Kempczynski and **Róg** *Biochimica et Biophysica Acta-Biomembranes* **2016,** *1858* (10), 2362-2379.
http://dx.doi.org/10.1016/j.bbamem.2016.02.038

Functionalized lipids and surfactants for specific applications

Mariusz Kepczynski[a] and Tomasz Róg[b,c]

[a] Faculty of Chemistry, Jagiellonian University, Ingardena 3, 30-060 Kraków, Poland

[b] Department of Physics, Tampere University of Technology, P.O. Box 692, FI-33101 Tampere, Finland;

[c] Department of Physics, Helsinki University, P.O. Box 64, FI-00014 Helsinki, Finland;

[*]**Please forward correspondence to kepczyns@chemia.uj.edu.pl, tomasz.rog@tut.fi;**

Abstract

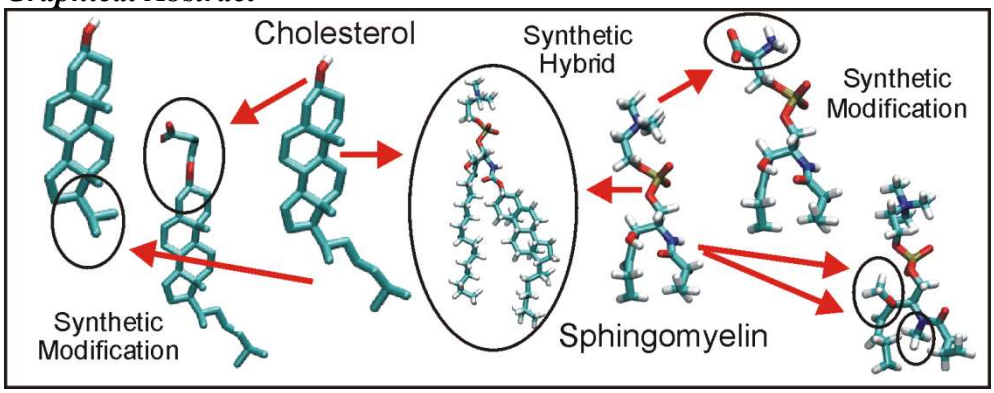
Synthetic lipids and surfactants that do not exist in biological systems have been used for the last few decades in both basic and applied science. The most significant applications of synthetic lipids and surfactants are drug delivery, gene transfection, reporting molecules, and as support for the structural biology of lipids. In this review, we describe the potential of the synergistic combination of computational and experimental methodologies to study the behavior of synthetic lipids and surfactants embedded in lipid membranes and liposomes. We focused on selected cases in which molecular dynamics simulations were used to complement experimental studies with an atomistic-level understanding of the structure and properties of new compounds. We also describe cases where molecular dynamics simulations were used to design new synthetic lipids and surfactants, as well as emerging fields for applications of these compounds.

Key words: synthetic lipids, molecular dynamics simulations, cholesterol, sphingomyelin, drug delivery, reporting molecules.

Highlights:

- Applications of synthetic lipids in applied and pure sciences are reviewed
- Drug delivery and gen transfection are large field of synthetic lipids applications
- Functionalized lipids are used as reporting molecules
- Synthetic lipids help to understand role of lipids structure
- MD simulations as a method of synthetic lipids studies and design is discussed

Graphical Abstract



1. Introduction

According to the definition taken from the biochemistry handbook, "Lipids are biological molecules insoluble in water and well soluble in organic solvents such as chloroform" [1]. Thus, the term "lipids" refers to naturally occurring compounds, such as triglycerides, phospholipids, sterols, and others. Thus, lipids are a large and diverse class of molecules. Recent studies of lipidomics have revealed the existence of thousands of lipid species. Lipids' profiles differ between organisms, cells types, cellular organelles, and among healthy and pathological cases [2]. For instance, exosomes were shown to be enriched in long tails of sphingomyelin (SPM) and phosphatidylserine in comparison to the cell from which they were derived [3]. Next, adipocytes from obese patients were shown to be enriched in, for example, ethanolamine plasmalogens that contain arachidonic acid in comparison to the patients' non-obese identical twins [4]. Taking into account the large number and diversity of lipids, one might ask why the addition of synthetic lipids is necessary. Clearly, however, synthetic lipids have countless applications in both applied and basic sciences.

The largest field of synthetic lipids applications is pharmacology. Lipids analogs might be directly used as drugs; for instance, lipase inhibitors or analogs of bacterial lipid A might be used to stimulate the immunological system. Currently, one of the most important research areas in pharmacology is drug delivery. Various lipid assemblies, such as liposomes, micelles, bicelles, nanodiscs, etc., are used as carriers for drug molecules. Properties of these carriers

have to pass rather high technical requirements such as having an optimal lifetime, a low permeability for drugs, the ability to prolong storage, etc. Not surprisingly, numerous designed lipids have been synthetized and tested for this purpose (for a recent review, see [5]). Likely, the most significant type of synthetic lipids is lipids functionalized with poly-(ethylene glycol) (PEG, PEGylated lipids). Molecular dynamics simulations have been extensively used to characterize the physicochemical properties of these lipids (e.g., [6]]).

Non-viral gene transfection, a method that carries significant scientific, medical, and technological importance, largely relies on synthetic lipids [7]. Synthetic lipids are used to form aggregates with DNA called *genosome* or, more commonly, *lipoplexes*. Since nucleic acids carry a large negative charge, lipids need to be positively charged. Cationic lipids do not exist in nature, so only synthetic lipids can be used for this purpose.

Basic research is another large field of synthetic lipids applications. Most straightforward and commonly used synthetic lipids are lipid-based reporting molecules. Specifically deuterated phospholipids are used in NMR studies of lipids to measure the order and dynamics of specific acyl tails segments [8]. Spin-labeled lipids are used in EPR measurements to describe a bilayer structure and dynamics [9], but also oxygen transport thorough membranes [10] and bilayer hydrophobic profiles [11]. Lipids with fluorescent labels are used in fluorescent spectroscopy to determine both structural and dynamics parameters and in microscopy to visualize various lipids compartments [12]. Finally, clickable lipids (having reactive groups that form covalent bonds with their nearest neighbors as a result of external stimuli such as light) are used in recognition of the lipid nearest neighbors [13]. Attachment of labels or clickable groups is expected to affect the properties of native molecules. Molecular dynamics (MD) simulations might be used to evaluate such effects and a large number of studies have provided insight into, for example, labels' effects on the native molecule location, orientation and structure providing background for the interpretation of experimental results (e.g., [14]). In the

case of spin labels, simulations might be used to calculate the EPR spectrum of the molecule providing direct validation of simulation methodologies [15].

An understanding of the importance of lipids' structure and the role of their various functional groups might be achieved via a comparison of naturally occurring lipids. Existing lipid diversity provides a large number of lipid variants allowing for such comparison but in some cases synthetic lipids are the only possible analogs. For example, the role of chirality can be studied only with synthetic lipids because, in the majority of cases, natural lipids have only one stereoisomer (e.g., glycerol moiety in all glycerol-based lipids adopts the *R* configuration of phosphatidylglycerol and sphingolipids always have a D-erythro enantiomeric configuration - 2S, 3R). Modifications of lipids' functional groups might be used for studies of the bulk properties of the lipid bilayers or to better understand lipids' interactions with proteins in macromolecular assemblies [16]. Modifications of functional groups in a lipid molecule could be seen as corresponding to site-directed mutagenesis, one of major tools in structural biology [17]. Synthetic surfactants also have applications in membrane proteins' extractions, biochemical characterization and crystallization. For example, a cholesterol (Chol) analog—cholesteryl succinate—is commonly used in G-coupled protein receptor studies [18]. Another example is linolein, a surfactant used in protein crystallization [19].

MD simulation is a method that provides information at the atomistic level in a time scale of picoseconds to milliseconds (for a more extensive review of MD, see [20]). In recent years, the quality of lipid models has greatly improved, due to the development of force-field parameters specifically for lipid molecules [21-25]. They have been shown to reproduce experimental data with better accuracy [26]. MD simulations seem to be particularly useful methods to study the properties of synthetic lipids; they are an inexpensive method and provide an understanding of a molecular structure at the atomistic level that is useful in the further design of lipid species with desired properties. In the past, MD simulations have been shown to have the ability to

correctly predict the effects of lipid modification [27]. In this article, we show examples of synthetic lipids and surfactants in all of the above-described applications. In particular, we discuss studies in which MD simulations have already been applied. We will concentrate on cases in which MD simulations are suitable methods to provide a background for the future development of synthetic lipids and their applications.

2. Pharmacological Applications

2.1. Drug Delivery

Drug delivery is challenging nanotechnology in which synthetic lipids are used intensively. Kohli et al. [5] pointed to three key steps in drug delivery that must be optimized and synthetic lipids might be a choice for such a task. The first step after the injection of liposomes into a blood vessel is its circulation. Liposomes composed of naturally occurring lipids are quickly removed from circulation by the immune system and their half-life in the bloodstream is about 1 hour. An extended circulation time increases the accumulation of the drug in tumors and is, thus, highly desired. As liposomes have to deliver their cargo, their stability and low permeability are important. The second step is the targeting of liposomes to specific cell types and the final step is the release of the drug, preferably in a controlled way. MD simulations are applicable to study all of these steps and are able to provide novel insight into the structure and properties of delivery systems.

The most commonly used method to extend liposomes' circulation time is the use of so-called stealth liposomes. Stealth liposomes are shielded from the immunological system by a layer of hydrophilic polymer that is covalently attached to lipids' headgroups. The most commonly used polymer is PEG attached to a phosphatidylethanolamine headgroup. PEGylated liposomes are extensively studied (for review, see [28]). MD dynamics provided a few novel observations. First of all, the PEG corona was shown to bind Na^+ cations; thus, PEGylated

liposomes carry a small positive charge [29]. The binding of K^+ to PEG was much weaker and the Ca^{2+} cations did not interact with PEG [30]. PEG chains were observed to penetrate the membrane hydrocarbon core for the case of a membrane in a liquid state, while it did not penetrate the membrane in the gel state [29].

Another interesting result obtained in MD simulation concern the location of a hydrophobic drug—porphyrin in this case—in the PEGylated bilayer. Simulations showed that porphyrin has two possible locations, one in the bilayer hydrocarbon core below carbonyl oxygens and the second in the PEG layer (see Fig. 1) [31]. This result was validated by a quenching experiment that showed the existence of two subpopulations of porphyrin—accessible and not accessible for the quencher. This observation is also in agreement with previous studies that show a higher binding constant of porphyrin to PEGylated liposomes than to a conventional one [32, 33]. Altogether, these results indicate that PEGylation increases the drug load efficiency of liposomes.

Chol is a key molecule regulating almost all properties of lipid bilayers (for review, see [34, 35]). From the point of view of drug delivery, the most important Chol effect on the structure of a lipid bilayer is the increase of the lipids' tail order, which results in the increased stability and lower permeability of liposomes. For this reason, Chol is a common component of drug delivery liposomes [36]. Chol, however, is a relatively mobile lipid [37, 38] and can be quickly exchanged between liposomes and lipoproteins or cells; thus, the stability of the delivery vesicle might decrease in the blood stream and the circulation time might be shortened. This unwanted effect might be overcome by using phosphatidylcholines (PCs) that are designed with one or two tails substituted by Chol molecules (Chol-PC lipids, see Fig. 2) [39]. Liposomes formed from these lipids were shown to be stable and characterized by permeability similar to liposomes formed with Chol [40]. A comparison of the monolayers formed from Chol-PC lipids

and a dipalmitoylphosphatidylcholine (DPPC) and Chol mixture showed that the area per DPPC-Chol unit is about 10 $Å^2$ smaller [41].

Chol in higher concentrations, together with phospholipids, forms the so-called liquid-ordered phase (Lo). The order of the tails in the Lo phase is similar to the order of the tails in the gel phase. MD simulations showed that PEG chains do not enter the hydrocarbon core of ordered bilayers in the gel state [29], but MD simulations of PEGylated bilayers with Chol showed, however, that PEG chains are able to enter the bilayer core [42]. Interestingly, PEG chains in each case entered the bilayer next to a Chol molecule. The penetration of the PEG chains into the bilayer core resulted in an increase of the surface area per lipid molecule; a bilayer with a 50 mol% area increased 6 $Å^2$ and the order of the hydrocarbon tails decreased. Experimental studies showed that Chol might be released from the lipid bilayer by PEGylated lipids [43]. MD simulations seem to agree with this result, as the observed behavior resembles the first step of Chol migration toward the PEG corona.

Targeting of drug delivery devices is another important problem in the entire delivery process [44]. The targeting of liposomes to selected cells may be achieved by the attachment of ligand-like carbohydrates, peptides or whole proteins. MD simulations have been used to complement experimental attempt of constructing a stealth delivery vesicle with an activated endothelium targeting peptide (AETP) that targets the vascular endothelium [45]. Although the AETP itself activated the endothelium, the constructed delivery system did not show improved targeting. MD simulations of an analogical system with an AETP and, additionally, with more hydrophilic RGD peptide showed that both peptides are covered by PEG chain and thus are not exposed and not available for receptors in the endothelium. Not surprisingly, more hydrophobic AETP was covered to the larger extent. These studies suggest that the use of more hydrophilic polymer instead of PEG might solve this problem.

The final step of drug delivery is the release of the carrier payload, preferably via a controlled mechanism. Triggering may be achieved via hit, light, ultrasound, pH, redox potential or enzymatic reaction (for more extensive reviews, see [46, 47]). Although triggered liposomes have not yet been successful in clinical tests, they are still promising and actively developed. An example of a triggered system studied both experimentally and in silico is liposomes doped with synthetic porphyrin-phosphatidylcholines [48]. These liposomes were shown to be triggered by near infrared light via a calcein-release test and their efficacy in mouse tumors was indicated. MD simulations showed that bilayers formed with 10 mol% of these lipids were stable and not significantly perturbed by the porphyrin. The porphyrin ring was shown to be located below the water membrane interface similar to porphyrins that were not functionalized with lipids [31, 49].

The optimization of the delivery vesicle might even go beyond the triggered release. In recent studies, van Hell et al. [50] designed and constructed liposomes doped with short-chain sphingolipid analogs *N*-hexanoyl-SPM and *N*-octanoyl-glucosylceramide and demonstrated that a new delivery system increased the cellular accumulation of the drug. The mechanism responsible for this phenomenon was explained by MD simulations, which demonstrated that doxorubicidin, the drug molecules used in these studies, together with short-tail lipid analogs formed transmembrane pores promoting the translocation of the drug thorough the membrane (see Fig. 3). Doxorubicidin translocation occurs in the μs time scale [51], while in a newly constructed system this time is reduced to the ns time scale. Tests performed on multidrug-resistant mammary tumor cells showed superior efficacy of the liposomes with new lipids analogs in comparison to traditional liposomes or a doxorubicidine solution. The proposed mechanism of antitumor activity can be split into 3 parts: (1) in physiological conditions, liposomes are known to accumulate in the tumors [52], increasing local doxorubicidin concentration which (2) further effectively translocated into tumor cells, due to the formation

of transient pores and lipids analogs. Finally (3), short-tail lipid analogs might be translocated to the other cell membrane by ABC-type drug efflux transporters [53] from where they are available for doxorubicidin to form a transient pore and flip back into the inner membrane. This might be seen as a recycling mechanism further enhancing the cellular uptake of doxorubicidine.

Transdermal drug delivery also might be improved by the application of synthetic lipids and might be studied with MD simulations. A few MD simulations of lipid bilayers with a composition similar to *Stratum Corneum,* the most external layer of the skin, showed a very rigid structure (see Fig. 4) [54-56] and high penetration barriers for small drug-like molecules [57, 58]. Synthetic lipids turned out to be useful in overcoming this barrier [58]. A ceramide analog with a 15-carbon sphingosine chain and 4- to 6-carbon acyl chain were able to increase the skin permeability up to 79 times.

2.2. Lipids as a pharmaceutical drugs

Lipids are involved in numerous cellular processes, so it is not surprising that they are both targets and potential drugs. In particular, lipids and synthetic lipid analogs have been extensively studied in the context of their anti-cancer properties [59]. In this context, analogs of fatty acids with trans-unsaturated double bonds were shown to have anti-cancer properties by activating the mitochondrial patchway of apoptosis [60]. Another example is ether glycolipids [61]. Moreover, it was proposed that targeting the sphingolipid metabolism might be useful in cancer therapy [62]. Unfortunately, the molecular mechanism of anti-cancer activity is not known for these lipids.

Lipid A is the main component of the outer-cell membrane of gram-negative bacteria since it is the anchor of lipopolysaccharide. Due to its importance for bacterial cells, Lipid A's biosynthetic pathway is a target for novel drugs (for extensive review, see [63]). Lipid A is considered as an endotoxin due to its ability to induce inflammation in picomolar concentration.

The activation of the immunological system is triggered via the tool-like receptor 4 (TLR4) which, with co-receptor myeloid differentiation factor and lipopolysaccharide, form dimers [64]. Lipid A has no therapeutic value due to its toxicity; however, its synthetic analogs are potential agonists or antagonists of TLR4. The synthetic analog of lipid A E5564 (eritoran) was tested in gram-negative septicemia, but it was not found to be effective [65]. However, E5564 was shown to effectively protect a mouse from a lethal influenza infection [66].

A lipid-drug conjugate is another application of synthetic lipids. For example, CMX001 is a conjugate of the acyclic nucleotide analog cidofovir (CDV) that is covalently bonded with a lipid which has already been tested in humans [67]. CDV is an antiviral drug used against dsDNA viruses [68]. The lipid conjugate CMX001 has clear advantages over CDV. CMX001 can be provided orally, as it is absorbed in the small intestine, while CDV must be administered by intravenous infusion. A high level of CDV in plasma is associated with nephrotoxicity, while due to the cellular uptake of CMX001, the CDV level in plasma is low. This leads to a higher concentration of the CDV in the targeted cells [69].

Intravenous lipids emulsions (ILEs) used for the treatment of drugs' toxic effects are another interesting direct application of lipids as medicament [70, 71]. ILEs are used as antidotes for drug overdoses or strong adverse reactions to medications. ILEs were tested for a large set of drugs, which includes local anesthetics, antiepileptic drugs, cardiovascular drugs, psychotropic medications, calcium channel blockers and β-blockers. One of the proposed mechanisms of ILEs' therapeutic properties is the so-called sink hypothesis, which assumes that lipophilic drugs would be adsorbed by ILE droplets. Few studies have confirmed this hypothesis [72-74]; however, it is not necessarily the only mechanism of ILE action. ILE is typically prepared from egg phospholipids, soybean oil, fish oil, or olive oil and little is known about the importance of specific ILE composition. Taking into account the specificity of the chemical structure of possible drugs to which an overdose might be treated by ILE, one would expect that that the

optimization of ILEs' composition might be of high relevance. For instance, it was shown that the synthetic liposomes composed of a mixture of PC and PG interacted stronger with amiodarone, ketamine and amitriptyline than with commercially available emulsions [75]. Additionally, the application of synthetic lipids, specifically designed for selected drugs, might reduce ILEs' adverse effects and their dose.

3. Gen transfection

3.1. Cationic lipids

Physiological lipids are usually negatively charged, neutral, or zwitterionic. Apart from a few exceptions, such as the intermediate species sphingosine and the rare cationic glycosphingolipids identified in the brain, positively charged lipids are not known to occur in biological systems [76]. However, due to potential applications, there is increasing interest in obtaining cationic lipoid compounds that are able to form a bilayer. Two methods are used to introduce permanent positive charges into lipid membranes: (i) chemical modifications of the natural lipids or (ii) the use of synthetic double-chained cationic surfactants. Both of these groups of compounds are known in the literature as "cationic lipids" and self-organize in the aqueous environments with the formation of vesicular structures known as cationic liposomes. The cationic liposomes have found a variety of applications in biotechnology or medicine. For example, cationic liposome-based vectors for gene delivery are one of the most promising alternatives to viral vectors [77]. Cationic lipids interact electrostatically with nucleic acids condensing them and forming so-called lipoplexes [78]. Their cationic nature facilitates interaction with the plasma membrane and they are, therefore, reminiscent of the cell-penetrating peptides that are typically strongly positively charged peptides and have been suggested as cellular delivery agents. In many (but not all) cases, the inclusion of "helper" lipids

in the cationic liposomes improves lipoplex efficiency [79]. Such lipids are usually phosphoethanolamines (PEs) or phosphocholines (PCs).

In this section we present the progress and recent advances that have been made in the area of the characteristics of cationic lipids, mainly during the last decade. We focused mainly on the most frequently studied cationic lipids. Special attention has been paid to their ability to form bilayers alone and the presence neutral or zwitterionic lipids that are used as the helper lipid.

3.2 Modified Natural Lipids

Zwitterionic PCs can be readily converted into cationic derivatives by the esterification of their phosphate groups. This modification deprives the lipid molecule of the negative charge located on the phosphate, leaving the positively charged choline unchanged. Fig. 5 shows 1,2-dioleoyl -*sn*-glycero-3-ethylphosphocholine (DOEPC) as an example of such alkylated phospholipids. This ethylphosphocholine has been used to obtained cationic liposomes [80]. The positively charged lipid vesicles were prepared from a mixture of a cationic lipid, DOEPC, and a helper lipid, the 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), which is the most frequently used in transfection. The DOEPC/DOPE liposomes were mainly spherical with a diameter of around 100 nm and a lipid bilayer with a thickness of approximately 4.4 nm, showing an appreciable percentage of multilamellar structures. Microscopic observations revealed that the mixed liposomes may present a variety of looped, twisted and invaginated structures, indicating a relatively high elasticity of the membrane. These vesicular structures were used as colloidal vectors to condense and compact genetic material. The results showed that the compaction of DNA by the cationic liposomes was mostly driven by the strong electrostatic interaction among the positively charged surfaces of the colloidal aggregates and the negatively charged DNA.

3.3 Double-Chained Cationic Surfactants

Dioctadecyldimethylammonium bromide (DODAB) (Fig. 5) is a synthetic, double-chained quaternary ammonium surfactant that assembles spontaneously in aqueous environments into bilayer structures [81]. However, the morphology of these structures, formed at room temperature, strongly depends on the method of preparation. Upon sonication, mostly bilayer fragments are formed [82], while the extrusion process results in the formation of nonspherical, faceted vesicle structures [83]. The DODAB vesicles have found widespread use both in fundamental studies on interfacial phenomena and in practical applications as DNA carrier systems for gene transfection and as vehicles for drug delivery [84]. MD simulations have been performed to obtain information on the molecular organization of the DODAB bilayer [84]. The results showed that the surfactant membrane arranges spontaneously into the rippled phase at 25 °C, where two distinct domains were present; the "zipped" domain, where the long alkane chains of the upper and lower monolayers are interdigitated with a thickness of 2.4 nm, and an "unzipped" domain, where the monolayers were separated by a distance with a thickness of 4.0 nm. The ordering within the chain fragment closest to the headgroup was relatively low and it increased significantly for the carbon atoms located in the center of the membrane. The calculated average density of the hydrated DODAB bilayer equaled 0.99 g cm^{-3}, which agreed well with the value determined experimentally (ca. 0.98 g cm^{-3}) [85]. Additionally, the calculated area per DODAB molecule of 0.58 nm^2 was in very good agreement with the experimental result of 0.56 mn^2, determined at 20 °C using Langmuir monolayer measurements [82]. The molecular organization of DODAB bilayer revealed in the MD simulations was used to explained anomalous transitions of DODAB using fast-scanning liquid calorimetry [86].

The structure and spectroscopic properties of water associated with the DODAX (X = F, Cl, Br, I) membranes as a function of the counterion have been studied using experimental methods and MD simulations [87]. The attenuated total reflection, Fourier-transformed infrared (ATR

FTIR) measurements of hydrated-oriented lipid multilayers of DODAX showed that the hydration of the surfactant headgroups was strongly altered by the halide anion exchange. At low hydration, the measurements demonstrated different hydrogen-bonded populations of water within the membrane environment. The variation of halide counterions leads to different hydrogen bonding strengths, changes of water stretch absorptions and corresponding molecular orientations. The MD simulations of the DODAB bilayer confirmed the existence of differently hydrogen-bonded and oriented water within the DODAB multilayers. Relatively slow rotation and confined diffusion were found for the surfactant-associated water molecules. Moreover, the MD simulations results were used for an assignment of water stretch band components to structures.

Gemini surfactants belong to a class of amphiphiles having two hydrophobic tails and two hydrophilic headgroups covalently linked by a spacer. The simplest and most-studied cationic gemini surfactants are the quaternary ammonium compounds that are present in two identical ammonium head groups connected by a saturated alkyl chain spacer with *s* carbons and two symmetric-saturated alkyl tails comprising *m* carbons [88]. Dimethylene bis(octadecyldimethylammonium bromide) (bisODAB), shown in Fig. 5, is an example of such gemini surfactants. A series of dicationic alkylammonium bromide gemini surfactants with different spacer (s = 2, 4, 6, 10, 12) and tail length (m = 12, 16 and 18) were synthesized and their effect on the structural and dynamic properties of the DODAB membranes was studied using differential scanning calorimetry (DSC) and MD simulations. It was shown that the thermotropic behavior of the mixed DODAB–gemini surfactant bilayers was strongly influenced by the molar fraction of the added surfactant, the spacer length and the chain length. MD studies revealed that the structure and order of the mixed bilayers were directly related to the vertical position of the gemini molecules. Gemini surfactants with long tails, compared with those of DODAB, were responsible for a higher atom density in the center of the bilayer, while

those with short tails reduced the respective density. In turn, a long spacer promoted a deeper insertion of the gemini molecule into the bilayer via a hydrophobic effect. This reduced the impact of short tails on the density of the hydrophobic region of the bilayer.

N-*t*-butyl-*N'*-tetradecyl-3-tetradecylaminopropionamidine (diC14-amidine, Fig. 5) is a fusogenic cationic lipid that forms stable vesicular structures under physiological pH and temperature. A pure diC14-amidine membrane and a series of DiC14-amidine/DMPC membranes have been simulated at 27 °C to gain insight into their structure and dynamic properties [89]. The area per lipid was found to increase from 0.564 to 0.833 nm^2, while the bilayer thickness decreased from 3.56 to 2.72 nm for the pure DMPC and diC14-amidine membrane, respectively. The diC14-amidine bilayer was in the fluid state with highly disordered, V-shaped lipid tails and, simultaneously, its leaflets were merged due to interdigitation. The presence of thermally accessible V-shaped lipids indicated a tendency for a strong bilayer curvature and fusion capabilities. Compared to more conventional lipids, diC14-amidine had a lowered affinity toward water and a smaller headgroup. Therefore, when mixed with physiological bilayers and in low quantities, the headgroups of diC14-amidine will be protected from contact with water via other lipids. Next, the properties of the diC14-amidine membrane were studied experimentally and compared to those of a zwitterionic lipid DMPC [90, 91]. It was shown that the diC14-amidine membrane had looser molecular packing in comparison to that of DMPC. As demonstrated with the MD simulations, below or around its transition temperature at 21 °C the membrane adopted an interdigitated structure. Above the transition temperature, a lamellar structure of the diC14-amidine membrane was observed [90]. At the phase transition, the interdigitation of the hydrocarbon chains was abolished, as observed by the jump-like increase of the membrane thickness (determined as the thickness of the hydrocarbon membrane core) from 1.78 to 2.02 nm and changes in the lateral area per molecule form 0.402 ± 0.002 to 0.732 ± 0.015 nm^2.

Cationic lipids with trimethylammonium-propane (TAP) headgroups and various fatty acid tails are another class of compounds used to construct non-viral vectors. Fig. 5 shows, for example, dioleoyltrimethylammonium propane (DOTAP). This compound is the most widely used cationic lipid. It is relatively inexpensive and efficient in both in vitro and in vivo applications [92]. MD simulations of mixed bilayers containing DOTAP and zwitterionic DMPC, a helper lipid, at different DOTAP fractions have been performed [93]. The simulations were focused on the specific effects of unsaturated lipid chains on structural and dynamic properties of mixed cationic bilayers. It was found that most structural parameters of the mixed bilayers, as area per lipid, demonstrated non-monotonic behavior with increasing cationic lipid fraction with the minimum observed at the fraction equal 0.4. Moreover, adding unsaturated DOTAP lipids into DMPC bilayers promoted strong lipid chain interdigitation and fluidization of bilayers, as indicated by enhanced lateral lipid diffusion. The formation of DMPC-DOTAP charge pairs as a result of electrostatic interactions within the membrane-water interface was observed. The PC-TAP pairs led to the formation of PC lipid clusters containing two or three PC lipids around TAPs.

4. Reporting molecules

Due to their sensitivity and versatility, fluorescence and electron paramagnetic resonance (EPR) spectroscopies are widely used as the main tools to study the biophysics of lipid bilayers and association of small molecules with the membranes [94] Using these methods, one can experimentally determine several important parameters: (i) the membrane properties, such as fluidity, permeability, polarity, organization and the dynamics of lipids, can be determined using appropriate molecular probes [95-98]; (ii) the lateral diffusion coefficients of lipid-soluble molecules can be estimated by fluorescence spectroscopy [99]; (iii) equilibrium partitioning (binding) of the molecule to the lipid bilayer can be estimated using a fluorescence titration

technique [100] and (iv) position of the molecule in the membrane can be determined using depth-sensitive fluorescent quenching analyses (FQA) [101].

Since membrane lipids are non-fluorescent and non-paramagnetic compounds, extrinsic membrane probes are widely used as so-called reporting molecules of the membrane properties. Unfortunately, the membrane probes are usually large foreign molecules inserted into the host lipid membrane. As a result, the perturbation of the bilayer structure and dynamics of bilayer components is inevitable, even at low probe concentrations. MD simulations present a convenient way to address these issues and have been increasingly used in recent years in this context. Computer simulations provide detailed information on the position and orientation of the reporting molecule inside the lipid bilayer, but also on changes in the organization of the lipid molecules after the incorporation of molecular probes [94]. To limit the perturbation of the membrane organization, the reporting molecules are generally constructed by modifying lipids. Therefore, the molecular probes are usually phospholipids with fluorophores or free radicals that are covalently linked to either the headgroup or one of the acyl chains, or sterols with fluorophores or free radicals attached to either the alkyl chain or the hydroxyl group. However, some lipoid dyes have been also proposed for reporting purposes.

In this review, we divided reporting molecules into two classes: (i) fluorescing molecular probes and (ii) spin-labeled molecular probes. We focused our attention on the most recent MD and experimental studies on the molecular probes.

4.1. Fluorescent molecular probes

Several lipid-like probes (lipoid dyes) have been developed and studied. The study involved two types of substances: (i) covalent conjugates of lipids and fluorophores, such as 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) or 7-nitrobenz-2-oxa-1,3-diazole (NBD) and (ii) amphiphilic dyes (fluorescent surfactants); for example, 1,1′-dioctadecyl-3,3,3′,3′-

tetramethylindocarbocyanine perchlorate (DiI). The chemical structures of these compounds are shown in Fig. 6.

Lipid Derivatives of BODIPY

BODIPY dyes tend to be strongly UV-absorbing small molecules that emit relatively sharp fluorescence peaks with high quantum yields [102]. They are relatively insensitive to the polarity and pH of their environment, stable to physiological conditions and show good photostability. For these reasons, the BODIPY dyes are widely used to label lipids. The dye can be substituted in three positions (see numbering in Fig. 6). However, the substitution position affects the important photophysical properties of the fluorophore, such as UV absorption maxima, fluorescence emission maxima and quantum yields [102].

Different BODIPY-Chol conjugates have been prepared and studied [103, 104]. Holtta-Vuori et al. proposed a *meso*-BODIPY-Chol probe, in which the sterol side chain was attached to the *meso* position of BODIPY, to visualize sterol trafficking in living cells and organisms [103]. MD simulations of Chol or *meso*-BODIPY-Chol embedded in DPPC or SPM membranes were performed to assess the potential of fluorescent Chol analogs to mimic the properties of endogenous Chol. The results indicated that the average area per lipid and membrane thickness were not affected by the BODIPY fluorophore, and the position of the steroid structure was very similar for both sterols. The perturbation of neighboring lipids in the DPPC membrane was slightly different for Chol and *meso*-BODIPY-Chol. However, in the more ordered SPM bilayer, the ordering properties of meso-BODIPY-Chol were essentially fully consistent with those of Chol. The BODIPY moiety had two preferred orientations with respect to the steroid structure. BODIPY was either standing along the steroid backbone with the long axis perpendicular to the bilayer normal or back-looping toward the head group region with the long axis oriented almost parallel to the membrane normal. The first orientation dominated in the more ordered SM membrane and the *meso*-BODIPY-Chol had a preference to stand in an

upright position with the BODIPY moiety located in the middle of the membrane, where its membrane perturbation effect is the smallest. It was concluded that *meso*-BODIPY-Chol closely mimics the ordering properties of Chol. The perturbations arising from the presence of the BODIPY moiety are minor and, importantly, become smaller as the membrane order increases. Further studies of BODIPY-Chol probes were presented by Solanko et al. [104], who proposed these conjugates to study intracellular Chol dynamics. Two probes (Fig. 6) were obtained by attaching Chol to BODIPY ring at *meso* or α position (α-BODIPY-Chol) [104]. It was shown experimentally that the orientation of the BODIPY moiety depended on the membrane's Chol content. In cell and model membranes containing Chol, the fluorophore in *meso*-BODIPY-Chol and α-BODIPY-Chol was perpendicularly oriented and almost parallel to the lipid acyl chains, respectively. Thus, the experimentally assessed alignment of fluorophore of *meso*-BODIPY-Chol is in agreement with that observed in the MD simulations [103]. The difference in orientation had a strong influence on the lateral diffusion constant of the Chol-analogs in cell membranes. It was demonstrated experimentally that α-BODIPY-Chol diffused significantly faster than *meso*-BODIPY-Chol in cell membranes, although there was no difference in the lateral diffusion in lipid model membranes. The authors speculated that the sterol orientation in α-BODIPY-Chol is less disturbed by the attached fluorophore than *meso*-BODIPY-Chol; however, to confirm this, some MD simulations are required.

Phospholipids have been tagged with BODIPY by incorporating the fluorophore into one of the acyl chains [105] or attaching it to the lipid headgroup [106]. They are ubiquitous in the studies of phase partitioning and also for understanding dynamics by using techniques such as fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS). The fluorescent lipid analog, 2-(5-butyl-4,4-difluoro-4-bora-3a,4adiaza-*s*-indacene-3-nonanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (BODIPY-PC, see Fig. 6), have been applied as a reporting lipid in single-molecule fluorescence measurements to provide insight

into DPPC chain tilting and order at different surface pressures in both monolayers and bilayers [107, 108] These studies utilized out-of-focus polarized total internal reflectance fluorescence microscopy (PTIRF-M) to characterize the orientation of BODIPY-PC, doped into the DPPC monolayer and membrane. It was shown that the tilt orientation of BODIPY-PC was very sensitive to the lateral surface pressure and addition of sterols. At low surface pressures, the long axis of BODIPY was largely parallel to the plane of the film and evolved linearly to an orientation normal to the surface as pressure was increased, indicating an increase in the acyl chain order. The tilt angle dependence on surface pressure was used to construct a calibration plot to find the equivalent surface pressure for bilayers. The experimental measurements were next complemented by 1.5 μs-long comparative MD simulations of DPPC monolayer and bilayer systems incorporating one BODIPY-PC molecule per leaflet at low (3 mN/m), medium (10 mN/m), and high (40 mN/m) lateral pressures [104, 105] These MD simulations were addressed to explore (1) the microscopic correspondence between monolayer and bilayer structures, (2) the fluorophore position within the membrane, and (3) the microscopic driving forces governing the fluorophore tilting. The MD simulations reveal very close agreement between the monolayer and bilayer systems, in terms of the dye orientations and lipid chain order, suggesting that monolayer experiments can be used to approximate bilayer systems. The simulations capture the trend of the reduced tilt angle of the fluorophore with increasing surface pressure, as seen in the single-molecule experimental results. Additionally, it was shown that the fluorophore in BODIPY-PC predominantly interacts with lipid acyl chains at any given surface pressure, although the fluorophore interaction with water and a headgroup slightly increases as the surface pressure decreases, which was not obtainable in the experiments. The BODIPY-PC molecule indeed changed its orientation in response to the changes in the acyl chain properties through such extensive interactions with lipid acyl chains. The simulations also revealed that the enthalpic contribution is dominant at 40 mN/m, resulting in smaller tilt angles

of the fluorophore, and the entropy contribution is dominant at lower pressures, resulting in larger tilt angles. However, the MD simulations did not definitively resolve the origin of the bimodal distributions of BODIPY-PC tilt angles observed in the single molecule experiments of the dye molecules doped into the DPPC monolayers at low and high surface pressures. The authors suggested that such experimental distributions may result from the lipid phase separation, rather than the distinct dye locations in the membranes.

Sachl et al. have investigated mono-palmitoyl- and dipalmitoyl-phosphatidylethanolamine probes labeled in the polar headgroup region by BODIPY fluorophores (referred to as BODIPY-mPE and BODIPY-dPE, respectively, Fig. 6) and solubilized in lipid systems that exhibit different curvatures [106]. Due to the bulky BODIPY groups, BODIPY-mPE has a conical shape, whereas BODIPY-dPE molecules tend to be cylindrical. The authors hypothesized that, due to its shape, conical-shaped lipids should exhibit a pronounced affinity to highly curved regions compared to the cylindrical-shaped lipids. However, a careful analysis of time-resolved resonance energy transfer experiments by means of analytical models, as well as Monte Carlo simulations, shows that BODIPY-mPE has a comparable affinity to highly curved and planar bilayer regions. Furthermore, the monoacyl probes were effectively closer to each other in a lipid bilayer as compared to the diacyl probes. This self-aggregation was suppressed when using the diacyl instead of the monoacyl derivative and/or by attaching BODIPY groups to the acyl chain. It seems that MD simulations can give molecular insight into the behavior of BODIPY-mPE and BODIPY-dPE probes.

Lipid Derivatives of NBD

NBD is another fluorophore that is often applied to label different lipid molecules. This dye has an excitation maximum at ca. 480 nm and emission maximum at ca. 540 nm. NBD-labeled sterols, such as commercially available 22-(7-nitrobenz-2-oxa-1,3-diazol-4-yl-amino)-23,24-bisnor-5-colen-3β-ol (22-NBDChol) and 25-[N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)methyl]-

amino]-27-norcholesterol (25-NBD-Chol, Fig. 5), are commonly used as fluorescent cholesterol analogs in membrane biophysics [109, 110]. However, some experimental reports questioned their ability to mimic the behavior of Chol in phospholipid bilayers. For example, the rapid reduction of NBD by dithionite was observed for both NBD-labeled sterols, indicating an upside-down orientation of the probes in membranes and NMR-ordered parameters showed a significantly lower condensation of lipid chains by these probes compared to Chol [111]. To clarify whether these probes are suitable to mimic Chol, atomistic MD simulations of systems containing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers doped with small concentrations of Chol, 22-NBD-Chol, or 25-NBD-Chol have been performed [110]. The results showed that the molecular probes had a tendency to adopt conformations with the NBD moieties that were oriented toward the membrane-water interface. In these molecules, the long sterol axis preferentially adopted orientations that were approximately parallel to the bilayer plane. In turn, these stretched conformations together with NBD-POPC interactions led to slow down the lateral diffusion of both fluorescent sterols compared to Chol. The order parameters and acyl chain tilts of the POPC chains were calculated for varying POPC-sterol distances. It was observed that the local ordering effect of sterol was altered in both fluorescent derivatives. In agreement with the reported experimental data, both fluorescent sterols were able to increase the order of POPC at a 20 mol% concentration (as some molecules adopted an upright conformation, which was possibly related to the formation of transbilayer aggregates), albeit to a smaller extent to that of Chol. Altogether, this study indicated that both 22- and 25-NBD-Chol were unable to mimic the most important features of the Chol behavior in the lipid bilayers.

1,2-Diacyl-*sn*-glycero-3-phosphoethanolamine labeled with NBD at the headgroup (NBD-diCnPE) with the acyl chain length n = 14, 16 and 18 are commercially available and widely used as fluorescent probes in the studies of dynamic properties of biological membranes [112]

Recently, Filipe et al. [113] performed MD simulations of a POPC bilayer doped with 3 mol% of NBD-diCnPE with varying chain length (n = 4 to 18). Several parameters, such as location and orientation of the fluorophore, acyl chain order parameters of both POPC and NBD-diCnPE, membrane electrostatic potential and lateral diffusion were calculated. The results showed that the NBD fluorophore was located near the lipid carbonyl region and adopted an orientation in which the NO_2 group was facing the bilayer interior. Most of these probes induced local disordering of the POPC acyl chains, which was on the whole counterbalanced by ordering resulting from the binding of sodium ions to lipid carbonyl/glycerol oxygen atoms. The exception was NBD-diC16PE, which displayed optimal matching with the POPC acyl chain and induced a slight local ordering of phospholipid chains. Moreover, due to the fact that NBD-diCnPE is negatively charged at a neutral pH, the inclusion of NBD-diCnPE probes increased the difference in the electrostatic potential between the bilayer interior and bulk water.

Various phospholipids with NBD-labeled acyl chains are also commercially available. However, it has been shown—both experimentally [114] and by MD simulations [115] that the average position of NBD was characterized by broad distributions with a maximum pointing to the probe location at the membrane-water interface. This behavior was explained by the back-looping of the fluorophore toward the aqueous phase. Due to its polarity and the acyl chain flexibility, the NBD moiety escaped from the membrane center to the headgroup region by bending the hydrocarbon chain to which it was attached and the nitro group was the most external part of the fluorophore. Such an arrangement of these probes caused considerable perturbation in the host membrane properties.

Pyrene-labeled lipids

The issue of optimal attachment of the external probe to a lipid chain in order to minimize the extent of host membrane perturbations has been addressed in a paper by Franová et al. [116]. The authors performed MD simulations of POPC membrane doped with 3 mol% of saturated

phosphocholines with pyrene (Pyr) moieties attached to the 6th, 8th or 10th carbon in both hydrocarbon chains (referred to as PC6-Pyr, PC8-Pyr and PC10-Pyr, Fig. 6) to explore how the position of Pyr moiety affected the membrane properties and the formation rate of Pyr dimers. The results showed that all the probes altered membrane order around them; thus, the dynamic properties of a Pyr-labeled lipid were different from those of native lipids. However, membrane perturbations leveled off within about one nanometer from the probe and are, therefore, of short range, affecting just the nearest neighbors of the probe lipid. The fluorophores in all derivatives were located in the membrane core, which agrees with the highly hydrophobic nature of Pyr molecules. However, in opposition to PC6-Pyr and PC8-Pyr, the Pyr groups in PC10-Pyr can locate the opposing leaflet, indicating quite profound interdigitation, and thereby also forming numerous dimers with pyrenes in the other leaflet. Thus, the formation of membrane-spanning pyrene dimers depends very strongly on the location of the fluorophore in its host lipid.

Fluorescent Natural Sterols

Intrinsically fluorescent sterols, dehydroergosterol (DHE) or cholestatrienol (CTL), can be used as molecular probes to mimic the behavior of Chol in membranes since both compounds have a quite similar chemical structure to that of Chol (Fig. 6). Three conjugated double bonds in the ring systems are responsible for their fluorescence emission. Moreover, DHE is a naturally occurring sterol, synthesized by the yeast *Candida tropicalis* and the sponge *Biemna fortis*. However, it was noticed that both reporting molecules suffer due to their unfavorable photophysical properties, which include absorption and emission in the UV region, relatively low molar absorption coefficient, low quantum yield, and rapid photobleaching [117]. All of these qualities can significantly limit their use in experimental work.

Recently, MD simulation studies have been performed to compare the behavior of the fluorescent Chol analogs in lipid bilayers [118, 119]. The MD simulations were used to study the POPC bilayers containing variable amounts of DHE or CTL (~1.6, 20, and 50 mol %) [118]

The ability of both sterols to mimic Chol in the different systems was evaluated by the assessment of the probe (location, orientation, dynamics) and phospholipid (area/lipid, bilayer thickness, acyl chain tilt and order parameters, dynamics) properties. The results showed that both DHE probes were adequate analogs of Chol, since their transverse location and orientation were similar to that of Chol. Probe rotational and translational dynamics were generally slightly faster than those of Chol, while the induced ordering of POPC bilayers was lower than that of Chol. The authors indicated that of the two studied probes, CTL was the one with behavior closest to that of Chol. This was related to the side-chain structure. CTL shares an identical side-chain with Chol and, therefore, it closely emulated the behavior of Chol, whereas DHE has a smaller ordering efficiency, which may be related to its modifications in the side-chain, an additional double bond and a methyl group. However, actual use of either sterol must also take into account the more limited availability of CTL compared to the naturally occurring DHE.

Further studies on the DHE behavior at membrane were performed by Pourmousa et al. [119]. They used a combination of time-resolved fluorescence spectroscopy, quantum-mechanical electronic structure computations, and MD simulations to characterize the perturbations induced by 20 mol% of DHE in a POPC membrane. It was shown that Chol was slightly stronger than DHE at both ordering and condensing membranes; the difference in the ordering effect of CHOL and DHE was more noticeable than the difference in their condensing effect. The area per lipid and the thickness of the POPC − DHE system differed by 3 and 2%, respectively, from those of the POPC − Chol system, while the order parameter of the POPC chains in the POPC − DHE system was 6% different from that of the POPC − Chol system. On the other hand, the interactions in the headgroup region were almost the same. Both sterols anchor similarly to the carbonyl, as well as the nitrogen groups of the POPC molecules, through H-bond and charge-pair interactions.

Fluorescent Surfactants

Surfactants with fluorescent polar groups can be used in labeling lipid membranes. A complete homologous series of fluorescent NBD-labeled fatty amines with varying alkyl chain lengths (NBD-Cn, n = 4 to 16, Fig. 7) has been studied experimentally in aqueous solution and associated with BSA or lipid bilayers [120]. It was found that all of the amphiphiles interacted efficiently with the POPC bilayers. The photophysical parameters of the amphiphiles inserted in the POPC bilayers showed no significant variation along the series, indicating that the NBD group was located in the same region of membrane, regardless of the hydrocarbon length. An exception was noted for NBD-C14, whose parameters were somewhat different from the trend observed. Several MD simulations of NBD-Cn probes incorporated in POPC [121, 122], POPC/Chol (1:1) or SPM/Chol (6:4) [123] membranes were performed to gain molecular insight into NBD-Cn – membrane systems.

Atomistic MD simulations of POPC bilayers containing 3 mol% of NBD-Cn revealed that the NBD fluorophore is located near the glycerol backbone/carbonyl region of POPC for all derivatives and establishes stable hydrogen bonding with POPC ester oxygen atoms [121]. Small differences observed in the transverse location of the fluorophore correlated with other calculated parameters, such as the area per lipid, POPC deuterium order parameter, NBD-Cn chain order parameter, bilayer thickness and with small discrepancies measured in the photophysical properties of the molecules [120]. In particular, the slightly deeper location of the fluorophore in NBD-C14 agreed with lower fluorescence quantum yield and anisotropy measured for this amphiphile. The longer-chained NBD-Cn amphiphiles showed a significant mass density near the bilayer midplane, and the chains of these derivatives interdigitated, to some extent, the opposite bilayer leaflet. This phenomenon led to a slower lateral diffusion for the longer-chained derivatives (n > 12). The perturbation induced by moderate concentrations (up to 3.1 mol %) of NBD-Cn was relatively mild, as small nonsystematic variations were

observed for parameters such as average area per lipid, bilayer thickness, POPC order parameters, and the lateral diffusion coefficient. Surfactants with chain lengths inside the $8 \leq n \leq 12$ range had lateral diffusion coefficients similar to that of POPC and, taking into account their relatively mild perturbing effect, can be useful fluorescent reporters of bilayer dynamics.

MD simulations of NBD-Cn embedded into POPC/Chol (1:1) or SpM/Chol (6:4) membranes showed that the incorporation of Chol resulted in the shallower location of the fluorophore than that observed for pure POPC bilayers [123]. The position of NBD at the water-membrane interface agreed with the lower fluorescent quantum yield, shorter fluorescence lifetime, and higher ionization constants (smaller pK_a) determined experimentally. The more external location was also consistent with the changes measured in steady-state fluorescence anisotropy from POPC to POPC/Chol (1:1) vesicles. Accordingly, the equilibrium location of the NBD group within the various bilayers was mainly governed by bilayer compositions, and was mostly unaffected by the length of the attached alkyl chain. Similar to the behavior observed in POPC bilayers, the longer-chained NBD-Cn amphiphiles show significant mass density near the mixed bilayers' midplanes and the alkyl chains of the longer derivatives penetrated the opposite bilayer leaflet to some extent. However, this effect was quantitatively less pronounced in these ordered bilayers than in POPC. Similar to the POPC bilayers, the effects of these surfactants on the structure and dynamics of the host lipid were found to be relatively mild.

DiI is another amphiphilic dye proposed for study of the membrane properties (Fig. 7). The fluorescence lifetime of DiI is linearly correlated with the local viscosity of the dye-surrounding environment, so the DiI fluorescence lifetime can be used as membrane tension reporter [124]. Fluorescence lifetime analysis of the DiI probe has been recently used to study phase transitions and macroscopic phase separation in live cells and giant plasma membrane vesicles [125]. Atomistic MD simulations of DiI-labeled DPPC bilayers under physiological lateral tensions

ranging from -2.6 mN m^{-1} to 15.9 mN m^{-1} were performed to quantify the precise relationship between tension, the structural properties of the membrane and the dynamics of lipids and a lipophilic reporter dye [126]. It was shown that DiI has systematically lower lateral and rotational diffusion coefficients compared to DPPC, but the increase in each with tension is quantitatively similar for DiI and DPPC. The lower dynamics of the probe is most likely due to the rigid and bulky structure of the DiI headgroup. Moreover, DiI is located near the lipid acyl chain region. Since the location of the dye with respect to lipid–water interface does not change with increased tension and water does not penetrate appreciably beyond lipid headgroups, no marked change in the DiI hydration is expected. This is important since the fluorescence lifetime of carbocyanine chromophores is sensitive to water accessibility and to the local microviscosity. These observations indicate that changes in the fluorescence lifetime of DiI due to membrane order are most likely attributed to changes in the viscosity near the headgroup, rather than due to changes in hydration. The authors concluded that the fluorescence lifetime of DiI, which depends on the lipid order near the headgroups, appears to be a good indicator of tension in membranes.

N-[[4′-N,N-diethylamino-3-hydroxy-6-flavonyl]-methyl]-N-methyl-N-(3-sulfopropyl)-1-dodecanaminium (F2N12S, Fig. 7) belongs to family of bright membrane-staining dyes, sensing polarity and hydration of lipid membranes via a mechanism involving an excited-state intramolecular proton transfer and the formation of hydrogen bonds with water, potentially accompanied by changes in molecular orientation [127]. Recently, this dye has been proposed as a molecular probe in fluorescence-detected linear dichroism (FDLD) measurements reporting on the molecular orientation and rotation of fluorescent molecules within the lipid membranes of liposomes and living cells [128]. The orientation of F2N12S molecules within POPC membrane was determined experimentally by using single- and two-photon polarization microscopy observations of linear dichroism. The results were consistent with a Gaussian-like

orientational distribution of the transition dipole moment of the dye, with a mean tilt angle of 53.2 ± 0.1° with respect to the bilayer normal and a standard deviation of 13.3 ± 0.6°. To confirm such alignment of the fluorophore in the POPC bilayer, MD simulations were performed. The calculated values were very similar a mean tilt angle of 48 ± 4° and a standard deviation of 13 ± 2°. The good agreement between the experimentally and computationally obtained values validated this fluorescent optical probe reporting on cellular events through changes in linear dichroism.

4.2. Spin-labeled probes

Nitroxide-free radicals can be attached as spin labels to proteins, lipids or synthetic macromolecules [129]. Such labeling methods allow the targeted introduction of an electron spin into otherwise diamagnetic systems. Several different nitroxide free radicals were proposed in various fields of membrane biophysics, while the rigid and conformationally unambiguous Doxyl groups (Fig. 8) can be introduced during the synthesis of lipids, steroids or surfactants. These electron spin labels can be observed either directly by electron paramagnetic resonance (EPR) or indirectly by NMR spectroscopy via its hyperfine interactions with nuclear spins or can serves as quenchers in fluorescence measurements.

Spin-labeled phospholipids are commonly used as fluorescence quenchers in depth-dependent quenching studies of membrane penetration of fluorescent molecules embedded in membranes [101, 130, 131]. This method allows for the calculation of the vertical position of a fluorophore in the bilayer by comparing the extent of quenching by two lipid-bound quenchers that are located at known, but different, vertical depths in the bilayer. Therefore, an accurate depth position of fluorophores relies on the use of several spin-labeled probes placed in the membrane at various positions. The depth position of the quenchers (spin probes) has to be determined independently; however, experimental determination of transverse distributions of spin-probe depths is difficult. MD simulations seem to be the most appropriate method to solve

these problems. They were used to study the membrane behavior and depth distributions of spin-labeled phospholipids in a POPC bilayer [131]. Five phosphocholines, in which a Doxyl moiety was covalently attached to *n*th carbon atoms (*n* = 5, 7, 10, 12, and 14) of the *sn*-2 stearoyl chain, namely 1-palmitoyl-2-stearoyl-(n-Doxyl)-*sn*-glycero-3-phosphocholine (n-Doxyl-PC), and phosphocholine with the Tempo group linked to a headgroup, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(TEMPO)choline (Tempo-PC), have been simulated. The results revealed that the chain-attached spin labels were broadly distributed across the model membrane and their environment was characterized by a high degree of mobility and structural heterogeneity. Despite the high thermal disorder, the depth distributions of the Doxyl labels were found to correlate well with their attachment positions, indicating that the distribution of the spin label within the model membrane was dictated by the depth of the *n*th lipid carbon atom, rather than by the intrinsic properties of the label. In the case of the Tempo-PC probe, a much broader and heterogeneous distribution was observed, probably due to the hydrophobic nature of the Tempo moiety, which favored partitioning from the headgroup region deeper into the membrane. Depending on the concentration of Tempo-PC lipids (11 or 29 mol%), the probable depth of the Tempo moiety could span a range from 1.44 to 1.82 nm from the membrane center.

Next, the tempo-PC and five n-Doxyl-PC probes have been used to investigate the immersion depth of the NBD fluorophore attached to the lipid headgroup in NBD-PE incorporated into a POPC bilayer [132]. A combination of MD simulations and depth-dependent fluorescence quenching was used to calibrate the methodology for extracting quantitative information on membrane penetration. The immersion depth of NBD was estimated by measuring steady-state and time-resolved fluorescence quenching with spin-labeled lipids co-incorporated into lipid vesicles. Using the methodology of Distribution Analysis, the immersion depth and the apparent half-width of the transversal distributions of the NBD moiety were estimated to be 1.47 and 0.67 nm, respectively, from the bilayer center.

This position was validated by atomistic MD simulations of the POPC bilayer with incorporeted NBD-PE. The MD simulations results showed good agreement with the experimental resutls.

A variety of nitroxide-labeled probes have been used in EPR measurements to characterize the dynamic structure on a molecular level of the liquid ordered (L_o) and liquid-disordered (L_d) coexisting phases in the brain-sphingomyelin/dioleoylphosphatidylcholine/cholesterol (SPM/DOPC/Chol) model lipid system [133]. The analysis of the EPR spectra of the probes provided the rotational diffusion rates and order parameters, which characterize the local molecular dynamics in the coexisting L_o and L_d phases. The use of 10 different spin-labels labeled along the acyl chain and within the headgroup of phospholipids, as well as a labeled Chol analog, allowed for analysis of both the hydrophobic and hydrophilic regions of the model bilayer. Based on the results, a molecular model of the L_o phase was proposed, including the condensing effect of Chol on the phospholipid acyl chain dynamics and ordering and the "umbrella model" of the phospholipid headgroup.

Spin-labeled fatty acids are commonly used for EPR and fluorescence studies of cell membranes to investigate physical properties such as phase transitions, fluidity, and the location of fluorophores inside lipid membranes. These compounds are commercially available and much cheaper compared to the spin-labeled phospholipids. The position and behavior of the quenching moieties of the spin-labeled stearic acids (SASLs) in the DPPC bilayer were determined previously by MD simulations [97]. Three SASL derivatives with the Doxyl group attached at the 5th, 10th or 16th carbon atom along the chain (5-SASL, 10-SASL, and 16-SASL, Fig. 8) were studied. It was shown that the incorporation of SASL molecules into the lipid bilayer resulted only in a small increase in order parameters and a related decrease of surface area, but it did not change the overall bilayer structure. The positions of the spin labels were calculated to be 1.27 ± 0.22, 1.02 ± 0.23, and 0.86 ± 0.29 nm for 5-SASL, 10-SASL and 16-SASL, respectively. This indicates that, similar to Doxyl-PCs, the distributions of locations

were rather broad and, depending on the attachment position, were shifted in comparison to the location of lipid acyl carbon atoms by two to eight positions toward the membrane surface. Opposite to the Doxyl-PC probes, SASLs can dissociate in the aqueous environments. The authors indicated that under experimental conditions, the carboxylic group of the SASL molecule should be dissociated. This can be achieved by increasing the pH to approximately 9.5, since the carboxyl group of 5-SASL in the membrane showed an apparent p*K*a of around 7.5. As shown with MD simulations, the uncharged form of SASL behaved drastically differently from that of charged SASL molecules. It was located deeper in the membrane and was able to diffuse between membrane layers during the simulation time.

The SASL probes have been applied with success in several experimental studies to determine the position of fluorescent molecules using fluorescence-quenching analysis (FQA). The obtained results were compared with MD simulations. For example, the complementary results of fluorescence and MD simulation studies on the behavior of small organic molecule, 2,6-bis(decyloxy)naphthalene, inside the POPC membrane were presented. Stable free radicals 16-SASL, 5-SASL, and 3β-Doxyl-5α-cholestane (CSL, Fig. 8) were used as quenchers. The quenching experiments showed that the naphthalene ring of the compound was located shallow in the membrane at approximately 1.4 nm from the center of the bilayer. The MD simulations provided more detailed information and revealed that the aromatic group resided in the upper acyl chain region near the headgroups of the bilayer and the hydrocarbon tails were directed to the center of the bilayer, which was in line with the experimental results. Recently, similar methodology has been used to study the effect of membrane PEGylation on the location and orientation of 5,10,15,20-tetrakis(4- hydroxyphenyl)porphyrin (p-THPP), a model hydrophobic compound [31]. The properties of p-THPP in the presence of different fluid PC bilayers and PEGylated membranes were considered. Both fluorescence-quenching and MD simulation results indicated that p-THPP within zwitterionic membranes were located at the interface

between the hydrocarbon chain region and the polar region or close to the water-membrane interface, whereas p-THPP solubilized in PEGylated liposomes was localized in two preferred positions: deep within the membrane (close to the center of the bilayer) and in the outer poly(ethylene glycol) corona.

In both papers [31, 94], CSL was used as a spin label with the quenching group located at the water-membrane interface. Since the position of CSL in the bilayer has not been previously examined, it was assumed that the depth of the nitroxide group of CSL is similar to the depth of the OH group of Chol. According to the finding by Rog and Pasenkiewicz-Gierula, such group is located in the region of the PC phosphate groups [134]. Although this assumption seems to be reasonable, MD simulations of the CSL behavior in lipid membranes are desirable since this probe is commercially available.

SASLs have been used as spin probes to determine the liposome microviscosity with EPR spectroscopy [98, 135]. The effective microviscosities were determined from the calibration of the EPR spectra of the probes in solvent mixtures of known viscosities. Next, by measuring the EPR-order parameter and correlation time of the SASL probes incorporated into the membrane, it was possible to quantify the value of effective microviscosity at different depths inside the liposome membrane. The developed methodology was proven to be useful to study changes in the membrane fluidity induced by temperature or the drug incorporation.

5. Synthetic lipids help to understand lipids structure-function relationship

Synthetic lipids are useful in studies concerning structure of lipids aiming to connect particular structural elements with properties of lipid bilayers formed by the studied lipid. The group might be removed or replaced by other groups with different chemical properties, such as a different charge or ability to form hydrogen bonds. In this context, Chol and sphingolipids were

particularly extensively studied; both experimentally and via MD simulations and we discussed them in more detail in next paragraph. Glycerol-based lipids were also studied with synthetic analogs (e.g. synthetic analogs with various sizes of headgroups) [136], or analog with altered chirality [137], and reverse charge were synthetized. In last paragraph we describe a case of reverse zwitterionic lipids.

5.1. Cholesterol synthetic analogs

Chol is the central molecule regulating the physical properties of lipid bilayers. Cholesterol affects lipids' phase behavior by decreasing the temperature of the main phase transition and decreasing its cooperativity in small and moderate concentrations. In large concentrations, Chol completely eliminates phase transition. In higher Chol concentrations, Chol with other lipids forms a Ld phase which, in certain temperatures and concentration ranges, may coexist with the Lo phase. Chol increases the mechanical strength of the lipid bilayer and decreases its permeability. Molecular mechanisms behind these Chol properties are its abilities to increase lipid tails' order and condense the lipid bilayer. Thus, it is not surprising that Chol modulates interactions between membrane lipids and membrane proteins, both integral and peripheral. Chol concentration in various types of membrane differs significantly from being lowest in mitochondrial membranes and highest in the cell membrane. For all of these reasons, Chol is one of the most intensively studied molecules; in particular, the importance of its structure has been in the spotlight for a long time. Already in 70-ties Chol structure was shown to be composed of three structural elements necessary for its membrane function: small polar headgroup 3β-OH, sterol ring and short isooctyl tail [138]. Changes of these structural elements typically decreases sterol's ordering and condensing abilities. This old observation was recently highlighted by post-analysis of published data on numerous sterols and proven to be statistically significant [139]. In the next paragraph, we show examples of synthetic sterols with modifications related to these three structural elements.

Enantiomeric cholesterol (ent-Chol) is a synthetic Chol analog not existing in nature in which all 8 chiral centers have reverted chirality compared to Chol. Ent-Chol was synthetized for the first time in 2002 by Jiang and Covey with 23-step synthesis and an overall yield of 2.6% [140]. More recent studies described 16-step synthesis, but with an overall yield of 2% [141]. Ent-Chol was shown to affect the lipid bilayer and monolayer properties to the similar extent as Chol [142-144]. Surprisingly in cell lines with impaired Chol biosynthesis, ent-Chol was able to substitute Chol and promote cell growth [145]. These abilities of ent-Chol make it useful to recognize whether or not the observed effect of Chol on membrane proteins' functions result from direct, specific interactions or is mediated through the bulk membrane properties [146]. For instance, two bacterial toxins streptolysin O and *Vibrio cholerae* cytolysin that are known to destabilize Chol-containing membranes were shown to be specific toward bilayers with Chol but not with ent-Chol [147]. Similarly, peptides known to affect the phase behavior of Chol-containing bilayers were not affecting bilayers with ent-Chol [148]. Ent-Chol, contrary to Chol, was found not to be able to activate acyl-CoA cholesterol acyltransferase [149]. Finally, ABC-type transporters were shown to be sensitive on sterol chirality [150]. However, for the case of a serotonin 1A receptor, ent-Chol was able to replace Chol and maintain receptor functions [151].

A Chol ring is asymmetric and has two faces: α-face with no substituents and β-face with two methyl groups and hydroxyl group pointing out from the β-face. Due to this configuration in the membrane plane, 3-fold symmetry is observed [152]. The first sterol at the Chol biosynthetic pathway lanosterol has 5 methyl groups located at both faces, thus synthesis steps and the removal of the methyl groups make sterols gradually smoother. Further, the ordering and condensing capability of sterols along the biosynthetic pathway gradually increase, suggesting that the biosynthetic pathway reflects evolutionary optimization of the sterol properties [153]. Building on this idea, we designed a new sterol with all methyl groups

removed - 18-19-di-nor-cholesterol (Dchol) (see Fig. 9b) [154] and a few sterols with a single-methyl group removed [155], expecting further improvement of sterol properties. MD simulations, however, showed that methyl groups are important elements that maintain sterol orientation in membranes and their removal actually decreases sterols' ordering abilities. Our initial results were recently confirmed experimentally. Dchol synthesis, although complicated, was shown to be possible. Synthesis required 18 steps from commercially available compounds and its yield is 3.5% [156]. Biophysical studies showed slightly lower-condensing properties of Dchol and generally weaker effects on bilayer properties [157]. A more expanded description of this case can be found in Rog et al. (2015) [27].

Only the polar part of a Chol molecule is a 3β- hydroxyl group, which is present in the majority of the natural membrane-active sterols (e.g., typical for fungus ergosterol or plant sterol camposterol). The only natural steroid present in significant amounts in a biological membrane with an altered head group is cholesterol sulphate (CS). Properties of CS showed that its effects on bilayer properties are weaker than that of Chol, likely due to a shift toward the water phase of the location of the polar part [158, 159]. Few steroids with a synthetically modified polar group are known and their properties were reported to be different from Chol. For example cholesteryl PC (see Fig. 9h) was shown to form bilayers with Chol, di-acyl-glycerol, and ceramide [160]. Next, the cholesteryl PC-ordering effect in the POPC bilayer was much weaker than of that of Chol. Thio-cholesterol (see Fig. 9k), a steroid with the OH group substituted with a slightly less polar SH group, was shown to have a slightly weaker effect on the bilayer properties [161]. In the next two paragraphs, we offer two examples of more extensively studied cases of sterols with modified a headgroup: epicholesterol and cholesteryl hemisuccinate.

Epicholesterol (see Fig. 9i), an epimeric form of Chol with 3α- hydroxyl group, does not occur in nature. A few older studies showed that epicholesterol effects on membrane ordering,

passive permeability and phase state are weaker in comparison to Chol [138, 162, 163]. The results of MD simulations' study were in line with these experimental data: epicholesterol ordering and condensing effects were shown to be weaker than effects of Chol [164]. The proposed mechanism of decreasing the strength of sterol-phospholipid interaction was to shift the polar part of the sterol toward the water phase. This mechanism is in line with the much higher spontaneous transfer rate between liposomes of epicholesterol than Chol [165]. More recent calorimetric studies, however, showed that the epicholesterol effect on phase behavior in comparison to Chol depends on sterol concentration [166]. In lower concentrations, epicholesterol affects the phase behavior more than Chol, but at higher concentrations (30-50 mol%), the effects of Chol are stronger. Not surprisingly, epicholesterol was shown not to be able to substitute Chol in its interaction with the serotonin 1A receptor necessary for receptor activity [151].

Cholesteryl hemisuccinate (see Fig. 9f) (CHS) is a detergent that is frequently used to substitute Chol in crystallography and in biochemical studies of G-protein couplet receptors [18, 167-170]. The choice of CHS instead of Chol is dictated by its higher solubility; thus, it is easier to handle in laboratory conditions. CHS was shown to stabilize liposomes [171, 172] and decrease the fluidity of the cell and model membranes [173, 174]. MD simulations of CHS embedded into saturated PCs showed that CHS affects lipid properties less than Chol, in particular its charge form, which is dominant in neutral pH and was less effective [175]. For the case of the unsaturated bilayer, the neutral form of CHS was almost as effective as Chol, but the effects of the charge form was clearly weaker than that of Chol [176]. Time-resolved fluorescence anisotropy of diphenylhexatriene measurements confirmed that CSH is less effective than Chol in ordering the lipid bilayer. MD simulations showed that the tilt of the sterol ring of CSH is larger than that of Chol, which is one of the determinants of sterols' ordering capability [177, 178]. CSH was also shown to protrude more into the water phase than

Chol, particularly in the charge form that is translocated more toward water. All of these effects showed that CSH is not able to fully substitute Chol. Cationic analog of CSH DC-Chol (3β-[*N*-(dimethylaminoethane)-carbamoyl]-cholesterol) (see Fig. 9j) was also synthetized and used, such as for immune-stimulating complex (ISCOM) formulation [179, 180] and in monolayer studies [180]; however, its bilayer properties are not well characterized. Another similar but neutral moiety is attached to the so-called exchangeable Chol (see Fig. 9g) used in nearest-neighbor recognition assay [181]. In this form of sterol, the short tail is ended with a reactive thiol group.

The last structural sterol element, the alkyl tail, shows certain diversity in naturally occurring sterols, but the isooctyl tail seems be superior over other natural variants. The characteristic feature of the Chol tail is branching at its end. The role of this feature was recently examined by using synthetic Chol analogs with unbranched tails that were 3–14 carbon atoms long (see Fig. 9d, e) [182]. Chol was shown to have the strongest ordering effect and sterol, with an unbranched, 12-carbon tail, had ordering properties closest to Chol in the POPC bilayer. In the DPPC bilayer, sterols with tails that varied in length from 7 to 13 contained ordered bilayers to a similar degree with Chol. Surprisingly, sterols' effect on bilayer permeability and lateral diffusion were not correlated with sterols' ordering capability. A clickable version of Chol (see Fig. 9c) with a click group located in the Chol tail is in use for Chol-protein studies, but the effect of this modification on the sterol behavior is not known [183].

5.2. Sphingomyelin

SPMs, a class of lipids with numerous biological functions [184], have a structure based on sphingosine, an amino alcohol with an 18-carbon-atom chain and a *trans* unsaturated bond at position 4–5 (see Fig. 10a, e). The hydrocarbon tail is attached via an amide bond and PC headgroup via an ester bond. Due to this, SPM differs significantly from glycerol-based lipids, which are the dominant lipids group. SPMs have hydroxyl and amide groups that are both

capable of forming H bonds as a donor; these groups in a lipid bilayer are located at the water membrane interface. A few naturally occurring modifications of sphingosine are possible; for example, a species lacking a *trans* unsaturated bond or a species with additional hydroxyl groups exist (for a more comprehensive review of SPM structure, see [185]). PC is not the only possible headgroup for sphingolipids, ceramide species in which the hydroxyl group is not esterified is also a base for glycolipids and inositols. Natural variants of SPM provide an opportunity to understand the importance of modified groups and were intensively studied (for review, see [186, 187]) but few synthetic species of SPM provided additional insight into the importance of the SPM structure.

The PC headgroup is connected with ceramide via an ester bond in all natural PSMs. The role of the chemical nature of this connection was examined via studies of species with oxygen atoms replaced with sulfur atom (PSM-S) (see Fig. 10l), NH (PSM-NH) (see Fig. 10h) and CH2 (PSM-CH2) groups (see Fig. 10d) [188]. For the PSM-S, an increase in the stability of the bilayer and decreased polarity at the membrane-water interface region were observed. Opposite results were noted for both PSM-NH and PSM-CH2; membrane stability was decreased and interface polarity increased. While interactions of PSM and its analogs with Chol were studied, it was found that all lipids form a Chol-rich domain; however, for the case of PSM-NH and PSM-CH2, lower thermal stability of the formed domain was observed.

PC is a relatively large headgroup, mainly due to the bulky nature of the choline group. The importance of the PSMs' headgroup size was studied with PSM analogs in which methyl groups of choline were substituted with hydrogen atoms one by one (modified PSM with 2 methyl group and 1 hydrogen CPE-Me2 (see Fig. 10b), with 1 methyl group and 2 hydrogens CPE-Me1 (see Fig. 10f), and with 3 hydrogens CPE (see Fig. 10j)), where the last lipid in the series is phosphatidylethanolamine [189]. Experimental calorimetric studies showed that with the increasing number of methyl groups, the temperature of the main-phase transition is lowered

while corresponding MD simulations showed that the area per lipid is larger and the order of the acyl tail is lower. Interactions of PSM and its analogs with Chol were also affected by the number of methyl groups— the affinity of sterols was higher for species with a larger number of methyl groups. MD simulations showed that the Chol ordering effect was larger in a bilayer composed of species with a larger number of methyl groups. Moreover, MD simulations showed an increasing number of polar interactions between Chol and lipids for the species with a larger number of methyl groups. These results are in agreement with the so-called umbrella model, one possible mechanism of Chol condensing and ordering effects [190]. In this mechanism, the PC headgroup acts as an umbrella preventing the Chol hydrophobic part from unfavorable interactions with water. Another lipid which has a small cross-section area of the polar groups compared to the hydrophobic tail is ceramide. Ceramide is known to have condensing properties and the umbrella model was also suggested to be mechanism of this effect [191]. For this reason, one could expect similarities to the Chol behavior described above, but differential scanning calorimetry and fluorescence spectroscopy showed that the size of headgroup—for the case of PSM and its analogs with a lower number of methyl groups—does not affect ceramide interaction with these lipids [192]. Moreover, it was shown that sphingomyelinase from *Bacillus cereus* was capable of degrading CPE-Me2, but not CPE-Me1 and CPE [193].

Phosphatidylserine is a frequent negatively charged headgroup in glycerol-based lipids, but is not known to occur as a headgroup of sphingolipids. Nevertheless, synthetic sphingolipid with phosphatidylserine (SSM) (see Fig. 10i) was synthetized and its interactions with Chol were examined [194]. SSM was found to form a Chol-rich domain slightly less effectively than PSM, contrary to glycerol-based phosphatidylserine.

SPM has two hydrogen bonding groups, OH and NH, which are located at the water membrane interface. The role of these groups was studied utilizing three synthetic analogs in

which these groups were methylated (PSM-Ome (see Fig. 10c), PSM-Nme (see Fig. 10g), and PSM-ONme (see Fig. 10k)). In one analog, both groups were methylated and, in two others, OH or NH groups were methylated [195]. The presence of methylation destabilized the gel phase and decreased the temperature of the main-phase transition. MD simulations showed that, in the fluid phase, methylation led to surface area expansion and a decrease of the order parameter, particularly for the case of an analog with both groups methylated. The methylation of the OH group almost completely abolishes the formation of the sterol-rich ordered domain, while methylation of NH does not abolish domain formation but, instead, domains were less thermostable. Chol affinity toward membranes formed by methylated analogs was much lower than for the case of SPM. Further MD simulations showed much weaker polar interactions between Chol and methylated analogs than SPM. Methylation of the NH group was found to be particularly important for interactions of PSM with ceramide [196]. Methylation of the OH group was not affecting the degradation of this lipid by sphingomyelinase from *Bacillus cereus*, but NH methylation was reducing the rate of degradation [193]. Finally, the methylation of any of the group abolished membrane pore formation by toxin sticholysin II derived from sea anemone *Stichodactyla heliantus* known to specifically interact with PSM [197].

N-cholesteryl sphingomyelin (SPM-Chol) is a synthetic sphingolipid with amide link tail substituted by Chol carbamate [198] similar to that discussed above (paragraph 2.1) in the context of drug delivery glycerol-based PC-cholesteryl constructs (see Fig. 2). SPM-Chol was found to be able to form vesicles of various radiuses that were resistant to Triton X-100. SPM-Chol in mixture with other lipids had ordering properties, although not as strong as Chol. Unlike SPM, SPM-Chol in mixture with POPC is not capable of forming sterol-rich ordered domains. Similar to glycerol-based PC-cholesteryl, SPM-Chol seems be a good candidate for use in drug delivery, due to its high stability, ordering properties and resistance to phospholipases A2 [198].

5.3. Invers zwitterionic lipids

Naturally occurring zwitterionic lipids have a phosphate group attached to a glycerol backbone and then a positively charged group (ethanolamine or PC) attached to a phosphate group. Due to this configuration, the positively charged group is more dynamic and protrudes more at the water phase. Inverse zwitterionic lipids, or lipids with reverse order of positively and negatively charged groups, do not exist in nature but were synthetized as a possible candidate for use in drug delivery system. Until now, however, their applications have been rather limited, but they provide an additional understanding of the natural lipids. In all, synthetized lipids' choline group was first attached to the glycerol backbone and next charge group – phosphate, methyl phosphate, carboxyl, sulfonate or sulfate (see Fig. 11) [199-201]. The phase behavior of these lipids differs significantly from PCs; the temperature of the main-phase transition is very high, which is not expected for lipids with large headgroups. Interactions of these lipids with cations are significantly reduced. This effect is particularly important for Ca++, as it is able to destabilize liposomes and induce fusion. MD simulations of inverse PC (with methyl phosphate) were in agreement with experimental studies [202]. Interactions with Na+, K+, Ca++ anions were reduced. Next, the water layer at the water membrane interface was differently ordered than in natural PCs and the profile of electrostatic potential was significantly altered. The reverse of the groups strongly affected the hydration of the bilayer. The most important observation concerns the carbonyl groups of lipids which attract water in natural PCs, but were almost dehydrated in the reverse lipid case. This might be the reason for the higher temperature of main phase transition since water in the carbonyl group significantly affects the bilayer order.

6. Summary

In this review, we have discussed several applications of synthetic lipids in pure and applied science with a focus on studies where atomistic MD simulations contributed for obtaining

understanding into atomistic level mechanisms associated with new functionalities of synthetic lipids. Examples discussed in this paper clearly document the usefulness of MD simulations. This provides additional information useful to the interpretation of experimental results (reporting molecules) and additionally suggests possible mechanisms responsible for both success and failure of synthetic lipids in practical applications like drug delivery, allowing us to better understand the relationship between lipids structure and their properties. Moreover, MD simulations have been shown to have predictive power, for example, the case of cholesterol analogs. The methodology of MD simulations is still under intensive development and from time to time spectacular failures have been described in the literature (discussed in the article by Karttunen in this issue of Biochim. Biophys. Acta). On the other hand, the accuracy of the force fields for lipids has been greatly improved over few last years (discussed in the article by Lyubartsev in this issue of Biochim. Biophys. Acta) and methodological issues seem to be well understood. Finally, time and size scales that can be achieved in simulations nowadays are definitely appropriate for kind of problems we have described. Taking into account all these facts one should expect further intensive study of synthetic lipids and surfactants with MD simulations as a routine method.

ACKNOWLEDGMENTS:

The European Research Council (Advanced Grant project CROWDED-PRO-LIPIDS) (TR), the Academy of Finland Center of Excellence program (TR), and the National Science Centre Poland (decision number DEC-2012/07/B/ST5/00913) (MK) are thanked for financial support. CSC − Finnish IT Centre for Scientific Computing (Espoo, Finland) is acknowledged for its computer resources. We would like to thanks Sami Risanen for help with figures preparation.

Figure Legend

Figure 1. "Images of the systems consisting of p-THPP molecules in DLPC/DLPE-PEG bilayers: four p-THPP molecules (system M3) at (a) $t = 0$ and (b) after 100 ns of simulation; two p-THPP molecules (system M4) at (c) $t = 0$ and (d) after 350 ns; and six p-THPP molecules (system M5) at (e) $t = 0$ and (f) after 350 ns. The porphyrin molecules are shown in red as a licorice representation. DLPC molecules are shown as blue sticks, with black spheres for phosphate groups. DLPE-PEG lipids are shown as beige sticks. For clarity, water and ions are not shown" (reprinted with permission from Dzieciuch et al. 2015).

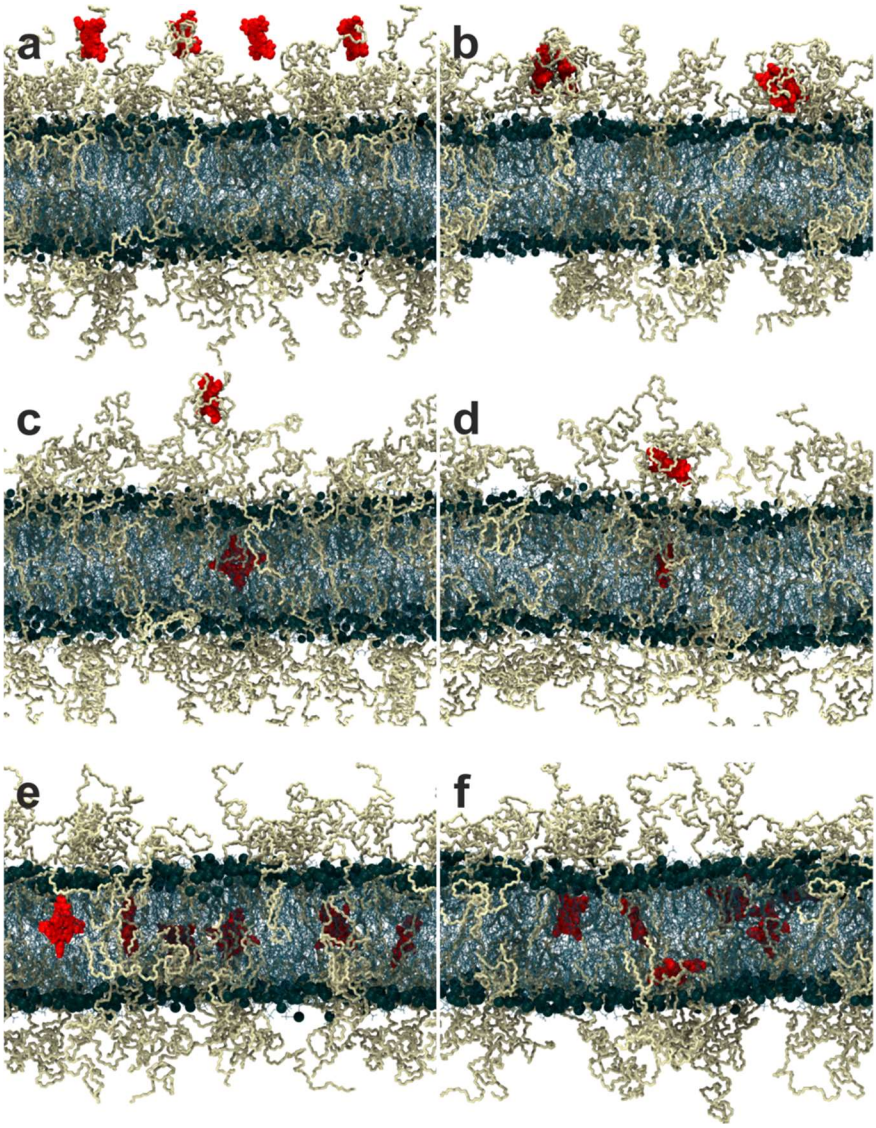


Figure 2. Structure of the cholesterol-modified lipids: (a) S*n2*-cholesteryl phosphatidylcholine; (b) di-cholesteryl phosphatidylcholine; (c) N-cholesteryl sphingomyelin. Color code: light blue – carbon, white – hydrogen, dark blue – nitrogen, red – oxygen, brown – phosphorus, and yellow/orange sulfur. Figure prepared with VMD [203].

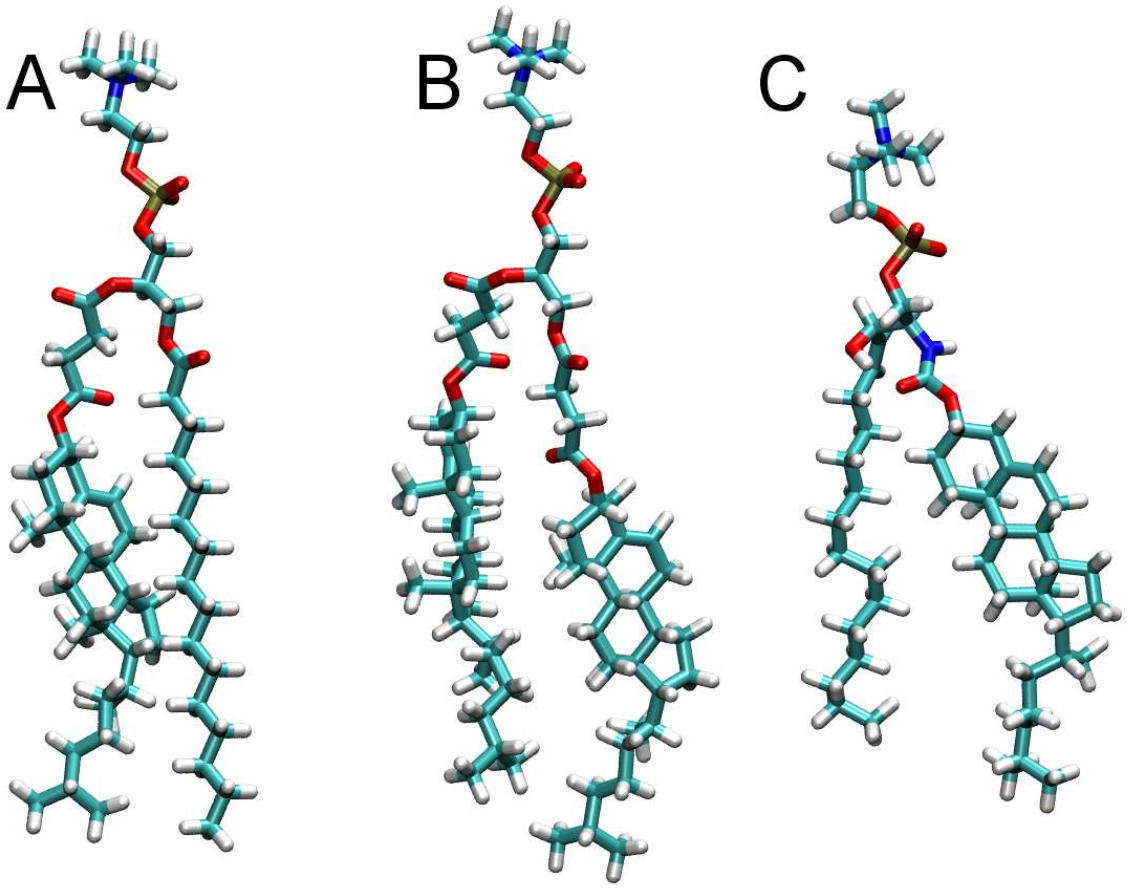


Figure 3. "Short-chain phosphatidylcholines dynamically assemble into a molecular gateway for doxorubicin. (A), (B) Snapshots of simulations of a pure DPPC bilayer (A) or a 451 DPPC:DOctPC bilayer (B). Doxorubicin was localized at the center of the bilayer by a harmonic potential. During doxorubicin traversal, the short-chain lipids assemble a transient membrane channel. Doxorubicin is in red, water in blue, DPPC in yellow and DoctPC in green; lipid headgroups are represented by a single bead at the phosphorus position. Increased therapeutic window of doxorubicin by GC-mediated membrane modulation. (C): From the liposomal vehicle, fDox (red) and free GC (green) leak into the interstitial fluids and partition into the (tumour) cell membrane (top cartoon). fDox plasma peak levels are much reduced by liposomal formulation (thinner red arrows left of membrane), but doxorubicin entry in the tumour cell is low in absence of GC (middle cartoon). GC, when co-inserted into the membrane, enhances membrane traversal of doxorubicin (thickened red arrow right of membrane) and thus, accumulation into tumour cell DNA" (reprinted with permission from van Hell et al. 2013).

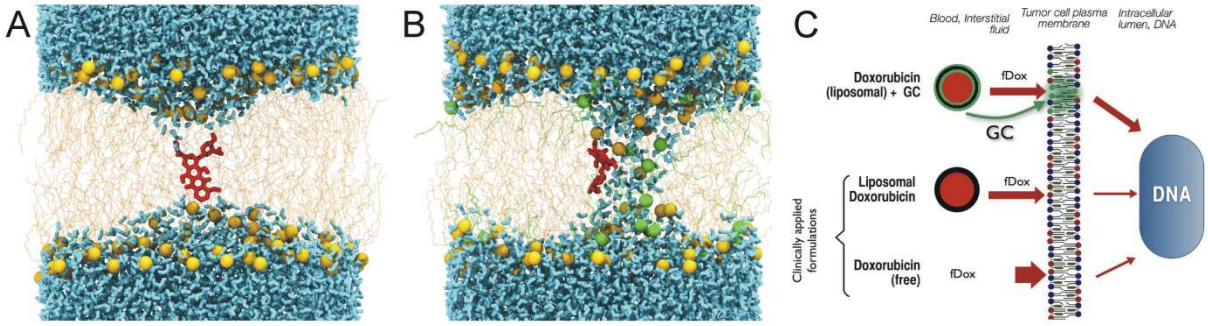


Figure 4. (a) "Lipid bilayer of a 1:1:1 ternary mixture of ceramide (green), cholesterol (yellow), and free fatty acid (blue) with 0.1000 mole fraction oleic acid (orange) (300 K) shown (reprinted with permission from Hoopes et al. 2011)." **(**b**)** Snapshots of the ceramide bilayer at 305 K and structure of the ceramide molecule (reprinted with permission from Guo et al. 2013).

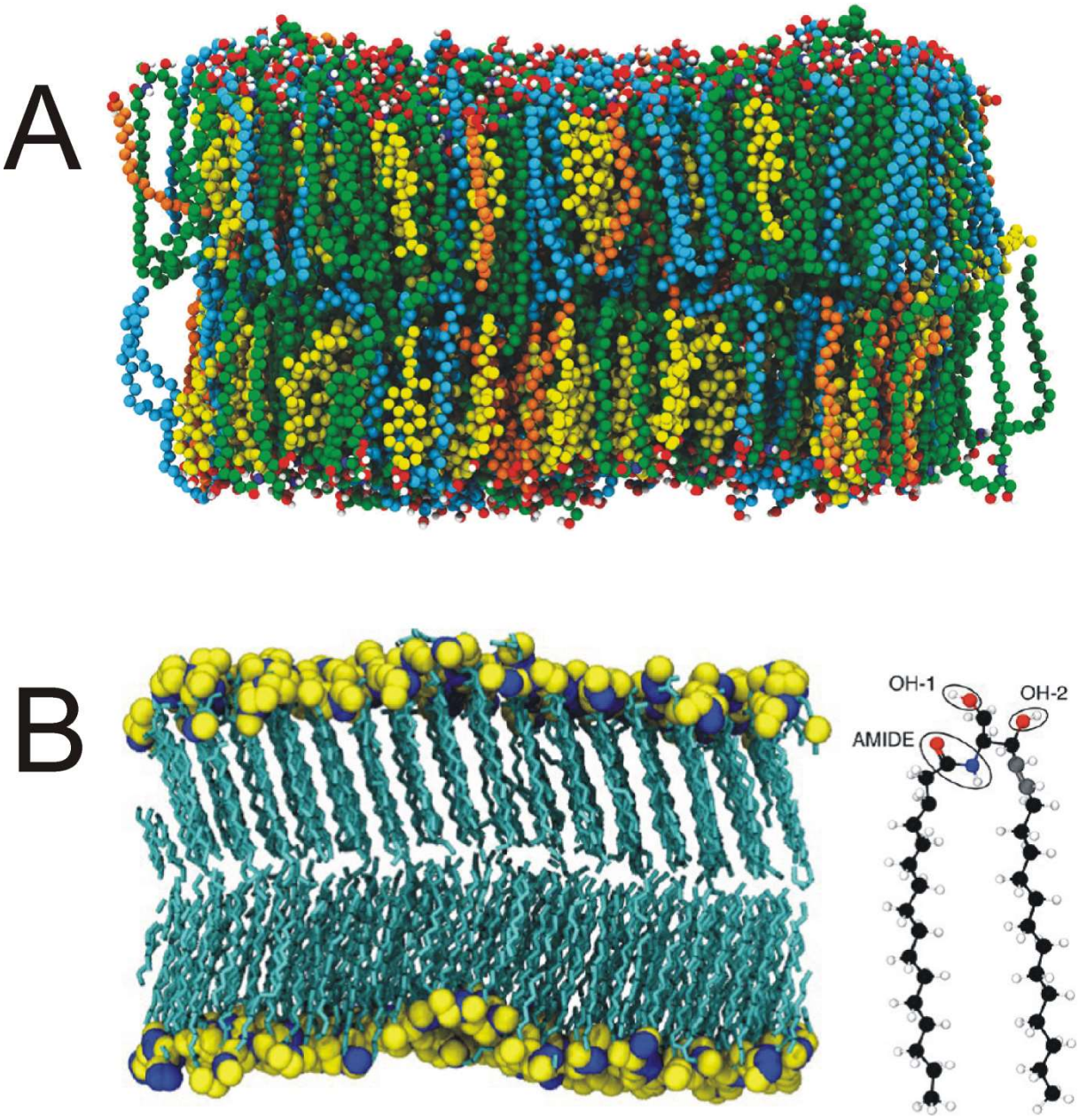


Figure 5. Structures of cationic lipids mentioned in this article.

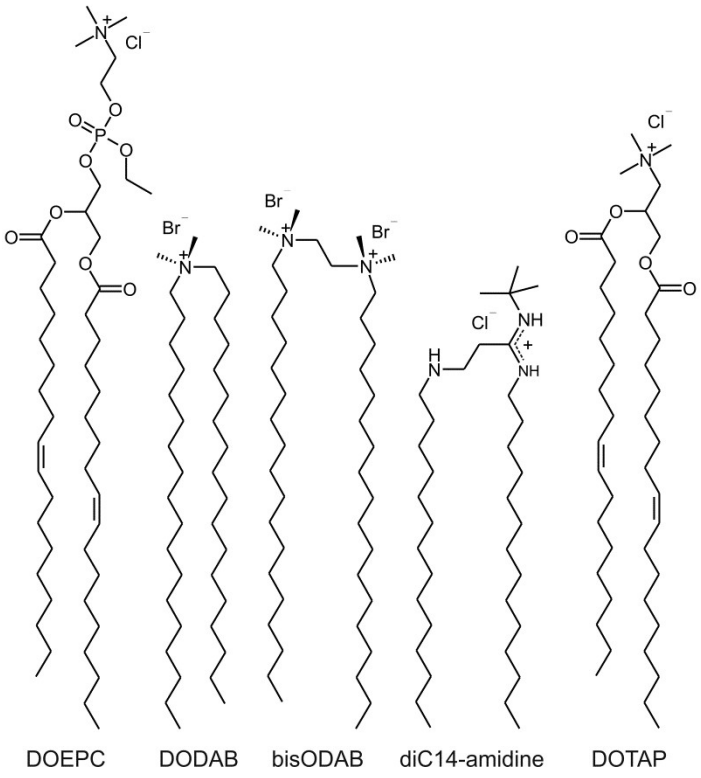


Figure 6. Structures of membrane probes mentioned in this article. BODIPY, NBD, and Pyr are shown in red, blue, and green, respectively. The numbering system for BODIPY dyes is also shown.

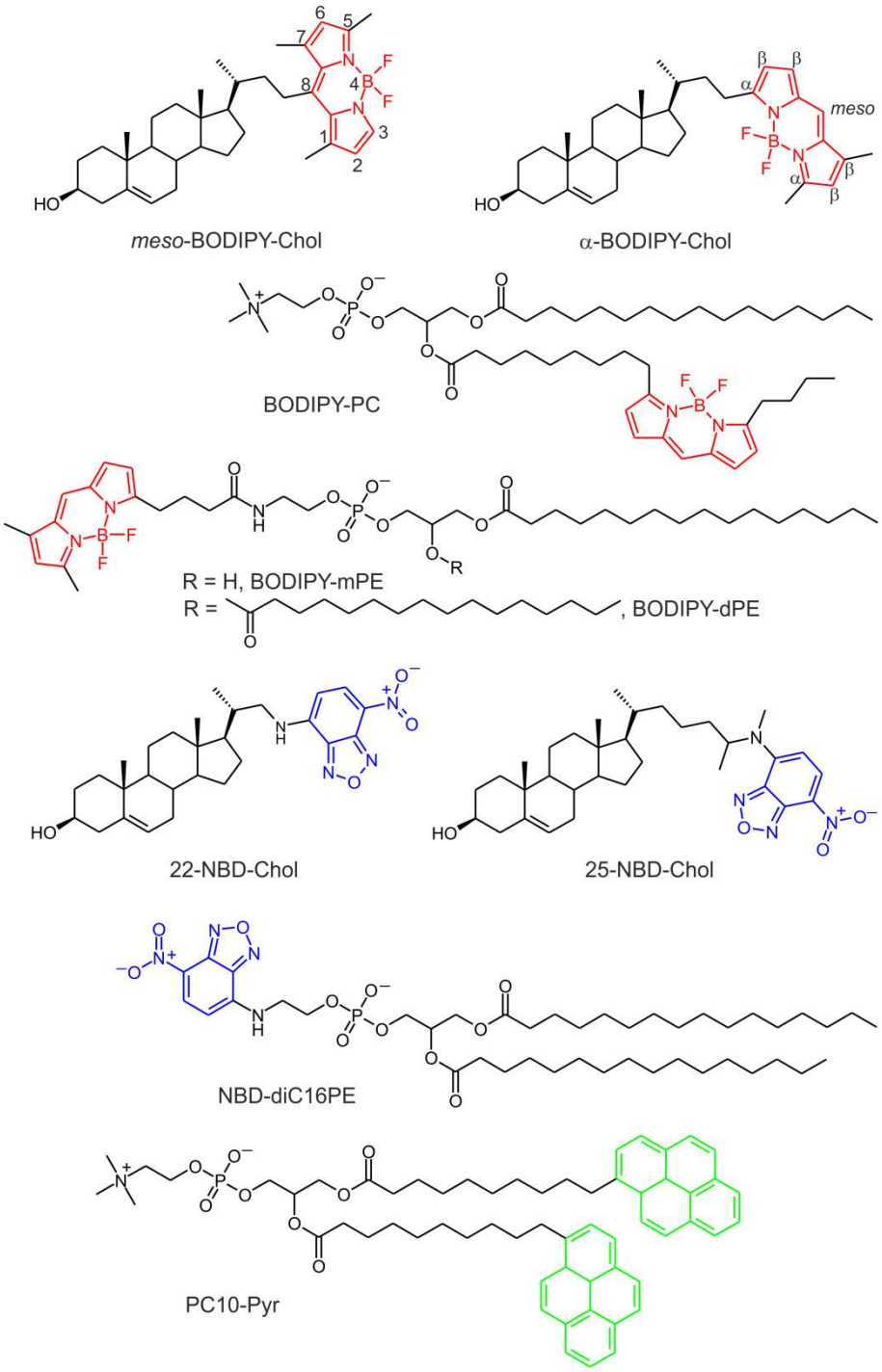


Figure 7. Structures of fluorescent cholesterol analogs and surfactants mentioned in this article. Hexatrien chromophore in the dehydroergosterol (DHE) or cholestatrienol (CTL) structures and NBD are shown in green and blue, respectively.

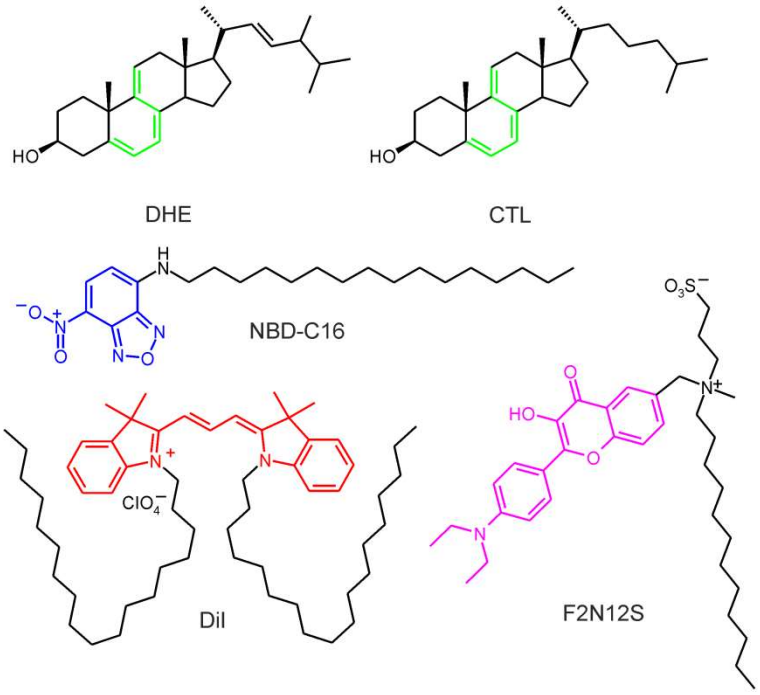


Figure 8. Structures of spin-labeled probes mentioned in this article. Tempo and Doxyl groups are shown in green and red, respectively.

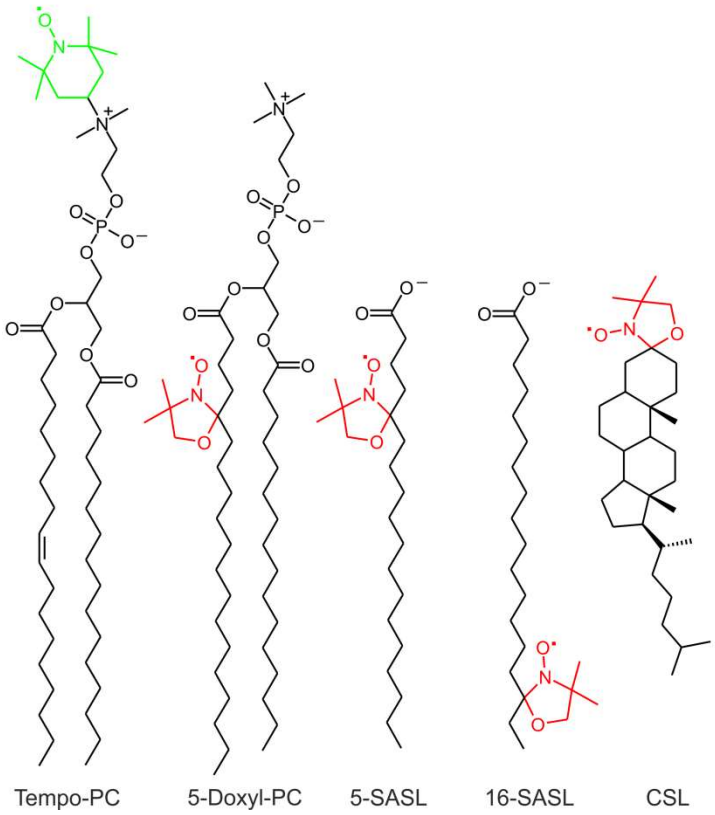


Figure 9. Structures of cholesterol (a), Dchol (b), clickable cholesterol (c), cholesterol with 3 carbons tail (d), cholesterol with 14 carbons tail (e), cholesteryl succinate (f), exchangeable cholesterol (g), DC-chol (h), epicholesterol (i), cholesteryl phosphatidylcholine (j), and thio-cholesterol (k). Color code is same as in Fig. 2. Figure prepared with VMD [203].

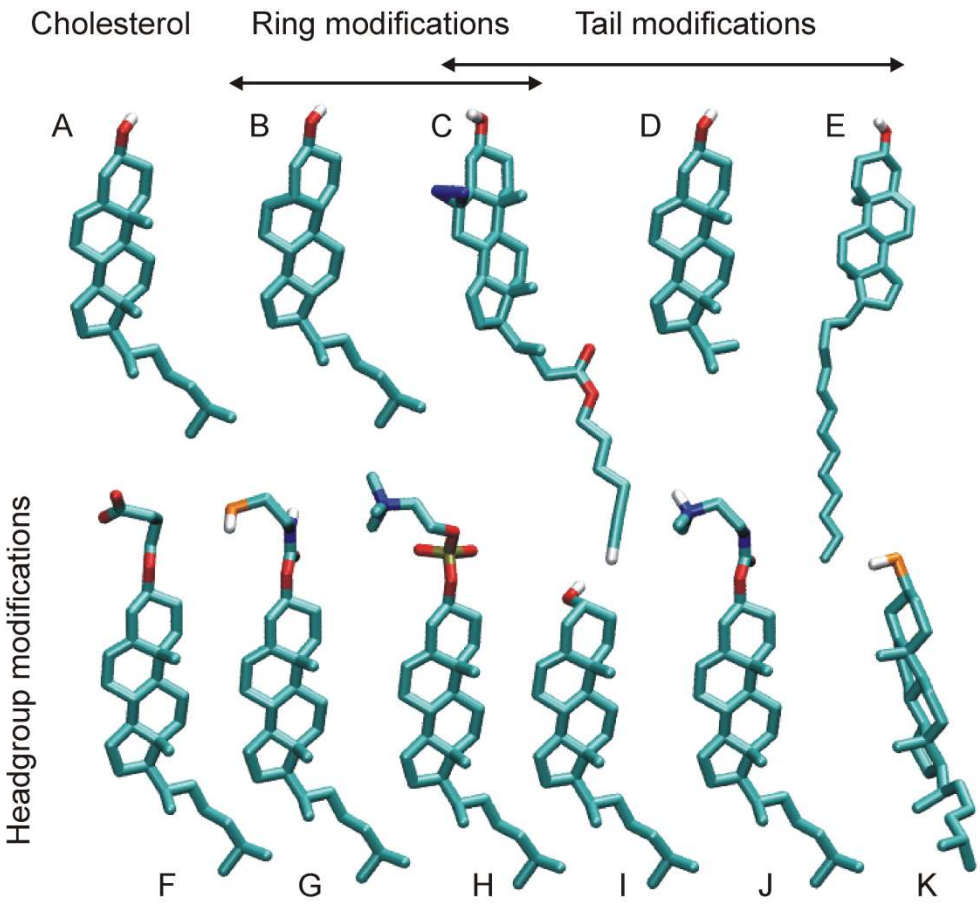


Figure 10. Structures of sphingomyelin (a, e) (double bond shown in green at panel (e)), CPE-Me2 (b), PSM-OMe (c), PSM-CH2 (d), CPE-Me1 (f), PSM-NMe (g), PSM-NH (h), SSM (i), CPE (j), PSM-ONMe (k), and PSM-S (g). Arrows indicates modified groups. Acyl tails are cut for clarity. Color code is same as in Fig. 2. Figure prepared with VMD [203].

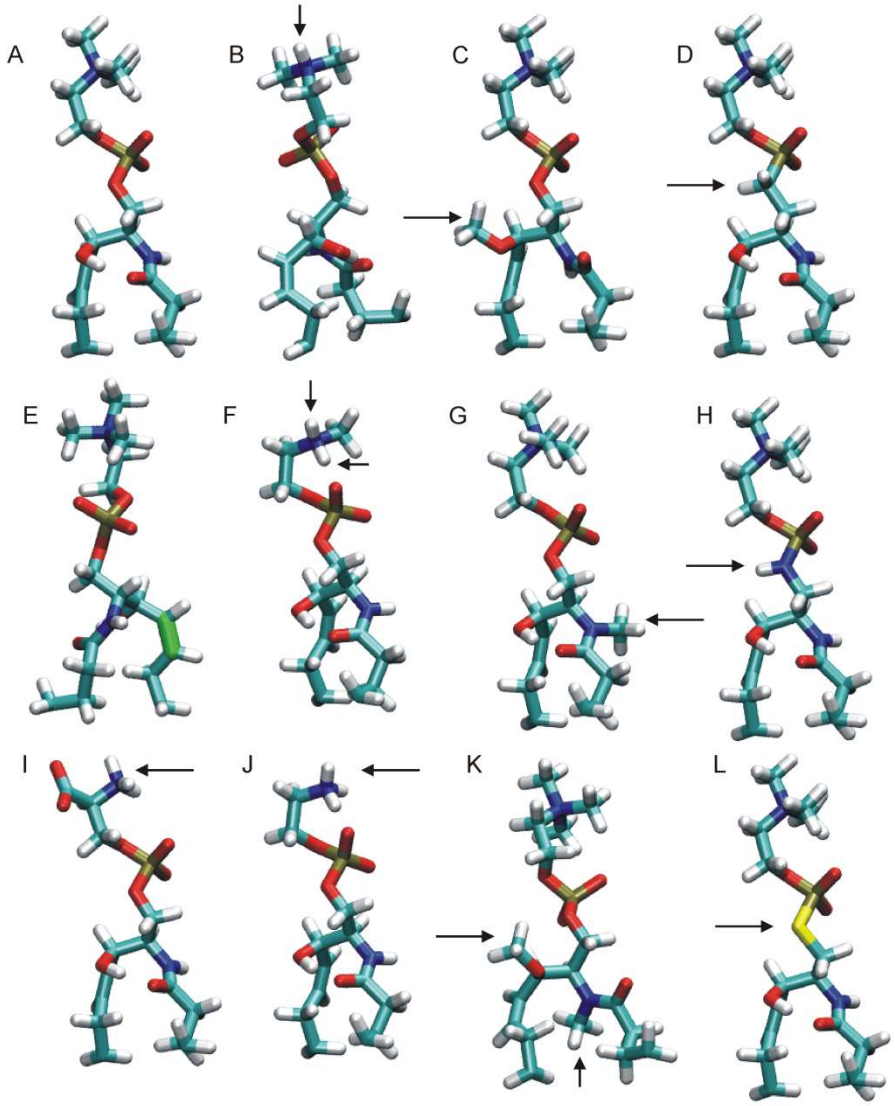
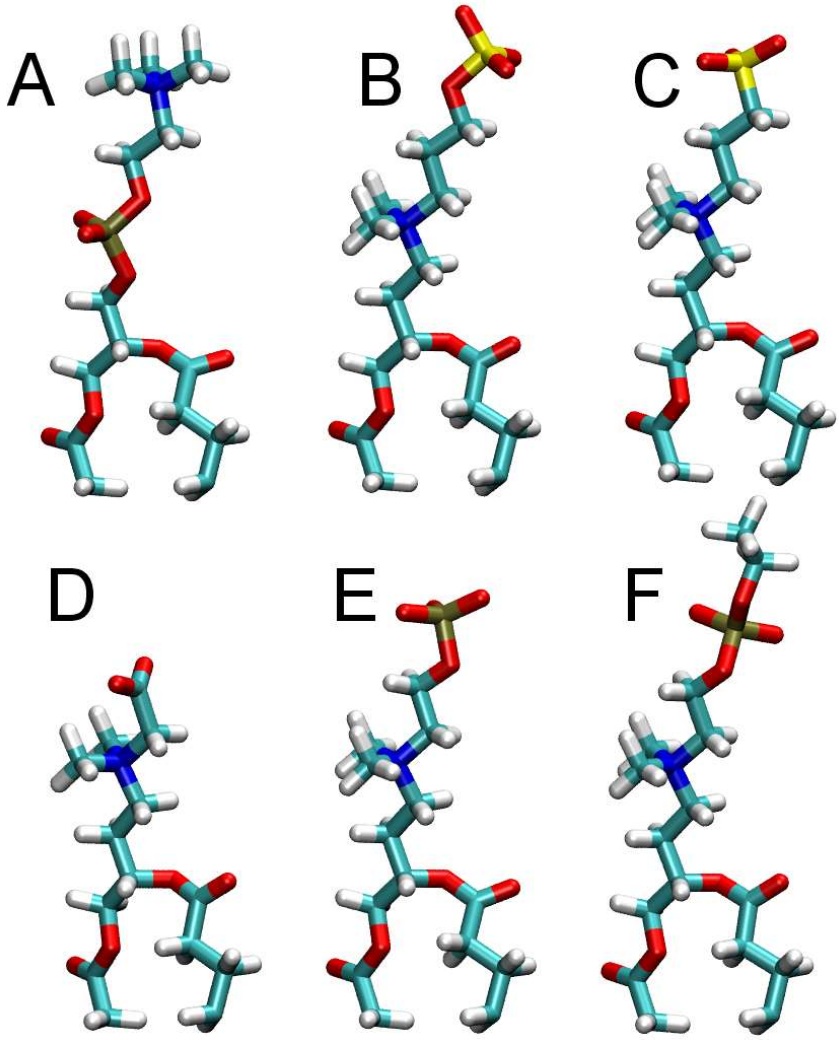


Figure 11. Structures of phosphatidylcholine (a) and charge reverse lipids: choline sulfates (b) choline sulfonates (c) choline carboxylate (d), choline phosphate (e), choline ethyl-phosphate (f). Acyl tails are cut for clarity. Color code is same as in Fig. 2. Figure prepared with VMD [203].



References

[1] J.M. Berg, J.L. Tymczko, L. Stryer, Biochemia, Wydawnictwo Naukowe PWN, Warszawa, 2005, p. 322.

[2] G. van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipids: where they are and how they behave, Nature Reviews Molecular Cell Biology, 9 (2008) 112-124.

[3] A. Llorente, T. Skotland, T. Sylvanne, D. Kauhanen, T. Rog, A. Orlowski, I. Vattulainen, K. Ekroos, K. Sandvig, Molecular lipidomics of exosomes released by PC-3 prostate cancer cells, Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids, 1831 (2013) 1302-1309.

[4] K.H. Pietilainen, T. Rog, T. Seppanen-Laakso, S. Virtue, P. Gopalacharyulu, J. Tang, S. Rodriguez-Cuenca, A. Maciejewski, J. Naukkarinen, A.-L. Ruskeepaa, P.S. Niemela, L. Yetukuri, C.Y. Tan, V. Velagapudi, S. Castillo, H. Nygren, T. Hyotylainen, A. Rissanen, J. Kaprio, H. Yki-Jarvinen, I. Vattulainen, A. Vidal-Puig, M. Oresic, Association of Lipidome Remodeling in the Adipocyte Membrane with Acquired Obesity in Humans, Plos Biology, 9 (2011).

[5] A.G. Kohli, P.H. Kierstead, V.J. Venditto, C.L. Walsh, F.C. Szoka, Designer lipids for drug delivery: From heads to tails, Journal of Controlled Release, 190 (2014) 274-287.

[6] A. Bunker, Poly(ethylene glycol) in drug delivery, why does it work, and can we do better? All atom molecular dynamics simulation provides some answers, Proceedings of the 25th Workshop on Computer Simulation Studies in Condensed Matter Physics, 34 (2012) 24-33.

[7] P.P. Karmali, A. Chaudhuri, Cationic Liposomes as non-viral carriers of gene medicines: Resolved issues, open questions, and future promises, Medicinal Research Reviews, 27 (2007) 696-722.

[8] J.P. Douliez, A. Leonard, E.J. Dufourc, RESTATEMENT OF ORDER PARAMETERS IN BIOMEMBRANES - CALCULATION OF C-C BOND ORDER PARAMETERS FROM C-D QUADRUPOLAR SPLITTINGS, Biophysical Journal, 68 (1995) 1727-1739.

[9] W.K. Subczynski, A. Wisniewska, Physical properties of lipid bilayer membranes: relevance to membrane biological functions, Acta Biochimica Polonica, 47 (2000) 613-625.

[10] W.K. Subczynski, J. Widomska, A. Wisniewska, A. Kusumi, Saturation-recovery electron paramagnetic resonance discrimination by oxygen transport (DOT) method for characterizing membrane domains, Methods in molecular biology (Clifton, N.J.), 398 (2007) 143-157.

[11] W.K. Subczynski, A. Wisniewska, J.J. Yin, J.S. Hyde, A. Kusumi, HYDROPHOBIC BARRIERS OF LIPID BILAYER-MEMBRANES FORMED BY REDUCTION OF WATER PENETRATION BY ALKYL CHAIN UNSATURATION AND CHOLESTEROL, Biochemistry, 33 (1994) 7670-7681.

[12] O. Maier, V. Oberle, D. Hoekstra, Fluorescent lipid probes: some properties and applications (a review), Chemistry and Physics of Lipids, 116 (2002) 3-18.

[13] S. Alam, D.S. Alves, S.A. Whitehead, A.M. Bayer, C.D. McNitt, V.V. Popik, F.N. Barrera, M.D. Best, A Clickable and Photocleavable Lipid Analogue for Cell Membrane Delivery and Release, Bioconjugate Chemistry, 26 (2015) 1021-1031.

[14] L.M.S. Loura, J.P. Prates Ramalho, Recent Developments in Molecular Dynamics Simulations of Fluorescent Membrane Probes, Molecules, 16 (2011) 5437-5452.

[15] P. Hakansson, P.O. Westlund, E. Lindahl, O. Edholm, A direct simulation of EPR slow-motion spectra of spin labelled phospholipids in liquid crystalline bilayers based on a molecular dynamics simulation of the lipid dynamics, Physical Chemistry Chemical Physics, 3 (2001) 5311-5319.

[16] A.L. Parrill, Synthetic lipids and their role in defining macromolecular assemblies (vol 191, pg 38, 2015), Chemistry and Physics of Lipids, 191 (2015) 39-39.

[17] A. Minnich, X. Collet, A. Roghani, C. Cladaras, R.L. Hamilton, C.J. Fielding, V.I. Zannis, SITE-DIRECTED MUTAGENESIS AND STRUCTURE-FUNCTION ANALYSIS OF THE HUMAN APOLIPOPROTEIN-A-I - RELATION BETWEEN LECITHIN-CHOLESTEROL ACYLTRANSFERASE ACTIVATION AND LIPID-BINDING, Journal of Biological Chemistry, 267 (1992) 16553-16560.

[18] A.A. Thompson, J.J. Liu, E. Chun, D. Wacker, H. Wu, V. Cherezov, R.C. Stevens, GPCR stabilization using the bicelle-like architecture of mixed sterol-detergent micelles, Methods, 55 (2011) 310-317.

[19] P.J. Loll, Membrane proteins, detergents and crystals: what is the state of the art?, Acta Crystallographica Section F-Structural Biology Communications, 70 (2014) 1576-1583.

[20] I. Vattulainen, T. Rog, Lipid Simulations: A Perspective on Lipids in Action, Cold Spring Harbor Perspectives in Biology, 3 (2011).

[21] J.B. Klauda, V. Monje, T. Kim, W. Im, Improving the CHARMM Force Field for Polyunsaturated Fatty Acid Chains, Journal of Physical Chemistry B, 116 (2012) 9424-9431.

[22] B.D. Madej, I.R. Gould, R.C. Walker, A Parameterization of Cholesterol for Mixed Lipid Bilayer Simulation within the Amber Lipid14 Force Field, Journal of Physical Chemistry B, 119 (2015) 12424-12435.

[23] J.P.M. Jambeck, A.P. Lyubartsev, Derivation and Systematic Validation of a Refined All-Atom Force Field for Phosphatidylcholine Lipids, Journal of Physical Chemistry B, 116 (2012) 3164-3179.

[24] A. Maciejewski, M. Pasenkiewicz-Gierula, O. Cramariuc, I. Vattulainen, T. Rog, Refined OPLS All-Atom Force Field for Saturated Phosphatidylcholine Bilayers at Full Hydration, Journal of Physical Chemistry B, 118 (2014) 4571-4581.

[25] W. Kulig, M. Pasenkiewicz-Gierula, T. Rog, Topologies, structures and parameter files for lipid simulations in GROMACS with the OPLS-aa force field: DPPC, POPC, DOPC, PEPC, and cholesterol, Data in brief, 5 (2015) 333-336.

[26] A. Botan, F. Favela-Rosales, P.F.J. Fuchs, M. Javanainen, M. Kanduc, W. Kulig, A. Lamber, C. Loison, A. Lyubartsev, M.S. Miettinen, L. Monticelli, J. Maatta, O.H.S. Ollila, M. Retegan, T. Rog, H. Santuz, J. Tynkkynen, Toward Atomistic Resolution Structure of Phosphatidylcholine Headgroup and Glycerol Backbone at Different Ambient Conditions, Journal of Physical Chemistry B, 119 (2015) 15075-15088.

[27] T. Rog, S. Poyry, I. Vattulainen, Building Synthetic Sterols Computationally - Unlocking the Secrets of Evolution?, Frontiers in bioengineering and biotechnology, 3 (2015) 121-121.

[28] S.M. Moghimi, J. Szebeni, Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties, Progress in Lipid Research, 42 (2003) 463-478.

[29] M. Stepniewski, M. Pasenkiewicz-Gierula, T. Rog, R. Danne, A. Orlowski, M. Karttunen, A. Urtti, M. Yliperttula, E. Vuorimaa, A. Bunker, Study of PEGylated Lipid Layers as a Model for PEGylated Liposome Surfaces: Molecular Dynamics Simulation and Langmuir Monolayer Studies, Langmuir, 27 (2011) 7788-7798.

[30] A. Magarkar, E. Karakas, M. Stepniewski, T. Rog, A. Bunker, Molecular Dynamics Simulation of PEGylated Bilayer Interacting with Salt Ions: A Model of the Liposome Surface in the Bloodstream, Journal of Physical Chemistry B, 116 (2012) 4212-4219.

[31] M. Dzieciuch, S. Rissanen, N. Szydlowska, A. Bunker, M. Kumorek, D. Jamroz, I. Vattulainen, M. Nowakowska, T. Rog, M. Kepczynski, PEGylated Liposomes as Carriers of Hydrophobic Porphyrins, Journal of Physical Chemistry B, 119 (2015) 6646-6657.

[32] K. Nawalany, A. Rusin, M. Kepczynski, A. Mikhailov, G. Kramer-Marek, M. Snietura, J. Poltowicz, Z. Krawczyk, M. Nowakowska, Comparison of photodynamic efficacy of tetraarylporphyrin pegylated or encapsulated in liposomes: In vitro studies, Journal of Photochemistry and Photobiology B-Biology, 97 (2009) 8-17.

[33] K. Nawalany, B. Kozik, M. Kepczynski, S. Zapotoczny, M. Kumorek, M. Nowakowska, B. Jachimska, Properties of polyethylene glycol supported tetraarylporphyrin in aqueous solution and its interaction with liposomal membranes, Journal of Physical Chemistry B, 112 (2008) 12231-12239.

[34] T. Rog, I. Vattulainen, Cholesterol, sphingolipids, and glycolipids: What do we know about their role in raft-like membranes?, Chemistry and Physics of Lipids, 184 (2014) 82-104.

[35] M.R. Krause, S.L. Regen, The Structural Role of Cholesterol in Cell Membranes: From Condensed Bilayers to Lipid Rafts, Accounts of Chemical Research, 47 (2014) 3512-3521.

[36] D.C. Drummond, C.O. Noble, M.E. Hayes, J.W. Park, D.B. Kirpotin, Pharmacokinetics and In Vivo Drug Release Rates in Liposomal Nanocarrier Development, Journal of Pharmaceutical Sciences, 97 (2008) 4696-4740.

[37] W.F.D. Bennett, J.L. MacCallum, M.J. Hinner, S.J. Marrink, D.P. Tieleman, Molecular View of Cholesterol Flip-Flop and Chemical Potential in Different Membrane Environments, Journal of the American Chemical Society, 131 (2009) 12714-12720.

[38] M. Neuvonen, M. Manna, S. Mokkila, M. Javanainen, T. Rog, Z. Liu, R. Bittman, I. Vattulainen, E. Ikonen, Enzymatic Oxidation of Cholesterol: Properties and Functional Effects of Cholestenone in Cell Membranes, Plos One, 9 (2014).

[39] Z. Huang, M.R. Jaafari, F.C. Szoka, Jr., Disterolphospholipids: Nonexchangeable Lipids and Their Application to Liposomal Drug Delivery, Angewandte Chemie-International Edition, 48 (2009) 4146-4149.

[40] Z. Huang, F.C. Szoka, Jr., Sterol-Modified Phospholipids: Cholesterol and Phospholipid Chimeras with Improved Biomembrane Properties, Journal of the American Chemical Society, 130 (2008) 15702-15712.

[41] F. Foglia, D.J. Barlow, F.C. Szoka, Jr., Z. Huang, S.E. Rogers, M.J. Lawrences, Structural Studies of the Monolayers and Bilayers Formed by a Novel Cholesterol-Phospholipid Chimera, Langmuir, 27 (2011) 8275-8281.

[42] A. Magarkar, T. Rog, A. Bunker, Molecular Dynamics Simulation of PEGylated Membranes with Cholesterol: Building Toward the DOXIL Formulation, Journal of Physical Chemistry C, 118 (2014) 15541-15549.

[43] V. Janout, T.A. Daly, L.L. Cline, L.J. Kulp, S.L. Regen, Stimulated Release of Cholesterol from Liposomal Membranes by a PEGylated Phospholipid, Bioconjugate Chemistry, 23 (2012) 336-339.

[44] P.P. Deshpande, S. Biswas, V.P. Torchilin, Current trends in the use of liposomes for tumor targeting, Nanomedicine, 8 (2013) 1509-1528.

[45] J. Lehtinen, A. Magarkar, M. Stepniewski, S. Hakola, M. Bergman, T. Rog, M. Yliperttula, A. Urtti, A. Bunker, Analysis of cause of failure of new targeting peptide in PEGylated liposome: Molecular modeling as rational design tool for nanomedicine, European Journal of Pharmaceutical Sciences, 46 (2012) 121-130.

[46] T.M. Allen, P.R. Cullis, Liposomal drug delivery systems: From concept to clinical applications, Advanced Drug Delivery Reviews, 65 (2013) 36-48.

[47] S. Bibi, E. Lattmann, A.R. Mohammed, Y. Perrie, Trigger release liposome systems: local and remote controlled delivery?, Journal of Microencapsulation, 29 (2012) 262-276.

[48] K.A. Carter, S. Shao, M.I. Hoopes, D. Luo, B. Ahsan, V.M. Grigoryants, W. Song, H. Huang, G. Zhang, R.K. Pandey, J. Geng, B.A. Pfeifer, C.P. Scholes, J. Ortega, M. Karttunen, J.F. Lovell, Porphyrin-phospholipid liposomes permeabilized by near-infrared light, Nature Communications, 5 (2014).

[49] M. Stepniewski, M. Kepczynski, D. Jamroz, M. Nowakowska, S. Rissanen, I. Vattulainen, T. Rog, Interaction of Hematoporphyrin with Lipid Membranes, Journal of Physical Chemistry B, 116 (2012) 4889-4897.

[50] A.J. van Hell, M.N. Melo, W.J. van Blitterswijk, D.M. Gueth, T.M. Braumuller, L.R.C. Pedrosa, J.-Y. Song, S.J. Marrink, G.A. Koning, J. Jonkers, M. Verheij, Defined lipid analogues induce transient channels to facilitate drug-membrane traversal and circumvent cancer therapy resistance, Scientific Reports, 3 (2013).

[51] R. Regev, D. Yeheskely-Hayon, H. Katzir, G.D. Eytan, Transport of anthracyclines and mitoxantrone across membranes by a flip-flop mechanism, Biochemical Pharmacology, 70 (2005) 161-169.

[52] J. Fang, H. Nakamura, H. Maeda, The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect, Advanced Drug Delivery Reviews, 63 (2011) 136-151.

[53] G. van Meer, D. Halter, H. Sprong, P. Somerharju, M.R. Egmond, ABC lipid transporters: Extruders, flippases, or flopless activators?, Febs Letters, 580 (2006) 1171-1177.

[54] M.I. Hoopes, M.G. Noro, M.L. Longo, R. Faller, Bilayer Structure and Lipid Dynamics in a Model Stratum Corneum with Oleic Acid, Journal of Physical Chemistry B, 115 (2011) 3164-3171.

[55] Z. Sovova, K. Berka, M. Otyepka, P. Jurecka, Coarse-Grain Simulations of Skin Ceramide NS with Newly Derived Parameters Clarify Structure of Melted Phase, Journal of Physical Chemistry B, 119 (2015) 3988-3998.

[56] S. Guo, T.C. Moore, C.R. Iacovella, L.A. Strickland, C. McCabe, Simulation Study of the Structure and Phase Behavior of Ceramide Bilayers and the Role of Lipid Headgroup Chemistry, Journal of Chemical Theory and Computation, 9 (2013) 5116-5126.

[57] M. Paloncyova, R.H. DeVane, B.P. Murch, K. Berka, M. Otyepka, Rationalization of Reduced Penetration of Drugs through Ceramide Gel Phase Membrane, Langmuir, 30 (2014) 13942-13948.

[58] B. Skolova, K. Vavrova, K. Hudska, K. Palat, Model Stratum Corneum Membranes with Sphingosine, Phytosphingosine and Dihydrosphingosine Ceramides: Permeability & Biophysics, Journal of Investigative Dermatology, 134 (2014) S48-S48.

[59] M. Murray, A. Hraiki, M. Bebawy, C. Pazderka, T. Rawling, Anti-tumor activities of lipids and lipid analogues and their development as potential anticancer drugs, Pharmacology & Therapeutics, 150 (2015) 109-128.

[60] M. Murray, H.R.E. Dyari, S.E. Allison, T. Rawling, Lipid analogues as potential drugs for the regulation of mitochondrial cell death, British Journal of Pharmacology, 171 (2014) 2051-2066.

[61] T. Rog, A. Koivuniemi, The biophysical properties of ethanolamine plasmalogens revealed by atomistic molecular dynamics simulations, Biochimica Et Biophysica Acta-Biomembranes, 1858 (2016) 97-103.

[62] J.-P. Truman, M. Garcia-Barros, L.M. Obeid, Y.A. Hannun, Evolving concepts in cancer therapy through targeting sphingolipid metabolism, Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids, 1841 (2014) 1174-1188.

[63] S.H. Joe, Lipid A as a Drug Target and Therapeutic Molecule, Biomolecules & Therapeutics, 23 (2015) 510-516.

[64] U. Ohto, K. Fukase, K. Miyake, T. Shimizu, Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2, Proceedings of the National Academy of Sciences of the United States of America, 109 (2012) 7421-7426.

[65] S.M. Opal, P.-F. Laterre, B. Francois, S.P. LaRosa, D.C. Angus, J.-P. Mira, X. Wittebole, T. Dugernier, D. Perrotin, M. Tidswell, L. Jauregui, K. Krell, J. Pachl, T. Takahashi, C. Peckelsen, E. Cordasco, C.-S. Chang, S. Oeyen, N. Aikawa, T. Maruyama, R. Schein, A.C. Kalil, M. Van Nuffelen, M. Lynn, D.P. Rossignol, J. Gogate, M.B. Roberts, J.L. Wheeler, J.-L. Vincent, A.S. Grp, Effect of Eritoran, an Antagonist of MD2-TLR4, on Mortality in Patients

With Severe Sepsis The ACCESS Randomized Trial, Jama-Journal of the American Medical Association, 309 (2013) 1154-1162.

[66] K.A. Shirey, W. Lai, A.J. Scott, M. Lipsky, P. Mistry, L.M. Pletneva, C.L. Karp, J. McAlees, T.L. Gioannini, J. Weiss, W.H. Chen, R.K. Ernst, D.P. Rossignol, F. Gusovsky, J.C.G. Blanco, S.N. Vogel, The TLR4 antagonist Eritoran protects mice from lethal influenza infection, Nature, 497 (2013) 498-+.

[67] W. Painter, A. Robertson, L.C. Trost, S. Godkin, B. Lampert, G. Painter, First Pharmacokinetic and Safety Study in Humans of the Novel Lipid Antiviral Conjugate CMX001, a Broad-Spectrum Oral Drug Active against Double-Stranded DNA Viruses, Antimicrobial Agents and Chemotherapy, 56 (2012) 2726-2734.

[68] E. De Clercq, Clinical potential of the acyclic nucleoside phosphonates cidofovir, adefovir, and tenofovir in treatment of DNA virus and retrovirus infections, Clinical Microbiology Reviews, 16 (2003) 569-+.

[69] K.Y. Hostetler, Alkoxyalkyl prodrugs of acyclic nucleoside phosphonates enhance oral antiviral activity and reduce toxicity: Current state of the art, Antiviral Research, 82 (2009) A84-A98.

[70] D. Bartlett, Intravenous Lipids: Antidotal Therapy for Drug Overdose and Toxic Effects of Local Anesthetics, Critical Care Nurse, 34 (2014) 62-67.

[71] M.S. Ozcan, G. Weinberg, Intravenous lipid emulsion for the treatment of drug toxicity, Journal of intensive care medicine, 29 (2014) 59-70.

[72] G. Weinberg, B. Lin, S. Zheng, G. Di Gregorio, D. Hiller, R. Ripper, L. Edelman, K. Kelly, D. Feinstein, Partitioning effect in lipid resuscitation: Further evidence for the lipid sink, Critical Care Medicine, 38 (2010) 2268-2269.

[73] J.-X. Mazoit, R. Le Guen, H. Beloeil, D. Benhamou, Binding of Long-lasting Local Anesthetics to Lipid Emulsions, Anesthesiology, 110 (2009) 380-386.

[74] T.L. Samuels, J.W. Willers, D.R. Uncles, R. Monteiro, C. Halloran, H. Dai, In vitro suppression of drug-induced methaemoglobin formation by Intralipid (R) in whole human blood: observations relevant to the lipid sink theory', Anaesthesia, 67 (2012) 23-32.

[75] J. Lokajova, J.M. Holopainen, S.K. Wiedmer, Comparison of lipid sinks in sequestering common intoxicating drugs, Journal of Separation Science, 35 (2012) 3106-3112.

[76] T. Hikita, K. Tadano-Aritomi, N. Iida-Tanaka, S.B. Levery, I. Ishizuka, S. Hakomori, Cationic glycosphingolipids in neuronal tissues and their possible biological significance, Neurochemical Research, 27 (2002) 575-581.

[77] S.L. Ginn, I.E. Alexander, M.L. Edelstein, M.R. Abedi, J. Wixon, Gene therapy clinical trials worldwide to 2012 an update, Journal of Gene Medicine, 15 (2013) 65-77.

[78] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielsen, LIPOFECTION - A HIGHLY EFFICIENT, LIPID-MEDIATED DNA-TRANSFECTION PROCEDURE, Proceedings of the National Academy of Sciences of the United States of America, 84 (1987) 7413-7417.

[79] D. Hirsch-Lerner, M. Zhang, H. Eliyahu, M.E. Ferrari, C.J. Wheeler, Y. Barenholz, Effect of "helper lipid" on lipoplex electrostatics, Biochimica Et Biophysica Acta-Biomembranes, 1714 (2005) 71-84.

[80] M. Munoz-Ubeda, A. Rodriguez-Pulido, A. Nogales, O. Llorca, M. Quesada-Perez, A. Martin-Molina, E. Aicart, E. Junquera, Gene vectors based on DOEPC/DOPE mixed cationic liposomes: a physicochemical study, Soft Matter, 7 (2011) 5991-6004.

[81] A. Orlowski, S. Kukkurainen, A. Poyry, S. Rissanen, I. Vattulainen, V.P. Hytonen, T. Rog, PIP2 and Talin Join Forces to Activate Integrin, Journal of Physical Chemistry B, 119 (2015) 12381-12389.

[82] M. Kepczynski, J. Bednar, D. Kuzmicz, P. Wydro, M. Nowakowska, Spontaneous Formation of Densely Stacked Multilamellar Vesicles in Dioctadecyldimethylammonium Bromide/Oleosiloxane Mixtures, Langmuir, 26 (2010) 1551-1556.

[83] A. lopes, K. Edwards, E. Feitosa, Extruded vesicles of dioctadecyldimethylammonium bromide and chloride investigated by light scattering and cryogenic transmission electron microscopy, Journal of Colloid and Interface Science, 322 (2008) 582-588.

[84] D. Jamroz, M. Kepczynski, M. Nowakowska, Molecular Structure of the Dioctadecyldimethylammonium Bromide (DODAB) Bilayer, Langmuir, 26 (2010) 15076-15079.

[85] P. Saveyn, P. Van der Meeren, M. Zackrisson, T. Narayanan, U. Olsson, Subgel transition in diluted vesicular DODAB dispersions, Soft Matter, 5 (2009) 1735-1742.

[86] L.P. de la Rama, L. Hu, M.Y. Efremov, E.A. Olson, P.F. Nealey, M.A. McLean, S.G. Sligar, L.H. Allen, Anomalous transitions of DODAB using fast scanning liquid calorimetry, Thermochimica Acta, 522 (2011) 72-76.

[87] L. Woiterski, D.W. Britt, J.A. Kaes, C. Selle, Oriented Confined Water Induced by Cationic Lipids, Langmuir, 28 (2012) 4712-4722.

[88] G.Y. Bai, J.B. Wang, H.K. Yan, Z.X. Li, R.K. Thomas, Thermodynamics of molecular self-assembly of cationic gemini and related double chain surfactants in aqueous solution, Journal of Physical Chemistry B, 105 (2001) 3105-3108.

[89] M.F. Lensink, C. Lonez, J.-M. Ruysschaert, M. Vandenbranden, Characterization of the Cationic DiC(14)-amidine Bilayer by Mixed DMPC/DiC(14)-amidine Molecular Dynamics Simulations Shows an Interdigitated Nonlamellar Bilayer Phase, Langmuir, 25 (2009) 5230-5238.

[90] T.R. Oliveira, E.L. Duarte, M.T. Lamy, M. Vandenbranden, J.-M. Ruysschaert, C. Lonez, Temperature-Dependence of Cationic Lipid Bilayer Intermixing: Possible Role of Interdigitation, Langmuir, 28 (2012) 4640-4647.

[91] G. Pabst, C. Lonez, M. Vandenbranden, J. Jestin, A. Radulescu, J.-M. Ruysschaert, T. Gutberlet, Stalk-free membrane fusion of cationic lipids via an interdigitated phase, Soft Matter, 8 (2012) 7243-7249.

[92] D. Simberg, S. Weisman, Y. Talmon, Y. Barenholz, DOTAP (and other cationic lipids): Chemistry, biophysics, and transfection, Critical Reviews in Therapeutic Drug Carrier Systems, 21 (2004) 257-317.

[93] W. Zhao, A.A. Gurtovenko, I. Vattulainen, M. Karttunen, Cationic Dimyristoylphosphatidylcholine and Dioleoyloxytrimethylammonium Propane Lipid Bilayers:

Atomistic Insight for Structure and Dynamics, Journal of Physical Chemistry B, 116 (2012) 269-276.

[94] M. Kepczynski, M. Kumorek, M. Stepniewski, T. Rog, B. Kozik, D. Jamroz, J. Bednar, M. Nowakowska, Behavior of 2,6-Bis(decyloxy)naphthalene Inside Lipid Bilayer, Journal of Physical Chemistry B, 114 (2010) 15483-15494.

[95] R. Saxena, S. Shrivastava, A. Chattopadhyay, Exploring the organization and dynamics of hippocampal membranes utilizing pyrene fluorescence, Journal of Physical Chemistry B, 112 (2008) 12134-12138.

[96] M. Kepczynski, K. Nawalany, M. Kumorek, A. Kobierska, B. Jachimska, M. Nowakowska, Which physical and structural factors of liposome carriers control their drug-loading efficiency?, Chemistry and Physics of Lipids, 155 (2008) 7-15.

[97] L. Stimson, L. Dong, M. Karttunen, A. Wisniewska, M. Dutka, T. Rog, Stearic acid spin labels in lipid bilayers: Insight through atomistic Simulations, Journal of Physical Chemistry B, 111 (2007) 12447-12453.

[98] M.A. Bahri, A. Seret, P. Hans, J. Piette, G. Deby-Dupont, M. Hoebeke, Does propofol alter membrane fluidity at clinically relevant concentrations? An ESR spin label study, Biophysical Chemistry, 129 (2007) 82-91.

[99] A.F.P. Sonnen, H. Bakirci, T. Netscher, W.M. Nau, Effect of temperature, cholesterol content, and antioxidant structure on the mobility of vitamin E constituents in biomembrane models studied by laterally diffusion-controlled fluorescence quenching, Journal of the American Chemical Society, 127 (2005) 15575-15584.

[100] M. Kepczynski, A. Karewicz, A. Gornicki, M. Nowakowska, Interactions of porphyrin covalently attached to poly(methacrylic acid) with liposomal membranes, Journal of Physical Chemistry B, 109 (2005) 1289-1294.

[101] R.D. Kaiser, E. London, Location of diphenylhexatriene (DPH) and its derivatives within membranes: Comparison of different fluorescence quenching analyses of membrane depth, Biochemistry, 37 (1998) 8180-8190.

[102] A. Loudet, K. Burgess, BODIPY dyes and their derivatives: Syntheses and spectroscopic properties, Chemical Reviews, 107 (2007) 4891-4932.

[103] M. Holtta-Vuori, R.-L. Uronen, J. Repakova, E. Salonen, I. Vattulainen, P. Panula, Z. Li, R. Bittman, E. Ikonen, BODIPY-Cholesterol: A New Tool to Visualize Sterol Trafficking in Living Cells and Organisms, Traffic, 9 (2008) 1839-1849.

[104] L.M. Solanko, A. Honigmann, H.S. Midtiby, F.W. Lund, J.R. Brewer, V. Dekaris, R. Bittman, C. Eggeling, D. Wuestner, Membrane Orientation and Lateral Diffusion of BODIPY-Cholesterol as a Function of Probe Structure, Biophysical Journal, 105 (2013) 2082-2092.

[105] K.C. Song, P.W. Livanec, J.B. Klauda, K. Kuczera, R.C. Dunn, W. Im, Orientation of Fluorescent Lipid Analogue BODIPY-PC to Probe Lipid Membrane Properties: Insights from Molecular Dynamics Simulations, Journal of Physical Chemistry B, 115 (2011) 6157-6165.

[106] R. Sachl, I. Mikhalyov, N. Gretskaya, A. Olzynska, M. Hof, L.B.A. Johansson, Distribution of BODIPY-labelled phosphatidylethanolamines in lipid bilayers exhibiting different curvatures, Physical Chemistry Chemical Physics, 13 (2011) 11694-11701.

[107] P.W. Livanec, H.A. Huckabay, R.C. Dunn, Exploring the Effects of Sterols in Model Lipid Membranes Using Single-Molecule Orientations, Journal of Physical Chemistry B, 113 (2009) 10240-10248.

[108] P.W. Livanec, R.C. Dunn, Single-Molecule Probes of Lipid Membrane Structure, Langmuir, 24 (2008) 14066-14073.

[109] S. Mukherjee, A. Chattopadhyay, Membrane organization at low cholesterol concentrations: A study using 7-nitrobenz-2-oxa-1,3-diazol-4-yl-labeled cholesterol, Biochemistry, 35 (1996) 1311-1322.

[110] J.R. Robalo, J.P. Prates Ramalho, L.M.S. Loura, NBD-Labeled Cholesterol Analogues in Phospholipid Bilayers: Insights from Molecular Dynamics, Journal of Physical Chemistry B, 117 (2013) 13731-13742.

[111] H.A. Scheidt, P. Muller, A. Herrmann, D. Huster, The potential of fluorescent and spin-labeled steroid analogs to mimic natural cholesterol, Journal of Biological Chemistry, 278 (2003) 45563-45569.

[112] M.J. Moreno, L.M.B.B. Estronca, W.L.C. Vaz, Translocation of phospholipids and dithionite permeability in liquid-ordered and liquid-disordered membranes, Biophysical Journal, 91 (2006) 873-881.

[113] H.A.L. Filipe, L.S. Santos, J.P. Prates Ramalho, M.J. Moreno, L.M.S. Loura, Behaviour of NBD-head group labelled phosphatidylethanolamines in POPC bilayers: a molecular dynamics study, Physical Chemistry Chemical Physics, 17 (2015) 20066-20079.

[114] D. Huster, P. Muller, K. Arnold, A. Herrmann, Dynamics of lipid chain attached fluorophore 7-nitrobenz-2-oxa-1,3-diazol-4-yI (NBD) in negatively charged membranes determined by NMR spectroscopy, European Biophysics Journal with Biophysics Letters, 32 (2003) 47-54.

[115] L.M.S. Loura, J.P.P. Ramalho, Location and dynamics of acyl chain NBD-labeled phosphatidylcholine (NBD-PQ in DPPC bilayers. A molecular dynamics and time-resolved fluorescence anisotropy study, Biochimica Et Biophysica Acta-Biomembranes, 1768 (2007) 467-478.

[116] M.D. Franova, J. Repakova, J.M. Holopainen, I. Vattulainen, How to link pyrene to its host lipid to minimize the extent of membrane perturbations and to optimize pyrene dimer formation, Chemistry and Physics of Lipids, 177 (2014) 19-25.

[117] D. Wustner, Fluorescent sterols as tools in membrane biophysics and cell biology, Chemistry and Physics of Lipids, 146 (2007) 1-25.

[118] J.R. Robalo, A.M.T. Martins do Canto, A.J. Palace Carvalho, J.P. Prates Ramalho, L.M.S. Loura, Behavior of Fluorescent Cholesterol Analogues Dehydroergosterol and Cholestatrienol in Lipid Bilayers: A Molecular Dynamics Study, Journal of Physical Chemistry B, 117 (2013) 5806-5819.

[119] M. Pourmousa, T. Rog, R. Mikkeli, I. Vattulainen, L.M. Solanko, D. Wustner, N.H. List, J. Kongsted, M. Karttunen, Dehydroergosterol as an Analogue for Cholesterol: Why It Mimics Cholesterol So Well-or Does It?, Journal of Physical Chemistry B, 118 (2014) 7345-7357.

[120] R.M.S. Cardoso, H.A.L. Filipe, F. Gomes, N.D. Moreira, W.L.C. Vaz, M.J. Moreno, Chain Length Effect on the Binding of Amphiphiles to Serum Albumin and to POPC Bilayers, Journal of Physical Chemistry B, 114 (2010) 16337-16346.

[121] H.A.L. Filipe, M.J. Moreno, L.M.S. Loura, Interaction of 7-Nitrobenz-2-oxa-1,3-diazol-4-yl-Labeled Fatty Amines with 1-Palmitoyl, 2-Oleoyl-sn-glycero-3-phosphocholine Bilayers: A Molecular Dynamics Study, Journal of Physical Chemistry B, 115 (2011) 10109-10119.

[122] H.A.L. Filipe, M.J. Moreno, T. Rog, I. Vattulainen, L.M.S. Loura, How To Tackle the Issues in Free Energy Simulations of Long Amphiphiles Interacting with Lipid Membranes: Convergence and Local Membrane Deformations, Journal of Physical Chemistry B, 118 (2014) 3572-3581.

[123] H.A.L. Filipe, D. Bowman, T. Palmeira, R.M.S. Cardoso, L.M.S. Loura, M.J. Moreno, Interaction of NBD-labelled fatty amines with liquid-ordered membranes: a combined molecular dynamics simulation and fluorescence spectroscopy study, Physical Chemistry Chemical Physics, 17 (2015) 27534-27547.

[124] T. Tabouillot, H.S. Muddana, P.J. Butler, Endothelial Cell Membrane Sensitivity to Shear Stress is Lipid Domain Dependent, Cellular and Molecular Bioengineering, 4 (2011) 169-181.

[125] I.-H. Lee, S. Saha, A. Polley, H. Huang, S. Mayor, M. Rao, J.T. Groves, Live Cell Plasma Membranes Do Not Exhibit a Miscibility Phase Transition over a Wide Range of Temperatures, Journal of Physical Chemistry B, 119 (2015) 4450-4459.

[126] H.S. Muddana, R.R. Gullapalli, E. Manias, P.J. Butler, Atomistic simulation of lipid and DiI dynamics in membrane bilayers under tension, Physical Chemistry Chemical Physics, 13 (2011) 1368-1378.

[127] A.S. Klymchenko, Y. Mely, A.P. Demchenko, G. Duportail, Simultaneous probing of hydration and polarity of lipid bilayers with 3-hydroxyflavone fluorescent dyes, Biochimica Et Biophysica Acta-Biomembranes, 1665 (2004) 6-19.

[128] S. Timr, A. Bondar, L. Cwiklik, M. Stefl, M. Hof, M. Vazdar, J. Lazar, P. Jungwirth, Accurate Determination of the Orientational Distribution of a Fluorescent Molecule in a Phospholipid Membrane, Journal of Physical Chemistry B, 118 (2014) 855-863.

[129] G. Jeschke, Conformational dynamics and distribution of nitroxide spin labels, Progress in Nuclear Magnetic Resonance Spectroscopy, 72 (2013) 42-60.

[130] A. Chattopadhyay, E. London, PARALLAX METHOD FOR DIRECT MEASUREMENT OF MEMBRANE PENETRATION DEPTH UTILIZING FLUORESCENCE QUENCHING BY SPIN-LABELED PHOSPHOLIPIDS, Biochemistry, 26 (1987) 39-45.

[131] A. Kyrychenko, A.S. Ladokhin, Molecular Dynamics Simulations of Depth Distribution of Spin-Labeled Phospholipids within Lipid Bilayer, Journal of Physical Chemistry B, 117 (2013) 5875-5885.

[132] A. Kyrychenko, M.V. Rodnin, A.S. Ladokhin, Calibration of Distribution Analysis of the Depth of Membrane Penetration Using Simulations and Depth-Dependent Fluorescence Quenching, Journal of Membrane Biology, 248 (2015) 583-594.

[133] A.K. Smith, J.H. Freed, Dynamics and ordering of lipid spin-labels along the coexistence curve of two membrane phases: An ESR study, Chemistry and Physics of Lipids, 165 (2012) 348-361.

[134] T. Rog, M. Pasenkiewicz-Gierula, Cholesterol effects on a mixed-chain phosphatidylcholine bilayer: a molecular dynamics simulation study, Biochimie, 88 (2006) 449-460.

[135] M.A. Bahri, B.J. Heyne, P. Hans, A.E. Seret, A.A. Mouithys-Mickalad, M.D. Hoebeke, Quantification of lipid bilayer effective microviscosity and fluidity effect induced by propofol, Biophysical Chemistry, 114 (2005) 53-61.

[136] S. Jaikishan, A. Bjorkbom, J.P. Slotte, Phosphatidyl alcohols: Effect of head group size on domain forming properties and interactions with sterols, Biochimica Et Biophysica Acta-Biomembranes, 1798 (2010) 1615-1622.

[137] W. Guyer, K. Bloch, PHOSPHATIDYLCHOLINE AND CHOLESTEROL INTERACTIONS IN MODEL MEMBRANES, Chemistry and Physics of Lipids, 33 (1983) 313-322.

[138] R.A. Demel, Bruckdor.Kr, Vandeene.Ll, EFFECT OF STEROL STRUCTURE ON PERMEABILITY OF LIPOSOMES TO GLUCOSE, GLYCEROL AND RB+, Biochimica Et Biophysica Acta, 255 (1972) 321-&.

[139] J.J. Wenz, Predicting the effect of steroids on membrane biophysical properties based on the molecular structure, Biochimica Et Biophysica Acta-Biomembranes, 1818 (2012) 896-906.

[140] X. Jiang, D.F. Covey, Total synthesis of ent-cholesterol via a steroid C,D-ring side-chain synthon, Journal of Organic Chemistry, 67 (2002) 4893-4900.

[141] J.D. Belani, S.D. Rychnovsky, A concise synthesis of ent-cholesterol, Journal of Organic Chemistry, 73 (2008) 2768-2773.

[142] D.A. Mannock, T.J. McIntosh, X. Jiang, D.F. Covey, R.N. McElhaney, Effects of natural and enantiomeric cholesterol on the thermotropic phase behavior and structure of egg sphingomyelin bilayer membranes, Biophysical Journal, 84 (2003) 1038-1046.

[143] E.J. Westover, D.F. Covey, H.L. Brockman, R.E. Brown, L.J. Pike, Cholesterol depletion results in site-specific increases in epidermal growth factor receptor phosphorylation due to membrane level effects - Studies with cholesterol enantiomers, Journal of Biological Chemistry, 278 (2003) 51125-51133.

[144] S. Bandari, H. Chakraborty, D.F. Covey, A. Chattopadhyay, Membrane dipole potential is sensitive to cholesterol stereospecificity: Implications for receptor function, Chemistry and Physics of Lipids, 184 (2014) 25-29.

[145] F. Xu, S.D. Rychnovsky, J.D. Belani, H.H. Hobbs, J.C. Cohen, R.B. Rawson, Dual roles for cholesterol in mammalian cells, Proceedings of the National Academy of Sciences of the United States of America, 102 (2005) 14551-14556.

[146] D.F. Covey, ent-Steroids: Novel tools for studies of signaling pathways, Steroids, 74 (2009) 577-585.

[147] A. Zitzer, E.J. Westover, D.F. Covey, M. Palmer, Differential interaction of the two cholesterol-dependent, membrane-damaging toxins, streptolysin O and Vibrio cholerae cytolysin, with enantiomeric cholesterol, Febs Letters, 553 (2003) 229-231.

[148] R.M. Epand, S.D. Rychnovsky, J.D. Belani, R.F. Epand, Role of chirality in peptide-induced formation of cholesterol-rich domains, Biochemical Journal, 390 (2005) 541-548.

[149] J. Liu, C.C.Y. Chang, E.J. Westover, D.F. Covey, T.Y. Chang, Investigating the allosterism of acyl-CoA : cholesterol acyltransferase (ACAT) by using various sterols: in vitro and intact cell studies, Biochemical Journal, 391 (2005) 389-397.

[150] J. Wang, F. Sun, D.-w. Zhang, Y. Ma, F. Xu, J.D. Belani, J.C. Cohen, H.H. Hobbs, X.-S. Xie, Sterol transfer by ABCG5 and ABCG8 - In vitro assay and reconstitution, Journal of Biological Chemistry, 281 (2006) 27894-27904.

[151] M. Jafurulla, B.D. Rao, S. Sreedevi, J.-M. Ruysschaert, D.F. Covey, A. Chattopadhyay, Stereospecific requirement of cholesterol in the function of the serotonin(1A) receptor, Biochimica Et Biophysica Acta-Biomembranes, 1838 (2014) 158-163.

[152] H. Martinez-Seara, T. Rog, M. Karttunen, I. Vattulainen, R. Reigada, Cholesterol Induces Specific Spatial and Orientational Order in Cholesterol/Phospholipid Membranes, Plos One, 5 (2010).

[153] K.E. Bloch, SPECULATIONS ON THE EVOLUTION OF STEROL STRUCTURE AND FUNCTION, Crc Critical Reviews in Biochemistry, 7 (1979) 1-5.

[154] T. Rog, M. Pasenkiewicz-Gierula, I. Vattulainen, M. Karttunen, What happens if cholesterol is made smoother: Importance of methyl substituents in cholesterol ring structure on phosphatidylcholine-sterol interaction, Biophysical Journal, 92 (2007) 3346-3357.

[155] S. Poyry, T. Rog, M. Karttunen, I. Vattulainen, Significance of cholesterol methyl groups, Journal of Physical Chemistry B, 112 (2008) 2922-2929.

[156] L. Mydock-McGrane, N.P. Rath, D.F. Covey, Synthesis of a Smoothened Cholesterol: 18,19-Di-nor-cholesterol, Journal of Organic Chemistry, 79 (2014) 5636-5643.

[157] M.R. Krause, M. Wang, L. Mydock-McGrane, D.F. Covey, E. Tejada, P.F. Almeida, S.L. Regen, Eliminating the Roughness in Cholesterol's beta-Face: Does it Matter?, Langmuir, 30 (2014) 12114-12118.

[158] C. Faure, J.F. Tranchant, E.J. Dufourc, Comparative effects of cholesterol and cholesterol sulfate on hydration and ordering of dimyristoylphosphatidylcholine membranes, Biophysical Journal, 70 (1996) 1380-1390.

[159] A.M. Smondyrev, M.L. Berkowitz, Molecular dynamics simulation of dipalmitoylphosphatidylcholine membrane with cholesterol sulfate, Biophysical Journal, 78 (2000) 1672-1680.

[160] M. Lonnfors, O. Langvik, A. Bjorkbom, J.P. Slotte, Cholesteryl Phosphocholine - A Study on Its Interactions with Ceramides and Other Membrane Lipids, Langmuir, 29 (2013) 2319-2329.

[161] M.G.K. Benesch, R.N.A.H. Lewis, R.N. McElhaney, On the miscibility of cardiolipin with 1,2-diacyl phosphoglycerides: Binary mixtures of dimyristoylphosphatidylglycerol and tetramyristoylcardiolipin, Biochimica Et Biophysica Acta-Biomembranes, 1848 (2015) 2878-2888.

[162] B. de Kruyff, R.A. Demel, L.L. van Deenen, The effect of cholesterol and epicholesterol incorporation on the permeability and on the phase transition of intact Acholeplasma laidlawii cell membranes and derived liposomes, Biochimica et biophysica acta, 255 (1972) 331-347.

[163] E.J. Dufourc, E.J. Parish, S. Chitrakorn, I.C.P. Smith, STRUCTURAL AND DYNAMICAL DETAILS OF CHOLESTEROL LIPID INTERACTION AS REVEALED BY DEUTERIUM NMR, Biochemistry, 23 (1984) 6062-6071.

[164] T. Rog, M. Pasenkiewicz-Gierula, Effects of epicholesterol on the phosphatidylcholine bilayer: A molecular simulation study, Biophysical Journal, 84 (2003) 1818-1826.

[165] Y. Nakagawa, S. Nojima, K. Inoue, TRANSFER OF STEROIDS AND ALPHA-TOCOPHEROL BETWEEN LIPOSOMAL MEMBRANES, Journal of Biochemistry, 87 (1980) 497-502.

[166] D.A. Mannock, M.Y.T. Lee, R.N.A.H. Lewis, R.N. McElhaney, Comparative calorimetric and spectroscopic studies of the effects of cholesterol and epicholesterol on the thermotropic phase behaviour of dipalmitoylphosphatidylcholine bilayer membranes, Biochimica Et Biophysica Acta-Biomembranes, 1778 (2008) 2191-2202.

[167] M. Zocher, C. Zhang, S.G.F. Rasmussen, B.K. Kobilka, D.J. Mueller, Cholesterol increases kinetic, energetic, and mechanical stability of the human beta(2)-adrenergic receptor, Proceedings of the National Academy of Sciences of the United States of America, 109 (2012) E3463-E3472.

[168] M.A. O'Malley, M.E. Helgeson, N.J. Wagner, A.S. Robinson, The Morphology and Composition of Cholesterol-Rich Micellar Nanostructures Determine Transmembrane Protein (GPCR) Activity, Biophysical Journal, 100 (2011) L11-L13.

[169] J. Oates, B. Faust, H. Attrill, P. Harding, M. Orwick, A. Watts, The role of cholesterol on the activity and stability of neurotensin receptor 1, Biochimica Et Biophysica Acta-Biomembranes, 1818 (2012) 2228-2233.

[170] K. Vukoti, T. Kimura, L. Macke, K. Gawrisch, A. Yeliseev, Stabilization of Functional Recombinant Cannabinoid Receptor CB2 in Detergent Micelles and Lipid Bilayers, Plos One, 7 (2012).

[171] G.J. Zhang, H.W. Liu, L. Yang, Y.G. Zhong, Y.Z. Zheng, Influence of membrane physical state on the lysosomal proton permeability, Journal of Membrane Biology, 175 (2000) 53-62.

[172] W.X. Ding, X.R. Qi, P. Li, Y. Maitani, T. Nagai, Cholesteryl hemisuccinate as a membrane stabilizer in dipalmitoylphosphatidylcholine liposomes containing saikosaponin-d, International Journal of Pharmaceutics, 300 (2005) 38-47.

[173] M.Z. Lai, N. Duzgunes, F.C. Szoka, EFFECTS OF REPLACEMENT OF THE HYDROXYL GROUP OF CHOLESTEROL AND TOCOPHEROL ON THE THERMOTROPIC BEHAVIOR OF PHOSPHOLIPID-MEMBRANES, Biochemistry, 24 (1985) 1646-1653.

[174] J.B. Massey, Effect of cholesteryl hemisuccinate on the interfacial properties of phosphatidylcholine bilayers, Biochimica Et Biophysica Acta-Biomembranes, 1415 (1998) 193-204.

[175] W. Kulig, J. Tynkkynen, M. Javanainen, M. Manna, T. Rog, I. Vattulainen, P. Jungwirth, How well does cholesteryl hemisuccinate mimic cholesterol in saturated phospholipid bilayers?, Journal of Molecular Modeling, 20 (2014).

[176] W. Kulig, P. Jurkiewicz, A. Olzynska, J. Tynkkynen, M. Javanainen, M. Manna, T. Rog, M. Hof, I. Vattulainen, P. Jungwirth, Experimental determination and computational interpretation of biophysical properties of lipid bilayers enriched by cholesteryl hemisuccinate, Biochimica Et Biophysica Acta-Biomembranes, 1848 (2015) 422-432.

[177] J. Aittoniemi, T. Rog, P. Niemela, M. Pasenkiewicz-Gierula, M. Karttunen, I. Vattulainen, Tilt: Major factor in sterols' ordering capability in membranes, Journal of Physical Chemistry B, 110 (2006) 25562-25564.

[178] G. Khelashvili, D. Harries, How Cholesterol Tilt Modulates the Mechanical Properties of Saturated and Unsaturated Lipid Membranes, Journal of Physical Chemistry B, 117 (2013) 2411-2421.

[179] D.G. Lendemans, J. Myschik, S. Hook, T. Rades, Cationic cage-like complexes formed by DC-cholesterol, Quil-A, and phospholipid, Journal of Pharmaceutical Sciences, 94 (2005) 1794-1807.

[180] J.-C. Wu, T.-L. Lin, C.-P. Yang, U.S. Jeng, H.-Y. Lee, M.-C. Shih, X-ray reflectivity and BAM studies on the LB film of mixed DPPC/DC-cholesterol monolayer, Colloids and Surfaces a-Physicochemical and Engineering Aspects, 284 (2006) 103-108.

[181] J. Zhang, H. Cao, S.L. Regen, Cholesterol-phospholipid complexation in fluid bilayers as evidenced by nearest-neighbor recognition measurements, Langmuir, 23 (2007) 405-407.

[182] T. Meyer, D.J. Baek, R. Bittman, I. Haralampiev, P. Mueller, A. Herrmann, D. Huster, H.A. Scheidt, Membrane properties of cholesterol analogs with an unbranched aliphatic side chain, Chemistry and Physics of Lipids, 184 (2014) 1-6.

[183] J.J. Hulce, A.B. Cognetta, M.J. Niphakis, S.E. Tully, B.F. Cravatt, Proteome-wide mapping of cholesterol-interacting proteins in mammalian cells, Nature Methods, 10 (2013) 259-264.

[184] J.P. Slotte, Biological functions of sphingomyelins, Progress in Lipid Research, 52 (2013) 424-437.

[185] B. Ramstedt, J.P. Slotte, Membrane properties of sphingomyelins, Febs Letters, 531 (2002) 33-37.

[186] B. Westerlund, J.P. Slotte, How the molecular features of glycosphingolipids affect domain formation in fluid membranes, Biochimica Et Biophysica Acta-Biomembranes, 1788 (2009) 194-201.

[187] J.P. Slotte, Molecular properties of various structurally defined sphingomyelins - Correlation of structure with function, Progress in Lipid Research, 52 (2013) 206-219.

[188] A. Bjorkbom, T. Yamamoto, S. Kaji, S. Harada, S. Katsumura, J.P. Slotte, Importance of the phosphocholine linkage on sphingomyelin molecular properties and interactions with cholesterol; a study with phosphate oxygen modified sphingomyelin-analogues, Biochimica Et Biophysica Acta-Biomembranes, 1778 (2008) 1501-1507.

[189] A. Bjorkbom, T. Rog, K. Kaszuba, M. Kurita, S. Yamaguchi, M. Lonnfors, T.K.M. Nyholm, I. Vattulainen, S. Katsumura, J.P. Slotte, Effect of Sphingomyelin Headgroup Size on Molecular Properties and Interactions with Cholesterol, Biophysical Journal, 99 (2010) 3300-3308.

[190] W.-C. Hung, M.-T. Lee, F.-Y. Chen, H.W. Huang, The condensing effect of cholesterol in lipid bilayers, Biophysical Journal, 92 (2007) 3960-3967.

[191] Y.W. Hsueh, R. Giles, N. Kitson, J. Thewalt, The effect of ceramide on phosphatidylcholine membranes: A deuterium NMR study, Biophysical Journal, 82 (2002) 3089-3095.

[192] I. Artetxe, C. Sergelius, M. Kurita, S. Yamaguchi, S. Katsumura, J.P. Slotte, T. Maula, Effects of Sphingomyelin Headgroup Size on Interactions with Ceramide, Biophysical Journal, 104 (2013) 604-612.

[193] C. Sergelius, S. Niinivehmas, T. Maula, M. Kurita, S. Yamaguchi, T. Yamamoto, S. Katsumura, O.T. Pentikainen, J.P. Slotte, Structure-activity relationship of sphingomyelin analogs with sphingomyelinase from Bacillus cereus, Biochimica Et Biophysica Acta-Biomembranes, 1818 (2012) 474-480.

[194] C. Sergelius, S. Yamaguchi, T. Yamamoto, O. Engberg, S. Katsumura, J.P. Slotte, Cholesterol's interactions with serine phospholipids - A comparison of N-palmitoyl ceramide phosphoserine with dipalmitoyl phosphatidylserine, Biochimica Et Biophysica Acta-Biomembranes, 1828 (2013) 785-791.

[195] A. Bjorkbom, T. Rog, P. Kankaanpaa, D. Lindroos, K. Kaszuba, M. Kurita, S. Yamaguchi, T. Yamamoto, S. Jaikishan, L. Paavolainen, J. Paivarinne, T.K.M. Nyholm, S. Katsumura, I. Vattulainen, J.P. Slotte, N- and O-methylation of sphingomyelin markedly affects

its membrane properties and interactions with cholesterol, Biochimica Et Biophysica Acta-Biomembranes, 1808 (2011) 1179-1186.

[196] T. Maula, M. Kurita, S. Yamaguchi, T. Yamamoto, S. Katsumura, J.P. Slotte, Effects of Sphingosine 2N- and 3O-Methylation on Palmitoyl Ceramide Properties in Bilayer Membranes, Biophysical Journal, 101 (2011) 2948-2956.

[197] T. Maula, Y.J.E. Isaksson, S. Garcia-Linares, S. Niinivehmas, O.T. Pentikainen, M. Kurita, S. Yamaguchi, T. Yamamoto, S. Katsumura, J.G. Gavilanes, A. Martinez-del-Pozo, J.P. Slotte, 2NH and 3OH are crucial structural requirements in sphingomyelin for sticholysin II binding and pore formation in bilayer membranes, Biochimica Et Biophysica Acta-Biomembranes, 1828 (2013) 1390-1395.

[198] C. Sergelius, S. Yamaguchi, T. Yamamoto, J.P. Slotte, S. Katsumura, N-cholesteryl sphingomyelin-A synthetic sphingolipid with unique membrane properties, Biochimica Et Biophysica Acta-Biomembranes, 1808 (2011) 1054-1062.

[199] E.K. Perttu, F.C. Szoka, Jr., Zwitterionic sulfobetaine lipids that form vesicles with salt-dependent thermotropic properties, Chemical Communications, 47 (2011) 12613-12615.

[200] E.K. Perttu, A.G. Kohli, F.C. Szoka, Jr., Inverse-Phosphocholine Lipids: A Remix of a Common Phospholipid, Journal of the American Chemical Society, 134 (2012) 4485-4488.

[201] A.G. Kohli, C.L. Walsh, F.C. Szoka, Synthesis and characterization of betaine-like diacyl lipids: zwitterionic lipids with the cationic amine at the bilayer interface, Chemistry and Physics of Lipids, 165 (2012) 252-259.

[202] A. Magarkar, T. Rog, A. Bunker, Molecular Dynamics Simulation of Inverse-Phosphocholine Lipids, Journal of Physical Chemistry C, 118 (2014) 19444-19449.