UTILIZING THIOCHOLESTEROL AS A HYDROPHOBIC PROBE TO CHARACTERIZE LIPID PHASE BEHAVIOUR

LEE YAN FEN

UNIVERSITI SAINS MALAYSIA

2017

UTILIZING THIOCHOLESTEROL AS A HYDROPHOBIC PROBE TO CHARACTERIZE LIPID PHASE BEHAVIOUR

by

LEE YAN FEN

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

March 2017

ACKNOWLEDGEMENT

I am deeply thankful for all the help and support that I have received throughout the long and challenging journey of my PhD research study. With this in mind, I would like to thank everyone who has provided me with their valuable help. This thesis would not have been possible without the precious contributions from so many people and the opportunities that have been given to me.

Foremost, I sincerely thank my main supervisor Prof. Dr. K. Sudesh Kumar. I appreciate all the great opportunities, thoughtful guidance and consistent support that he has given to me in the past 7 years as a research member of the Ecobiomaterial Laboratory and as an exchange student to RIKEN, Japan under the International Program Associate scheme. His interdisciplinary knowledge and wise experience in scientific research and human resources management are always an example for many of us to follow. His comments are always critically beneficial to all his students.

It is an honour for me to thank gratefully Dr. Tech. Peter Greimel, my co-supervisor based in Wako, RIKEN. I am grateful to him for leading me towards lipid membrane research and organic chemistry with his passionate and patient guidance in the past 3 years, together with his great knowledge in many perspectives of science and life. He once told me never to give on achieving my dreams or goals. I benefited greatly from his advice and these 3 years have been one of the greatest learning periods of my life.

I would also like to deliver my deepest appreciation to Prof. Toshihide Kobayashi, Prof. Dr. Surash Ramanathan and Prof. Atsushi Miyawaki for their warm hosting in their respective esteemed laboratories, and their support and advice for experiments and publication of my work. I also thank Prof. Peter Slotte (Finland) and

Dr. Hiroshi Takahashi (Japan) for our successful collaboration and their contribution towards data analysis in part of this thesis.

Special thanks to all the current and previous members of research laboratories that I have encountered for their kind dedication in sharing time and ideas with me; especially Dr. Nathini Sridewi, Dr. Chuah Jo-Ann, Dr. Neval Yilmaz, Ms. Lim Shi Rou, Dr. Sabrina Kargoll, Dr. Francoise Hullin-Matsuda, Dr. Wong Yoke Ming, Mr. Terick Chia Kim Hou, and Dr. Manoj Lakshmanan. Also, sincere thanks to all the secretaries including Ms. Ferryn Ooi, Dr. Chee Jiun Yee, Dr. Diana C'hng, Ms. Ng Ko-Sin, Ms. Yoko Ogura, Ms. Yoshiko Toyoda and Ms. Reiko Saito for their great help with official documents during my stay in Japan. Deep thanks to the USM fellowship and International Associate Program for providing financial support to perform and complete my PhD research.

To my parents and siblings, I am blessed and thankful for all the love, understanding and encouragement from you all in every single decision that I have made. Thank you for giving me a sweet and warm home filled with lovely family that I could lean on, especially during the stresses of completing this study. Thanks to God for giving me such a great family. To my dearest and special lovely soulmate, Dr. Nicholas Thomson, thank you for your companionship and big arms during my bright and dark days along this journey. You were always there for me, independent of country borders and time zone differences, and you encouraged me when there was a time when I thought it was impossible to finish. I must thank you for your great editing and formatting skills in shaping this thesis. Lastly, my gratitude goes to all my friends who have joined my life up to this moment, especially the bff team, Ms. Chong Yoong Fang and Ms. Chai Yu Xuan. Thank you all for the encouragement and sweet support.

TABLE OF CONTENTS

CHAPTER 3: MATERIALS AND METHODS

CHAPTER 4: RESULTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Area
ACN	Acetone
CCD	Charge-coupled device
CHARMM	Chemistry at Harvard macromolecular mechanics
Chol	Cholesterol
$CHCl_3$	Chloroform
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
pSM	*N*-palmitoyl-D-*erythro*-sphingosylphosphorylcholine
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry
DTNB	5-5´-Dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
Et_3N	Triethylamine
EtOH	Ethanol
FID	Free induction decay
HCl	Hydrochloric acid
Hex	n-hexane
K	rate constant
K_2HPO_4	Di-potassium hydrogenphosphate
KH_2PO_4	Potassium dihydrogen phosphate

LB	Langmuir Blodgett
L_β	Gel state
L_d	Liquid disordered phase
L_o	Liquid ordered phase
LUV	Large unilamellar liposomes
MD	Molecular dynamic
MeOH	Methanol
MLV	Multilamellar liposomes
M_w	Melting temperature
N	Constant number of particles
NAMD	Nanoscale molecular dynamics
NMR	Nuclear magnetic resonance
P	Pressure
PC	Phosphatidylcholine
P_β	Ripple phase
RT	Room temperature
SAXS	Small angle X-ray scattering
SM	Sphingomyelin
SUV	Small unilamellar vesicles
T	Temperature
tChol	Thiocholesterol
T_m	Melting temperature
TKE	Tris-Phosphate-EDTA buffer
tPA	Trans parinaric acid
$t_{1/2}$	half-life or half-time

VMD	Visual molecular dynamics
^2H_2O	Deuterium oxide
7SLPC	1-palmitoyl-2-stearoyl-(7-doxyl)-sn-glycero-3-Phosphocholine
^{31}P NMR	Phosphorus-31 nuclear magnetic resonance

LIST OF UNITS AND SYMBOLS

g	Gram
h	Hour
L	Litre
ºC	Degree Celsius
M	Molar
mM	Millimolar
s	Second
min	Minute
v	Volume
θ	Angle
λ	Wavelength
π	Pi
µL	Microlitre
mm	Millimetre
mm^2	Square millimetre
µm	Micrometer
nm	Nanometer
mN/m	Surface pressure
sin	Sine function
Hz	Frequency
k	kilo
µs	microsecond

PENGGUNAAN THIOKOLESTEROL SEBAGAI PROB HIDROFOBIK UNTUK PENCIRIAN SIFAT FASA LIPID

ABSTRAK

Lipid seperti sfingomielin, gliserofosfolipik dan kolesterol merupakan komponen utama yang membentuk serta mengekalkan kestabilan dan dinamik membran plasma eukariotik. Ciri-ciri fisikokimia dalam membran plasma amat dipengaruhi oleh kandungan kolesterol (Chol). Di dalam projek ini, kesan thiokolesterol (tChol) terhadap sifat fasa fosfolipik diterokai serta dibandingkan dengan Chol. Secara umumnya, tChol mengandungi 3-thiol sebagai kumpulan berfungsi di manakala Chol mengandungi 3-hidroksil. Ini membolehkan penjelajahan pengaruhan kumpulan fungsi 3-hidroksil dalam Chol pada interaksi antara lipid dan kolesterol. Di samping itu, tChol juga digunakan sebagai prob untuk memahami pengaruhan matrik fosfolipik terhadap Chol flip-flop. Pelbagai teknik biofizikal seperti kalorimetri pengimbasan perbezaan (DSC), spektroskopi pembelauan sinar-X sudut lecil(SAXS), resonans magnet nukleus(NMR), “Langmuir-Blodgett” (LB) dan simulasi dinamik molekul telah digunakan untuk menilai kesan tChol terhadap sifat fasa lipid dalam liposom yang mengandungi fosfahidilkolina (PC) dan sfingomielin (SM). Berdasarkan hasil kajian, di dalam model membran yang mengekalkan fasa cecair teratur (Lo), tChol menunjukkan ciri-ciri yang serupa dengan Chol seperti kelakuan dan susunannya. Kajian memaparkan perubahan fasa daripada fasa cecair teratur (L_o) ke fasa cecair tidak teratur (L_d) dapat diperhatikan dalam membran yang mengandungi tChol. Perubahan fasa tersebut berlaku pada suhu kira-kira 10 ºC di bawah suhu lebur fosfolipid tulen yang ditunjukkan oleh 1,2-dipalmitoyl-sn-glisero-3-fosfokolina (DPPC), 1,2-dimyristoyl-sn-glisero-3-fosfokolina (DMPC), 1,2-distearoyl-sn-glisero-3-fosfokolina (DSPC) and N-palmitoyl sphingosine (pSM).

Berbeza dengan tChol, membran yang mengandungi Chol menunjukkan tiada perubahan fasa semasa proses pemanasan... Sementara itu, orientasi dan pergerakan tChol didapati tidak menyerupai Chol dalam fasa L_d. Fenomena ini dapat diperhatikan dengan jelas melalui interaksi antara tChol dengan 1-palmitoyl-2-oleoyl-sn-glisero-3-fosfokolina (POPC) and 1,2-dioleoyl-sn-glisero-3-fosfokolina (DOPC) dwilapis membran. Pergerakan tChol di dalam membran yang menunjukkan fasa L_d adalah lebih dinamik berbanding dengan Chol. Berdasarkan keputusan hasil kajian yang mengenai ciri- ciri tChol, satu pendekatan kimia yang merujuk kepada cerakin Ellman telah digunakan untuk menentu kelajuan pergerakan melintang tChol merentasi dua lapis membran. Yang menariknya, keputusan kajian menunjukkan kelajuan pergerakan tChol merentasi membran DPPC and pSM adalah hampir serupa. Kejadian sebegini kemungkinan adalah disebabkan hasilan daripada padanan yang bagus antara lipid dan kolesterol. Sebaliknya, pergerakan tChol adalah lebih laju semasa merentasi ketidak padanan lipid-Chol membran seperti DSPC, DMPC, DOPC dan POPC.

UTILIZING THIOCHOLESTEROL AS A HYDROPHOBIC PROBE TO CHARACTERIZE LIPID PHASE BEHAVIOUR

ABSTRACT

Lipids, such as sphingomyelins, glycero-phospholipids and cholesterol, represent the primary component of the cellular plasma membrane and provide the necessary stability and dynamics to support protein function. The physicochemical properties of the cellular plasma membrane are strongly influenced by cholesterol (Chol) content. In the present work, the effect of thiocholesterol (tChol) on phospholipid phase behavior was assessed and compared to Chol. In general, tChol features a 3-thiol group compared to the 3-hydroxyl function of Chol. This allowed dissecting the influence of the 3-hydroxyl group from the rigid hydrophobic core of Chol on the lipid-Chol interaction. Additionally, tChol was established and utilized as a probe to assess the influence of the phospholipid matrix on Chol flip-flop. Utilizing an array of biophysical techniques, including differential scanning calorimetry (DSC), small-angle X-ray diffraction spectroscopy (SAXS), Langmuir Blodgett monolayer studies and nuclear magnetic resonance (NMR), combined with molecular dynamics (MD) simulations, the effect of tChol on the lipid phase behavior in phosphatidylcholine (PC) and sphingomyeline (SM) rich liposomes was evaluated. Based on the results, tChol exhibited a similar behavior and position as Chol in the liquid ordered (L_o) phase of model lipid bilayers. During heating, the change of lipid phase from L_o to liquid disordered phase (L_d) in membranes containing high content of tChol was not prevented, in contrast to Chol-containing membranes. In general, a more or less pronounced phase transition was observed at approximately 10 ºC below the melting temperature of the respective pure

phospholipid, such as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and N-palmitoylsphingosine (pSM). However, tChol orientation and movement in L_d phase did not mimic its hydroxylated counterpart Chol, as clearly evidence in the different interaction with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipid bilayers. The movement of tChol was more dynamic in membranes adopting L_d phase. Based on this detailed understanding of tChol behavior, a chemical approach based on the Ellman's assay was then employed to determine the rate of transversal movement (flip-flop) of tChol between membrane leaflets. Interestingly, tChol flip-flop speed was similar in DPPC- and pSM-containing membranes, possibly due to good lipid-Chol matching effects. On the other hand, tChol flipped faster in lipid-Chol mismatched membranes, such as DSPC, DMPC, DOPC and POPC.

1 INTRODUCTION

Biological membranes are composed of a diverse mix of lipids and proteins, in part heavily decorated with carbohydrates. The plasma membrane as well as intracellular membranes act as a barrier, separating the outer environment from their respective inner environment, while providing selective permeability via integrated proteins for nutrient uptake and signal transduction (Luckey, 2008). The cell membrane is primarily composed of two categories of phospholipids: phosphatidylcholines (PC), a glycerophospholipid, and sphingomyelins (SM), a sphingolipid. Both lipids feature the same hydrophilic phosphorylcholine headgroup and two long hydrocarbon chains, commonly referred to as lipid tails. To minimize exposure of the hydrophobic tails of lipid molecules, lipids dispersed in water spontaneously assemble into a bilayer arrangement with a hydrophobic core and a hydrophilic surface. In each of the layers, the lipids are oriented with their tails packed together, facing towards the lipid tails of the opposite leaflet forming the hydrophobic core. At the same time the hydrophilic head groups point towards the bilayer surfaces establishing the hydrophilic surface character (Luckey, 2008; Ohvo-Rekila *et al*., 2002). In general, a lipid bilayer is a fluidic arrangement of lipids, providing free 2D movement within each membrane leaflet.

The third major component of cellular membranes aside from PC and SM are sterols (steroid alcohols). In mammalian membranes, the major sterol is cholesterol (Chol), which is present for example in both leaflets of the plasma membrane. Chol is constructed of a tetracyclic fused ring skeleton, with a hydroxyl group (OH) at carbon position 3 of the A-ring and an iso-octyl hydrocarbon side chain attached to the D-ring (Ohvo-Rekila *et al*., 2002). Chol has attracted significant scientific interest due to its ability to modulate the physicochemical properties of lipid membranes, such as phase behaviour, mechanical and structural properties. Additionally, Chol has been reported to interact with membrane proteins and also acts as a precursor for steroid hormones and bile acids in eukaryotes (Silvius, 2003; Marc and Magali, 2010; Bennett and Tieleman, 2013).

In the lipid bilayers, Chol and structurally related analogues, tend to engage in more or less tight sterol-lipid interactions, sometimes referred to sterol-lipid complexes (Jain 1975; Oppenheimer and Cordes 1981; Smaby *et al*., 1994). Importantly, the interaction of Chol with its respective lipid partner strongly depends on the characteristics of the interfacial region of the lipid partner. For example, PC with its glycerol backbone featuring two fatty acid esters can only act as hydrogen bond acceptor. In contrast, SM featuring an amide bond can additionally act as a hydrogen bond donor (Ohvo-Rekila *et al*., 2002). As a consequence, the behaviour of the interfacial region of SM is strongly different between PC and SM, enabling a tight interaction between SM and Chol. Due to their tight interaction and ability to induce lateral segregation, SM:Chol complexes are often referred to as ‘rafts’. These lipid rafts have been proposed to be involved in many cellular activities in mammalian cells, such as immune responses, delivering proteins and newly-synthesized lipids to the cell surface or organelles (membrane tracfficking) and distributing across the surface, and serving as a hub for receptor-mediated signal transduction. In addition, various disease-causing bacteria and viruses have been shown to target lipid rafts in order to infiltrate host cells (Simons and Ikonen, 1997; Briggs *et al*., 2003; Parton and Richards, 2003; London 2005). Nevertheless, the exact nature of lipid rafts in living cells still remains somewhat controversial due to difficulties in directly visualizing the small-scale structure of ordered domains by current techniques (Huang and London, 2016).

Model membranes, composed of simple lipid mixtures forming multilamellar and unilamellar vesicles, have been utilised as an important tool to characterize the physical properties of lipid bilayers and the underlying factors of raft formation. In this context, lipid rafts are often referred to as the coexistence of liquid-ordered (L_o) (Chol and sphingolipids rich domains) and liquid disordered (L_d) domains (depleted in Chol) (Simons and Ikonen, 1997; London, 2002). The phase behaviour of lipid bilayers, particularly its main phase transition or chain-melting transition (Mouritsen and Bagatolli, 2015), has been proposed to be an important factor affecting lipid raft formation. A key effect of Chol on bilayer phase behaviour is the elimination of the gel phase (L_β) to liquid ordered (L_o) phase transition. In the absence of Chol, lipid bilayers tend to exhibit a gel state (L_β) at lower temperatures and a liquid crystalline or liquid disordered (L_d) phase at higher temperatures. In the presence of relatively high concentrations of Chol, an intermediate phase between the L_β and L_d phase is formed. Further increase of Chol content gives rise to regions featuring a L_o phase (McMullen *et al*., 2004).

Phase transition is strongly influenced by temperature changes. For example, below the melting temperature (T_m) of the individual lipid species present in the model system, the lipid bilayers exhibit tight packing and a high degree of acyl chain order, commonly referred to as L_o phase. Above the T_m, reduced acyl chain order indicates the transition to L_d phase, along with a change in membrane thickness and lateral lipid distribution. Consequently, the influence of lipid phase on cellular events, such as signal transduction, is significant (Heimburg, 2007).

In addition to the influence of Chol on the lipid phase, rapid translocation of Chol between the leaflets of the membrane (flip-flop) has also been proposed as a potential mechanism for the formation of lipid raft (Collins, 2008). This led to the hypothesis that the incidence of lipid phase separation caused by the presence of Chol in one leaflet of an asymmetric bilayer prompts raft domain formation in the opposing leaflet. In addition, lipid flip-flop has an impact on the regulation of cell growth and intercellular signalling. However, it is still not fully understood exactly how Chol influences the membrane structure to mediate the formation of lipid rafts (Edidin, 2001). In particular, probing the interactions between lipids and cholesterol still remains the subject of a compelling and ongoing debate after decades of studies via many physical methods and molecular dynamic simulations using model membrane assemblies (Chiu *et al*., 2002; Pandit *et al*., 2004). Examples of the employed biophysical methods include the use of differential scanning calorimetry (DSC) (Koynova *et al*., 1985), nuclear magnetic resonance (NMR) (Parkes *et al*., 1982), Langmuir-Blodgett (LB) monolayer film studies (Smaby *et al*., 1994) and small angle X-ray scattering (SAXS) (Takahashi *et al*., 2007). DSC is one of the classic methods to study the phase transition of lipid bilayers. In the meantime, NMR and SAXS are always coupled with DSC to obtain insightful information on the structure of both bilayers and the lipid molecules themselves, including the polymorphism, bilayer thickness and orientation of lipid molecules in the bilayer. To add an extra layer of understanding, LB measurements can be used to obtain the average occupied area per molecule. This method is useful to determine the condensing effect of Chol on membrane bilayers.

In parallel with the technical methods, many chemical probes have been developed to study the individual role of lipids (especially Chol) and intracellular transport in membrane bilayers. Such probes include cholesterol oxidase, filipin, dehydroergosterol, Bora-diaza-indace (bodipy)-cholesterol, NBD-cholesterol, spin-labelled compound 25-doxyl-cholesterol, azidocholesterol, benzophenone-containing photoreactive cholesterol and others (Gimpl and Gehrig-Burger, 2011). Of these probes, some were observed to induce alterations in the membrane properties. For example, the membrane-perturbing effect caused by filipin has been used for visualization of filipin-sterol complexes by ultrastructure (Clark *et al*., 1987). Further development of novel Chol probes remains important for determining the role of Chol in membrane bilayers while minimizing the disturbance of lipid-lipid interaction by the novel probes.

To date, Chol analogues (Maxfield and Wüstner, 2012), which have a structure closely resembling that of Chol are widely regarded to behave in a similar way to Chol in membranes. Thiocholesterol (tChol) is a synthetic Chol analog in which the OH group at carbon 3 is replaced by a sulfhydryl (thiol, SH) group. Owing to the lower electronegativity of sulphur compared to oxygen atoms, the tChol molecule is known to be much more hydrophobic than Chol. This results in a reduction in hydrogen bond formation between water and tChol (Armstrong and Carey, 1987). Head group modified tChol was successfully integrated into liposomes and exhibited similar miscibility with phospholipids to Chol (Huang *et al*., 2005). In addition, tChol coupled to proteins was also inserted into lipid vesicles without causing significant damage to the lipid structure while retaining the protein activity (Wallach, 1991). Additionally, tChol and other Chol analogues were utilized to illuminate the importance of the hydroxyl group of Chol to support solubilization of phospholipids in triolein (Oppenheimer and Cordes, 1981). This research is focused to explore the lipid-Chol interaction and Chol flip-flop utilizing tChol as a suitable probe. tChol is not naturally present in bilayers and can selectively be detected by chemical approaches. Therefore, the rationale to employ tChol as a probe is to minimize the interference of larger (fluorescent) probes on the surrounding lipid phase behaviour while maintaining simple measurement of the tChol flip-flop rate.

1.1 Objectives

Motivated by the interest in developing Chol probes to study Chol flip-flop, the main objective of this research was to explore the potential utilization of tChol as a probe in Chol flip-flop and lipid-cholesterol studies using model membrane assemblies. Several aims have been accomplished to understand and evaluate the changes in membrane properties induced by tChol prior to probing Chol movement across membrane bilayers. These aims were to illuminate the: -

a. Effect of tChol on the membrane properties of binary mixtures comprised of phospholipids and sterols, via biophysical methods and computer simulations.
b. Arrangement and position of tChol in phospholipid bilayer membranes, on the molecular level, via biophysical methods and computer simulations.
c. Parallel relationship between sterol solubility and phospholipids by quantifying the maximum solubility of sterols in lipid bilayers.
d. Flipping rate of Chol in lipid bilayers by utilizing tChol as a probe, through a chemical approach.

2 LITERATURE REVIEW

2.1 Lipids

Lipids are classified as amphiphilic molecules, consisting of a hydrophilic (Greek: friend of water) or polar head and a hydrophobic (Greek: in fear of water) or non-polar tail (Alberts *et al*., 2002). As the hydrophobic section tends to exceed the polar moieties of a lipid molecule, they tend to be more readily soluble in organic solvents than in water. In biological systems, a very large number of different lipid classes are synthesized by cells. In eukaryotes, most cell types (but particularly adipocytes) contain lipid storage organelles, which consist of a lipid droplet surrounded by a protein shell (Martin and Parton, 2006) . Despite the general use of the term lipid reservoir, these organelles play an active role in regulating lipid metabolism in addition to simply storing lipids for future use. The exact nature and composition of lipid reservoirs remain elusive, in part due to the incredibly large number of lipid species that they contain. The complexity introduced by the variety of lipids means that the function of specific molecules still represents an intriguing topic in current lipid research.

In general, the function of lipids can be categorized into three groups; namely energy storage, compartmentalization (forming the cellular membrane matrix) and signaling, as lipids are known to act as first and second messengers in signal transduction and molecular recognition processes (van Meer *et al.*, 2008). For a long time, the role of lipids in membranes has been more or less neglected in the field of biology. In this project, biophysical properties of lipids are studied in association with membrane research. Hence, membrane research is the main focus of this review.

To date, the main effort of membrane research has been focused on membrane organization and more recently on the dynamics of membrane organization, which still remains a highly challenging topic. Utilizing a wide array of newly developed and well established methods originating from a wide array of disciplines, the previously poorly accessible lipids have gradually been brought back to the center of the spotlight. Lipids can be more directly probed via new methodologies and consequently the detailed role of lipids in membranes will gradually be explored (Simons, 2016).

2.2 Lipids to bilayers

In general, the eukaryotic cell membrane (also known as the plasma membrane) is composed of lipids and proteins. Lipids are associated with the formation of the membrane matrix for membrane associated and integrated proteins to function. They also act as a permeability barrier of the cell, to retain valuable nutrients and prevent toxic material from entering. Intriguingly, lipids are typically self-assembled into a bilayer structure (Dowhan, 1997) and thus form without the help of an elaborate cellular machinery. That means lipid molecules assemble themselves into two thin sheets by orienting their hydrophobic tails pointing inwards while the hydrophilic heads are exposed to the water interface. Figure 2.1 displays a simple cartoon of the orientation of lipid molecules in a lipid bilayer.

Lipid bilayers are generally considered as two-dimensional fluids with a typical thickness of about 5 nm in eukaryotic cells. Based on the historic development of the field, the evolution of the lipid bilayer concept had already been observed and proposed by Gorter and Grendel in 1925 through the study of chromocytes from biological blood samples on a Langmuir air-water isotherm. Later, in 1972, the well-known fluid mosaic model was then proposed by Singer and Nicolson (Figure 2.2). The fluid mosaic model suggested the oriented proteins and glycoproteins are

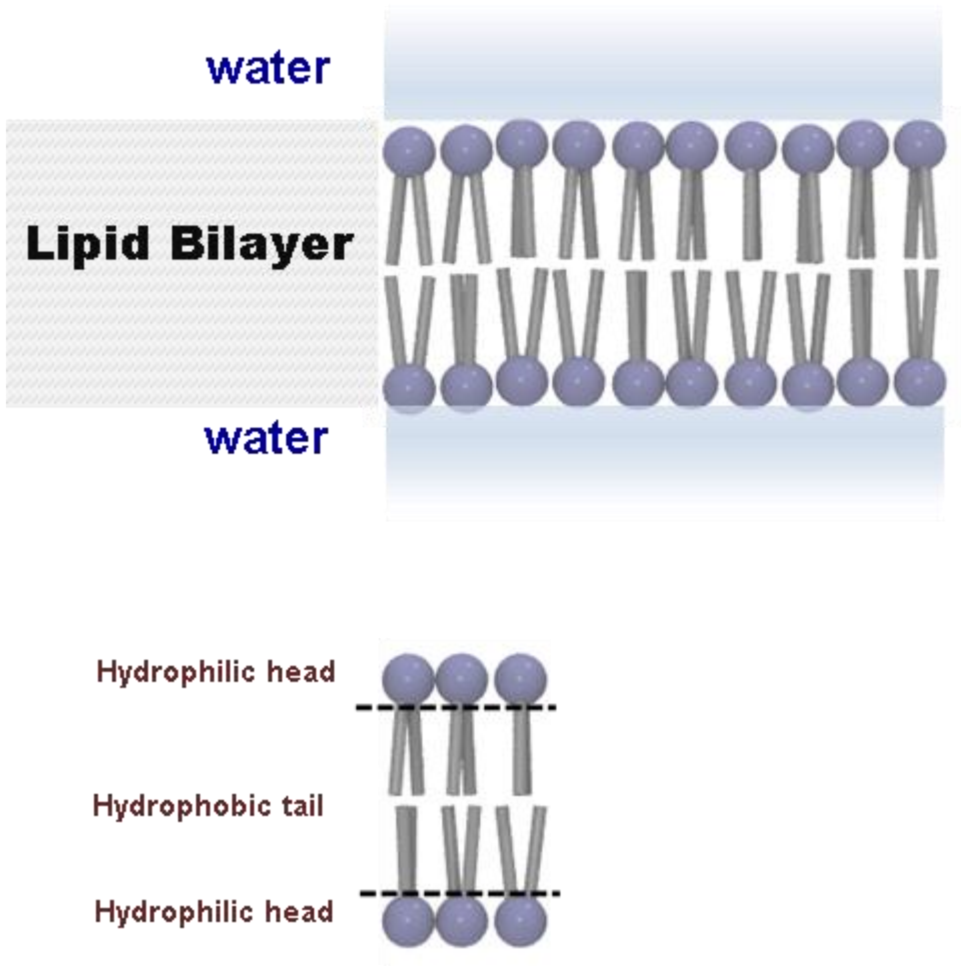

Figure 2.1: Simple cartoon illustration of lipid bilayers. A lipid bilayer is defined as two layers of lipid molecules arranged by orientating the hydrophilic head so that it is exposed to water, while the hydrophobic tails point towards each other to form a hydrophobic core.

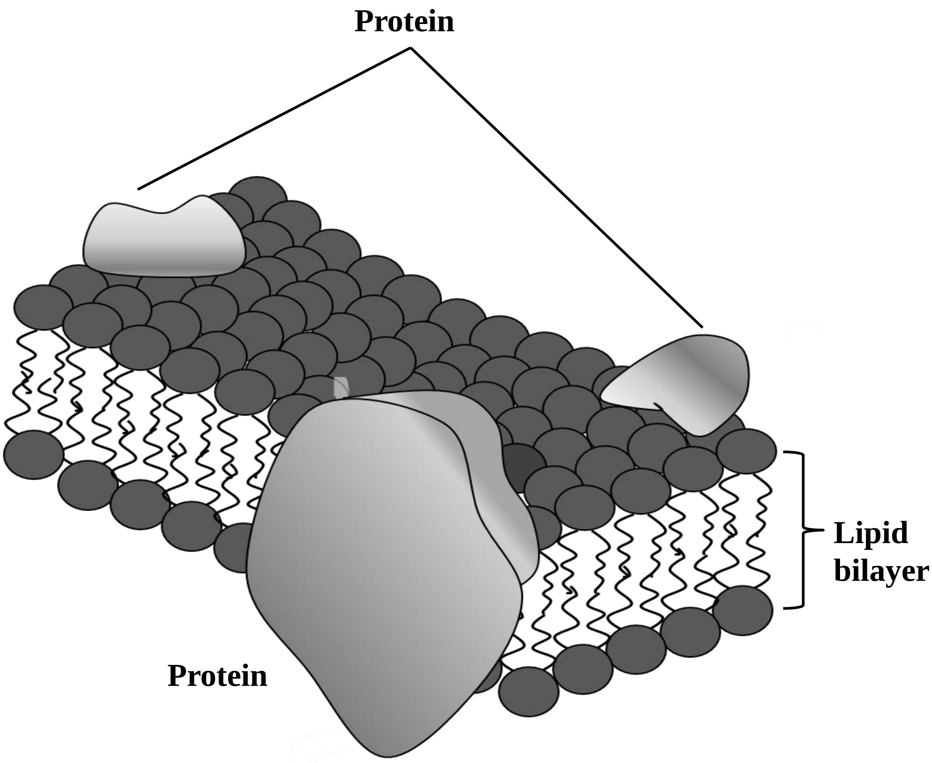

Figure 2.2: Fluid-mosaic model proposed by Singer and Nicolson, (1972). The proteins are arranged so that they either span the whole bilayer or rest within a single leaflet.

likely organized into the two-dimensional structure in a homogenous fluid phase. The model also discussed the concept of bilayer asymmetry. While the model was well developed for its time, more data on lipid behavior increased criticism and new suggestions have been brought forward to extend the proposed model. This led to the development of the current membrane model, that is adopted based on the concept proposed by Simons and Ikonen in 1997 (Figure 2.3).

In cell membranes, the lipids are intriguingly organized into lipid rafts or small-scale domains. The heterogeneity of lipid compositions in phase-separated micro-domains is likewise attributable to the regulation of physical properties in biological membranes. In addition, detailed information on the progress in lipid research and updates on the proposed fluid mosaic model membrane (Singer and Nicolson, 1972) have been well summarized by Nicolson (2014). In his review, Nicolson and co-authors propose an updated model (Figure 2.4) including the findings that have been revealed since the 1970s. These findings include the current information about lipid rafts, membrane domains and cytoskeletal fencing.

A lipid raft (Figure 2.3) is postulated as an area that is enriched with a high content of glycosphingolipids coexisting with cholesterol. In other word, these areas are lipid-based microdomains. According to the current understanding, the lateral interactions between the glycerosphingolipids based on their chemical structures lead to the segregation of highly oriented lipid regions (Liquid-ordered, L_o phase) from the surrounding glycophosphatidyl lipids that exhibit a more fluid phase (Liquid-disordered, L_d phase) (Simons and Ikonen, 1997; Parton and Richards, 2003; Mouritsen and Bagatolli, 2015). Such microdomains are widely hypothesized to be involved in various cell functions such as endocytosis, cell signaling, post-Golgi trafficking, and virus assembly processes, to name just a few (Parton and Richards,

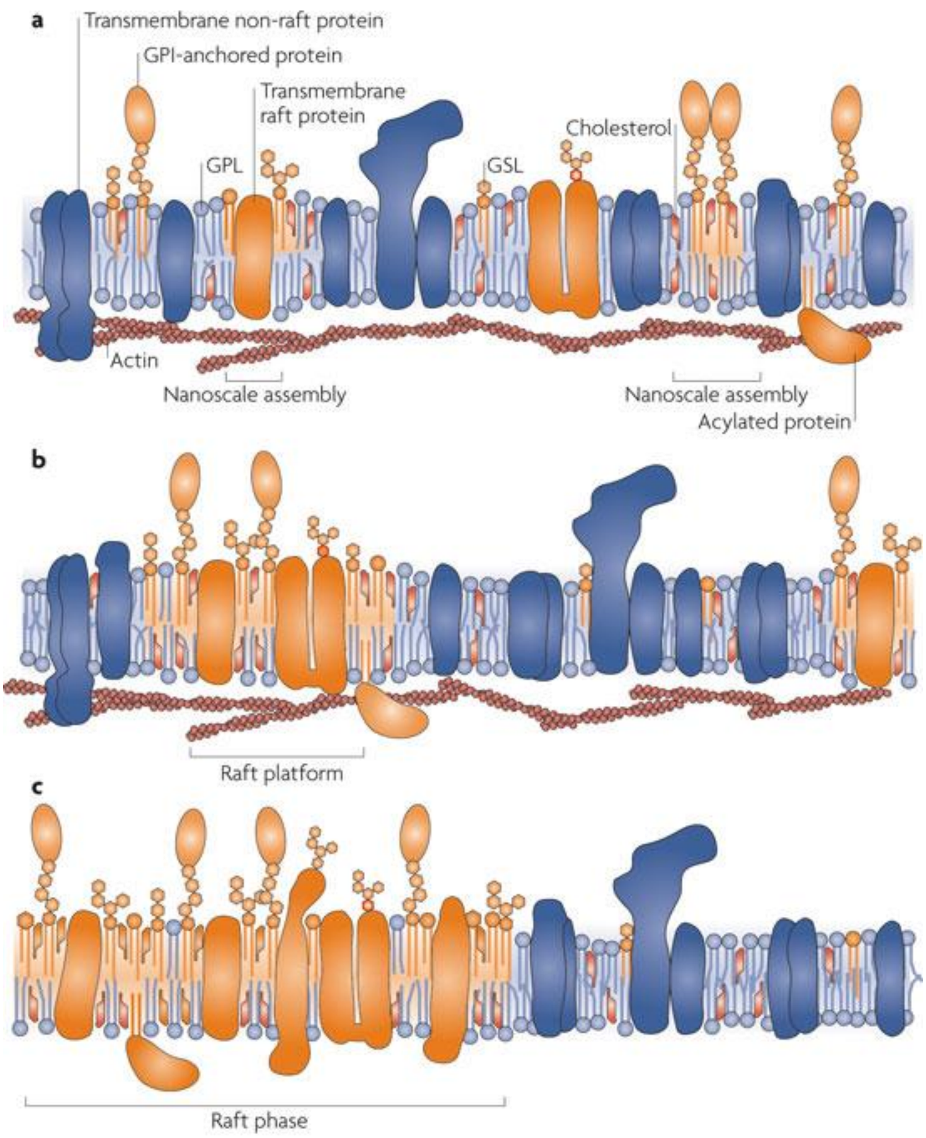

Figure 2.3: Cell membrane structures with the presence of small scale domains called rafts. (a) Organization of membranes with lipids and proteins, highlighting the different ways that proteins can be incorporated into a membrane. (b) Proteins, including transmembrane proteins and GPI-anchored proteins, can bind to the raft platform together with doubly acylated protein. (c) A high lipid content with glycolipid-enriched complexes (DIGs) results in the formation of a raft phase, containing glycosphingolipids (GS) and is characterized by cholesterol or glycerophospholipid-cholesterol clustering. The lipid bilayer in rafts is proposed to be asymmetric. The figure is taken from the published work by Mouritsen and Bagatolli (2015) referring to the concept proposed by Simon and Ikonen (1997).

.

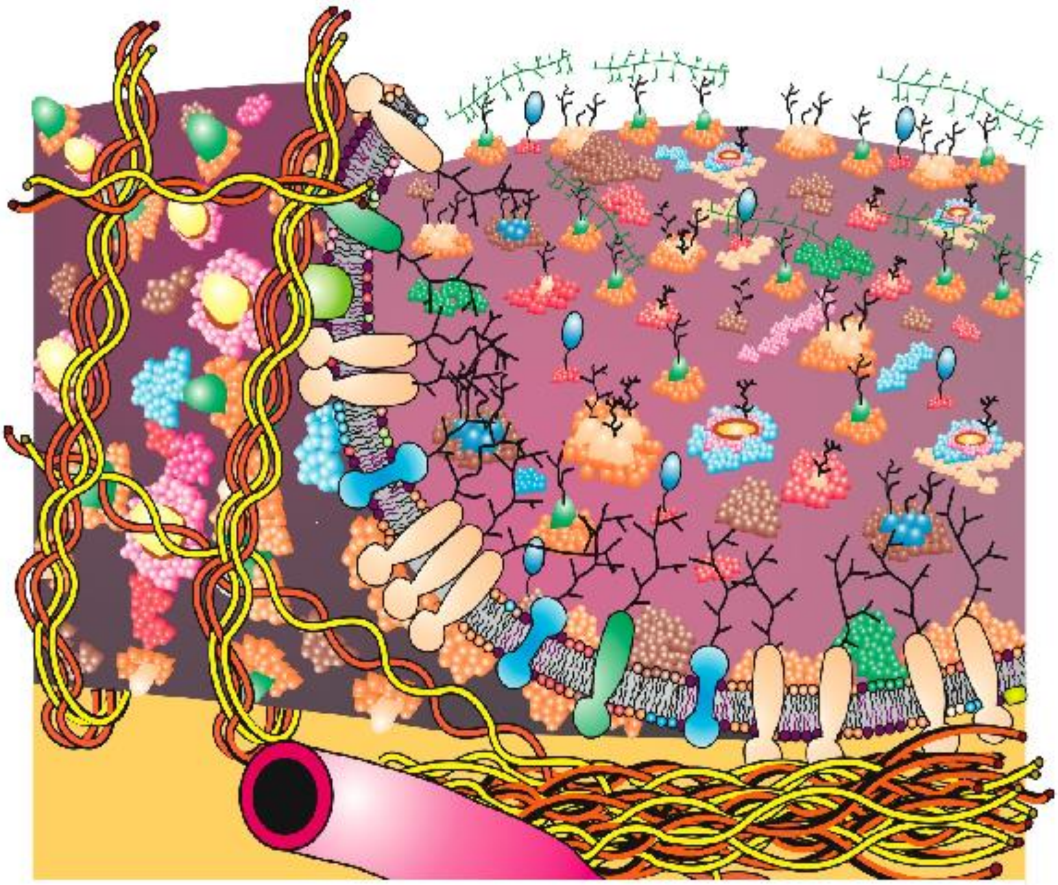

Figure 2.4: The updated fluid mosaic membrane proposed by Nicolson. Lipids, oligosaccharides and proteins are presented in different colors. The updated model accounts for the presence of membrane domain structures, membrane-associated cytoskeletal features and extracellular structures. To the left of the figure: the bottom or inner membrane surface is pictured with the membrane-associated skeletal structures (corrals) forming a fence. This consequently restricts some integral membrane proteins to move laterally. Those structures also interact with integral membrane proteins that present at the inner membrane surface, and with matrix components at the outer surface. The figure is taken from Nicolson (2014).

2003; Simons and Sampaio, 2016). In this context, lipid bilayers represent the basic structure of membranes and are crucial to allow the cell membrane to carry out its essential roles, forming the most important barrier in the biological world.

2.3 Model cell membranes

2.3.1 Diversity of lipids

Many problems in advanced studies of biological membranes arise from the complexity of biological membranes and the incredible diversity of lipids present in cellular membranes So far, hundreds of lipid species have been identified and more than 10^8 different lipid molecular structures are thought to exist across the cellular membrane (Janmey and Kinnunen, 2006). Among lipids, phospholipids are the most abundant in biological membranes, particularly in eukaryotes. Glycerol (a 3-carbon alcohol) or sphingosine (an amino alcohol) are commonly forming the backbone of phospholipids and allowing differentiation into glycero-based and sphingoid-based phospholipids. The chemical structure of a glycerophospholipids is composed of two molecules of fatty acids – either identical or different in structure – that are esterified to the backbone, while a phosphate (phosphatidic acid) is attached to the remaining backbone hydroxyl function (Working and Andrews, 1941).

Glycero-based phospholipids are particularly predominant in eukaryotic cellular membranes. The phosphatidic acid residue of glycerophospholipids is generally esterified with choline, ethanolamine, serine or inositol, leading to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), or phosphatidylinositol (PI) (van Meer *et al*., 2008), respectively Of these lipids, PC (Figure 2.5 A) is recognized as the primary phospholipid in animals and plants, but absent or only rarely present in prokaryotic membranes.

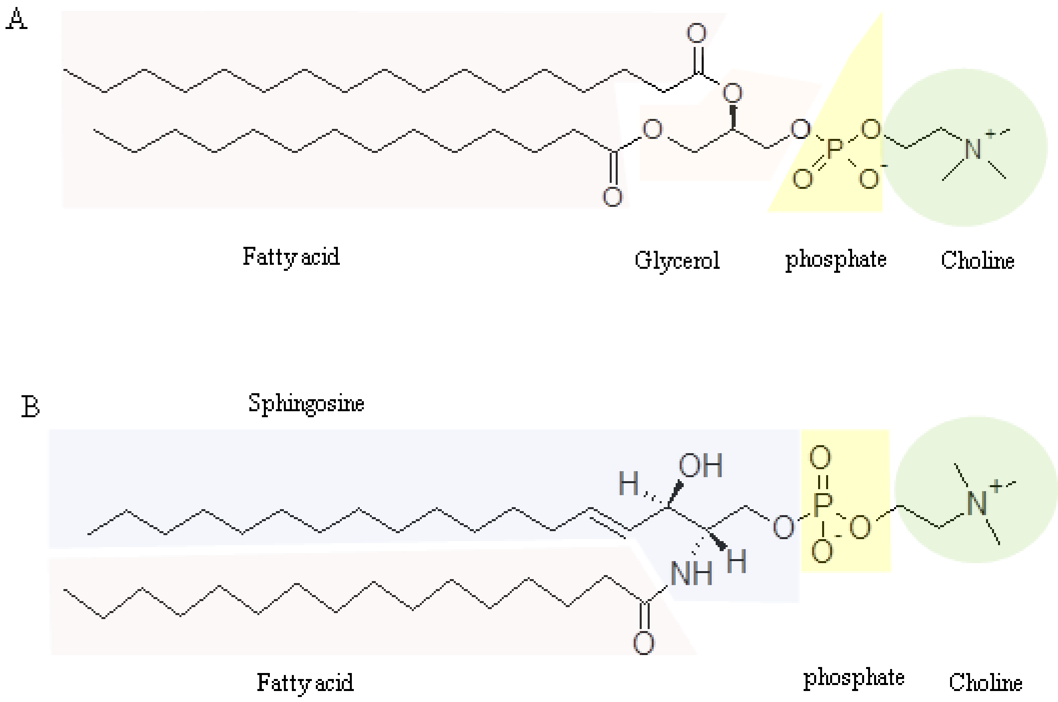

Figure 2.5: The chemical structure of (A) glycerophospholipids, with phosphatodycholine (PC) as an example (B) sphingolipids, with sphingomyelin (SM) as an example.

In the past, PC was often referred to as lecithin and has been shown to constitute up to 50 % of the total lipid composition. PCs are typically neutral or zwitterionic phospholipids. The ionic state of the molecule refers to the polar head group, which in the case of zwitterionic phospholipids contains an anionic phosphate group and a cationic quaternary ammonium center. The different ionic properties cause PC molecules to exhibit distinct electrophoretic velocities in solutions depending on the pH. PC can be found with saturated and/or unsaturated fatty acid chains as well as different acyl chain lengths in animal tissues and organelles. PCs are also thought to share some of their membrane functions with sphingomyelins, in part due to their similar structure (Christie, 2014).

Other than glycerophospholipids, the sphingoid-based lipids such as sphingomyelin (SM) and glycosphingolipids (gangliosides) also occupy a major fraction of eukaryotic membranes. In contrast to glycerophospholipids, the backbone of sphingolipids such as (SM) contains sphingosine linked to a fatty acid chain (van Meer *et al*., 2008). The structural features of sphingolipids and glycerophospholipids are compared in Figure 2.5, as both belong to the category of phospholipids. In general, SM comprises about 2-15 % of the total proportion of phospholipids in animal tissues. Some distinct features of the SM structure are important for their role in biological membranes. These include an asymmetric molecular structure, the ability to form hydrogen-bonds with neighboring molecules, and a low degree of unsaturation. (Slotte and Ramstedt, 2007).

Apart from phospholipids, cholesterol (Chol) is well known as a component of the eukaryotic plasma membrane (Simons and Sampaio, 2016). Cholesterol belongs to a separate class of lipids known as sterols. Unlike the high diversity of phospholipid structures found in mammalian membranes, sterols display less variation in their structure. The Chol molecule has a hydrophilic 3*β*-hydroxyl head, a rigid body of 4 fused, planar rings with two *β*-oriented methyl groups attached at C-10 and C-13, and a hydrocarbon tail branched at C-17 (Figure 2.6) (Róg *et al.*, 2009). In particular, Chol is an important component in lipid raft theory. The lipid-lipid interactions between phospholipids and Chol induce so called lipid rafts, sometime also called micro domains, which are enriched in Chol and SM as mentioned before.

The headgroups of glycerol- and sphingolipids are believed to shield the more hydrophobic Chol molecules when Chol is added to the phospholipid bilayer. This is known as the "umbrella model". According to this model, the small headgroup of Chol is protected by the large headgroup of phospholipids to reduce the contact of Chol with the water interface (Huang and Feigenson, 1999). Consequently, this increases Chol-lipid interaction, while disfavors Chol-Chol interaction. Chol orients its headgroup towards the ester or amide carbonyl oxygen of phospholipids, while its tail is oriented parallel to the phospholipids acyl chain. In this context, Chol can affect the conformational order of neighbouring lipid acyl chains, and thus can induce a change towards the liquid ordered (L_o) phase when added in sufficiently high concentrations to membranes otherwise adopting the liquid disordered (L_d) phase. In part due to favorable van der Waals interactions with neighboring lipid molecules, the presence of Chol leads to a decreased area per lipid molecule exposed to the water interface. This effect is also known as condensing effect. In another words, Chol influences membrane permeability and regulates membrane lateral organization, lipid-lipid interactions and lipid-protein interactions for example by inducing changes in the membrane thickness (Maxfield and Wüstner, 2012; Róg and Vattulainen, 2014).

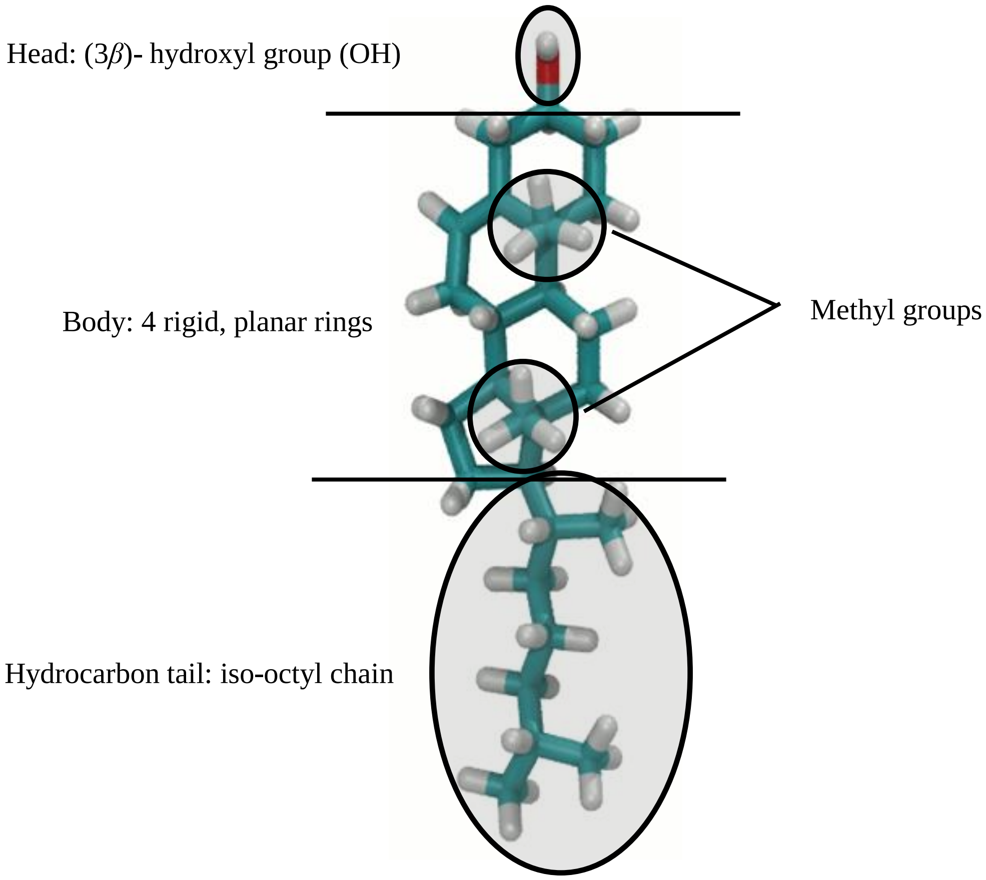

Figure 2.6: The structure of cholesterol molecules.

A broad range of experimental and computational studies have been conducted to explore the ability and properties of phospholipids and sterols to generate synthetic model membranes and to gain more insight into the behavior of lipids in membranes. These simplified systems also encourage the study of individual lipid components including the organization and dynamic structure of lipids. For these studies, a variety of techniques to prepare and analyze synthetic model lipid membranes, such as lipid monolayers and liposomes, have been developed and employed (Peetla *et al.*, 2009).

2.3.2 Lipid monolayers

One of the most widely used techniques to study lipid behavior is to prepare a layer of lipid molecules on a Langmuir Blodgett (LB) trough, also known as Langmuir monolayers. In this technique, lipids or amphiphilic molecules are initially dissolved in a suitable organic solvent and then spread on the surface of a liquid sub-phase (usually water or an aqueous pH buffer). Subsequently, the molecules under study self-assembles into a lipid monolayer on the water surface (or, more precisely, on the water-air interface) in the LB trough, constituting a 2D model system. The hydrophilic, polar head group faces towards the water interface, while the hydrophobic tail points outwards to the air interface (Kaganer *et al.*, 1999). This setup allows vary parameters such as temperature and surface pressure, and enables to study lipid-lipid interactions in the monolayer instead of a more complex bilayer. Nevertheless, information from monolayer studies can be extended to bilayers (with some restrictions), as the bilayers are essentially constructed of two lipid monolayers. In addition, the lateral intermolecular interactions in both bilayers and monolayers are likely to be similar (Stefaniu *et al.*, 2014). Therefore, LB monolayer models allow the study of some membrane properties including polymorphism or structural organization of monolayers under full control of temperature, pressure and pH.

As an example of the type of experiment that are routinely carried out in an LB trough is the compression study. Here, the surface pressure of assembled monolayer at the air-water interface is measured and can be plotted against the mean molecular area. Upon the compression of monolayers by reducing the size of the water-air interface, induced by slowly pushing together opposite sides of the monolayer trough, different physical states of fluidity in the monolayer can be observed. These usually involve gaseous, liquid expanded, liquid-condensed and solid-like states. Remarkably, the changes in the physical behavior of monolayers are correlated with the level of conformational order of the molecules at the interface. This could be due to the presence of intermolecular interactions (lipid-lipid interactions) in monolayers (Marc and Magali, 2010). The possible physical states usually encountered during monolayer compression are summarizes in Figure 2.7. Already in earlier LB based studies, the significant influence of sterols, especially Chol on bilayer fluidity and packing, such as the condensing effect on lipid bilayers, were proposed (Demel *et al*., 1972).

2.3.3 Liposomes

Additionally to lipid monolayers, common artificial lipid model membrane system used in membrane research studies employ liposomes (Sessa and Weissmann, 1968). By simple definition, liposomes are typically described as spherically-shaped vesicles composed of single or multiple lipid bilayers (lamellas), containing an internal aqueous partition (Laouini et al., 2012). The discovery of liposomes is usually ascribed to Bangham in the 1960s. He supposedly discovered that