INTERACTIONS OF CHOLESTEROL REDUCING AGENT SIMVASTATIN WITH CHARGED PHOSPHOLIPID MODEL MEMBRANES

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INTERACTIONS OF CHOLESTEROL REDUCING AGENT SIMVASTATIN WITH CHARGED PHOSPHOLIPID MODEL MEMBRANES

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ABSTRACT

INTERACTIONS OF CHOLESTEROL REDUCING AGENT SIMVASTATIN WITH CHARGED PHOSPHOLIPID MODEL MEMBRANES

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Interactions of cholesterol reducing agent simvastatin with charged model membranes were investigated. Effects of cholesterol reducing agent simvastatin on the phase transition behaviour and physical properties of the anionic dipalmitoyl phosphatidylglycerol (DPPG) multilamellar liposome were studied as a function of temperature and simvastatin concentration. Moreover the effect of acyl chain length on the simvastatin model membrane interactions was monitored using dipalmitoyl phosphatidylglycerol (DPPG) and dimyristoyl phosphatidylglycerol (DMPG) lipids. All experiments were carried out by two non-invasive techniques namely Fourier Transform Infrared (FTIR) Spectroscopy and Differential Scanning Calorimetry (DSC).

The observations made in the this study clearly showed that simvastatin interacts with the lipids of multilamellar liposomes and induces some variations in the structure of membranes. These effects are seen in the thermotropic phase transition profile of the membranes, on membrane order, acyl chain flexibility, lipid head group structures and membrane fluidity.

The analysis of the C-H stretching region of FTIR spectra showed that, as simvastatin concentration increased, the phase transition curve broadened, pretransition temperature diminished, membrane order and membrane fluidity increased for anionic DPPG membrane. Moreover analysis of the
C=O stretching and PO$_2^-$ stretching bands showed that simvastatin caused dehydration effect by decreasing of hydrogen bonding capacity in the glycerol backbone and also around the lipid head groups.

DSC studies showed that as the simvastatin concentration increased, DSC curves broadened. In addition, simvastatin-induced lateral phase separation was observed in the DSC thermograms.

In the second part of the study, the effect of acyl chain length on the simvastatin - membrane interactions was investigated for DPPG and DMPG lipid membranes. All parameters used in the FTIR studies are compared for DMPG and DPPG membranes. Similar results were observed for both membranes, except for the CH$_2$ antisymmetric stretching band frequency at gel phase,. Results showed that there are no significant effect of acyl chain length on simvastin - membrane interactions.

**Key words:** FTIR spectroscopy, DSC, simvastatin, DPPG, DMPG
ÖZ

KOLESTEROL DÜŞÜRÜCÜ AJAN SIMVASTATİNİN YÜKİLİ FOSFOLİPİD MODEL MEMBRANLARLA ETKİLEŞİMİ

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Bu çalışmada yapılan değerlendirmelerde simvastatinin kullanılan model membranlarla etkileşime girdiği ve membran yapısında fiziksel değişimlere yol açtığı tespit edildi. Bu etkiler membranların termotrofik faz değişim davranışlarında, membran düzenliliği (açılı zincir esnekliği), lipitlerin kafa grup yapılarında ve membran akışkanlığında görülmüştür.

Sonuçlarımızda göre FTIR spektrumun C-H bölgesinin analiz edilmesiyle simvastatin konsantrasyonu yükseldikçe, faz geçiş eğrisinin genişlediği ve ön faz geçiş sıcaklığı tamamen yok olduğu, membran düzensizliğinin azaldığı, membran akışkanlığının arttığı görülmüştür. Ayrıca C=O gerilme ve PO₂
gerilme bantlarının analiziyle simvastatin kafa gruplarına yakın gliserol iskeletinde ve PO\textsubscript{2} fonksiyonel gruplarının etrafında hidrojen bağ yapma kapasitesinin azaldığı diğer bir deyimle simvastatin kaynaklı dehidrasyon oluştuğu gözlemlenmiştir.

Simvastatin konsantrasyonu arttıkça DSC tepe egrileri genişlemiş ve daha düşük sıcaklık değerlerine kaymıştır. Ayrıca DSC termogramlarında simvastatin sebepli yanal faz ayrımı görülmüştür.

Çalışmanın ikinci kısmında, simvastatin-model membran etkileşimlerinde asıl zincir uzunluğunun etkisi DPPG ve DMPG lipitleri kullanılarak araştırılmıştır. FTIR spectroskopisi çalışmalarında kullanılan parametreler DMPG ve DPPG membranları için karşılaştırılmış ve CH\textsubscript{2} asimetrik gerilme bandı jel fazı frekans değerleri dışında benzer sonuçlar gözlemlemiştir. Yapılan çalışmalarda genel olarak zincir uzunluğunun değişimi simvastatinin membranlar üzerinde etkisinde anlamlı değişikliklere yol açmadığı anlaşılmıştır.

**Anahtar Kelimeler:** FTIR spektroskopisi, DSC, simvastatin, DPPG, DMPG
To my grandmother,
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CHAPTER 1

INTRODUCTION

1.1. Scope and Aim of This Study

The plasma membrane encloses the cell, defines its boundaries, and maintains the essential differences between the cytosol and extracellular environment. All biomembranes form closed structures, separating the lumen on the inside from the outside, and are based on a similar bilayer structure. They control the movement of molecules between the inside and the outside of a cell and into and out of the organelles of eukaryotic cells (Lodish et.al., 2004). Because most of the drugs used for medication must be taken into the cell passing through the plasma membrane, we should understand the interaction of these drugs with membranes.

The physical properties and functional roles of individual lipid species in membranes are exceedingly difficult to ascertain in an intact biological membrane due to the complex lipid composition. In order to gain insight into the roles of individual components, it is necessary to construct model membrane systems that contain the lipid species of interest (Vance, 1991). In most membrane systems, the balance of charged phospholipids favors the zwitterionic phospholipids, as less than 30% of the membrane phospholipids are anionic. Evidence from a broad range of organisms and systems supports the involvement of anionic phospholipid head groups in the membrane association of many cytoplasmic proteins (Dowhan, 1997). Phosphatidylglycerols (PGs) are one of the anionic lipids mainly found in the bacterial cell membranes, eukaryotic mitochondria and chloroplast thylakoid membranes. Therefore model membranes composed of phosphatidylglycerols as anionic lipids are one of the most widely studied.
Cholesterol is a major precursor for the synthesis of many biologically important molecules like bile acids and steroid hormones in the body (Mathews et al., 2000). It is also one of the components of the cell membrane and determines the physico-chemical properties of the membrane (Lodish et al., 2004). Cholesterol synthesis occurs in all types of cells but mainly in liver cells (Evans and Rees, 2002). An early and rate-limiting step of the synthesis is catalyzed by HMG CoA reductase (Hamelin and Turgeon, 1998). The group of drugs called statins are HMG-CoA reductase inhibitors and they stop the cholesterol synthesis at the rate limiting state in the mevalonate pathway (Päivä et al., 2005). Because of this inhibition the blood plasma levels of LDL-cholesterol and triglycerides decrease while the level of HDL-cholesterol increases (Schachter, 2004). Thus, cholesterol metabolism is regulated. Due to these properties statins are widely used in the treatment of hyperlipidemic patients (Wierzbicki et al., 2003).

Simvastatin is one of the member of statin drugs. The discovery of statins led to important improvements in the primary and secondary prevention of coronary artery disease (CAD). Subsequent large prospective clinical trials have provided unequivocal evidence that cholesterol-lowering therapy with this class of compounds not only reduces the major coronary event rate in primary and secondary prevention, but also reduces all-cause mortality in secondary prevention (Vaughan et al., 2000). Despite its vital effects, it is reported that simvastatin has pleitropic effects on many different tissues (Almuti et al., 2006).

In one of the previous studies that was conducted in our lab, the effect of simvastatin on zwitterionic phospholipids, dipalmitoyl phosphatidylcholine (DPPC), model membranes was studied (Koçak, 2007). As an extension of this work, to achieve more information about drug-membrane interactions, in the current study the interaction between simvastatin and model membranes constructed with anionic lipid dipalmitoyl phosphatidylglycerol (DPPG) and dimyristoyl phosphatidylglycerol (DMPG) was investigated. In this context, the effect of simvastatin on the lipid organization, fluidity of membrane and phase transition profiles were explored. In addition, the location of
simvastatin in the model membrane was estimated. This study is going to be important because it is the first study involving the interaction of simvastatin with the charged phospholipid model membranes which will help together with previous study on zwitterionic membranes to better understand the interaction of the drug with biological membranes. For this purpose, different non-perturbing biophysical techniques; namely, Fourier transform infrared (FTIR) spectroscopy, and Differential Scanning Calorimetry (DSC) were used. Second part of the study involves the comparison between DPPG and DMPG model membranes which contain different acyl chain length lipids and how this difference influences the interaction of simvastatin with the anionic model membranes.

1.2. Biological Membranes and Diversity in Lipid Structure

It is widely recognized that phospholipids play multiple roles in cell processes. Their primary function is to define the permeability barrier of cells and organelles by forming a phospholipid bilayer. This bilayer serves as the matrix and support for a vast array of proteins involved in important functions of the cell such as energy transduction, signal transduction, solute transport, DNA replication, protein targeting and trafficking, cell-cell recognition, secretion, etc. Phospholipids do not play a static role in these processes but are active participants that influence the properties of the proteins associated (Dowhan, 1997).

Despite their differing functions, all biological membranes have a common general structure: each is a very thin film of lipid (fatty) and protein molecules held together mainly by noncovalent interactions (see Fig. 1).
Figure 1. Three views of a cell membrane (A) An electron micrograph of a plasma membrane (human red blood cell) seen in cross section. (B) and (C) these drawings show two dimensional and three dimensional views of a cell membrane (Adopted from Alberts, 2008).

Cell membranes are dynamic and fluid structures and most of their molecules move in the plane of the membrane. The lipid molecules are arranged as a continuous double layer of about 5 nm thick. Amphipathic phospholipids, like other amphiphilic molecules, are subject to two conflicting forces: the hydrophilic head is attracted to water, while the hydrophobic hydrocarbon moiety avoids water and seeks to aggregate with other hydrophobic molecules (like-dissolves-like). This dichotomy is elegantly resolved by the formation of a lipid bilayer in an aqueous environment (Tien and Ottova, 2000).

A typical biomembrane is assembled from phosphoglycerides, sphingolipids, and steroids. All three classes of lipids are amphipathic molecules having a polar (hydrophilic) head group and hydrophobic tail. The hydrophobic effect and van der Waals interactions cause the tail groups to self-associate into a bilayer with the polar head groups oriented toward water. Although the common membrane lipids have this amphipathic character in common, they differ in their chemical structures, abundance, and functions in the membrane. Phosphoglycerides, the most abundant class of lipids in most membranes, are derivatives of glycerol 3-phosphate (Fig. 2). A typical phosphoglyceride molecule consists of a hydrophobic tail composed of two
fatty acyl chains esterified to the two hydroxyl groups in glycerol phosphate and a polar head group attached to the phosphate group. The two fatty acyl chains may differ in the number of carbons that they contain (commonly 16 or 18) and their degree of saturation (0, 1, or 2 double bonds) (Lodish et.al., 2004).

Figure 2. The parts of a phosphoglyceride molecule. This example is a phosphotidylcholine molecule represented (A) schematically (B) by a formula (C) as a space filling model and (D) as a symbol (Adopted from Alberts et.al., 2008).
By combining several different fatty acids and head groups fatty acids and head groups, cells make many different phosphoglycerides. The head groups of the phospholipids (Fig. 3) extend the diversity of lipids defining phosphatidic acid (PA, with OH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), and cardiolipin (CL). Archaebacteria analogs exist with head groups of glycerol and glyceromethylphosphate as well as all of the above except PC and CL. Archaebacteria, also have neutral glycan lipid derivatives in which mono- and disaccharides (glucose or galactose) are directly linked to sn-1-archaeol. Plants (mainly in the thylakoid membrane) and many gram-positive bacteria also have high levels of neutral diacylglycerol glycans with mono- or disaccharides linked to the 3-carbon of sn-3-glycerol. Added to head group.

**Figure 3.** Structure of glycerophosphate-based lipids (Adopted from Vance et.al., 2008)
diversity is the range of alkyl chains of the lipids. Fatty acid chain lengths can vary from 12 to 18 in eubacteria and contain double bonds. Some gram-positive bacteria contain odd-numbered branched-chain fatty acids rather than unsaturated fatty acids. Eukaryotic lipids contain fatty acid chains up to 26 carbons in length with multiple or no double bonds. Therefore, the diversity of glycerol-based lipids in a single organism is significant, but the diversity throughout nature is enormous (Dowhan, 1997).

The bilayer in a typical eukaryotic cell has a continuous surface area of hundreds of square microns, containing hundreds of different lipid types. The types of lipids in the inner and outer leaflets are very different. Hundreds of different lipid species are present in the plasma membrane (Balasubramanian and Schroit, 2003; Boon and Smith, 2002; Hammond et al., 2006). Many of the rare but important signaling phospholipids, such as polyphosphoinositides, seem to be exclusively generated or delivered to the cytoplasmic face (inner leaflet) of the plasma membrane and to specific classes of internal membranes. By contrast, cholesterol accounts for a large fraction of both the inner and outer leaflets but seems to be more abundant in the outer leaflet. This asymmetry is not strictly conserved, and different cell types, organelles and cells at different states of activity are likely to change the lipid distribution. The transbilayer asymmetry is produced primarily in the trans-Golgi network (TGN), but also at the plasma membrane by several proteins that require ATP hydrolysis (Devaux et al., 2006; Boon and Smith, 2002). ATP binding cassette (ABC) transporters seem to move (flop) phospholipids from the inner to the outer leaflet. Aminophospholipid translocases that control movement and retrieval (flipping) of lipids to the inner leaflet include P-type ATPases. Several candidate flippases have been identified in yeast, with various specificities for different lipids (Natarayan et al., 2004).

The ability of lipids to diffuse laterally in a bilayer indicates that it can act as a fluid. The degree of bilayer fluidity depends on the lipid composition, structure of the phospholipid hydrophobic tails, and temperature. As already noted, van der Waals interactions and the hydrophobic effect cause the nonpolar
tails of phospholipids to aggregate. Long, saturated fatty acyl chains have the greatest tendency to aggregate, packing tightly together into a gel-like state. Phospholipids with short fatty acyl chains, which have less surface area for interaction, form more fluid bilayers. Likewise, the kinks in unsaturated fatty acyl chains result in their forming less stable van der Waals interactions with other lipids than do saturated chains and hence more fluid bilayers. When a highly ordered, gel-like bilayer is heated, the increased acyl chain flexibility of the fatty acyl tails cause it to undergo a transition to a more fluid, disordered state (Lodish et.al., 2004).

1.3. Model membrane Systems

The physical properties and functional roles of individual lipid species in membranes are exceedingly difficult to ascertain in an intact biological membrane due to the complex lipid composition. In order to gain insight into the roles of individual components, it is necessary to construct model membrane systems that contain the lipid species of interest.

A number of techniques have been developed for producing model membranes from lipids (Hope et.al., 1986). Preparation of the simplest model system involves the straightforward hydration of a lipid film by mechanical agitation, such as vortex mixing. In the case of bilayer-forming lipids, this hydration results in a macromolecular structure which is composed of a series of concentric bilayers separated by narrow aqueous spaces (Bangham et.al., 1965). Such structures are usually referred to as liposomes or multi-lamellar vesicles (MLVs) and have been used for many years as models for the bilayer matrix of biological membranes. Their use is mostly restricted to physical studies on bilayer organization and the motional properties of individual lipids within a membrane structure. MLVs are not ideal models for the study of other aspects of lipids in membrane structure and function, mainly because as little as 10% of the total lipid of a MLV is contained in the outermost bilayer. As a result, methods have been sought by which unilamellar (single bilayer) model membranes can be obtained either directly or from MLVs.
Small unilamellar vesicles (SUVs) can be made from MLVs by subjecting the MLVs to ultrasonic irradiation or by passage through a French press. However, their small size limits their use in model membrane studies. The radius of curvature experienced by the bilayer in SUVs is so small that the ratio of lipid in the outer monolayer to lipid in the inner monolayer can be as large as 2:1. As a result of this curvature, the packing constraints experienced by the lipids perturb their physical properties in comparison with less highly curved systems. This restricts the use of SUVs for physical studies on the properties of membrane lipid. A more useful membrane model is the large unilamellar vesicle (LUV) system where the distribution of lipid between the outer and inner monolayers is closer to 1:1. These preparative procedures usually include the use of detergents or organic solvents, although LUVs can be produced directly from MLVs (Szoka and Papahadjopoulos, 1980).

A synthetic bilayer made from a single type of phospholipid changes from a liquid state to a two dimensional rigid crystalline (or gel) state at a characteristic freezing point (see Fig. 4). This change of state is called a phase transition, and the temperature at which it occurs is lower (that is, the membrane becomes more difficult to freeze) if the hydrocarbons chains are short or have double bonds. A shorter chain length reduces the tendency of the hydrocarbon tails to interact with one another, in both the same and opposite monolayer, and cis-double bonds produce kinks in the hydrocarbon chains that make them more difficult to pack together so that the membrane remains fluid at lower temperatures (Alberts et.al., 2008).
Figure 4. Schematic illustration of membrane phases (A) Gel phase (B) Liquid crystalline phase

1.4. Lipid Bilayer Domains

Because a lipid bilayer is a two dimensional fluid, it is supposed that most types of lipid molecules in it to be randomly distributed in their own monolayer. The Van der Waals attractive forces between neighboring hydrocarbon are not selective enough to hold groups of phospholipid molecules together. With certain lipid molecules, however, different lipids can come together transiently, creating a dynamic patchwork of different domains. In synthetic lipid bilayers composed of phosphatidylcholine, sphingomyelin, and cholesterol, Van der Waals forces between long and saturated hydrocarbon chains of the molecules can be just strong enough to hold the adjacent molecules together transiently (Kahya et.al., 2004).
Figure 5. Lateral phase separation in artificial lipid membranes (A) Giant liposomes produced from a 1:1 mixture of phosphatidylcholine and sphingomyeline. (B) Liposomes produced from a 1:1:1 mixture of phosphatidylcholine, sphingomyelin and cholesterol form bilayers with two immiscible phases (Kahya et.al. 2004).

The results of recent studies have challenged the long-held belief that lipids are randomly mixed in each leaflet of a bilayer. The first hint that lipids may be organized within the leaflets was the discovery that the residues remaining after the extraction of plasma membranes with detergents contain two lipids: cholesterol and sphingomyelin. Because these two lipids are found in more ordered, less fluid bilayers, it is hypothesized that they form microdomains, termed lipid rafts, surrounded by other more fluid phospholipids that are easily extracted by detergents. Biochemical and microscopic evidence supports the existence of lipid rafts in natural membranes. For instance, fluorescence microscopy reveals aggregates of lipids and raft-specific proteins in the membrane. The rafts are heterogeneous in size but are typically 50 nm in diameter. Rafts can be disrupted by methyl-β-cyclodextrin, which depletes the membrane of cholesterol, or by antibiotics, such as filipin, that sequester cholesterol; such findings indicate the importance of cholesterol in maintaining the integrity of these rafts. Besides their enrichment by cholesterol and sphingolipids, lipid rafts are enriched for many types of cell-surface receptor proteins, as well as many signaling proteins that bind to the receptors and are activated by them.
These lipid–protein complexes can form only in the two-dimensional environment of a hydrophobic bilayer and they are thought to facilitate the detection of chemical signals from the external environment and the subsequent activation of cytosolic events (Simons, 2000).

1.5. Cholesterol Biosynthesis and Statins

The cholesterol biosynthetic pathway is a complex pathway of over 40 cytosolic and membrane-bound enzymes, some of which are subject to feedback regulation by the end product, cholesterol, and oxygenated forms of cholesterol (called oxysterols). Factors that regulate cholesterol synthesis have been found to also control the synthetic pathways for fatty acids, triacylglycerols, and NADPH. Furthermore, the evidence is rapidly building that cholesterol’s precursors and metabolites might serve as biologically active signaling molecules (Vance et al., 2008).

![Figure 6. Overview of the metabolic and transport pathways that control cholesterol levels in mammalian cells.](image-url)
Essential non-steroidal isoprenoids, such as dolichol, prenylated proteins, heme A, and isopentenyl adenosine-containing tRNAs, are also synthesized by this pathway. In extrahepatic tissues, most cellular cholesterol is derived from de novo synthesis (Dietschy and Turley, 2001) whereas hepatocytes obtain most of their cholesterol via the receptor-mediated uptake of plasma lipoproteins, such as low-density lipoprotein (LDL). LDL is bound and internalized by the LDL receptor and delivered to lysosomes via the endocytic pathway, where hydrolysis of the core cholesteryl esters (CE) occurs. Normal mammalian cells tightly regulate cholesterol synthesis and LDL uptake to maintain cellular cholesterol levels within narrow limits and supply sufficient isoprenoids to satisfy metabolic requirements of the cell. Regulation of cholesterol biosynthetic enzymes takes place at the level of gene transcription, mRNA stability, translation, enzyme phosphorylation, and enzyme degradation. Cellular cholesterol levels are also modulated by a cycle of cholesterol esterification mediated by acyl-CoA:cholesterol acyltransferase (ACAT) and hydrolysis of the CE, by cholesterol metabolism to bile acids and oxysterols, and by cholesterol efflux.

Sterols are synthesized from the two-carbon building block, acetyl-CoA. The soluble enzyme acetoacetyl-CoA thiolase interconverts acetyl-CoA and acetoacetyl-CoA, which are then condensed by 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase to form HMG-CoA. There are two genes for HMG-CoA synthase (F.G. Hegardt, 1999). HMG-CoA reductase catalyzes the reduction of HMG-CoA to mevalonate, utilizing two molecules of NADPH. HMG-CoA reductase is a 97-kDa glycoprotein of the endoplasmic reticulum (ER) (L. Liscum et.al., 1985) and peroxisomes (Olivier and Krisans, 2000).

HMG-CoA reductase is the rate-determining enzyme of the cholesterol biosynthetic pathway and, like HMG-CoA synthase, is highly regulated by the supply of cholesterol. Thus, the enzyme has received intense scrutiny as a therapeutic target for treatment of hypercholesterolemia. The enzyme is inhibited by a class of pharmacological agents, called statins, which have an HMG-like moiety and a bulky hydrophobic group (Gotto and Larosa, 2005). Statins occupy the HMG-binding portion of the active site, preventing HMG-
CoA from binding (Istvan and Deisenhofer, 2001). Also, the bulky hydrophobic group causes disordering of several catalytic residues (see Fig. 7). Thus, statins are potent, reversible competitive inhibitors of HMG-CoA reductase with \( K_i \) values in the nanomolar range. Elevated plasma cholesterol levels are a primary risk factor for coronary artery disease, and statin inhibition of HMG-CoA reductase effectively reduces plasma cholesterol levels and decreases overall mortality. Statins also exert cholesterol-independent, or ‘pleiotropic’, effects that improve endothelial function, attenuate smooth muscle cell proliferation, and reduce inflammation (Liao, 2005).

**Figure 7.** Chemical structures of HMG-CoA and several statin inhibitors of HMG-CoA reductase.

Although these statin molecules have similar chemical structures, they have some different physicochemical characteristics. Under physiological pH conditions of 7–7.4, the relative lipophilicity of various statins currently in clinical use was: simvastatin > cerivastatin > lovastatin > fluvastatin
atorvastatin>>pravastatin, where pravastatin is 70- to 300-times more hydrophilic than the other statins. Simvastatin is 100 times more lipophilic than pravastatin and lovastatin is 40 times more lipophilic than pravastatin (Joshi et.al., 1999; Sarr et.al, 2008).

A pharmacologic agent may have some actions other than those for which the agent was specifically developed. These effects, which are called pleiotropic effects, may be undesirable, neutral or beneficial. The pleiotropic effects are either related or unrelated to the primary mechanism of action of the drug, and they are usually unanticipated. Many recent studies reported that the effects of statins extend beyond their cholesterol-lowering capacity (Takemoto et al., 2001) and most of their multiple activities are mediated by the ability to block the synthesis of isoprenoid products. Understanding the pleiotropic effects of statins is important to optimize their use in treatment and prevention of cardiovascular disease (Tchapda, 2005).

By inhibiting L-mevalonic acid synthesis, statins also prevent the synthesis of other important isoprenoid intermediates of the cholesterol biosynthetic pathway, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) (Goldstein and Brown, 1990). These intermediates serve as important lipid attachments for the posttranslational modification of a variety of proteins, including the γ subunit of heterotrimeric G proteins, Heme-a, nuclear lamins, and the small GTP-binding protein Ras and Ras-like proteins, such as Rho, Rab, Rac, Ral, and Rap (Van Aelst and D’Souza, 1997). Thus, protein isoprenylation permits the covalent attachment, subcellular localization, and intracellular trafficking of membrane associated proteins. Members of the Ras and Rho GTPase family are major substrates for posttranslational modification by prenylation (Van Aelst and D’Souza, 1997; Hall, 1998). Because Rho is a major target of geranylgeranylation, inhibition of Rho and its downstream target, Rho kinase, is a likely mechanism mediating some of the pleiotropic effects of statins on the vascular wall (Laufs et.al., 2000). Each member of the Rho family serves specific functions in terms of cell shape, motility, secretion, and proliferation, although overlapping functions between the members could be observed in
overexpressed systems. Indeed, evidence suggests that inhibition of Rho isoprenylation mediates many of the cholesterol-independent effects of statins not only in vascular wall cells but also in leukocytes (Singh et al., 1999) and bone (Mundy et al., 1999).

Statins have been shown to inhibit platelet function (Huhle et al., 1999). Potential mechanisms include a reduction in the production of thromboxane A2 and modifications in the cholesterol content of platelet membranes (Vaughan et al., 2000; Lijnen et al., 1996). The cholesterol content of platelet and erythrocyte membranes is reduced in patients undergoing statin therapy. This may lead to a decrease in the thrombogenic potential of these cells. Plaque rupture is a major cause of acute coronary syndromes (Libby et al., 1997). The atherosclerotic lesion contains highly thrombogenic materials in the lipid core that are separated from the bloodstream by a fibrous cap (Fernandez-Ortiz et al., 1994). Lipid lowering by statins may contribute to plaque stability by reducing plaque size or by modifying the physiochemical properties of the lipid core (Fukumoto et al., 2001). This effect of statins may reduce the incidence of acute coronary syndromes by lessening the propensity for plaque to rupture.

Inflammatory cytokines secreted by these macrophages and T lymphocytes can modify endothelial function, SMC proliferation, collagen degradation, and thrombosis (Libby et al., 1995). An early step in atherogenesis involves monocyte adhesion to the endothelium and penetration into the subendothelial space (Ross, 1999). Recent studies suggest that statins possess anti-inflammatory properties by their ability to reduce the number of inflammatory cells in atherosclerotic plaques (Vaughan et al., 2000).

An intriguing result of large clinical trials with statins is the reduction in ischemic stroke (Crouse et al., 1998). Some studies show that cholesterol lowering activity has nothing associated with the reductions in ischemic strokes (Sythkowski et al., 1990). It appears likely that there are pleiotropic effects of statins that are beneficial in ischemic stroke. Some of these
beneficial effects of statins in ischemic stroke may be due, in part, to their ability to upregulate eNOS expression and activity (Laufs et al., 1998).

1.6. Simvastatin Membrane Interactions

There are limited number of studies conducted on erythrocyte cells showing the effects of simvastatin on biological membranes. These studies mostly involved the treatment of simvastatin to erythrocyte cells and inhibition of cholesterol synthesis on those cells thereby analyzing the effects of simvastatin on the cell membranes (Vevera et al., 2005; Franiak-Pietryga et al., 2009). In the recent study of Franiak-Pietryga 2009, it was observed that simvastatin directly causes the reduction of cholesterol content in erythrocytes plasma membranes in hypercholesterolemic patients. At higher concentration of simvastatin, the lower membrane cholesterol concentration was observed. Their data showed that simvastatin in patients with hypercholesterolemia causes a normalization of plasma membrane structure. It led to the decline in the level of peroxidation products (thiobarbituric acid-reactive substances), the increase in erythrocyte plasma membrane fluidity and decrease in cholesterol content in plasma membranes (Koter et al., 2003; Broncel et al., 2005). In another study conducted by Morita et al in 1997, showed that in 40% reduced cholesterol levels was reached in endothelial cells by simvastatin treatment and this leads to increasing of the membrane dynamics which is detected by FRAP (Florescence recovery after photobleaching) Technique (Morita et al., 1997).

Moreover, Djaldetti et al. in 2006 explained an increase in the phagocytic capacity of the macrophages due to the simvastatin-induced increase in the membrane fluidity. Djaldetti also stated that alterations in membrane bilayer lipid composition and particularly its cholesterol content may affect phagocytic cell function either by changes in membrane rigidity, or by redistribution of the cholesterol in the cell membrane (Djaldetti et al., 2006). Furthermore, related about the membrane fluidity and neutrophil functions Spika et al in 1996 stated that diminished membrane fluidity due to increased
membrane stability decreased neutrophil functions, such as phagocytosis and chemotaxis (Spika et al., 1996).

1.7. Electromagnetic Radiation and Optical Spectroscopy

Electromagnetic radiation is considered as two mutually perpendicular electric and magnetic fields, oscillating in single planes at right angles to each other. These fields are in phase and are propagated as a sine wave as shown in Figure 8, where E is the direction of the electric field while B is the direction of the magnetic field (Stuart, 1997).

![Figure 8. An electron magnetic wave](image)

The energy of the wave is:

\[ E = \frac{hc}{\lambda} = h\nu \]  

(1)

where “h” is Planck’s constant, “c” is the velocity of light, “\(\lambda\)” is the wavelength, and “\(\nu\)” is the frequency. When such a wave encounters a
molecule, it can be either scattered (i.e., its direction of propagation changes) or absorbed (i.e., its energy is transferred to the molecule) (Freifelder, 1982). This equation can be used to identify a common spectroscopic unit called wave number, which is denoted by \( \tilde{\nu} \). Wavenumber is defined as the reciprocal of the wavelength as follows:

\[
\tilde{\nu} = \text{wavenumber} = \left(1/\lambda\right) \quad \text{[has a unit of cm}^{-1}\text{]} \tag{2}
\]

Thus,

\[
E = h \nu = h c \tilde{\nu} \tag{3}
\]

From these equations, it is clear that both wavenumber and frequency are directly proportional to energy.

The relative probability of the occurrence of each process is a property of the particular molecule encountered. If the electromagnetic energy of the light is absorbed, the molecule is said to be excited or in an excited state. A molecule or part of a molecule that can be excited by absorption is called a chromophore. This excitation energy is usually converted into heat (kinetic energy) by the collision of the excited molecule with another molecule (e.g., a solvent molecule). With some molecules it is reemitted as fluorescence. In both cases, the intensity of the light transmitted by a collection of chromophores is less than the intensity of the incident light. An excited molecule can possess any one of a set of discrete amounts (quanta) of energy described by the laws of quantum mechanics. These amounts are called the energy levels of the molecule. A typical energy-level diagram describing these energy levels is presented in Figure 5. The thin horizontal lines represent vibrational energy levels. Accordingly, the long arrow exemplifies an electronic transition, while the short arrow typifies a vibrational transition. The lowest electronic level is called the ground state and all others are excited states (Freifelder, 1982).
Figure 9. Typical energy-level diagrams showing the ground state and the first excited state. Vibrational levels are shown as thin horizontal lines. A possible electronic transition between the ground state and the fourth vibrational level of the first excited state is indicated by the long arrow. A vibrational transition within the ground state is indicated by the short arrow (Freifelder, 1982).

For most purposes, it is convenient to treat a molecule as if it possesses several distinct reservoirs of energy. The total energy is given by:

$$E_{\text{total}} = E_{\text{transition}} + E_{\text{rotation}} + E_{\text{vibration}} + E_{\text{electronic}} + E_{\text{electron spin orientation}} + E_{\text{nuclear spin orientation}}$$

Each $E$ in the equation represents the appropriate energy as indicated by its subscript. In solution, a molecule can translate, rotate and vibrate. The energies associated with each of these are quantized (Campbell, 1984). The separations between the neighboring energy levels corresponding to $E_{\text{vibration}}$ and $E_{\text{electronic}}$ are associated with the infrared and ultraviolet-visible region of the electromagnetic spectrum, respectively (Campbell and Dwek, 1984). The means of study for these energy transitions is standard absorption spectroscopy for electronic transition, infrared and Raman spectroscopy for vibrational and rotational transitions and nuclear magnetic resonance for nuclear spin orientation (Freifelder, 1982).
Spectroscopy is defined as the study of the interaction of electromagnetic radiation with matter. Spectroscopic techniques involve irradiation of a sample with some form of electromagnetic radiation, measurement of the scattering, absorption, or emission in terms of some measured parameters, and the interpretation of these measured parameters to give useful information.

1.8. Fourier Transform Infrared Spectroscopy

Infrared spectroscopy has proved to be a powerful tool for studying biological molecules and the applications of this technique to biological problems is continually expanding, particularly with the advent of Fourier Transform Infrared Spectroscopy in recent decades. One of the great advantages of infrared spectroscopy is that virtually any sample, in virtually any state, can be studied. Liquids, solutions, pastes, powders, films, fibers, gases, and surfaces can all examined by a judicious choice of sampling technique. Biological systems such as proteins, peptides, lipids, biomembranes, carbohydrates, pharmaceuticals, foods, and both plant and animal tissues have all been successfully characterized by using infrared spectroscopy (Stuart, 1997).
Transitions between vibrational levels of the ground state of a molecule result from the absorption of light in the infrared (IR) region: from 103 nm to 105 nm (see Figure 10). These vibrational levels and, hence, infrared spectra are generated by the characteristic motions (bond stretching, bond bending, and more complex motions) of various functional groups (e.g., methyl, carbonyl, amide, etc.). Figure 11 shows various types of vibrations for linear and nonlinear molecules. The value of infrared spectral analysis comes from the fact that the modes of vibration of each group are very sensitive to changes in chemical structure, conformation, and environment. Infrared spectroscopy is thought of as being different principally from visible and ultraviolet spectroscopy because it has a somewhat different technology and because it is used to examine chemical groups not accessible to ultraviolet and visible-light absorption spectroscopy (Freifelder, 1982).
An infrared spectrum is commonly obtained by passing infrared radiation through a sample and determining what fraction of the incident radiation is absorbed at a particular energy. For a molecule to show infrared absorptions it must possess a specific feature, i.e. an electric dipole moment of the molecule must change during the vibration. This is the selection rule for infrared spectroscopy. For example C=O functional group has carbon and oxygen atoms. Because of the difference in electronegativity between carbon and oxygen, the carbonyl group is permanently polarized. Stretching this bond will increase the dipole moment and, hence, C=O stretching is an intense absorption (Stuart, 1997).

1.9. Infrared Spectroscopy in Membrane Research

FT-IR can be regarded as a valuable tool for structural determination of biological membranes. Since membranes are mostly composed of lipids, it is advantageous to use FTIR as a visualization tool. The infrared spectra of phospholipids can be divided into the spectral regions that originate from the molecular vibrations of the hydrocarbon tail, the interface region and the head group (Sahin et.al, 2007; Watts and De Pont, 1986; Lewis and McElhaney, 2002). Structural parameters like order-disorder state,
thermotropic phase transition behaviors and dynamics properties of membranes are most frequently studied by physical techniques like spectroscopic methods which are useful in determining molecular motions and molecular moieties. Fourier Transform Infrared Spectroscopy (FTIR) has proved to be a powerful tool for studying biological molecules and the applications of this technique to biological problems is continually expanding (Stuart, 1997). The hydrocarbon tail gives rise to acyl chain modes. The most intense vibrations in the infrared spectra of lipid systems are the CH2 stretching vibrations and these give rise to bands in the 3100 to 2800 cm\(^{-1}\) region. In certain phospholipid membranes that contain unsaturated acyl chains, the typical lamellar liquid crystalline phase converts to a micellar non-lamellar phase upon heating (Jackson and Mantsch, 1993). Temperature studies of the infrared spectra of phospholipids provide a sensitive means of studying such transitions in lipids. Spectral modes arising from the head group and interfacial region also provide valuable information (Mushayakarara and Levin, 1982, Korkmaz et al., 2005 (a)). There are plenty of studies, which use FTIR as an investigation tool, involving membranous structures and interaction of variety of drugs, proteins, peptides, vitamins etc. with membranes (Lefevre and Subirade, 2000; Severcan et al., 2005 (b); Korkmaz et al., 2005 (a)). These studies mostly search for the membrane phase transition profiles, structural information like orneriness of the membrane, dynamic information about the membrane, membrane water interface region molecular bonding.

1.10. Differential Scanning Calorimetry and Membrane Research

Differential scanning calorimetry or DSC is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference are measured as a function of temperature. Both the sample and reference are maintained at nearly the same temperature throughout the experiment. Generally, the temperature program for a DSC analysis is designed such that the sample holder temperature increases linearly as a function of time. The reference sample should have a well-defined heat capacity over the range of temperatures to
be scanned. The term DSC was coined to describe this instrument which measures energy directly and allows precise measurements of heat capacity (Wunderlich, 1990).

The basic principle underlying this technique is that, when the sample undergoes a physical transformation such as phase transitions, more or less heat will need to flow to it than the reference to maintain both at the same temperature. For example, as a solid sample melts to a liquid it will require more heat flowing to the sample to increase its temperature at the same rate as the reference. This is due to the absorption of heat by the sample as it undergoes the endothermic phase transition from solid to liquid. Likewise, as the sample undergoes exothermic processes (such as crystallization) less heat is required to raise the sample temperature. By observing the difference in heat flow between the sample and reference, differential scanning calorimeters are able to measure the amount of heat absorbed or released during such transitions (Dean, 1995; Skoog et.al., 1998).

The result of a DSC experiment is a curve of heat flux versus temperature or versus time. There are two different conventions: exothermic reactions in the sample shown with a positive or negative peak; it depends on the different kind of technology used by the instrumentation to make the experiment. This curve can be used to calculate enthalpies of transitions. This is done by integrating the peak corresponding to a given transition (Pungor, 1995). For first order phase transitions such as the bilayer gel to liquid-crystalline transition, the transition temperature, $T_m$, is where the heat capacity, $C_p$, reaches its maximum value. The value of the calorimetric enthalpy ($\Delta H_{cal}$) for the phase transition is determined by integrating the area under the peak.

$$\Delta H_{cal} = \int C_p dT$$

From these values, the entropy of the phase transition is determined:

$$\Delta S = \Delta H_{cal} / T_m$$
Comparison of $\Delta H_{\text{cal}}$, $\Delta S$ and $T_m$ shows the effect of a structural modification (e.g. chain length) on the thermodynamics of the phase transition. However, unlike a simple organic compounds crystal to liquid melting transition, the phase transition in bilayers involves more than just the initial and final states. In fact, intermediate “states” are formed during the transition and a “nontwo-state” model is necessary for phospholipids in liposomes (Mason, 1998; Sturtevant, 1997). These intermediate states result from the formation of domains (e.g. disordered, mobile areas within the gel phase) before the phase transition temperature, and are due to lateral movement of the phospholipids within the bilayer. The asymmetric shape of the DSC peak reflects the fact that a non-two-state transition is occurring.

In a recent study of Schwieger and Blume (2009), they have tested how electrostatic interactions influence the binding of poly(L-arginine) (PLA) to anionic DPPG bilayer membranes. Phase transition profiles of the membranes containing different ratios of lipid to peptide are studied with the DSC method (Schwieger and Blume, 2009). In an other study performed by Vautrin et.al (2004), DSC technique was used to find out how the balance of pH and ionic strength influence “salt-free” mixtures of single-tailed cationic and anionic surfactants, called “catanionic systems” (Vautrin et.al, 2004).
CHAPTER 2

MATERIAL AND METHODS

2.1. Reagents

- Simvastatin (2,2-dimethyl-,1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)-ethyl]-1-naphthalenyl ester, [1S-[1\_3\_7\_8(2S\^*,4S\^*),-8a_]]): was purchased from Calbiochem (Merck), Darmstadt, Germany
- 1,2-Dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) (DPPG), 1,2-Didymystoyl-sn-glycero-3-phospho-rac-(1-glycerol) (DMPG): was purchased from Sigma, St. Louis, MO, USA
- Ethanol (C\_2H\_5OH), Sodium Hyroxide (NaOH), Hydrochloric acid (HCl), Chloroform (CHCl\_3), Sodium Phosphate (Na\_2HPO\_4): was purchased from Merck KGaA, Darmstadt, Germany

All reagents were used without further purification.

2.2. Phosphate Buffer Preparation

A concentration of 10 mM phosphate buffer with pH 7.4 was prepared by using Na\_2HPO\_4 powder with a molecular weight of 141.96 g. First, 0.2839 g of Na\_2HPO\_4 powder was added to 150 ml double distilled water. Second, obtained solution was stirred up until the powder was completely dissolved. Third, pH value was adjusted to 7.4 at 25 °C either by adding hydrogen chloride HCl or sodium hydroxide NaOH. During the pH adjustment solution was stirred up. Finally, sufficient double distilled water was added to bring the volume up to 200 ml.
2.3. Simvastatin Stock Solution Preparation

Simvastatin stock solution was prepared by dissolving 5 mg simvastatin powder with a molecular weight of 418.6 g in 1 mL pure ethanol and treating with 0.813 mL of 1 N NaOH in a glass tube. Later, the pH of the solution was adjusted to 7.2 by adding small quantities of 1 N HCl. Thus, the inactive form of simvastatin was converted to the active open ring structure form by a reaction with NaOH. This solution should be used immediately after preparation, otherwise simvastatin may lose its active form.

2.4. Preparation of Model Membranes

DMPG and DPPG multilamellar liposomes were prepared according to the procedure reported by Toyran and Severcan (2003). To prepare simvastatin containing liposomes desired amount of simvastatin was taken from the stock solution and put in an eppendorf tube. The excess ethanol was evaporated by nitrogen stream and then desired amount of DMPG or DPPG was added and dissolved with chloroform in the same eppendorf tube. Since simvastatin is not soluble in water it should be added the system before preparation of liposomes to induce its interaction with the membrane lipids. Again the excess chloroform was evaporated by nitrogen stream and the remaining solvent in the eppendorf tube was removed by spin vacuum drying for 2 hours. The dry thin films were then hydrated by adding phosphate buffer, pH 7.4 with the desired amount of hydration. Multilamellar liposomes were formed by vortexing the mixture for 20 minutes at 20°C above the Tm of DMPG (~43°C) and the Tm of DPPG (~62°C). Pure DMPG or DPPG multilamellar liposomes were prepared by following the same procedure only without using the simvastatin stock solution.

2.5. Sample Preparation For FTIR Experiments

5 mg of DMPG or DPPG was used to prepare thin films. Obtained dry thin films were hydrated by adding 25 μL of 10 mM phosphate buffer, pH 7.4. Infrared spectra were recorded on a Perkin Elmer Spectrum 100 FT-IR
spectrometer. 20 μL of liposome samples were placed between water insoluble CaF$_2$ windows with 12 μm sample thickness. Interferograms were averaged for 20 scans at 2 cm$^{-1}$ resolution. Temperature of samples was regulated by Graseby Specac temperature controller unit. Samples were scanned between 20-60°C with 2°C intervals but above the 50°C interval was increased to 4°C. Samples were incubated for 5 minutes at each temperature before the spectrum acquisition. Experiments are repeated two times for temperature dependent studies of DPPG.

2.6. FTIR Spectrum Analysis

The FTIR spectrums of phospholipid membranes were analyzed elaborately and 4 lipid bands were characterized (Casal and Mantsch, 1984; Cameron and Charette, 1981). Two of them are caused by carbon-hydrogen stretching vibrations and known as antisymmetric and symmetric CH$_2$ stretching modes in the spectral region of 3100-2800 cm$^{-1}$. The CH$_2$ antisymmetric stretching located at 2920 cm$^{-1}$ and CH$_2$ symmetric stretching located at 2851 cm$^{-1}$ are strong lipid bands which are sensitive to conformational changes. Frequency shifts in different regions or changes in the widths of corresponding peaks can be used to extract information about various physicochemical processes taking place in the systems. For example, the frequencies of the CH$_2$ stretching bands of acyl chains depend on the degree of conformational disorder and hence the frequency values can be used to monitor the average trans/gauche isomerization in the systems. The shifts to higher wavenumbers correspond to an increase in number of gauche conformers. Furthermore the bandwidths of the CH$_2$ stretching bands give dynamic information about the system (Severcan, 2005 (a)). One of the most useful infrared band for probing the polar part of the membrane is that of band due to the ester group vibrations at 1730 cm$^{-1}$ (C=O stretching). Any change in the structure of lipid molecule can be monitored by analyzing this sensitive interfacial region. Examining C=O stretching band gives information about the strength of hydrogen bonding (Mendelsohn and Mantsch, 1986). The other band for probing directly the head group of DPPG and DMPG is PO$_2^-$ antisymmetric
double stretching band which is located at 1260 - 1200 cm\(^{-1}\) region. Figure 12 shows the FTIR spectrum bands which were analyzed in this study.

The O-H stretching bands due to buffer appear in the regions of 3400-3200 cm\(^{-1}\) and 1800-1500 cm\(^{-1}\). However, the region of 3400- cm\(^{-1}\) overlaps with the region of C-H stretching band and the region of 1800-1500 cm\(^{-1}\) overlaps with the region of C=O stretching band. Therefore, spectrum of the buffer without simvastatin and lipids was taken at different temperatures and it was subtracted from the spectra of liposomes at corresponding temperatures. Subtraction process was done manually by using Perkin Elmer software programme. Figure 12 shows the infrared spectra of DPPG liposomes respectively before and after water subtraction. Moreover, molecules in the air interfere with the spectra of samples. To prevent this interfering, spectrum of the air was recorded as a background spectrum and subtracted automatically from the spectra of samples by using the same software programme of FTIR spectroscopy.

![FTIR spectrum of DPPG in the 3200 – 1000 cm\(^{-1}\) region. (A) Blue: Before buffer spectrum is subtracted (B) Black: Buffer spectrum (C) Red: After buffer buffer spectrum is subtracted](image-url)

**Figure 12.** FTIR spectrum of DPPG in the 3200 – 1000 cm\(^{-1}\) region. (A) Blue: Before buffer spectrum is subtracted (B) Black: Buffer spectrum (C) Red: After buffer buffer spectrum is subtracted
2.7. Sample Preparation For DSC Experiments

2 mg of DMPG or DPPG was used to prepare thin films. Obtained dry thin films were hydrated by adding 50 μL of 10 mM phosphate buffer, pH 7.4. Liposomes were prepared as described before. 50 μL of liposome samples were placed in high volume pans and these pans were sealed by a special sample encapsulating press. An empty pan was used as a reference pan during the experiment. Heating rate was chosen as 1°C/min. DMPG liposome samples were heated between 0-50°C and DPPG liposome samples were heated between 20-70°C. Experiments were carried out with TA DSC Q100.

2.8. DSC Thermo gram Analysis

Lipids can form a large variety of phase structures as a function of the chemical composition (including length and instauration of the chains), temperature, pressure, hydration, etc. Typical phases at low temperature are baltlayers in different sub gel, gel, and ripple phases. These phases have stretched acyl chains (i.e., in all-trans conformation) giving rise to wax-like properties. At the main transition or melting temperature, $T_m$, the ordered phase is transformed into the liquid crystalline (or fluid) phase. Since the pioneering studies of Chapman (Ladbrooke and Chapman, 1969; Chapman and Urbina, 1974) and others, the standard technique to monitor these phase transitions is Differential Scanning Calorimetry (DSC). Pure lipids usually have very sharp melting transitions. Since impurities tend to broaden the transition, the width can be considered an indicator of purity. Strong membrane curvature in small vesicles as well as undulations or shape fluctuations of large unilamellar vesicles also broaden the transition, and may slightly shift its maximum to lower temperature. Over the years, a wealth of lipid melting data has been collected and the effects of chain length and unsaturation, head group and backbone structure, etc on $T_m$ and $\Delta H$ have been thoroughly studied and modelled (Heerklotz, 2004). In this study we have used the peak maximum taken as the main phase transition temperature.
DSC records the temperature-dependent isobaric heat capacity, $C_p (T)$, of a sample:

$$C_p = \frac{\partial H}{\partial T} \bigg|_p$$  \hspace{1cm} (7)

Equation (7) shows that the integral of a DSC curve from an appropriate baseline yields the enthalpy change of the transition, $\Delta H$. Another useful parameter used in the analysis of DSC curves of lipid membranes is the width at half height of phase transition curve. Moreover, the width of its DSC peak is an index of the cooperativity of this conversion: the narrower the peak, the higher the cooperativity (Arouri et.al., 2009; Zhang et.al., 1995).

Figure 13 shows the parameters analyzed in this study on the DSC curve of pure DPPG MLVs.

![DSC curve of DPPG pure sample](image)

**Figure 13.** DSC curve of DPPG pure sample
CHAPTER 3

RESULTS

The results of the studies performed by Fourier Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC) were discussed in this chapter. DPPG and DMPG Multilamellar Liposomes (MLVs) containing 5 concentrations of simvastatin (1mol%, 6mol%, 12mol%, 18mol% and 24mol%) and pure MLVs were used in this study to monitor interactions of simvastatin with charged phospholipid membranes.

3.1. FTIR Studies

The FTIR studies have been separated into two parts. In the first part, temperature dependent FTIR studies of DPPG model membranes were analyzed. And similar results were obtained from two times repeated experiments. FTIR spectra of DPPG liposomes were analysed with respect to the CH\textsubscript{2} symmetric and antisymmetric, C=O stretching and PO\textsuperscript{2-} antisymmetric bands. During the analyses of these infrared absorption bands, wavenumber values of the all of the bands and bandwidth values of the CH\textsubscript{2} symmetric and antisymmetric bands were investigated. The variations on these parameters were measured and plotted on a graph as a function of temperature to interpret the results. These data were used to understand the simvastatin induced effect on the anionic DPPG model membranes.

The second part of the study include the comparison between simvastatin induced changes on the DPPG and DMPG lipids which have different fatty acid chain lengths of 14 methylene and 12 methylene groups. Again the
same absorption bands and parameters were used for the analyses of FTIR spectra of DMPG or DPPG liposomes. The measured parameters were plotted on column graphs as a function of simvastatin concentrations. And these graphs were given for only two temperatures corresponding to the gel and liquid crystalline phase of the lipids. For DMPG liposomes the selected temperatures were 10°C and 40°C. For DPPG liposomes the selected temperatures were 30°C and 50°C. Every experiment at each temperature was repeated 5 times and the results were averaged. Later, Mann-Whitney U test was applied. Final results which were statistically significant were shown by an asterisk on the graphs.

3.1.1. Temperature Dependent FTIR Studies of DPPG Model Membranes

The FTIR spectra which shows the CH\textsubscript{2} symmetric and antisymmetric stretching bands that is C-H stretching region (3000-2800 cm\textsuperscript{-1}) of DPPG liposomes at different temperatures is seen in the Fig. 14. As you can see from the figure, the frequency values shifted to higher values and bandwidth was increased at higher temperatures. Fig. 15 shows the C-H stretching region of FTIR spectra (3000-2800 cm\textsuperscript{-1}) of DPPG liposomes in the presence and absence of high and low concentrations of simvastatin at 52°C (liquid crystalline phase). The spectra of pure and simvastatin containing DPPG liposomes at high concentration are very different from each other in terms of frequency and bandwidth values.
Figure 14. FTIR spectra of pure DPPG multilamellar liposomes in the C-H stretching region at different temperatures. The spectra were normalized with respect to the CH$_2$ antisymmetric stretching band located at 2920 cm$^{-1}$. (A.U: Arbitrary Unit)

Figure 15. FTIR spectra of DPPG liposomes in the presence and absence of low and high concentrations of simvastatin in the C-H stretching region at 52°C. The spectra were normalized with respect to the CH$_2$ antisymmetric stretching band located at 2920 cm$^{-1}$.
FTIR spectra of DPPG liposomes in the presence and absence of low and high concentrations of simvastatin in the 1800-1000 cm\(^{-1}\) are seen in the fig. 16. This region involves the C=O ester carbonyl stretching band located at 1735 cm\(^{-1}\) and PO\(_2^-\) antisymmetric double stretching band located at 1230 cm\(^{-1}\). Simvastatin-induced frequency shifts and variations in signal intensity values of the bands are seen in the figure.

![FTIR Spectra](image)

**Figure 16.** FTIR spectra of DPPG liposomes in the presence and absence of low and high simvastatin concentrations in the 1800-1000 cm\(^{-1}\) region at 52°C. The spectra were normalized with respect to the C=O stretching band located at 1735 cm\(^{-1}\).

The frequencies of the CH\(_2\) stretching bands of acyl chains depend on the degree of conformational disorder and hence the frequency values can be used to monitor the average trans/gauche isomerization in the systems. The shifts to higher wavenumbers correspond to an increase in number of gauche conformers (Severcan et.al., 2005 (a); Korkmaz and Severcan, 2005 (b)). Ordering state of the system is reflected in the spectrum as a decrease in the frequency of the C-H stretching mode. Figure 17 shows temperature dependence of the frequency of the CH\(_2\) symmetric stretching mode of
DPPG multilamellar liposomes in the presence and absence of different simvastatin concentrations. The frequency values were measured at centre of the bandwidth at 80% of the height of the peaks. The main phase transition temperature ($T_m$) of pure DPPG liposomes was around 40°C and the pretransition temperature was around 33°C (Dorohoi and Severcan, 2008; Sahin et.al, 2007).

**Figure 17.** Temperature dependence of the frequency of the CH$_2$ antisymmetric stretching mode of DPPG liposomes in the absence and presence of simvastatin in varying concentrations
As it can be seen in the figures 17 and 18, the phase transition curve broadened and frequency values shifted to lower values both in the gel and liquid crystalline phase as simvastatin concentration increased. It seems that simvastatin ordered the system for anionic DPPG membranes especially at higher concentrations of simvastatin (12mol% sim, 18mol% sim and 24mol% sim). Broadening of the phase transition curve implies that cooperativity between lipid molecules decreases as penetration of simvastatin into the cooperativity region (C2–C8) of the fatty acyl chains molecules locates in the bilayer (Jain, 1977). Similar trends were observed for both the CH₂ antisymmetric and symmetric stretching bands.

**Figure 18.** Temperature dependence of the frequency of the CH₂ symmetric stretching mode of DPPG MLVs in the absence and presence of simvastatin in varying concentrations
Figure 19. Temperature dependence of the bandwidth of the CH$_2$ antisymmetric stretching mode of DPPG liposomes in the absence and presence of simvastatin in varying concentrations.

Figure 20. Temperature dependence of the bandwidth of the CH$_2$ symmetric stretching mode of DPPG liposomes in the absence and presence of simvastatin in varying concentrations.
Fig. 19 and Fig. 20 show the temperature dependence of the bandwidth of the CH$_2$ antisymmetric and symmetric stretching band of DPPG MLVs, respectively, in the absence and presence of different simvastatin concentrations. Bandwidth was measured at 0.75 · peak height position. The variation of the bandwidth gives information about the dynamics of the system. An increase in the bandwidth of the mentioned band indicates an increase in lipid dynamics (Casal and Mantsch, 1984; Kazanci et al., 2001). As you can see from the figures bandwidth values increase as simvastatin concentration increases for both the CH$_2$ antisymmetric and symmetric stretching bands. This implies that simvastatin gradually increases the fluidity of the membrane as its concentration increases. This increase in dynamics of the membrane is more effective at high simvastatin concentrations (12mol%, 18mol%, 24mol%).

One of the most useful infrared band for probing the polar part of the membrane is the ester group vibration band at 1730 cm$^{-1}$ (C=O stretching). Temperature dependence of the frequency of the C=O stretching modes of DPPG multibilayers in the absence and presence simvastatin is shown in Fig.
21. According to the empirical rules, a decrease in the frequency of this band implies either the strengthening of existing hydrogen bonding or formation of new hydrogen bonding between the components (Severcan, 2005 (a); Frengeli 1976). In our case we have observed two different effect of simvastatin in gel and liquid crystalline phases for C=O stretching mode. Simvastatin increase the frequency values in gel phase for all but more dramatically for 24 mol% simvastatin. In the gel phase simvastatin decreases the number of hydrogen bonds around the carbonyl groups in other words increases dehydration around this group. On the other hand in the liquid crystalline phase, for 12 mol%, 18 mol% and 24 mol% concentrations of simvastatin, the frequency values decrease which indicates that simvastatin increases the strength of hydrogen bonding around this functional group.

**Figure 22.** Temperature dependence of the frequency of the \( \text{PO}_2^- \) stretching mode of DPPG MLVs in the absence and presence of simvastatin in varying concentrations

\( \text{PO}_2^- \) molecules are highly sensitive to changes in hydration (Casal, 1987). Temperature dependence of the frequency of the \( \text{PO}_2^- \) antisymmetric double stretching modes of DPPG multibilayers in the absence and presence of simvastatin is shown in Fig. 22. The increase of the frequency values of this
band is an indication of the dehydration effect and decreasing of the strength of hydrogen bonding around this functional group with water molecules (Casal et al., 1989; Severcan, 2005 (a)). In the present study, the frequency values increased as simvastatin concentration is increased. This increase is more clearly seen with the higher concentrations of simvastatin (12mol% sim, 18mol% sim and 24mol% sim) at liquid crystalline phase implying dehydration around these functional groups in the polar part of the lipids.

3.1.2. FTIR Studies to Monitor the Acyl Chain Length Effect Between DMPG and DPPG Lipids

This part of the study involves the effect of acyl chain length difference on the simvastatin membrane interactions by using two lipids which have different chain lengths. These lipids are DMPG and DPPG which have chemical formulas of C_{34}H_{66}O_{10}P^- ve C_{38}H_{74}O_{10}P^-, respectively. The sole difference between these DMPG and DPPG lipids is that DPPG has two more CH_2 (methylene) group in its fatty acid chain. The same FTIR absorption bands in the temperature dependent study of DPPG - simvastatin interactions were also used in this part of the study. We have chosen two temperatures for each of the DMPG and DPPG lipids, namely 10 °C and 40 °C for DMPG, and 30 °C and 50 °C for DPPG MVLs which monitors the gel and liquid crystalline phases, respectively. Experiments were repeated 5 times at each temperature and average of the results were calculated. Later, Mann-Whitney U test was applied to measured frequency and bandwidth values. Final results which have statistically significant differences in between with the pure DMPG and DPPG samples were shown by an asterisk on the graphs.

In figure 23, the spectra of pure DMPG and DPPG membranes in their gel phases are presented. As seen in the figure, the spectra of DMPG and DPPG membranes have some differences in terms of frequency and bandwidth values.
Figure 23. FTIR spectra of pure DMPG and DPPG liposomes in their gel phases in 3000-2800 cm$^{-1}$ region. The spectra were normalized with respect to the CH$_2$ antisymmetric stretching band located at 2920 cm$^{-1}$.

Fig. 24 and Fig. 25 show the variation of the frequency of the CH$_2$ antisymmetric mode of the DMPG and DPPG liposomes, respectively in their gel phase for varying simvastatin concentrations. Order–disorder state of the system was studied by the frequency analysis of C-H stretching region of FTIR spectra (Severcan et.al., 2005 (a)). As it can be seen in the figures, the frequency values of the mentioned band for samples containing 24 mol% sim and 18 mol% sim have changed significantly, however simvastatin induced opposite effects for DMPG and DPPG membranes. At high simvastatin concentrations order of the system increased for DPPG membranes whereas order of the system decreased for DMPG membranes. Moreover simvastatin induced a reverse effect at the concentrations of 6 mol% and 12 mol% simvastatin in which it ordered the system for DMPG membranes. In the gel phase, the acyl chain length difference between DPPG and DMPG caused simvastatin to induce opposite effects on the order-disorder state of the system. But the results are different for the liquid crystalline phase. Figures 26 and 27 show the variations in the frequency of the CH$_2$ antisymmetric stretching modes of DMPG and DPPG liposomes, respectively in their liquid
crystalline phase for different simvastatin concentrations. As it can be seen in both figures, the frequency values of the samples decreased implying that orders of DMPG and DPPG lipids in the liquid crystalline phase increased as the simvastatin concentration in the liposomes increased. These figures were in the same trend for both DMPG and DPPG liposomes implying that acyl chain length difference between DMPG and DPPG lipids has no effect on the order-disorder state of the model membranes in this study in the liquid crystalline phase.

Figure 24. Simvastatin concentration dependence of the frequency of the CH₂ antisymmetric stretching mode for DMPG liposomes at 10°C. (*) P < 0.05.
Figure 25. Simvastatin concentration dependence of the frequency of the \( \text{CH}_2 \) antisymmetric stretching mode for DPPG liposomes at 30°C. (*) \( P < 0.05 \)

Figure 26. Simvastatin concentration dependence of the frequency of the \( \text{CH}_2 \) antisymmetric stretching mode for DMPG liposomes at 40°C. (*) \( P < 0.05 \).
Figure 27. Simvastatin concentration dependence of the frequency of the CH\textsubscript{2} antisymmetric stretching mode for DPPG liposomes at 50°C. (*) P < 0.05

Figures 28 and figure 29 show dependence of the bandwidth of the CH\textsubscript{2} antisymmetric stretching band of DMPG and DPPG liposomes respectively to simvastatin concentration in their gel phase. Membrane fluidity is monitored by analyzing bandwidths of CH\textsubscript{2} stretching modes of these two MLVs in different simvastatin concentrations (Toyran and Severcan, 2003; Kazancı et.al., 2001). An increase in the bandwidth is the indication of an increase in dynamics. The bandwidth values increased as simvastatin concentration increased for both of the DMPG and DPPG liposomes, implying an increase in the membrane fluidity for both of the lipids used. Acyl chain length difference has no significant effect on membrane dynamics in the gel phase.

Figures 30 and figure 31 show dependence of the bandwidth of the CH\textsubscript{2} antisymmetric stretching band of DMPG and DPPG liposomes respectively to simvastatin concentration, in their liquid crystalline phase. Similar results were obtained for DMPG and DPPG liposomes at the temperatures of 40 °C and 50 °C respectively as in their gel phase. In liquid crystalline phase at high simvastatin concentrations (12mol%, 18mol% and 24%mol) bandwidth...
values increased significantly when compared to the samples containing no simvastatin. Again in liquid crystalline phase simvastatin induce an increase in the membrane fluidity for both of the membranes used. These results imply that there is no effect of acyl chain length difference on membrane dynamics.

Figure 28. Simvastatin concentration dependence of the bandwidth of the CH$_2$ antisymmetric stretching mode for DMPG liposomes at 10°C. (*) P < 0.05.

Figure 29. Simvastatin concentration dependence of the bandwidth of the CH$_2$ antisymmetric stretching mode for DPPG liposomes at 30°C. (*) P < 0.05
Figure 30. Simvastatin concentration dependence of the bandwidth of the CH$_2$ antisymmetric stretching mode for DMPG liposomes at 40°C. (*) P < 0.05.

Figure 31. Simvastatin concentration dependence of the bandwidth of the CH$_2$ antisymmetric stretching mode for DPPG liposomes at 50°C. (*) P < 0.05

Simvastatin concentration dependence of the frequency of the C=O stretching modes of DMPG and DPPG multibilayers in their gel phase are shown in Fig. 32 and Fig 33 respectively. The C=O stretching band was
analyzed to examine the interaction of simvastatin with glycerol backbone near the head group of phospholipids in interfacial region (Toyran and Severcan, 2003). An increase in the frequency of oscillation is characteristic of free ester carbonyl groups and indicates reducing the strength of hydrogen bonds near the glycerol skeleton closer to the head groups (Hubner, 1994; Lewis, 1990). As it is seen in the figures, the frequency values of the simvastatin containing samples increased especially at high concentrations (18 mol% sim and 24 mol% sim) when compared with the pure samples. Simvastatin reduces the strength of hydrogen bonds around glycerol group and causes the formation of free carbonyl groups for both anionic membranes DMPG and DPPG in their gel phases.

Figures 34 and figure 35 show simvastatin concentration dependence of the frequency of the C=O stretching band of DMPG and DPPG liposomes respectively, in their liquid crystalline phase. Similar results are observed in the liquid crystalline phase as in the gel phase. Same trends are observed for DMPG and DPPG membranes. There is no effect of acyl chain length difference on the hydrogen bonding of glycerol backbone.
Figure 32. Simvastatin concentration dependence of the frequency of the C=O stretching mode for DMPG liposomes at 10°C. (*) P < 0.05.

Figure 33. Simvastatin concentration dependence of the frequency of the C=O stretching mode for DPPG liposomes at 30°C. (*) P < 0.05.
Figure 34. Simvastatin concentration dependence of the frequency of the C=O stretching mode for DMPG liposomes at 40°C. (*) P < 0.05.

Figure 35. Simvastatin concentration dependence of the frequency of the C=O stretching mode for DPPG liposomes at 50°C. (*) P < 0.05.
The interaction between simvastatin and head group of DMPG or DPPG multilamellar liposomes was monitored by analysing the PO$_2^-$ antisymmetric double stretching band. Figures 36 and Figure 37 show simvastatin concentration dependence of the frequency of the PO$_2^-$ antisymmetric double stretching modes of DMPG and DPPG liposomes respectively in their gel phase. In the gel phase the frequency values of the %24 mol and %18 mol simvastatin containing samples decreased significantly for both membranes (DMPG and DPPG) implying strengthening of hydrogen bonding between the head groups of liposomes with water molecules around (Casal et.al., 1987). Similar trends were observed for DMPG and DPPG membranes which means that there is not any effect of acyl chain length on simvastatin head group interactions in the gel phase.

On the contrary, our results in the liquid crystalline phase are quite different from that of gel phases results. Figures 38 and Figure 39 show simvastatin concentration dependence of the frequency of the PO$_2^-$ antisymmetric double stretching modes of DMPG and DPPG liposomes respectively in their liquid crystalline phase. As seen from the figures, the frequency values for 24 mol% simvastatin-containing samples increased significantly for both the anionic DMPG and DPPG membranes, implying dehydration around this fuctional group. Similar trends were observed for DMPG and DPPG membranes which means that there is not any effect of acyl chain length on simvastatin head group interactions in the liquid crystalline phase.
Figure 36. Simvastatin concentration dependence of the frequency of the PO$_2^-$ antisymmetric double stretching mode for DMPG liposomes at 10°C (*) P < 0.05.

Figure 37. Simvastatin concentration dependence of the frequency of the PO$_2^-$ antisymmetric double stretching mode for DPPG liposomes at 30°C (*) P < 0.05.
Figure 38. Simvastatin concentration dependence of the frequency of the PO$_2^-$ antisymmetric double stretching mode for DMPG liposomes at 40°C (*) P < 0.05.

Figure 39. Simvastatin concentration dependence of the frequency of the PO$_2^-$ antisymmetric double stretching mode for DPPG liposomes at 50°C (*) P < 0.05.
3.2. DSC Studies

We performed a series of DSC experiments to study the influence of simvastatin on the phase behavior of DMPG and DPPG membranes. Calorimetric investigations of DMPG or DPPG multilamellar liposomes in the presence and absence of simvastatin were carried out for a temperature range of 0-50°C and 20-70°C, respectively. Figure 40 and Figure 41 show the corresponding heating flow as a function of temperature. Our current study is an agreement with the previous studies performed about phase transition profiles of the anionic lipid vesicles DMPG and DPPG (Zhang et al., 1997). These studies reports that pretransition and main phase transition ($T_m$) occur, at physiological conditions, around 34°C and 40°C for DPPG liposomes and 14 °C and 24 °C for DMPG liposomes, respectively. (Schwieger and Blume, 2009; Prenner et al., 1999). In our current study we have found that the pretransition and main transition temperatures for DMPG are 13.61°C and 20.23 °C. On the other hand pretransition and main transition temperatures for DPPG membrane was found to be 35.03 °C and 39.65 °C which are in good agreement with the previous findings.

![Figure 40. DSC thermogram of DMPG liposome.](image)

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Figure 41. DSC thermogram of DPPG liposome.

Figure 42 and Figure 43 show DSC thermograms of DMPG and DPPG liposomes respectively, in the absence and presence of varying simvastatin concentrations. As seen from the figures, for DMPG membranes the main transition temperatures changed irregularly with the addition of different concentrations of simvastatin that is varying between 20-22 °C. But for DPPG membranes, up to the concentration of 18 mol% simvastatin, $T_m$ slightly decreased 1 or 2 °C. $T_m$ was found to be 38.04 °C for 18 mol% simvastatin containing DPPG membrane. But most severe shifting of $T_m$ was observed for the 24 mol% simvastatin containing sample, which was 34.3 °C. There was also broadening of the phase transition peak for both membranes. This broadening may result from the loss of cooperativity between lipid molecules. More than one peak was observed for the high simvastatin concentrations for both DMPG and DPPG liposomes (12 mol% sim, 18 mol% sim and 24 mol% sim). These type of behaviors are indicating the presence of domains in the bilayer structures in which simvastatin causes lateral phase separation in the bilayers of anionic lipids (Korkmaz and Severcan, 2005 (b)).
Figure 42. DSC thermogram of DMPG liposomes in the absence and presence of simvastatin in varying concentrations with their main transitions temperatures.

Figure 43. DSC thermogram of DPPG liposomes in the absence and presence of simvastatin in varying concentrations with their main transitions temperatures.
Table 1 and Table 2 show the width at half heights ($\Delta T$), transition temperatures ($T_m$) and enthalpy of the transitions ($\Delta H$) for DMPG and DPPG liposomes respectively. The broadening of the main phase transition peak was observed because the width of the transition peak increased as simvastatin concentration in the liposomes increased. $\Delta H$ enthalpy of transitions decreased as simvastatin concentration increased especially for DPPG liposomes.

Table 1. Width at half height ($\Delta T$), transition temperatures ($T_m$) and enthalpy of the transitions ($\Delta H$) for DMPG liposomes

<table>
<thead>
<tr>
<th>Samples</th>
<th>$\Delta T$ (°C)</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H$ (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPG</td>
<td>1.78</td>
<td>20.23</td>
<td>31.92</td>
</tr>
<tr>
<td>DMPG + 1mol% simvastatin</td>
<td>1.68</td>
<td>21.75</td>
<td>30.05</td>
</tr>
<tr>
<td>DMPG + 6mol% simvastatin</td>
<td>2.91</td>
<td>21.26</td>
<td>32.52</td>
</tr>
<tr>
<td>DMPG + 12mol% simvastatin</td>
<td>3.53</td>
<td>21.68</td>
<td>41.32</td>
</tr>
<tr>
<td>DMPG + 18mol% simvastatin</td>
<td>3.98</td>
<td>21.94</td>
<td>38.64</td>
</tr>
<tr>
<td>DMPG + 24mol% simvastatin</td>
<td>3.00</td>
<td>22.16</td>
<td>22.47</td>
</tr>
</tbody>
</table>
Table 2. Width at half height ($\Delta T$), transition temperatures ($T_m$) and enthalpy of the transitions ($\Delta H$) for DPPG liposomes

<table>
<thead>
<tr>
<th>Samples</th>
<th>$\Delta T$ (°C) At half height</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H$ (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPG</td>
<td>1.01</td>
<td>39.65</td>
<td>44.47</td>
</tr>
<tr>
<td>DPPG + 1mol% simvastatin</td>
<td>1.32</td>
<td>39.01</td>
<td>40.51</td>
</tr>
<tr>
<td>DPPG + 6mol% simvastatin</td>
<td>2.09</td>
<td>39.12</td>
<td>36.63</td>
</tr>
<tr>
<td>DPPG + 12mol% simvastatin</td>
<td>2.96</td>
<td>37.56</td>
<td>36.33</td>
</tr>
<tr>
<td>DPPG + 18mol% simvastatin</td>
<td>1.70</td>
<td>38.04</td>
<td>36.75</td>
</tr>
<tr>
<td>DPPG + 24mol% simvastatin</td>
<td>3.83</td>
<td>34.3</td>
<td>35.74</td>
</tr>
</tbody>
</table>
CHAPTER 4

DISCUSSION

In the present study FTIR spectroscopy and Differential Scanning Calorimetry (DSC) techniques were used to investigate the effects of cholesterol reducing agent simvastatin on the phase transition profile, order, dynamics and hydration states of the head and the region near the aqueous region of anionic dipalmitoyl phosphatidylglycerol (DPPG) and dimyristoyl phosphatidylglycerol (DMPG) multilamellar vesicles (MLVs). These parameters were monitored as a function of temperature and simvastatin concentration. The study was divided into two parts: first part of the study includes the detailed examination of the interaction of simvastatin with the DPPG MLVs as a function of temperature to study the parameters that has been written above and second part include the study of effect of acyl chain length on the simvastatin - anionic membrane interactions using two different anionic lipids. These are DMPG and DPPG which have chemical formulas of $C_{34}H_{66}O_{10}P^{-}$, $C_{38}H_{74}O_{10}P^{-}$ respectively. The sole difference between these DMPG and DPPG lipids is that DPPG has 2 more CH$_2$ (methylene) group in its fatty acid chain.

Under physiological conditions, most cell membranes have a negative charge due to the presence of acidic lipid head groups. In view of the potential importance of the membrane negative character in many biological processes, anionic phospholipids have been widely used as model systems for possible anionic domains in membranes. Phosphatidylglycerol (PG) is the most abundant anionic phospholipid head group present in prokaryotic cell membranes, and has been extensively studied as a model for negatively charged membranes (Seelig et al., 1987; Biaggi et al., 1997; Fernandez and
Lamy-Freund, 2000). Because anionic lipids impart a negative charge to the surfaces of lipid bilayers, it has been assumed that one of the specific functions of anionic lipids is the regulation of membrane lipid surface charge density (Christiansson et al., 1985; Clementz and Christiansson, 1986; Clementz et al., 1987). Indeed, lipid bilayer surface charge density can have major effects on the passive permeability of biological membranes to ions and charged metabolites and on the activity of a number of membrane-bound enzymes and transport proteins (Huunan-Seppalla, 1971; Schafer and Rowohl-Quisthoudt, 1976; Theuvenet et al., 1976 a, b; Wojtczaj and Nalecz, 1979). DPPG is the anionic counterpart of the neutral lipid dipalmitoyl phosphatidylglycerol (DPPC) which is present widely among mammalian cell membranes. Therefore, in the present study we used DPPG as a charge model lipid although it is not available in the mammalian cell membranes to compare the results of the current study with our previous study on simvastatin-DPPC model membrane interaction (Koçak, 2007).

In the first part of the study, liposomes containing simvastatin at concentrations varying from 1 to 24 mol% are used. For FTIR spectral analysis, the C-H stretching modes at 3000-2800 cm\(^{-1}\), the C=O stretching band located at 1735 cm\(^{-1}\) and PO\(_2^-\) antisymmetric double stretching band located at 1240-1220 cm\(^{-1}\) were considered. Simvastatin-induced shifts in the peak position of the FTIR bands and changes in the bandwidths of several bands were determined in comparison to those of pure DPPG liposomes. In the present work, we were particularly careful in distinguishing between structural parameters describing molecular order and motion parameters such as bandwidth describing molecular dynamics as suggested by others (Severcan et al., 2005 (a); Korkmaz et al., 2005 (a)).

CH\(_2\) stretching vibrations in FTIR spectra used to gain structural information and visualize phase transition behaviors and encode information about the conformational disposition of the hydrocarbon chains (Sahin et al., 2007; Korkmaz and Severcan, 2005 (b), Zhang et al., 1997). In the CH\(_2\) stretching region of the infrared spectrum (2800–3000 cm\(^{-1}\)), the hydrocarbon chains which contain gauche conformers absorb infrared radiation at higher
frequencies than those that contain all-trans conformers. Thus, the increase in hydrocarbon chain conformational disorder is accompanied by an increase in the frequencies of infrared absorption bands arising from the symmetric and asymmetric stretching vibrations of lipid hydrocarbon chains. This property can thus be used as a probe of changes in lipid hydrocarbon chain conformational disorder and has frequently been used for the detection and/or monitoring of lipid hydrocarbon chain-melting phase transitions (Mantsch, 1991; Jackson, 1993). The abrupt shift in the frequency of CH$_2$ antisymmetric and symmetric stretching modes (Fig. 17 and Fig. 18) which takes place around 40°C monitors the well known main cooperative endothermic phase transition of DPPG liposomes and has been associated with the change from all-trans to gauche conformers (Casal and Mantsch, 1984). According to our results we have observed phase transition curve broadening in the presence of simvastatin which means that simvastatin perturb extensively the cooperativity region (C$_2$–C$_8$) of the fatty acyl chains and probably simvastatin molecules are located in the hydrophobic interior of the bilayer (Jain, 1997; Korkmaz and Severcan, 2005 (b)). High simvastatin concentrations caused decreasing of the frequency values of CH$_2$ antisymmetric and symmetric stretching modes at both gel and liquid crystalline phase implying that simvastatin orders the system for anionic DPPG membranes. According to our previous study, simvastatin increased the degree of disordering in neutral DPPC membranes (Koçak, 2007). Simvastatin seems to induce opposite effects for neutral DPPC and anionic DPPG membranes. In a previous study performed by our group, it was reported that simvastatin has an ordering effect in the skeletal muscle membranes which is in accordance with our results (Ozek et.al. 2010). DPPG is an anionic lipid which contains an anionic (phosphate) head group. Anions are attracted to the positive end of a dipole. The charges on ions and the charge separation in polar molecules explain the fairly strong interactions between them, with very strong ion–ion interactions, weaker ion–dipole interactions and considerably weaker dipole–dipole interactions (Sahin et. al. 2007). Polar molecules can interact with ions and interaction between DPPG and polar groups on simvastatin molecule may be ion–dipole interaction.
The variation of the bandwidth of CH\textsubscript{2} antisymmetric and symmetric stretching gives information about the dynamics of the system. An increase in bandwidth is the indication of an increase in dynamics (Casal and Mantsch, 1984). And fluidity at a certain degree is required for normal and optimal activity of membrane associated proteins (Yi and McDonald, 1973). Analysis of the bandwidths showed that simvastatin increase the bandwidth values of the DPPG membranes at high concentrations (12 mol\% sim, 18 mol\% sim and 24 mol\% sim) which implies that simvastatin increase the membrane fluidity. This type of behavior has been previously reported for interaction of ciprofloxacin with the DPPC and DPPG membranes (Bensikkaddour et.al, 2008). In our previous study, it was reported that simvastatin decreased the fluidity of neutral DPPC membranes (Kocak, 2007). Opposite results are observed for neutral DPPC membrane when compared with anionic DPPG membrane. But most of the other studies are in agreement with our findings in which simvastatin increases the membrane fluidity in several biological membranes including erythrocyte plasma membranes, endothelial cell membranes and skeletal muscle membranes (Koter et.al., 2003; Broncel et.al., 2005; Morita et.al., 1997; Ozek et.al., 2010).

The effect of simvastatin on the interfacial region of DPPG has been studied from the carbonyl stretching mode region, which ranges from 1800 to 1650 cm\textsuperscript{-1}. The C=O stretching vibration arising from the ester groups of phospholipids is sensitive to hydrogen bonding (Blume and Hübner, 1988). In our case we observed opposite effect of simvastatin on the C=O stretching vibrations of DPPG lipids for the gel and liquid crystalline phases. In the gel phase, the frequency of this band increased for all of the simvastatin concentrations, which implies that there is a decrease in the strength of hydrogen bonding and is explained by the presence of free carbonyl groups in the system. However in the gel phase, low and high concentrations of simvastatin produce different effects on the membrane (Fig.21). In the liquid crystalline phase, the addition of simvastatin produces a remarkable decrease in the frequency, which indicates that simvastatin increases the hydrogen bonding around this functional group. This type of behavior also
observed in the interaction of β-Lactoglobulin with DPPC membranes (Lefevre and Subirade, 2000).

Information about the hydration state of the polar head groups of the phospholipids can be monitored by the analysis of the frequency of the PO$_2^-$ antisymmetric double stretching band, located at 1200-1240 cm$^{-1}$. An increase in the frequency of this functional group reflects dehydrated phosphate group, and a decrease in the frequency corresponds to the hydrated phosphate group (Korkmaz and Severcan, 2005 (b); Lopezgarcia et al., 1993; Severcan, 2005 (a)). Our data show that addition of simvastatin did not change frequency values of DPPG liposomes so much in the gel phase, however frequency values increased for simvastatin concentrations of 6 mol%, 12mol%, 18 mol% and 24 mol% in the liquid crystalline phase significantly. This implies that there is dehydration around the phosphate group.

In the second part of the study we have used two anionic lipids namely DMPG and DPPG which have two CH$_2$ (methylene group) difference in their fatty acid chain. We have observed some variations in the interaction of simvastatin with the DMPG and DPPG liposomes. Firstly analysis of CH$_2$ antisymmetric stretching band shows that in the gel phase simvastatin at high concentrations (18 mol% sim and 24 mol% sim) increased the order for DPPG membranes but it disordered the DMPG membranes at the same concentrations (Fig. 24 and Fig. 25). This different effect of simvastatin on DMPG membranes can be explained as follows. Low ionic strength DMPG dispersions were found to present a thermal behavior rather different from that presented by dispersions under physiological conditions (Lamy-Freund, 2003). DMPG has a different melting regime in which it has an intermediate phase conducting mesoshophic behaviour (Riske et.al, 2004). At the start of this Intermediate phase ($T_{m,\text{on}}$) density fluctuations destroy the long-range order between hydrocarbon chains. Isolated gauche rotations may occur, but there is not yet a decrease in the overall bilayer thickness, which requires a collective rearrangement of the disordered chains, with defined kinks. With such mobility, polar heads separate due to the electrostatic repulsion,
allowing penetration of water and possibly ions more deeply into the membrane. But this is not the case in the liquid crystalline phase because simvastatin ordered the system for both of the DMPG and DPPG lipids. In the case of charged DMPG the process occurs along a melting regime. Pores are formed, which coalesce into stable fissures upon increasing the temperature, while the chain-melting process gradually takes place (Riske et.al. 2004). Fluidity is affected in the same way for both the lipids and increased in gel and liquid crystalline phases with the simvastatin addition. And also simvastatin influence on the hydrogen bonding of the head groups and hydration states are similar for DMPG and DPPG membranes. Since DMPG has a shorter fatty acid chain location of simvastatin into the cooperativity region (C_2-C_8) may not be as it is for DPPG membranes. But the interactions of the head groups are similar since both of the membranes having a charged polar head. Phase transition temperatures increased slightly for DMPG membranes with increasing concentrations of simvastatin according to our DSC results (Table 1). In phospholipid membranes with charged lipids, an increase of the chain melting temperature can be explained in terms of either (i) a decrease in the ionization state of the polar headgroups, (ii) a screening of the electrostatic repulsion by high ionic strength, or (iii) an increase in the lipid-lipid hydrophobic attraction following a decrease in the polar head hydration (Cevc, 1987). In our case, with low ionic strength (0.03 M), the T_m decreased for DPPG liposomes but T_m increased 1 to 2 °C for DMPG liposomes with the addition of simvastatin. Here, the total energy gain from the hydrogen bonding between protonated and deprotonated headgroups is larger than the energy loss due to the electrostatic repulsion between charged headgroups (Vautrin et.al., 2004).

Our DSC data revealed that simvastatin changes the phase transition behaviors of both of the lipids. DSC curves broadened and half widths are increased and moreover, the total enthalpy associated with both transitions decreases with increasing simvastatin concentration, particularly at higher simvastatin concentrations for both the DMPG and DPPG membranes (Table 1 and Table 2). In the case of pure DMPG, the main transition is not symmetric and exhibits a marked high-temperature shoulder, which is due to
the low ionic strength used in this experiment (Andrushchenko, 2007). At low ionic strength DMPG liposomes were found to present a thermal behavior rather different from that presented by dispersions under physiological conditions. DMPG membranes in the presence of 100 mM NaCl present a gel-fluid transition profile rather similar to that presented by the zwitterionic lipid dimyristoyl phosphatidylcholine (DMPC), with a sharp differential scanning calorimetry (DSC) peak in the heat capacity profile at 23.8 °C. At low ionic strength conditions these lipids present a broader phase transition peaks like that is shown in fig. 40 which is ranging from 20 °C to 30 °C (Lamy-Freund and Riske, 2003; Riske et.al., 2004; Salonen et.al., 1989). The decreasing of the enthalpy of the main phase transition at high concentrations of simvastatin is mainly due to the disruption of van der Waals interactions between the fatty acid chains, and perturbations on this transition are indicative of intercalation of the simvastatin molecules between the fatty acid chains (Alves et.al. 2008). There is also broadening in the phase transition peak for both membranes used. This broadening results from the loss of cooperativity between lipid molecules. There are also shoulders and more than one peak is observed for the high simvastatin concentrations for both DMPG and DPPG liposomes (12 mol% sim and 24 mol% sim). These type of behaviors indicates the presence of domains in the bilayer structures in which simvastatin cause lateral phase separation in the bilayers of anionic lipids (Korkmaz and Severcan, 2005).

This study is planned to be continued with incorporation of cholesterol into the membrane systems, because simvastatin seems to locate membranes at the position where cholesterol originally located in natural biological membranes. And the effect of acyl chain length will be studied in detail using other lipids, in addition to DMPG and DPPG, which have different acyl chain length.
CHAPTER 5

CONCLUSION

In the present work we have investigated the effects of cholesterol reducing agent simvastatin on the anionic DPPG membrane’s physical properties like membrane order, phase transition behaviour, hydrogen bonding, hydration status and dynamic properties like fluidity of the membrane as a function of simvastatin concentration and temperature. Moreover acyl chain length effect on simvastatin – charged membrane interactions were studied by comparing the results of two anionic liposomes DMPG and DPPG which have 2 CH₂ difference in their fatty acid chains. Two non-invasive methods FTIR spectroscopy and Differential Scanning Calorimetry were used. By using FTIR we were able to monitor different parts of the membrane such as the head group, acyl chain and the interfacial region. We were able to obtain information about these regions of the membrane in terms of interactions. DSC enabled us to monitor the main phase transition of the membranes directly.

According to our results we can say that simvastatin has a strong interaction with anionic lipid membranes. This interaction changes the phase transition behavior of the membranes. For example cooperativity of the lipid molecules decreased by simvastatin addition which has been located in the hydrophobic interior of the membrane. Order and fluidity of the membrane increased for anionic DPPG liposomes by simvastatin addition. Simvastatin caused decreasing of the hydrogen bonding capacity near the carbonyl groups and induced dehydration around phospholipid head groups. Since simvastatin molecules located in the interior of the membrane, it decreases the Van der Waals interactions between molecules, and therefore the loss in cooperativity.
and broadening in the phase transition profile were observed. The Enthalpy of transitions was lowered and pretransition temperature was diminished for both of the DMPG and DPPG membranes. The secondary peaks and shoulders were also observed in the phase transition profiles of the DMPG and DPPG membranes which imply the formation of domains upon addition of simvastatin and simvastatin induces lateral phase separation. Chain length difference effect did not show so much variation in the analyzed parameters of simvastatin-DMPG and DPPG membrane interactions except in the CH$_2$ antisymmetric stretching gel phase frequency results. Therefore no significant effect of acyl chain length effect on simvastatin anionic membrane interactions was observed.
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