In the results and discussion section a description is given of the analysis. However, since this was not done in this study here we only describe how to get an estimate of \( \Delta G \). The difference between the energy associated with being in the water phase and the lowest energy in the system is an estimate of \( \Delta G \). From \( \Delta G \) the partition coefficient (Kp) can be computed. Kp is known experimentally for NBD-C4.

Contacts analysis After an initial visual inspection, the measure of phase separation of the ternary mixture is quantified by calculating the dilinoleyl-phosphatidylcholine (dipc) - dipalmitoyl-phosphatidylcholine (dppc) contact fraction \( c_{\text{dipc-dppc}} \), which is defined in equation 1: \[5\]

\[
\text{Fraction}_{\text{mix}} = \frac{c_{\text{dipc-dppc}}}{c_{\text{dipc-dppc}} + c_{\text{dipc-dipc}}} \quad \text{equation 1}
\]

This is the fraction of the dipc-dppc contacts divided by the total number of contacts of dipc with all lipids, excluding cholesterol. \( c \) is the number of contacts between the lipids in the subscript. For this only the PO4 beads, representing the head groups of the lipids, are taken into account. A cutoff of 1.1 nm was used, as done by Barnoud et al. \[5\] For ideal mixing the \( c_{\text{dipc-dppc}} \) is 0.61. This number equals the molar fraction of dppc with respect to all phospholipids. For a completely phase separated system the contact fraction will be 0.

To study the partitioning into Ld or Lo phase of cholesterol and nbd-cholesterol the contact fractions were calculated with respect to dipc as shown in equation 2. (x represents (nbd)chol)

\[
\text{Fraction}_{\text{mix}} = \frac{c_{\text{x-dppc}}}{c_{\text{x-dppc}} + c_{\text{x-dipc}}} \quad \text{equation 2}
\]

For the contacts between (nbd)chol and the lipids all the beads in the molecule were used. This was done because the cholesterol can flipflop. The distance cutoff was 0.7 nm. The contact fractions were analysed to find the lateral distribution of (nbd)chol. If the (nbd)chol does not show a preference then the contact fraction will be equal/close to the molar fraction 0.39 of dipc with respect to all phospholipids. A contact fraction lower than 0.39 indicates preference of (nbd)chol for partitioning to dppc, a higher contact fraction shows preference for dipc. All contact fractions were calculated separately for every frame, and then averaged.

Model building – design considerations The design for a coarse-grained model for NBD-C4 was made using the not yet published MARTINI 3 beads. The third version of MARTINI has seen the introduction of tiny beads, which are very suitable for building flat aromatic ring structures such as NBD. In the first model, model A, with two or three heavy atoms per bead (figure 4), we tried to maintain the symmetry of NBD. With this in mind the heterocyclic ring was cleft in two through the oxygen. Except the two connecting the 4 carbon chain to the rest of the molecule, all bonds were constrained to lengths obtained from the mapped
atomistic model. In an attempt to keep the ring structure flat improper dihedrals were added but then the system became numerically unstable. The solvent accessible surface area of this cg model was analysed using gmx sasa, an analysis tool provided by GROMACS. [33-41] But since this model was also not completely satisfactory (see results and discussion) a new design was required.

For the new design we looked at the structure of NBD again, and we decided to go for a better ratio of 2 to 1 mapping for the tiny beads. Additionally, the mapping of the beads representing the heterocyclic ring was changed from the centre of mass to the centre of geometry. This shifted the beads further apart, which was necessary because they were very close, 0.15 nm, in model A. To keep the molecule stiff and flat, many constraints and improper dihedrals were added. Unfortunately this made the model very unstable. To solve this bead 7 was made a virtual particle. After every time step the bead is placed in the centre of the five beads forming a ring. This modification, together with the introduction of improper dihedrals allows the model to be stiff and stable. The altered model, model B, is shown in figure 5. When analysing the solvent accessible surface area (SASA) it was clear that using a normal C1 bead for the four carbon chain did not reflect the size shown by the atomistic model. Therefore a model was also made with a smaller SC1 bead. The distribution of angles and dihedrals of the chosen coarse grained models were compared to the atomistic mapped model. By making adjustments iteratively the resemblance of the cg NBBD-C4 compared to the atomistic mapped model was increased.

**Calculation of solvent accessible surface area (SASA)** The SASA of a molecule is the area that is accessible to a solvent. In this study a sphere with a radius of 0.185 nm is used to represent the solvent. This sphere probes the surface of the molecule. 4800 dots were used to construct the surface. Using more dots gives higher accuracy. The whole trajectory of the atomistic and coarse grained simulations was used when calculating the SASA of the respective model. In this study the resulting SASAs were used to compare the various models of NBD that were created. The sasa of the atomistic model was used as a reference, a standard that we wanted to achieve with the coarse grained model.

*Figure 4: Model A of NBD-C4.*

*Figure 5: The improved cg model of NBD-C4.*
Materials and Methods

All simulation images were constructed with VMD [SOURCE].

Atomistic simulations NBD-C4 Atomistic simulations were performed with the GROMACS 2018 package [33-41] with force field parameters that are compatible with the GROMOS 54A7 force field. [44] A time step of 2 fs was used. A Berendsen thermostat was used. The reference temperature was 300K. No pressure coupling was applied. The reference pressure was 1 bar.

The atomistic trajectory was mapped onto the cg topology to provide a reference for comparing SASA and angle and bond distributions. Mapping was done using the program Backward. [49] Atoms in the atomistic model were assigned to cg beads according to the topology of the cg model.

Coarse grained simulations NBD-C4 Coarse-grained simulations were performed with the GROMACS 2018 package [SOURCE] with the MARTINI 3.0 force field [SOURCE??] For the parametrization simulations in vacuum and water a v-rescale [SOURCE] thermostat was used, and no pressure coupling. The reference temperature was set to 341K. A time step of 30 fs was used for the vacuum simulation. For the parametrization simulations run in water a time step of 20 fs was used. The reference pressure was 1 bar in all directions. The box shape was rectangular, and periodic boundary conditions were applied. Constraints that were defined in .itp files were constrained with the LINCS algorithm. [SOURCE] For the vacuum simulations a stochastic dynamics (sd) integrator was used. For the simulations in water a molecular dynamics (md) integrator was used.

All parametrization systems were minimized first with a short run with the steep integrator. Then equilibration was done with a short time step of 5 fs and v-rescale temperature coupling.

Angle and bond distributions were found with gmx angle and gmx distance [SOURCES] respectively. Gmx sasa was used to find the solvent accessible surface area.

Coarse grained simulations of ternary lipid mixture with NBD-cholesterol The simulations with NBD-cholesterol in a ternary lipid mixture were accomplished with GROMACS 2018 [SOURCE] and the Martini 3.0 force field [SOURCE]. The system consisted of dilinoleyl-phophatidylcholine (DIPC), dipalmitoyl-phosphatidylcholine (DPPC), and cholesterol (CHOL) in a 42:28:30 molar ratio. Four cholesterol molecules were replaced by NBD tagged cholesterol, using the topology that was designed in this project.

Potential of Mean Force (PMF) computation: This was done with umbrella sampling. A system was set up with 128 POPC molecules and 1995 water molecules. Energy minimization was performed for 100 steps with the steepest descent integrator. Then it was run for 20000 steps at 10 fs time step at a reference temperature of 300 K. V-rescale temperature coupling and Berendsen pressure coupling was used. Semisotropic coupling was used for all membrane systems. The pressure was kept the same in the plane of the membrane (0) whilst this was not kept the same as to the normal of the membrane (3e-4). The reference pressure was 1 bar. The NBD model was put at the chosen umbrella positions using the pull option in the mdp file. The force used was 1000 kJ mol⁻¹ nm⁻². The spacing between adjacent umbrella positions was 0.1 nm. The positions went from the center of the bilayer to the center of the water, passing the interface. The covered distance was 3.8 nm. This system was run at 300 K. Then energy minimization and equilibration was done at this position. Temperature and pressure settings were not changed. Pull
settings were kept the same. First steepest descent with 20 fs step and 1000 steps was done twice. Then equilibration with sd integrator with 10 fs time step for 10000 steps. Finally, a production run was done with sd integrator, time step of 20 fs for 250000 steps. V-rescale temperature coupling was used for reference temperature of 300 K. Berendsen pressure coupling was used. This was semiisotropic with same parameters as before. Pull force constant was still 1000 kJ mol\(^{-1}\) nm\(^{-2}\).

Results and discussion

**Parametrization results and discussion** For the molecular dynamics study of NBD-cholesterol, NBD and cholesterol were designed separately. The coarse grained model for cholesterol was made by Jonathan Barnoud. For NBD two coarse grained models were created which were both compared with an atomistic model of NBD. Firstly, the models were assessed regarding angle and bond distributions. The distributions discussed here are shown in figure 6. The first model, model A (figure 4) seemed promising, but some distributions did not quite overlap with the atomistic reference. An example is the distribution of angle 452, which is broader than the atomistic counterpart and does not peak at the same value. However, most distributions looked like bond 23 or angles 654, 564, and 523. These are not perfect, but seem reasonable for a coarse grained model. The beads of model A were kept together with constraints, so some distributions are very narrow. Bond 34 catches the attention because the length of this bond is only 0.12 nm. This length is very short for the Martini force field. Another distribution that does not look promising is dihedral 2345, which measures the flatness of the four beads resembling the aromatic rings of NBD.

![Figure 6: Angle and bond distributions of model A. In red the atomistic values, in black the coarse grained values. On the x axis (nm), y axis (probability)](image)

8
atomistic distribution shows a sharp peak between 0 and -20 degrees, whilst the cg distribution is a blur between -50 and 50. When it was tried to improve the flatness the model became numerically unstable.

In hindsight, the mapping was not according to the 2 atoms to 1 bead mapping that Martini 3.0 tiny beads are meant to represent. This inaccuracy, combined with the knowledge that bond 34 was very short and the impossibility of improving the flatness led to the design of model B (figure 7). For this model we adhered as much as possible to the 2 to 1 mapping. Since there are 13 heavy atoms (excluding the four carbons chain) it is not possible to have exactly 2 heavy atoms to 1 cg bead. The mapping of model B places the beads 4 and 5 that represent the heterocyclic ring further apart by setting the centre of the beads on the nitrogens instead of using the standard centre of mass assignment. At first this model was not numerically stable. A visual inspection with VMD (figure 8) indicated that the beads 4 and 5 were flying all over the place, not connected to the rest of the molecule. The relevant bond distribution shown in figure 9 confirmed this, since these bonds of model B were approximately 3 nm long, ten times longer than expected. Furthermore, beads 3 and 7 were out of the plane of the other beads, 1245, that represent the aromatic rings. Initial attempts to solve these problems resulted in more crashes. With the help of some tips given by Paulo Telles de Souza the system was able to run at 20 fs timestep without crashing. He suggested introducing a virtual bead to reduce some of the strain on the system. Bead 7 was made a virtual bead, which has no mass and is placed in the geometric middle of the five surrounding beads after every timestep. The model B is kept together with bonds with high force constants in contrast to model A, which has many constraints. The topology files can be viewed in appendix 1 and 2. Too many constraints can make the model numerically unstable, but on the other hand high force constants can result in very high frequency bond oscillations. The model B was then further assessed with angle and bond distributions that are shown in figure 10. The angles regarding the position of bead 7 did not match the atomistic distributions well. This mismatch can be explained by the fact that bead 7 is a virtual particle and that its position is determined by the five surrounding beads. This position does not match the position of the atoms mapped to bead 7 in the atomistic model.

Model B’s distribution of angle 326 does not match the atomistic angle distribution either. This was done on purpose since the carbon tail of the atomistic model appears to stand upright with a right angle to the
aromatic rings after a visual inspection. Because the mapping was done using the centre of mass, in the cg model B the bead representing the carbon chain, bead 6, needs a longer bond length and more acute angle to better resemble the atomistic model visually. Further on, analysis of the solvent accessible surface area (SASA) is described. This SASA analysis confirmed that the carbon tail stands in the upright position that was initially suspected. Based on this observation it was decided to keep the longer bond and acuter angle in the cg model B. However, it is not clear why the carbon tail stays on its preferred side of the molecule. Since the bonds connecting it to the rest of the molecule are single bonds there is no straight forward reason for the bond not to rotate freely. Therefore it would be beneficial to investigate whether the atomistic model is made to be this way using constraints or force constants, or whether it is a property of the NBD – C4 molecule.

The dihedral distributions of model B indicate that the beads representing the aromatic rings of NBD are kept flat in this model. The distributions lie around zero and 180 degrees, corresponding to the dihedral between overlapping planes and side by side planes respectively.

**Sasa results** To evaluate the different models of NBD-C4 the solvent accessible surface area (SASA) was analysed. The SASA of the coarse grained models were compared to the SASA of the atomistic model. The choice of normal, small or tiny beads in the coarse model results in different SASA, because these beads have different sizes. The sizes are defined in Martini 3.0 as 0.264, 0.225 and 0.185 nm, respectively. For model A for the carbon chain a small bead was used, and all other beads were tiny. The SASA of model A averaged over all conformations in the trajectory of 200 ns was 4.2 nm$^2$. This is smaller than the SASA of the atomistic model obtained from the conformations of the 2 ns long trajectory, which is 4.8 nm$^2$. A visual inspection allows us to compare where this size difference is caused (figure 11). From a side and top view it appears that the carbon tail sticks out of the plane of the aromatic rings more, resulting in a larger surface area. Furthermore, the bottom and front view show that model A’s aromatic rings take up less area in their plane than the atomistic equivalent. This could be because model A does not adhere to the 2-1 mapping

![Figure 10: Angle and bond distributions of model B. In red the atomistic values, in black the coarse grained values. On the x axis (nm), y axis (probability) (Image)](image-url)
recommended for the tiny beads. Instead, thirteen heavy atoms are mapped to five beads, averaging to 2.6 atoms mapped to one bead. In model B thirteen heavy atoms are mapped to six beads, resulting in 2.17 atoms per bead. Therefore model B might give a SASA closer to the atomistic value. The results show that this is the case (figure 11). Model B with a small SC1 bead used for the carbon chain has a SASA of 4.5 nm\(^2\). Model B with a normal sized C1 bead in this position gives a SASA of 4.8 nm\(^2\), which is the same as the atomistic value. Not only the overall area, but also the local size is relevant for comparing models. For example, the extent to which the carbon chain sticks up is reflected by the surface area in that region. When comparing the coarse grained models to the atomistic model it appears that using a SC1 bead for the carbon chain, as done in model A and one version of model B, results in greater similarity with the atomistic model. However, using the C1 bead, as done in one version of model B, the overall SASA is a better match. It was mentioned in the parametrization results section that it is unclear why the carbon chain sticks up in the atomistic model in this way. When investigating this it might be useful to analyse what the SASA looks like if all restraints regarding the orientation of the carbon chain are removed in the itp file.

Another topic of discussion regarding the SASA is the solvent that is used in the system. The coarse grained models were simulated in water, which is not the preferred environment according to experimental data. [50] This data indicates that NBD-C4 preferentially partitions into the membrane rather than into the water phase. It might be the case that the molecule takes a different shape in a bilayer so it might be beneficial to investigate whether a membrane environment results in a different SASA. The atomistic value of the SASA was calculated from a trajectory with no solvent. The absence of a solvent might also have some effect on the SASA. Furthermore, in this project the atomistic SASA is used as a reference for the coarse grained model. Therefore it is better if both SASA measurements are performed under the same conditions. Unfortunately, because there was only a limited time available for this project it was not possible to repeat the calculations with different conditions.

Figure 11: Images of the SASA, made with VMD. In red the atomistic areas, in blue the coarse grained areas. The most left set is the comparison of atomistic with model A, the middle and right set are the comparison of atomistic with model B with a SC1 or C1 bead, respectively, for the carbon chain.
Potential of mean force (PMF) The coarse grained models were further tested by comparing their partitioning with experimental data. This data was acquired by Cardoso et al. by measuring the concentration of NBD-C4 in the water phase and in large unilamellar vesicles (LUV) of 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipids. The concentration was measured using a version of the Bartlett phosphate assay. From the respective concentrations the partition coefficient was calculated, and thus the difference in Gibbs free energy associated with the partition from water to POPC bilayer was also known. The partition coefficient for NBD-C4 was found to be $1.1 \times 10^3 \pm 0.1$. This can be further computed to give a value of $-17 \text{kJ/mol}$ for the difference in Gibbs free energy.

The PMF of the coarse grained models was measured with umbrella sampling, as explained before. For model B several bead types were examined, as shown in figure 12, based on several considerations regarding the beads. A table with partitioning data of the Martini 3.0 beads (appendix 3) was shared with me by Paulo Telles de Souza. This table gave a good indication of which beads are more hydrophobic and hydrophilic. If a bead is more hydrophobic it will have a higher preference for the membrane over the water phase. For a hydrophilic bead this is reversed. The experimental partitioning data suggests that NBD-C4 prefers to partition into the membrane so mostly hydrophobic beads were required. The properties of the chemical groups of NBD-C4 were also not ignored. For example, the amine can donate a hydrogen, and the nitro group is quite polar, whilst the carbon chain is more hydrophobic. Based on these chemical properties bead types were selected for each bead and several combinations were measured. Only one set of bead types was tested for model A.

![Figure 12: (above) All bead types that were considered for model B.](image)

**Table 1:**

<table>
<thead>
<tr>
<th>model</th>
<th>ΔG (kJ)</th>
</tr>
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<tbody>
<tr>
<td>Grey</td>
<td>-21</td>
</tr>
<tr>
<td>Magenta</td>
<td>-16</td>
</tr>
<tr>
<td>Yellow</td>
<td>-12</td>
</tr>
<tr>
<td>Blue</td>
<td>-18</td>
</tr>
<tr>
<td>Green</td>
<td>-14</td>
</tr>
<tr>
<td>Red</td>
<td>-12</td>
</tr>
<tr>
<td>Black</td>
<td>-13</td>
</tr>
</tbody>
</table>

![Figure 13: PMF results. The black horizontal line shows the experimental ΔG value. In the legend, after the colour the bead types are displayed. They follow the numbering of the beads. The grey set is model A, all others model B.](image)
A graph showing all PMF data sets can be seen in figure 13. The data sets start at zero nm, which corresponds to the centre of the POPC bilayer. At approximately 1.6 nm the head groups of the POPC lipids are found and from approximately 2.2 nm onwards the water phase begins. A lower energy value indicates being in that environment is more favourable. It appears that all combinations and models have the same interaction with the water phase, whilst the other interactions are different. Model A shows the greatest affinity to the centre of the bilayer, and also to the membrane head groups. To make an initial judgement of the difference in Gibbs free energy ($\Delta G$) between the environments we can measure the difference between the energy associated with the water phase and the minimum of the graph, which lies at the polar head groups for all the data sets. A table with the values is shown in table 1. It appears that model A and the magenta and blue versions of model B lie closest to the experimental value of -17 kJ/mol. Further on a description is given of how these measurements can be compared in a more accurate way.

The models corresponding to the most interesting PMFs were examined more closely. Model A clearly prefers the membrane centre more than the versions of model B. When NBD is attached to cholesterol experimentally it is expected/hoped that the NBD will sit in the centre of the membrane, not interfering with the natural orientation of cholesterol in the membrane. However, from the experimental partitioning data is it unknown at what position in the membrane NBD-C4 prefers to stay. Furthermore, the preliminary $\Delta G$ value for model A is -21 kJ which is more than 2.5 kJ away from the experimental value. Within a 2.5 kJ range, values are seen to be acceptable. The magenta and blue versions of model B show much less preference for the centre of the membrane, whilst they have minimum values within the 2.5 kJ range. The difference between these two data sets is (primarily) in the beads representing the nitro group and the carbon chain. The magenta set has a TN1a bead for the nitro group, whilst this is a TN3a bead for the blue set. Both these beads have hydrogen acceptor properties. For the carbon chain a C1 and SC1 bead is used, respectively. The TN1a bead is more hydrophobic than the TN3a bead, which is compensated by the more hydrophilic SC1 bead used in the magenta set.

Although the carbon chain is not relevant since it is not a part of the NBD-cholesterol moiety when NBD is attached to cholesterol, a final model was chosen that best represents NBD-C4. Based on the angle and bond distributions, SASA, and PMF the blue set is chosen as the final model for NBD-C4. This is a version of model B which uses a SC1 bead for the carbon chain and best matches the atomistic SASA. The $\Delta G$ value of X kJ is within the accepted range, and the beads representing the aromatic rings are flat. It must be noted that there is a lot of weight attached to the SASA in this decision, since the magenta model is very similar, except for the SASA.

In the article by Hinner et al. [51] an excellent explanation is given of how to relate the experimental partitioning constant $K_p$ to the simulated PMF. A relative probability profile for the membrane-bound molecule and for the membrane-unbound molecule is made by integrating along the axis for the respective phases. The membrane-bound integral must be multiplied by two, since the membrane has two leaflets. To find the partition coefficient, divide the relative probability of the molecule being membrane-bound by the length of the membrane, and relate this to the relative membrane-unbound probability. From the partition coefficient it is possible to calculate the Gibbs free energy difference, as described earlier. Unfortunately, there was no time in this project to follow this procedure. Therefore, the results of the PMF analysis are indicative, but must be viewed with some scrutiny.

**NBD-cholesterol** The final chosen NBD-C4 model was attached to a recently developed Martini 3.0 cholesterol model, made by Jonathan Barnoud. As mentioned earlier, there is no four carbon chain in this model since NBD is connected directly to cholesterol. No initial testing was performed on this combined model. It might be beneficial to make an atomistic model of NBD-cholesterol and to compare angle and
bond distributions with the coarse grained model. Furthermore, some research has been done on the behaviour of NBD-cholesterol in POPC bilayers. [1] Investigating the behaviour of our NBD-chol model in that system with their model might give more weight to the performance of our model.

**Ternary mixture** For the investigation of the partitioning of NBD-chol into Lo or Ld phase, a ternary lipid mixture was used. This system was made by Jonathan Barnoud, with a mixture of Dipalmitoylphosphatidylcholine (dppc), dilinoleyl-phosphatidylcholine (dipc) and cholesterol (chol). The same molar ratio was used as in an earlier paper [5]. This ratio is 42:28:30 (dppc: dipc : chol) However, the earlier system used different Martini 2.0 beads, whilst for this project the molecules were defined using Martini 3.0 beads. This change created some problems which will be discussed here.

At temperatures below 320 K some phase separation occurred, but the Lo phase became very rigid. At temperatures of 320K and 330K Lo and Ld phase separation was achieved with some motion of the molecules in all phases. These temperatures are different from the 300 K that all other simulations in this project were performed at. The angle and bond distributions of the final cg model were measured at a temperature of 330 K. The results were exactly the same as at 300 K. This was not checked for the umbrella sampling measurements, because there was not time to do so. Therefore it is possible that the model for NBD that was designed is not optimal for use in this ternary system. A more quantitative analysis of the phase separation in the system was performed at different temperatures than 320 K and 330 K, which made it more difficult to compare the phase separation in the system with and without NBD-chol. Furthermore, there was no time for such a comparison in this project.

**Contact fraction** Four NBD-cholesterol molecules were inserted into the ternary mixture manually. They were placed in the membrane at the position of four cholesterol molecules that were replaced by NBD-chol. From a visual inspection (figure 14) it became clear that these four molecules were not in each other’s close proximity. Visually it was also clear that there was some phase separation, as shown in figure 15. However, because the two membrane leaflets are not in sync it is difficult to see the different domains. In the image with the white background the dipc lipids are shown. These lipids normally partition into the Ld phase. [5] The difference between the blue and red colour is the position on the axis to the normal of the plane. Therefore the blue and red lipids belong to the two different membrane leaflets.

To quantify the phase separation into ordered and disordered domains in the system, the number of contacts between dppc and dipc \( (\text{C}_{\text{dipcdppc}}) \) were counted. An average was made over the whole trajectory of 2000 ns. In this case a contact is counted when the distance between the beads representing the head groups of the phospholipids is less than 1.1 nm. This cutoff is also used in [5]. From the number of contacts
between the phospholipids a contact fraction was calculated as stated earlier. The fraction was calculated for every time step of the trajectory, and then an average was calculated from all fractions. The fraction $c_{dppo_dppc}$ was found to be 0.40 at 320 K, which is closer to ideal mixing (0.61) than to complete phase separation (0). At 330 K this value was 0.47, which indicates that there was more phase separation at 320 K. These values do show that there was some phase separation, which means that there was a possibility for (NBD-)cholesterol to show a preference for either Lo or Ld domains, or a preference for neither. This preference was found by counting the number of contact between the whole (nbd-)cholesterol and the whole phospholipids with a cutoff of 0.7 nm, as done in [5]. The contact fraction was then calculated as explained earlier. The numbers of contacts in all measurements did not change after 200 ns, which indicates that the system had equilibrated after that time. Displayed in table X are the contact fractions at 320 and 330 K. As the molar ratio of dppc with all phospholipids is 0.39, any contact fraction that is higher than 0.39 indicates that the relevant molecule prefers the liquid disordered phase. A value lower than 0.39 indicates a preference for the liquid ordered phase. At 320 K and 330 K, $c_{cho_{dipc}}$ was 0.2 and 0.24 respectively, showing a preference of cholesterol for the Lo phase, as expected. [28] $c_{NBDcho_{dipc}}$ was 0.5 for both temperatures, which indicates that NBD-cholesterol has a preference for the disordered phase. However, since the value is quite close to 0.39 it could also mean that NBD-chol makes similar number of contacts with dppc and dipc. Then NBD-chol positions itself at the interface between the phases, or it sits in both phases with a slight preference for the Ld phase. Visually it is not easy to see which scenario is the case. An analysis of the density landscapes might give a more quantitative image of where NBD-chol goes in the system. Once this is known it would be interesting to know why NBD-cholesterol partitions in the way it does. For example, based on simulations of NBD-chol in POPC bilayers that show looping [24] a theory might be that NBD-chol does not fit well in the structure of the Lo phase the way that cholesterol does. If it is shown that looping is an issue attaching the NBD to the hydroxyl group of the cholesterol might prevent the looping. In a recent study NBD was attached to the head group of phosphatidylethanolamines (PE). This change did not alter the preferred location, although it did result in a different orientation and H-bond interactions. [25] After some searching on google and smartcat no literature was found about attaching NBD to the hydroxyl group of cholesterol. It would also be interesting to measure the contact fraction of NBD which is connected to cholesterol with the phospholipids. This might give information on whether the NBD is pulling the molecule into a certain phase.

Experimental research has shown that NBD is sensitive to the polarity of the environment [1], and that altering the structure of cholesterol can decrease its partitioning into the Lo phase. [25,26] These investigations already point to the direction that NBD-cholesterol might not be an ideal fluorescent probe. Another study confirmed this by showing that NBD-cholesterol prefentially partitions into the Ld phase. [28] From experimental data it seems clear that NBD-chol does not partition in the same way as cholesterol, and this molecular dynamics study seems to confirm that. Therefore it appears that NBD is not a good fluorescent marker to indicate Lo domains in membranes. This does not mean that NBD is useless, for example if it turns out that NBD-chol prefers the interface between Lo and Ld, the molecule could be used as a marker to show the interface between domains. Another point to be brought to the attention is that there are only four NBD-chol molecules added to the system. Therefore it is not certain that the values

Figure 15: Dipc lipids shown. The nearer leaflet is blue, the further
found correspond to NBD-cholesterol used experimentally. It would be interesting to study how NBD-cholesterol behaves at higher concentrations. For example, are agglomerations formed?

**Conclusion** Experimental research indicated that NBD-cholesterol does not partition as cholesterol. In this project we wanted to find a model that would correctly represent NBD-cholesterol, preserving important features of the molecule. For example, the structure was modelled based on atomistic simulations, whilst the interaction with the membrane and water environment was checked with umbrella sampling. Then, the model for NBD-cholesterol was placed in a ternary mixture with dppc, dipc and chol. From the results obtained in that study it appears that NBD-cholesterol does not partition in the same way as cholesterol in a ternary lipid mixture. However, this statement must be regarded with some scepticism. Firstly, the NBD-cholesterol has not been compared with an atomistic model, or tested against experimental partitioning data. NBD-C4 was tested, but there were some shortcomings. The orientation of the carbon chain is relevant because the cholesterol is attached to NBD in the same way. This orientation was modelled after the atomistic mapped model, but there is always some uncertainty as to the correctness of these models. Furthermore, the PMF was not fully analysed because there was not enough time in the project. Therefore the result is only an estimate. The ternary mixture that was used to find the partitioning of (NBD-)cholesterol was not optimized and the results of this project cannot be compared to other phase separation quantifications done for the system. However, cholesterol was found to partition into the liquid ordered phase, as expected from earlier research. And since NBD-cholesterol behaved differently under precisely the same conditions, this does strongly suggest that cholesterol and NBD-chol have different interactions with the membrane. It would be beneficial to measure more features, such as the contact fraction of NBD, with the lipids in the system, and the density landscape of the mixture. These results might give insight into how and why NBD-cholesterol behaves in the way it does. When discussing this project we must mention the relevance for experimental research. This research was trying to establish whether NBD-cholesterol behaves like cholesterol, and if it can justifiably be used as a fluorescent probe. Earlier experimental studies have suggested that NBD-cholesterol does not behave as cholesterol. Molecular dynamics simulations can provide a tool as to how and why this is so. This research study has produced a coarse grained model that shows good correspondence to earlier experimental data. However, it was not possible within the time scale to study the behaviour of the model any further. After testing the model for the shortcomings mentioned just now, it will be interesting to see what new information the model can provide.

**Literature**


Appendix

Appendix 1: topology of model A

moleculetype ]
molname    nrexcl
NBD4       1

atoms ]
type      resnr      residue      atom      cgnr      charge      mass
1 TC4      1            NBD4      NO2      1            0.0
2 TC4      1            NBD4      R1       2            0.0
3 TC4      1            NBD4      R2       3            0.0
4 TC4      1            NBD4      R3       4            0.0
5 TN3d     1            NBD4      R4       5            0.0
6 SC1      1            NBD4      C4       6            0.0

bonds ]
assuming 100 40 40 degree triangles
i  j  funct  length  force
4  6  1     0.460    5000
5  6  1     0.340    5000

angles ]
i  j  k  funct  angle  force
1  2  3  2     65.0    80.0
4  6  5  2     48.0    40.0
3  4  5  2     127.0   100.0
5  2  1  2     130.0   100.0
6  5  4  2     90.0     70.0
4  3  1  2     139.0   100.0
4  3  2  2     85.0     100.0
5  2  3  2     70.0     70.0
6  5  2  2     100.0    60.0
1  2  3  2     60.0     60.0
4  5  2  2     70.0    100.0
5  6  4  2     40.0    80.0

constraints ]
i  j  funct  length
1  2  1   0.323
2  3  1   0.366
3  4  1   0.120
1  3  1   0.353
2  5  1   0.300
4  5  1   0.302

[ bonds ]
i  j  funct  length  force
1  3  1   0.363   100000
2  3  1   0.293   80000
2  5  1   0.287   80000
5  4  1   0.220   100000
4  1  1   0.329   80000
2  6  1   0.400   100000

[ angles ]
i  j  k  funct  angle  force
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5  4  1  1   130      100.0
4  1  3  1   77      70.0
1  3  2  1   120      70.0
3  2  5  1   96      90.0
6  2  7  1   90     200.0
1  7  4  1   70     130.0
7  4  5  1   57      90.0
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7  5  4  1   56      90.0
1  3  7  1   63      70.0
1  7  3  1   76     150.0
3  1  7  1   41      100.0
2  3  7  1   57    120.0
2  7  3  1   70     70.0
3  2  7  1   53      70.0
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5  2  6  1   90     150.0

[ exclusions ]
i  j  k
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2  3  2 4 5 2 6 2 7
3  4  3 5 3 6 3 7
4  5  4 6 4 7
5  6  5 7
6  7

[dihedrals ]
i  j  k  l  funct  angle  force
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4  1  5  3  2   180.0   50.0
4  2  3  1  2   0.0    50.0
4  2  3  1  2   0.0    50.0
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[ virtual_sites ]
site  funct  from
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Appendix 2: topology of model B

```
;;Martini topology for 22-NBD-C4 cholesterol

Description:

\[ N^2 --- NO_a \]
\[ R^1 \cdot \cdot \cdot R^2 \]
\[ NH ---- NO_b \]
\[ C4 \]

[ moleculetype ]
molname  nrexcl
NBD4  1

[ atoms ]
type  resnr  residue  atom  cnr  charge  mass
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2  TN3d  1  NBD4  NH  2  0.0  36
3  TC4  1  NBD4  R1  3  0.0  36
4  TC6  1  NBD4  NOa  4  0.0  36
5  TC6  1  NBD4  NOb  5  0.0  36
6  C1  1  NBD4  C4  6  0.0  72
7  TC4  1  NBD4  R2  7  0.0  0

[bonds]
t  j  funct  length  force.c.
1  3  1  0.363  100000
2  3  1  0.293  80000
2  5  1  0.287  80000
5  4  1  0.220  100000
4  1  1  0.329  80000
2  6  1  0.400  100000

[angles]
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5  7  4  1  60  70.0
7  5  4  1  56  90.0
1  3  7  1  63  70.0
1  7  3  1  76  150.0
3  1  7  1  41  100.0
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2  7  3  1  70  70.0
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## Appendix 3: properties of Martini 3.0 beads

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