STRUCTURAL AND FUNCTIONAL INVESTIGATION OF THE INTERACTION OF AGOMELATINE WITH MODEL MEMBRANES

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ABSTRACT

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Depression is one of the most commonly seen psychiatric diseases in the population in recent years. Treatment of depression is mainly carried out by psychiatric drugs. In the past few years, agomelatine which is released to the market with a trade name, Valdoxane, has been thought to have far less side effects due to its non-addictive nature, not having trouble when the drug is quitted, and also due to its property of binding only to the specific receptor that the drug interacts with. The action mechanism of agomelatine on the membrane structure has not been clarified yet, for instance, no study has been found in the literature about the interaction of agomelatin with the lipids of biological membranes. In this current study, the interaction of agomelatine with the model membranes of dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylgylcerol (DPPG) and sphingomyelin (SM) is examined by Fourier transform infrared spectroscopy (FTIR) and Differential scanning calorimetry (DSC).

DSC and FTIR studies show that, agomelatine shifts the phase transition temperature of DPPC and DPPG multilamellar membrane to the lower degrees, however, it shifts the phase transition temperature of SM membrane to the higher degrees. Agomelatine addition increases the lipid order of the DPPC and SM liposome, whereas, it decreases the lipid order of DPPG liposome. Moreover this drug enhances the membrane fluidity among all types of liposome studied. The increase of

lipid order and increase of fluidity at DPPC and SM liposome indicates domain formation upon drug addition (Vest et al., 2004). This was also confirmed by DSC studies.

Agomelatine enhances H bonding capacity of all types of liposomes have been studied. However it has different effects on glycerol backbones of the DPPC and DPPG liposomes. At low agomelatine concentrations the increase in the frequency values indicates a decrease in the hydrogen bonding capacity of the glycerol skeleton of DPPC. In contrast, at high concentrations of agomelatine, a decrease in the frequency values was observed as an indicator of the enhancement of the hydrogen bonding capacity. So it enhances H-bonding capacity at gel phase but lowers it at liquid chrystalline phases. A progressive decreases in Tm was observed at DPPG and DPPC liposomes where it increased the Tm at SM. The pretransition peak is abolished and the Tm peak becomes broad, indicating a larger perturbation to the membrane. These observations indicate the possible interaction of agomelatine with the head group as well. The shoulder seen at the thermograms of DPPC and DPPC liposomes at high doses may indicate the lateral phase separation in to drug-rich and drug-poor domains (D'Souza et al., 2009). These results may indicate that agomelatine is partially buried in the hydrocarbon core of the bilayer, interacting primarily with the C2-C8 methylene region of the hydrocarbon chains. All these results highlight the fact that agomelatine interacts around the head group in such a manner that it destabilizes the membrane architecture to a large extent.

Keywords: Agomelatine, DPPC, DPPG, Sphingomyelin, FTIR, DSC

ÖZ

AGOMELATİN'İN MODEL MEMBRANLAR İLE ETKİLEŞMESİNİN YAPISAL VE İŞLEVSEL AÇIDAN İNCELENMESİ

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Depresyon son yıllarda halk arasında en sık gözlenen psikiyatrik hastalıklar arasında yer almaktadır. Depresyonun tedavisi temel olarak psikiyatrik ilaçlar ile yürütülmektedir. Geçtiğimiz son birkaç yıl içerisinde Valdoksan ticari adıyla piyasaya sürülen agomelatin'in bağımlılık yapıcı etkisinin olmaması, ilaç bırakma durumunda hastanın sorun yaşamaması ve ilacın yalnızca etki ettiği özel reseptörlere bağlanması gibi nedenlerle diğer psikiyatrik ilaçlara göre çok daha az yan etkiye sahip olduğu düşünülmektedir. agomelatin'in membran yapısı üzerindeki etki mekanizması henüz tam olarak netlik kazanmamıştır, örneğin literatürde agomelatin'in biyolojik membran lipitleri ile etkileşimi üzerine herhangi bir çalışma bulunmamaktadır. Bu çalışmamızda, agomelatin'in dipalmitolfosfatidilkolin (DPPC) ve spingomiyelin (SM) model membranları ile etkileşimi kızıl ötesi Fourier transform (FTIR) spektroskopi ve diferensiyel tarama kalorimetresi (DSC) teknikleri ile incelenmiştir.

DSC ve FTIR spektroskopisi çalışmaları agomelatin'in DPPC ve DPPG çok tabakalı membranın faz geçiş sıcaklığını düşük değerlere, SM membranda ise yüksek değerlere kaydırdığını göstermiştir. FTIR çalışmalarında CH_2 gerilme bandlarının frekans değerlerinin incelenmesi, agomelatin'in SM ve DPPC lipozomlarında lipit esnekliğini azalttığı DPPG lipozomlarında ize artırdığını göstermektedir (Vest ve ark., 2004). Diğer bir deyişle DPPC ve SM'de lipit düzenini artırdığını, DPPG'de ise

azalttığını göstermiştir. Bunun yanı sıra agomelatin ilavesi tüm dozlarda, her iki fazda da DPPC lipozomlarının bant genişliğini artırmaktadır ki bu da lipit dinamiğini arttırdığını belirtmektedir. Lipit dinamiğinin ve düzeninin artması ilaç eklenmesine bağlı ilaç-zengin bölgeler oluştuğunu göstermektedir. DPPC lipozomlarının C=O gerilme bantlarının frekans değerlerinin incelenmesi, agomelatin'in yüksek dozlarda gliserol iskeletinde hidrojen bağı yapma kapasitesini artırdığını, düşük dozlarda ise azalttığını göstermektedir.Agomelatin DPPG lipozomlarının H bağı yapma kapasitesini jel fazda artırmakta fakat sıvı kristal fazda azaltmaktadır. Gözlemlediğimiz PO_2^- antisimetrik bant frekansındaki azalma, agomelatinin fosfat grupları civarındaki hidrojen bağlanmasını arttırdığını gösterir. Agomelatin, tüm lipozomlarda kafa gruplarındaki hidrojen bağlanmasının kuvvetini, artırmaktadır. DPPG ve DPPC lipozomlarında Tm değerlerinde azalma SM lipozomlarında artma görülmüştür. Hem DSC ve FTIR çalışmaları faz geçiş eğrisinin genişlediğini göstermektedir. Bu genişleme ilacın membran yapısında bozulmaya neden olduğunu göstermektedir. Elde ettiğimiz bu sonuçlar agomelatinin aynı zamanda kafa grubuyla da etkileşime girdiğini göstermektedir. Her üç tip lipit membran için ilacın, C_2-C_8 bölgelerinde konumlandığını göstermiştir.

Anahtar Kelimeler: Agomelatin, DPPC, DPPG, Spingomiyelin, FTIR, DSC

To my dearest parents Aytaç and Nadir Ergün,

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DPPC	Dipalmitoylphosphatidylcholine
FTIR	Fourier Transform Infrared Spectroscopy
DPPG	Dipalmitoylphosphatidylglycerol
SM	Sphingomyelin
DSC	Differential Scanning Calorimetry
MLVs	Multilamelar vesicles

CHAPTER 1

INTRODUCTION

1.1. Depression

Depression is a highly disabling health problem, very frequently seen all around the world (Kessler et al, 2003). In 6·6% of the cases, major depressive disease has a 12-month prevalence, and in a lifetime there is 16•2% of probability to suffer from this disorder. Women have the double of probability to endure this disorder respect to men. It causes important impairment states. (Kessler et al, 2007). The disease causes reductions in health that are similar to those of other chronic disorders (eg, angina, arthritis, asthma, and diabetes), but also worsens mean health results more when it's comorbid with these diseases, than when the diseases happen occur (Moussavi et al, 2007).

Major depressive disorder (MDD) (also known as recurrent depressive disorder, clinical depression, major depression, unipolar depression, or unipolar disorder) is a mental disorder defined by an all-inclusive low mood, low self-esteem, loss of interest or pleasure in normally pleasant activities. In the American Psychiatric Association's diagnostic manual's 1980 edition, this set of symptoms was named, described and classified as one of the mood diseases. The term "depression" is often used to describe this syndrome, but could also be used about other mood disorders or to lower mood states with lacking clinical meaning. MDD is a disabling state of mind, that affects in a strongly negative way, a person's family, work or school life, sleeping and eating habits, and general health.

MDD's diagnosis is based on the patient's self-reported experiences, his behavior reported by relatives or friends, and on a examination about his mental status. There

is no laboratory test to measure major depression, although physicians generally request tests for physical conditions that may cause similar symptoms. The most common period in life of onset is between the ages of 20 and 30 years, with a later peak between 30 and 40 years (Kessler et al, 2005).

A major depressive episode is defined by a low mood or an inability to experience pleasure (anhedonia), or both, for more than 2 weeks, combined with several symptoms of cognitive and vegetative nature, with the happening of distress or impairment (APA, 1994) states. MDD's diagnosis can be made when a person suffers at least one of such episode without experiencing mania disorders.

The Prefrontal cortex, the amygdala, and mostly the hippocampus are the structures in the brain that are most deeply examined about depression. Magnetic resonance studies show that a decreasing in the volume's brain of depressed patients, when compared to healthy people's, and also large volume decreasing in the anterior cingulate and orbitofrontal cortex and mild decreasing in the hippocampus, the putamen and the caudate (Koolschijn et al, 2009) areas. PET studies have shown blood flow abnormalities in the regional cerebral and glucose metabolism, in several prefrontal cortical and limbic structures that are involved in emotional processing (Drevets, 1998).

Table 1.1.: Functional and structural evolutions in the limbic and PFC areas involved in MDD (Palazidou, 2012).

Substrate	**Volume**	**Histological changes**	**Metabolic Activity**	**Antidepressant effects**
Orbital/Ventromedial PFC	↓	↓	↑	
Anterior cingulate cortex	↓			Metabolic activity ↓
Hippocampus	↓	↓		Volume ↑
Amygdala	↓			Metabolic activity ↓
Dorso-lateral PFC	↓	↓	↓	

Table 1.2.: Neurochemical/hormonal abnormalities in MDD (Palazidou, 2012).

Substrate	**Concentration/activity**
Cortisol, corticotrophin releasing Hormone (CRH)	↑
Proinflammatory cytokines	↑
BDNF	↓
5-HT (Serotonin) neurotransmission	↓
NA neurotransmission	↓

Tables 1.1 and 1.2 recapitulate the most important discovers from the neuroimaging, histopathological, neurochemical and pharmacological researches. Additional neuroanatomic sites about mood state are the medial thalamus and the ventral striatum and pallidum as well as the hypothalamus and these are widely linked with the MPFC (Figure 1.1). This system also unite relevant structures in the midbrain/brainstem. This circuit is in charge of the regulation of the emotional stability and of the right answers to emotional stimuli, of the regulation of neurotransmission, as well about autonomic and neuroendocrine function. However, in the base of the available material, the hypothesis has been done that in the depressed state, the right equilibrium between the structures amongst the neuron circuit, may be interrupted as the consequence of reduced activity in the PFC which disturbs its action of regulation, on the limbic structures, which gets then overactive. This impairment could be the cause of the clinical depressive syndrome and the associated autonomic, neuroendocrine disturbances, and other visceral functions (Price & Drevets, 2010).

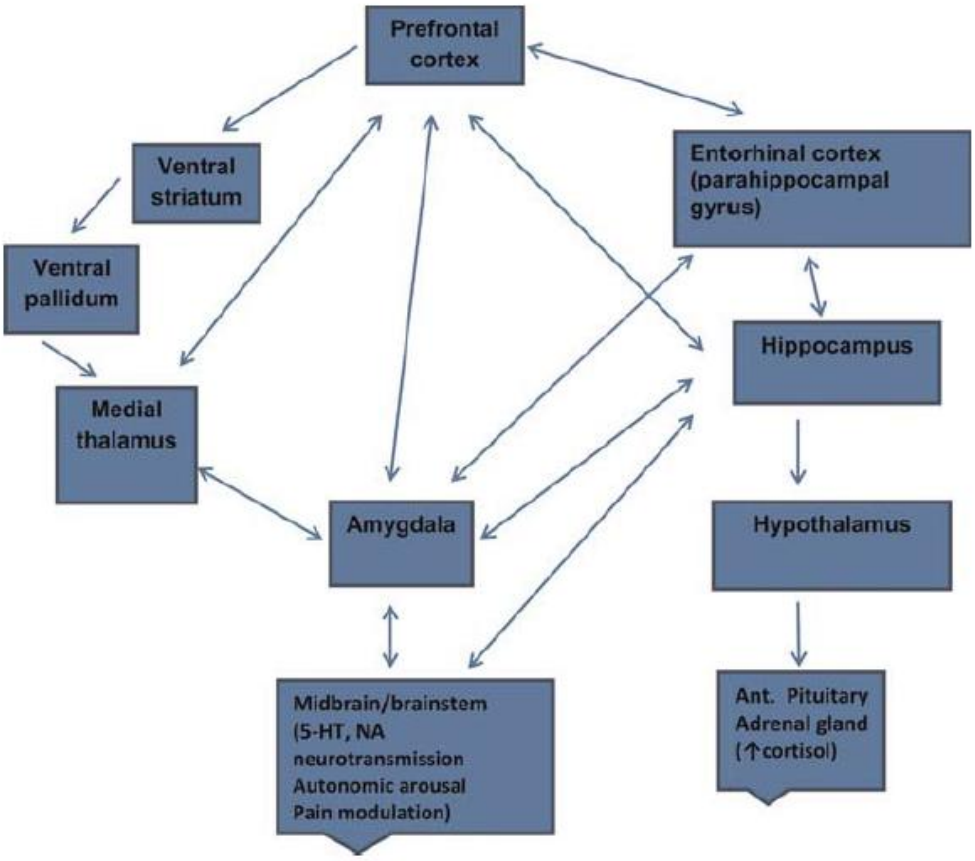

Figure 1.1. Schematic connections between the pre-frontal cortex and limbic structures among the limbic-cortico-striato-pallido-thalamic circuits correlated to the medial and orbital prefrontal cortex networks involved in depression. A reduction in the control of inhibition about the limbic structures by the PFC, is correlated to cognitive, behavioral and other symptoms of depression, and also as loss of normalities in the neuroendocrine function, in the modulation of the pain sensation and in the neurotransmitter activity (having a repercussion on the raphe, serotonergic nuclei and NA-ergic nucleus coeruleus), across its links with the hypothalamus and the midbrain, most importantly, with the periaqueductal zone (Palazidou et al., 2012).

1.1.1 Molecular Mechanisms Involved in Depression

Searches in neurobiology field about MDD, are most of the time, centered on the monoamine neurotransmitters serotonin and norepinephrine. The monoamine hypothesis stated at the beginning, that depressed patients have a tendency to possess low levels of these neurotransmitters because a large range of antidepressant drugs increase dramatically these neurotransmitters levels (Hirschfeld, 2000). Even if monoaminergic antidepressants are used most of the time for a cure in first-line, they do not show quickly their clinical benefit, and do not even provide any benefit for some people.

Understanding the mechanism of action of the antidepressant drugs could open the door to understand the pathogenesis of depression. One of the first theory spread, which survives to the present, the 'monoamine hypothesis of depression', stated that depression is the consequence of a lack of the monoamines, noradrenaline, serotonin or both, in the brain and that antidepressant drugs take back these to the normal level. Pharmacological progress in the nowadays techniques take the attention from the neurotransmitters to their receptors. The 'beta receptor down regulation hypothesis' was proposed on the base of the strong and numerously duplicated discovery, in particular in animal tests, that chronic but not severe cure with all effective antidepressant administrations, combined with electroconvulsive treatment, were correlated to a reduction in the density of post-synaptic beta 1 adrenergic receptors; even more, this result is in line with the timing of clinical enhancement (Charney & Manji, 2004). However, further animal tests showed sensitivity and density evolutions in some other adrenergic and serotonergic receptors, localized on the neuronal terminal, soma, and also as post-synaptically. This suggests that the receptor evolutions may be mechanisms of adaption in reaction to the augmentation of the availability of the neurotransmitter. Tests amongst humans that demonstrate an obvious effect of antidepressant drug cure on the adrenergic system output in both non depressed and depressed patients, showed data in augmentation, and non in diminution (Palazidou et al, 1992a);(Palazidou et al, 1992b) was not sustaining the

beta receptor down-regulation hypothesis. Ulterior animal tests showed that in spite of the decrease in density of the post-synaptic beta adrenoceptors, the post-receptor signal transduction cascade and correlated intracellular activity like protein synthesis, were in an obvious augmentation. These discoveries stress out the monoamine hypothesis demonstrating that antidepressants function by the augmentation of monoamine neurotransmission in the brain.

Numerous studies show low concentrations of serotonin and noradrenaline metabolites in plasma and cerebrospinal fluid disturbed neuroendocrine reactions on stimulus of noradrenergic and serotonergic receptors and a return of symptoms after antidepressant drug cure, with tryptophan or alpha-methyl-paratyrosine depletion, that decrease the amount of serotonin or noradrenaline concentration, respectively, (Ruhe et al, 2007) endorse the hypothesis that adrenergic and serotonergic activity is dysregulated in depression. All the available drugs showing a real impact, at least until now, MDD's cure, cause also the augmentation on the activity of one or both the systems above. Noradrenergic as well as serotonergic pathways are projected from their midbrain nuclei; the nucleus coeruleus and the raphe nuclei are located respectively, into the limbic and prefrontal zones and the hippocampus, near to the structures involved in the MDD's neurocircuit. How much could be relevant the role of these monoamine systems, in the complicated MDD's pathophysiology, is still to to be seen as other neurotransmitter systems as GABAergic, glutamatergic, that are also implicated in the circuit network. Until now, these pathways are still the main targets of the antidepressant cure and it seems that the therapeutic effects of antidepressants are obtained by a modulatory effect on the disturbed neurocircuit. Further studies and the development of drugs with new mechanisms of action, targeting systems outside the monoaminergic pathways, could be an efficient tool of clarification about their relevance.

Further studies implicate signal transduction intracellular in the pathophysiology of depression. Bunch of mechanisms involving phosphorylation enzymes, include protein kinase A (PKA) and C (PKC) (Figure 1.2.). The transmitter binding induces the activation of g-protein coupled receptor (Gs and Gq) with second messenger

enzymes like adelylate cyclase (AC) or phospholipase C (PLC). After activation those messengers catalyse the formation of the second couriers cyclic amp and diacylglycerol (DAG). Then they attach to PKA and PKC respectively, these enzymes eases phosphorylation (Nestler, 1984).Cell surface receptors like norepinephrine ß receptors, are in match with provocatory G (Gs) proteins. While binding process to transmitter, Gs drives adenylate cyclase (AC), inducing the catalization process of the formation of cyclic AMP from ATP. Cyclic AMP is broken down to AMP with phosphodiesterases (PDE), wheras inactivation of the cyclic AMP signal occurs. Cyclic AMP attaches with protein kinase A's (PKAs) regulator subunits (R), results with conformational alterations and the discharging of two catalytic subunits (C). After this process the C subunits are able to phosphorylate serine and threonine residues on aimed polypeptides. Protein like that is cyclic AMP response element binding protein (CREB), functions as a transcriptional factor. As a result after CREB phosphorylation the translocation of CREB-P to the nucleus and fortunate binding to CRE-containing gene promoter regions activate expression. A co-occuring process, illustrated with 5-HT2A transductional cascade, matches to Gq (another stimulatory G protein), activates phospholipase C (PLC) catalises the transformation of phosphotidylinositol (PI) to diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 enlists Ca^{2+} administration of intracellular stores. DAG matches with PKC wchich encompasses the phosphorylation of aimed proteins; which are both CREB and PKA. The PKA phosphorylation controls the enzyme action. Thereafter protein phosphatase 2A dephosphorylates substrates which are phosphorylated. CREB comes back to the cytosol within this process. Protein kinase inhibitor (PKI) is a different controller of PKA action; when it comes to fibroblasts subject, they are known as PKIγ isoform. PKA and PKC can be glutathionylated as well, this process protects them from oxidatively degraded. The activation chronicals of this two enzymes are phosphorylated with phosphoinositide dependent kinase 1 (PDK1).

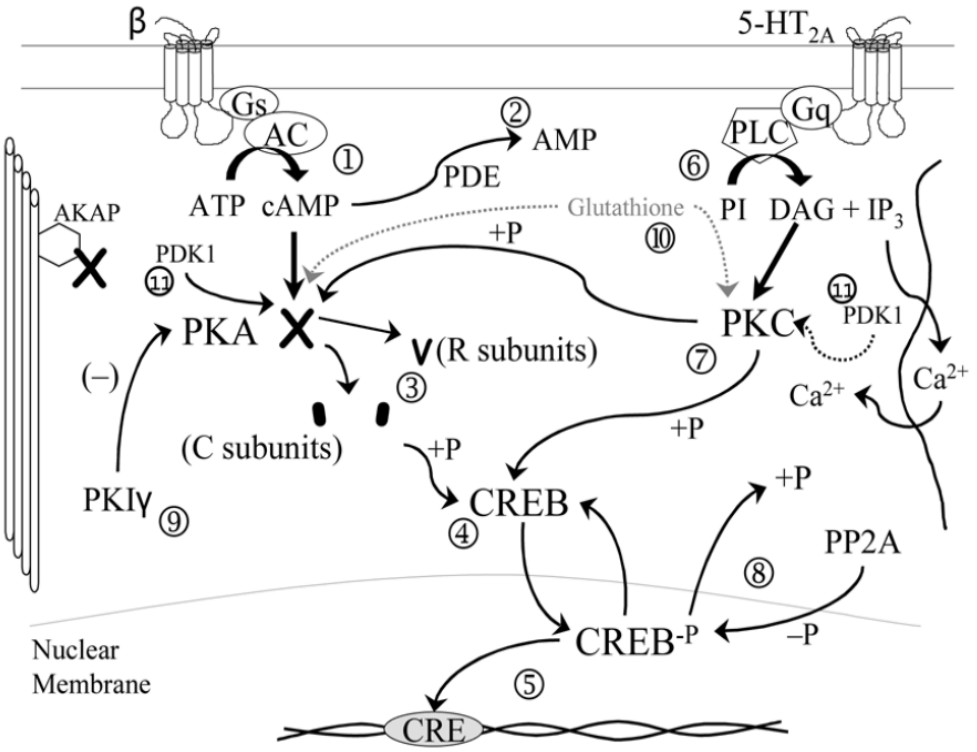

Figure 1.2.: Representative intracellular g-protein coupled signal transduction cascades. Protein kinases are critical elements of stimulus-response coupling (Hyman & Nestler, 1996).

One critical effect is the subsequent phosphorylation of the transcriptional factor cyclic AMP response element binding protein (CREB). Phosphorylation of CREB may perform a mutual action target of more noradrenergic and serotonergic antidepressants because CREB phosphorilation is related with both norepinephrine- (NE) and serotonin- (5-HT) linked pathways (Hyman & Nestler, 1996). Phosphorylated CREB attaches with cyclic AMP response element (CRE) at promoter region of genes, controlling expression of genes (Yamamoto et al, 1988). Showing an incorporate bunch of actions in such case, antidepressants proceeds through either NE or 5-HT can target a mutual bunch of genes and their resulting protein fruits. Diverse amount of studies has shown impairments in PKA and PKC in some depressives (Pandey & Dwivedi, 2005). For instance, Akin et al. reported that, compared to normal ones, particular depressed patients showed impaired PKA and PKC proteins, (Akin et al, 2004); (Akin et al, 2005) smaller incidence of bonding between cyclic AMP and PKA, (Manier et al, 2000) decreased CREB

phosphorylation, (Manier et al, 2000) and changed expression of gene motifs (Shelton et al, 2004). The common expectation about reduction at the action of these two vital enzymes would cause changes at genes expression which have CRE elements at their promoters; which may include important proteins at regulation process of the stress response in brain, which include brain derived neurotrophic factor (BDNF),(Shieh et al, 1998); (Meller et al, 2002); (Karege et al, 2004) the BDNF receptor trk-b, (Deogracias et al, 2004) and glucocorticoid receptors (GR (Barrett & Vedeckis, 1996). Whatsmore, GR exerts its function like a transcriptional factor and manipulates the expression of further different genes, especially shows an inhibitory effect on corticotrophin releasing hormone (CRH) (Malkoski & Dorin, 1999). Also, decreased activity of such vital enzymes would improve the probability stress reaction through different regulation of specified gene expression.

1.2. Antidepressants in the Treatment of Depression

Drugs related to the uptake process of biogenic amines are used to cure depression for about several decades. Figure 1.3. is representing major drugs considering their clinical effectiveness, up to now their utility is decreased for their adverse effects. The intention of discovering SSRIs or selective NE reuptake inhibitors (NRIs) was to obtain drugs as useful as TCAs. Those were pickier towards a monoamine transporter and with lesser adverse effects.

5-HT and NE inhibitors (SNRIs) recognized as twofold uptake inhibitors lately. Also 5-HT and NE inhibitors (SNRIs) represent a late action onset and adverse effects changing depressed patients' assent. What more, the effectiveness of every antidepressant used nowadays is interrogating as a result of a meaningful number of drug-resistant patients and drug/placebo effect is a matter in hand. With the production of the triple uptake inhibitors a novel improvement has been acquired. The triple uptake inhibitors are contemplated to block the uptake of every three neurotransmitters, 5-HT, NE, and DA, obviously related to depression (Figure 1.4.). Triple uptake inhibitors (SNDRIs) are thought to be the next generation chemicals for the curing of MDD (Chen & Skolnick, 2007).

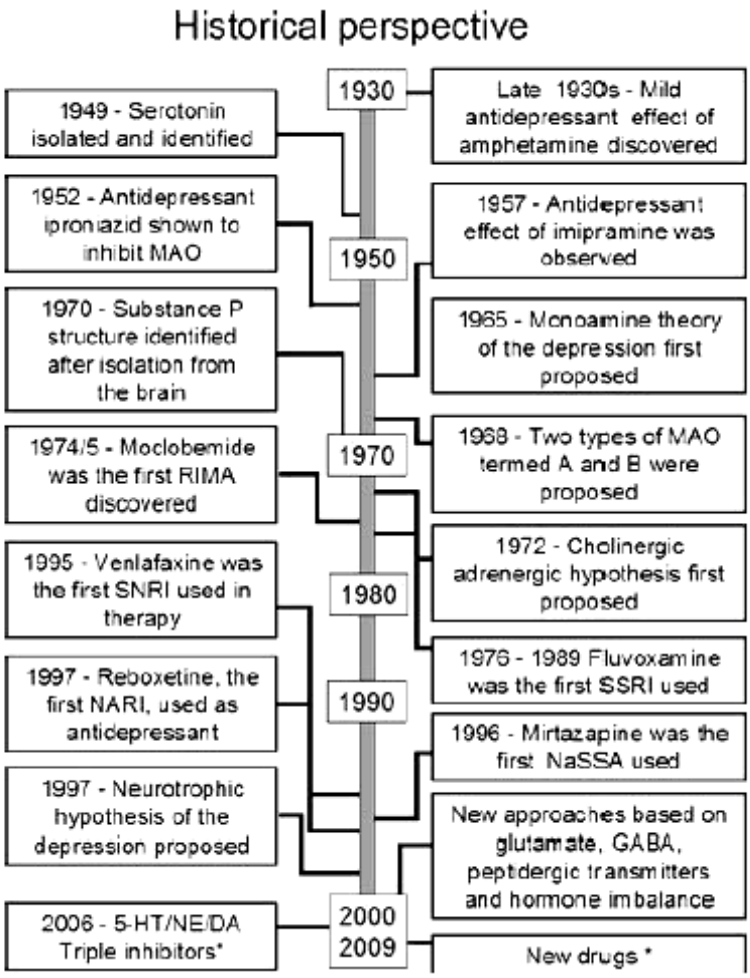

Figure 1.3.: Historical perspective of the different hypotheses on depression ethiopatology and of related drugs (Lanni et al, 2009).

Preclinical researches show that they can reproduce a quicker action onset and hold better effectiveness than widely used antidepressants (Chen & Skolnick, 2007). DOV 216,303 [(±)-1-(3,4-dichlorophenyl)- 3-azabicyclo-[3.1.0]hexane hydrochloride], the main product of the chemical mixtures worked as triple uptake inhibitors, has been shown to be working properly in prediced tests of antidepressant activity, like the mouse forced-swim test and locomotor depression (Skolnick et al, 2006) (Figure 1.3.). Up to today, as a result of lower adverse effects compared to other antidepressants, SSRIs impersonate the group of drugs to be chosen for curing of MDD. But, a considerable amount of depressed people take advantage partly only or not at all from curing process. Reaction to drug may be affected by genes and some

physiological and environmental aspects, containing age, renal and liver function, nutritional status, smoking and alcohol consumption. Genetic polymorphisms might denote an important role in giving a response to SSRI treatment especially (Lotrich & Pollock, 2005); (Kato, 2007); (Maier & Zobel, 2008); (Camilleri, 2007). Nowadays focal point of studies is to get a better knowledge of the 5-HT transporter (SERT) promoter regions' functional polymorphism in the. Polymorphism at SERT (known as 5-HTTLPR) includes the shortage or not shortage of a 44 base-pair segment, which is involved in the production of a long (L) or short (S) allele. The short one comes up with a reduction in transporter expression and, as a result reduced uptake of 5-HT (Lesch, 1998). 5-HTTLPR has been linked with depression vulnerability, but there are important amount of heterogeneity between researches (Caspi et al, 2003). Also the S allele is linked with decreased reaction to some number of SSRIs compared to the L allele in some research (Yu et al, 2002); (Serretti et al, 2004) also it may cause increased SSRI adverse effect vulnerability (Murphy et al, 2003). Patterning the genes that are linked to an antidepressant answer could be important to identify to obtain the patient-focused treatment.

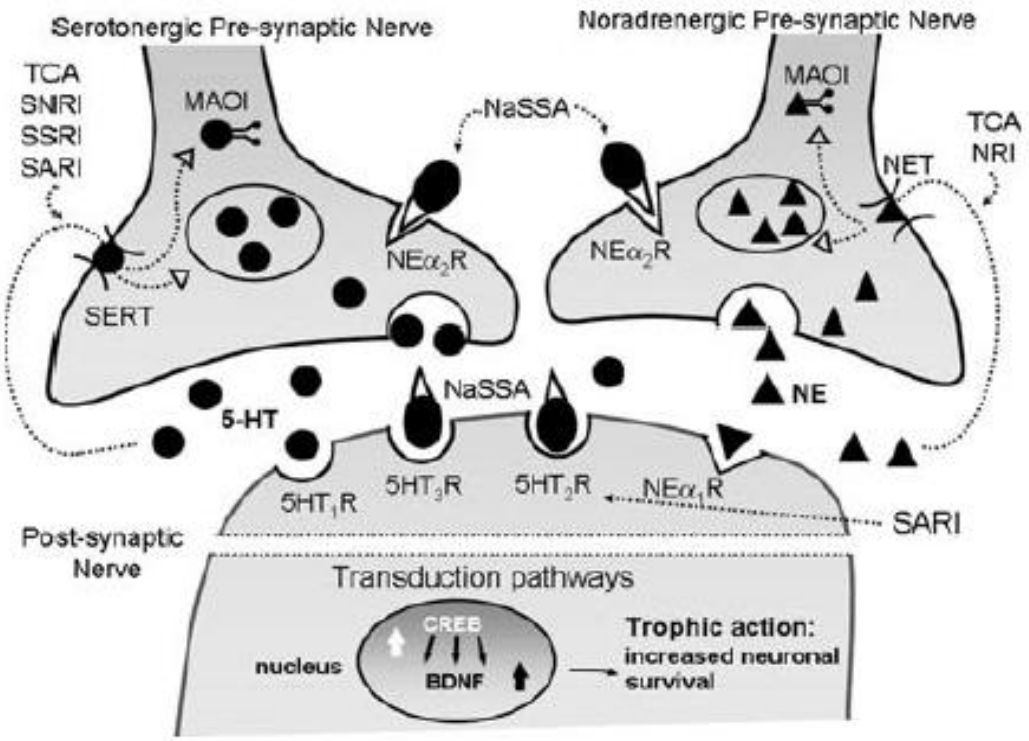

Figure 1.4.: A model for some antidepressant action at noradrenergic and serotonergic nerve endings.

1.2.1. Antidepressant Drug Agomelatine

The guidebooks formerly posted (Kennedy & Emsley, 2006) denoted that the SSRIs, SNRIs, and novel agents were firstly selected drugs to treat MDD. The reason for this situation is they thought to be safer and more tolerable characteristics compared to previously used drugs e.g. TCAs and MAO inhibitors.

The reproduction of novel antidepressants may be thought not like effective mechanistic originality but like variations on a theme (Sartorius & Force, 2008) (Millan, 2006). Nevertheless, improving monoaminergic antidepressants would be vital because, they have around 30 receptors waiting to be researched (Millan, 2006) (Morilak & Frazer, 2004). What more, the relation with monoaminergic antidepressant and an complementary mechanism, e.g. administration of an SSRI with lithium for resistant depression, created a claimed alternative (Millan, 2006) (Morilak & Frazer, 2004) (Wong et al, 2008).

An optional understanding to obtaion better cure for MDD can focus on melatonin, which is a vital tuner of circadian rhythms, that is impaired in MDD (Duncan, 1996) (Germain & Kupfer, 2008). Improvement and characterization of new melatonergic antidepressant called agomelatine, representing two main meaningful aspects: the very first antidepressant which as a main target of countering the impaired biological rhythms matched with depressed conditions, and the very first clinically reachable antidepressant exerting a non-monoaminergic action mechanism.

Previous to 1980s, the main melatonin function, was believed to be involved in the reproduction controlling processes (Morgan et al, 1994) (Macchi & Bruce, 2004). But, it had been understood that melatonin acts like a non-photic 'messenger' interacting with photic signals as a controller of circadian and diurnal rythms (Figure 1.5.). Where this is an especially significant situation since plasma levels of melatonin exhibit important circadian periodicity. To sum upl, melatonin recently shown to have a vital part in the synchronization of circadian rhythms (Arendt, 1998) (Morgan et al, 1994) which are inorganized in CNS diseases like MDD (Germain & Kupfer, 2008).

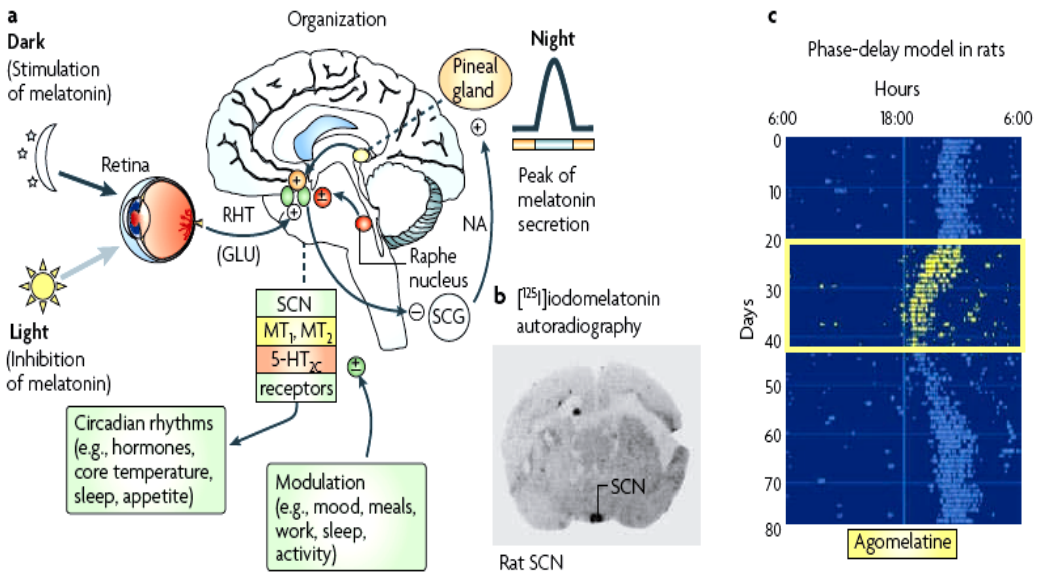

Figure 1.5.: The contact with melatonin, the SCN and circadian rhythms: melatonergic actions of agomelatine in vivo (de Bodinat et al, 2010).

The thought of utilizing melatonin matching ligands like drug-like agents (Guardiolalemaitre & Delagrange, 1995) was eagerly caught and a bunch of chemicals; melatonin-like derived naphthalene were made (Adam et al, 1992) (Adam et al, 1992). Such structures were thought to be act like melatonin. Also, the naphthalene ring of the derived chemicals is more lipophilic compared to the indole of melatonin, adding one more target which is improving getting through the brain. By the time, any of melatonergic receptors were cloned to make research and apply tests to perform matching studies on rodents unable to describe. As a consequence, radioligand [125I] iodomelatonin were used, to study the relation melatonin with of ligand binding sites placed at the sheep posterior pituitary (Vanecek et al, 1987). As shown in figure 1.6. melatonergic receptors bind through a Gα i/o protein to adenylyl cyclase. Furthermore, functional activity of ligands studied in vitro by figuring out their effects on forskolin-stimulated cyclic AmP reproduction in ovine pars tuberalis cells (Yous et al, 1992). Also, in electrophysiology studies, agonist properties of the chemicals affirmed at the important number of melatonergic receptors placed in the SCN. Those studies resulted that the derivatives of naphthalene of melatonin acted like high-affinity agonists. The chemical compound named as S20098 —after named agomelatine — was thought like the most auspicious drug basing on its features (Yous et al, 1992) (Ying et al, 1996) (Audinot et al, 2003). Agomelatine preferably matches with melatonin receptors, pressurizes cAmP reformation and copies the activity of melatonin by depending on its amount inhibits the firing rate of SCN neurons (Ying et al, 1996). Those findings were later proove when it was found that agomelatine also preferably reactivates cloned human melatonin 1 (MT1) and MT2 receptors (Audinot et al, 2003) (Figure 1.6.).

Around 1990s, scientists thought to examine the prospective alterations of agomelatine on 5-HT2C receptors, because they just detected an interaction via standardized binding screen. Actually, to reform structurally dissimilar isoforms 5-HT2C receptors post-transcriptional modification via editing mRNA had just been found (Burns et al, 1997) (Millan, 2006) (Aloyo et al, 2009). Untouched 5-HT2C receptors have constituent action, such that inverse agonists attenuates baseline signaling and improve the moving of 5-HT2C receptors to the plasma membrane

from the cytoplasm (Aloyo et al, 2009) (Chanrion et al, 2008). Inversely, neutral antagonists seem to be inactive alone, but it blocks the activities of agonists and inverse agonists. One more interesting characteristic of 5-HT2C sites were used to attach to some other cellular so called ligand-biased signaling (Millan, 2006) (Berg et al, 1998). However, the well established 5-HT2C receptors' signalling pathway was $G\alpha_q$-mediated activation of phospholipase C (Figure 1.7.). As a result of this, after authenticating that agomelatine replaces the radiolabelled antagonist, [3H] mesulergine, from recombinant human 5-HT2C receptors, proving performing a rivalry to antagonize the activation of $G\alpha_q$ and phospholipase C by serotonin (Figure 1.7.). Those examinations were broadened to some more G protein, $G\alpha_i$, asking that agomelatine acts like a broad-based antagonist at 5-HT2C receptors matched to broad signalling pathways (Figure 1.7.). Furthermore, at 5-HT2C sites agomelatine normalized signalling, different than pushing it under basal levels, coherent to neutral antagonist characteristics (Millan, 2006; Millan et al, 2008).

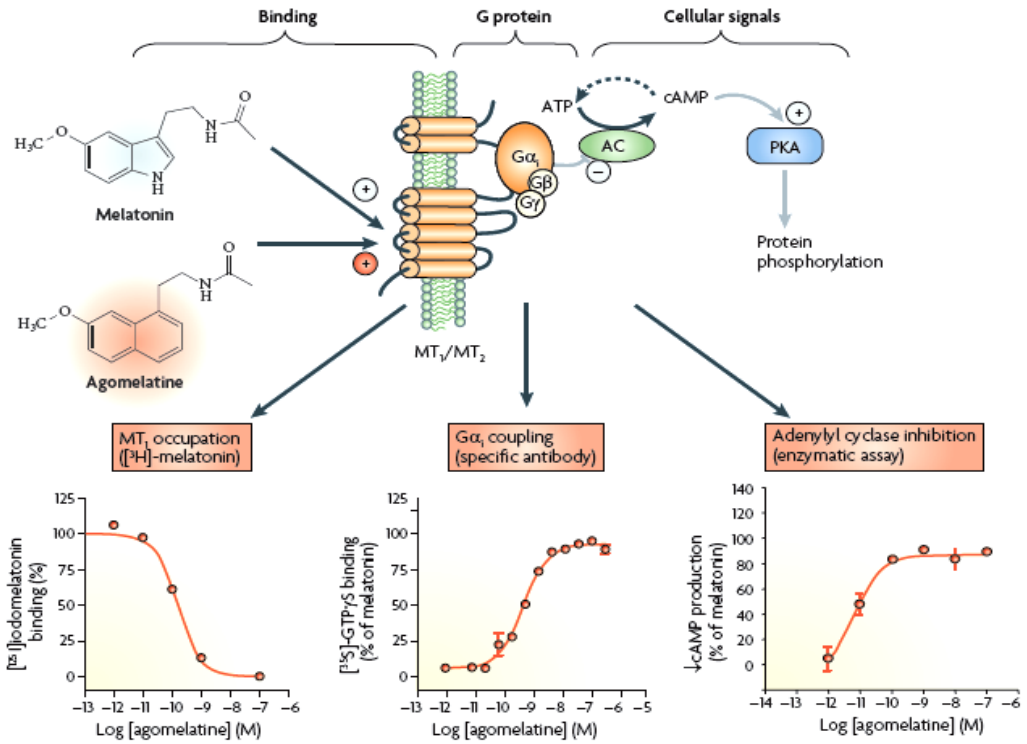

Figure 1.6.: Agonist functions of agomelatine at melatonergic receptors matching through $G\alpha_i$ to inhibit adenylyl cyclase.

The examination that agomelatine locks 5-HT2C receptors was a important thought like they congest important parts in the controlling of mood and the answer to stress (Giorgetti & Tecott, 2004) (Millan, 2005). What more, serotonin production is greatly circadian, the SCN is intentionally triggered by serotonergic pathways proceeding from the raphe nucleus, and 5-HT2C receptors placed on SCN participate to the matching of photic and non-photic alteration of circadian rhythms (Kennaway & Moyer, 1998) (Barassin et al, 2002) (Varcoe & Kennaway, 2008) (Cuesta et al, 2009) (Figure 1.5.). On account of differences among species, the 5-HT2C receptors accurate role on modulating SCN action in humans is still unclear (Cuesta et al, 2009) (Gannon & Millan, 2006). But, the activity of agomelatine on MT and 5-HT2C receptors both placed on the SCN may take part in its effects on circadian rhythms and on resynchronizing actions in depression. The invention of 5-HT2C antagonist characteristics for agomelatine was extremely valuable. But, it was important to know the thought of the difference among its high-level affinity for human MT1 and human MT2 sites and its importantly attenuated affinity at human 5-HT2C receptors. Even though to making comparison among potential of agonism

at one site with antagonism at another is hard, the matter of even if the tendency of agomelatine for 5-HT2C receptors is biologically significant had to be answered. The better way to ask this problem was only be solved via in vivo experiments with numerous behavioural and neurochemical tests.

It was proven that in rodents that agomelatine, rather than melatonin, was able to block 5-HT2C receptors at cerebral parts (Millan, 2005). Those impacts of agomelatine were seen when it was administrated to the body around 2.5–40.0 mg per kg, intraperitoneally, producing lower micromolar grades in brain, comparable to its affinity for 5-HT2C receptors. Moreover, in comparison with others correlated well with its matching tendency for 5-HT2C receptors in vitro (Millan, 2005). Actually, it is normal that such mild strenght for a drug. For instance, the SNRI venlafaxine has only micromolar tendency for NA transporters, but still exerts adrenergic activity in rats and humans (Millan, 2006) (Morilak & Frazer, 2004) (Stahl et al, 2005). Hopefully, the dissimilarity among the doses on important 5-HT2C antagonist and melatonergic agonist activity of agomelatine that are proven at doses 1.0–3.0 mg per kg, intraperitoneally, as shown far lower uttered in vivo than in vitro. A very elevated occupancy level of melatonergic parts is required for their sudden activation (Jockers et al, 2008). Apart from the substantiation, at the high doses of agomelatine, both melatonergic and 5-HT2C receptors becomes active and locked, and this is most likely accounts for humans (Figure 1.8.). Last magnetic resonance imaging studies has proven that agomelatine locks 5-HT2C parts in the rat brain (Linnik et al, 2009).

It was shown that agomelatine, but not melatonin, blocked cerebral populations of 5-HT2C receptors in rodents (Millan, 2005). These effects of agomelatine were seen at doses (2.5–40.0 mg per kg, intraperitoneally) yielding low micromolar levels in the brain, comparable to its affinity for 5-HT2C receptors. Moreover, in comparison with other correlated well with its affinity for 5-HT2C receptors in vitro (Millan, 2005). In fact, there is nothing unusual about such modest potency for a drug. For example, the SNRI venlafaxine possesses only micromolar affinity for noradrenaline transporters, yet exerts adrenergic actions in rats and humans (Millan, 2006) (Morilak & Frazer,

2004) (Stahl et al, 2005). Encouragingly, the difference between the doses at which substantial 5-HT2C antagonist and melatonergic agonist actions of agomelatine which are shown at doses 1.0–3.0 mg per kg, intraperitoneally, were observed was far less pronounced in vivo than in vitro. A very high degree of occupation of melatonergic sites is needed for their robust activation (Jockers et al, 2008). Irrespective of the explanation, at the dose range over which agomelatine possesses antidepressant properties in rodents, both melatonergic and 5-HT2C receptors should be activated and blocked, respectively, and this probably also applies for humans (Kasper & Hamon, 2009) (Figure 1.8.). Recent magnetic resonance imaging work has confirmed that agomelatine blocks 5-HT2C sites in the rat brain (Linnik et al, 2009) and a similar study is planned in humans.

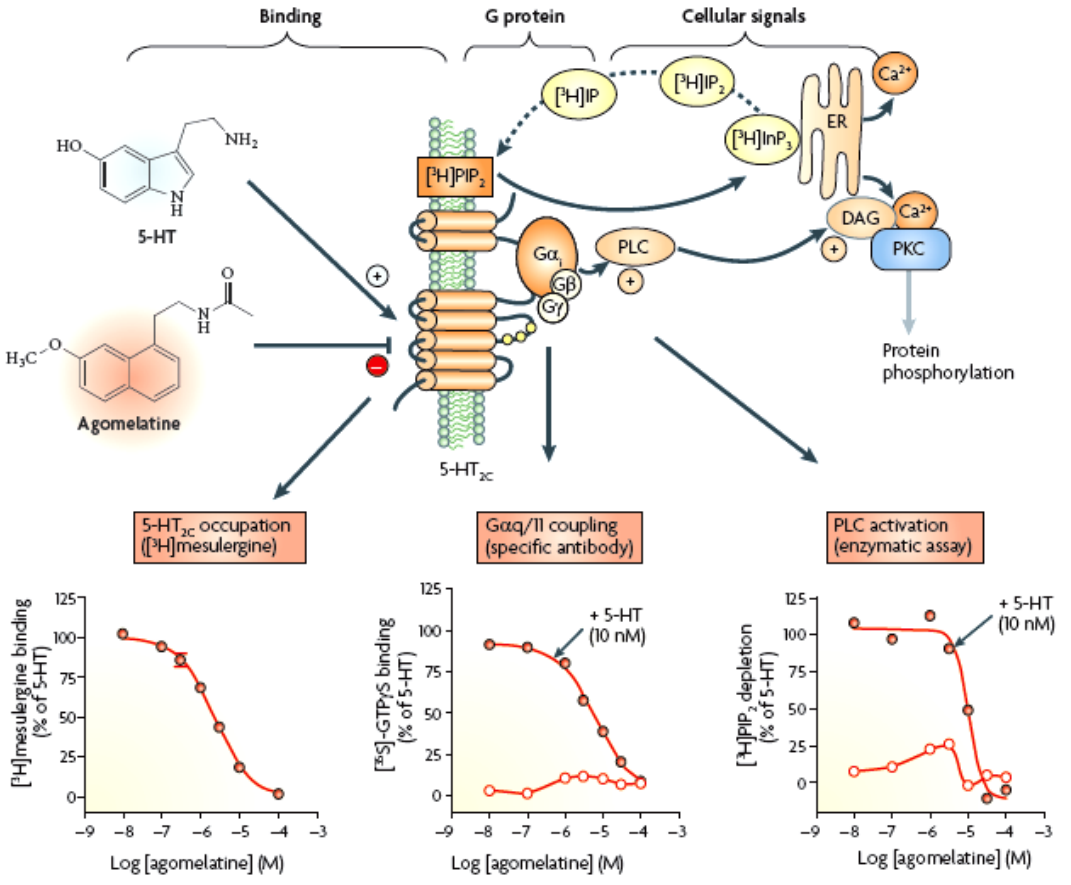

Figure 1.7.: Antagonist functions of agomelatine at 5-HT2c receptors matched through Gαq to activate phospholipase c. (Bodinat et al., 2010) .

Agomelatine shows an novel way to cure MDD (Kasper et al., 2009) since it is the prime approved moderating agent to integrate a non-monoaminergic mechanism. Agomelatins antidepressant action through a wide range of experimental process in animal models and its extraordinary therapeutic profile in humans presumably mirrors a synergistic interaction of its melatonergic (agonist) and 5-HT2C (antagonist) characteristics. Broad clinical trials have found that the short-term and long-term effects of agomelatine in MDD in mild and severe ill patients, with an better of sleep quality, sexual function preservation, no gain of weight and better tolerability. What more, giving up of drug does not results with withdrawal symptoms.

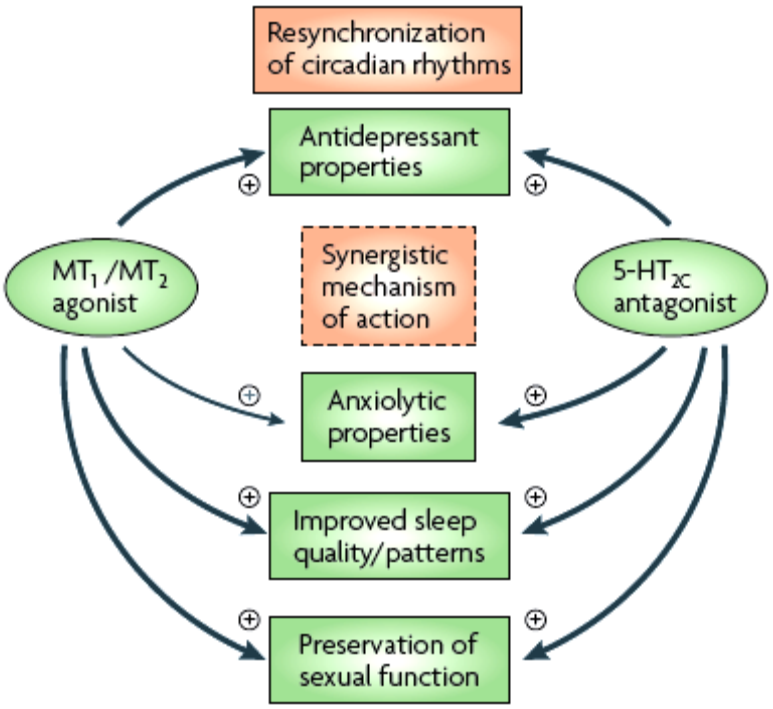

Figure 1.8.: Summary of the action mechanism of agomelatine. (Bodinat et al., 2010).

1.3. Biological Membranes

Plasma membrane or the cell membrane is the structure which separates the cell from the external world. Membranes have various functions. Their one major function is compartmentalization. Because of compartmentalization many chemical activities proceed without any disturbance from the outside and their regulation can be done independently. Also membranes provide a selectively permeable barrier from one side to the other side of the cell.

Moreover, membranes provide the communication between the compartments that they separate. Transportation is another major function of the cell membrane. They have the required mechanism in their structure for the physical transportation of substances from one side to the other side. Membranes have receptors in their structure. These receptors form a complementary structure with specific molecules known as ligands. Thus, membranes have an important role in signal transduction. Energy transduction, taking part in intercellular interaction and providing a suitable space for biochemical activities are some other main funtions of the cell membrane. (Karp, 1999).

All biological membranes are lipid-protein assemblies and the components are held together by noncovalent bonds. Membranes also contain short chains of carbohydrates. In early 1970s, S. J. Singer and G. Nicolson suggested fluid-mosaic model to demonstrate the chemical structure of biological membranes. Figure 1.9 shows fluid-mosaic model of a cell membrane. Regarding to fluid-mosaic model, lipid bilayers mainly serves as a structural framework for the membrane. The lipid molecules are in a fluid state and they can change their positons laterally inside the membrane plane. The proteins are like a mosaic of heterogeneous particles that penetrate deeply into, and even completely through, the lipid sheet. The most important property of the fluid mosaic model is presenting cellular membranes are like dynamic structures in which the ingredients are mobile and can come together to attach in numerous types of transient or semi-permanent interference. The proteins of the membrane perform some specific function, however, lipid bilayer provides a

barrier preventing the indiscriminate motions of water-soluble materials into and to the outside of the cell. The ratio of lipid to protein varies considerably depending on the type of cellular membrane, the type of organism and the type of cell (Karp, 1999) (Smith, 1983).

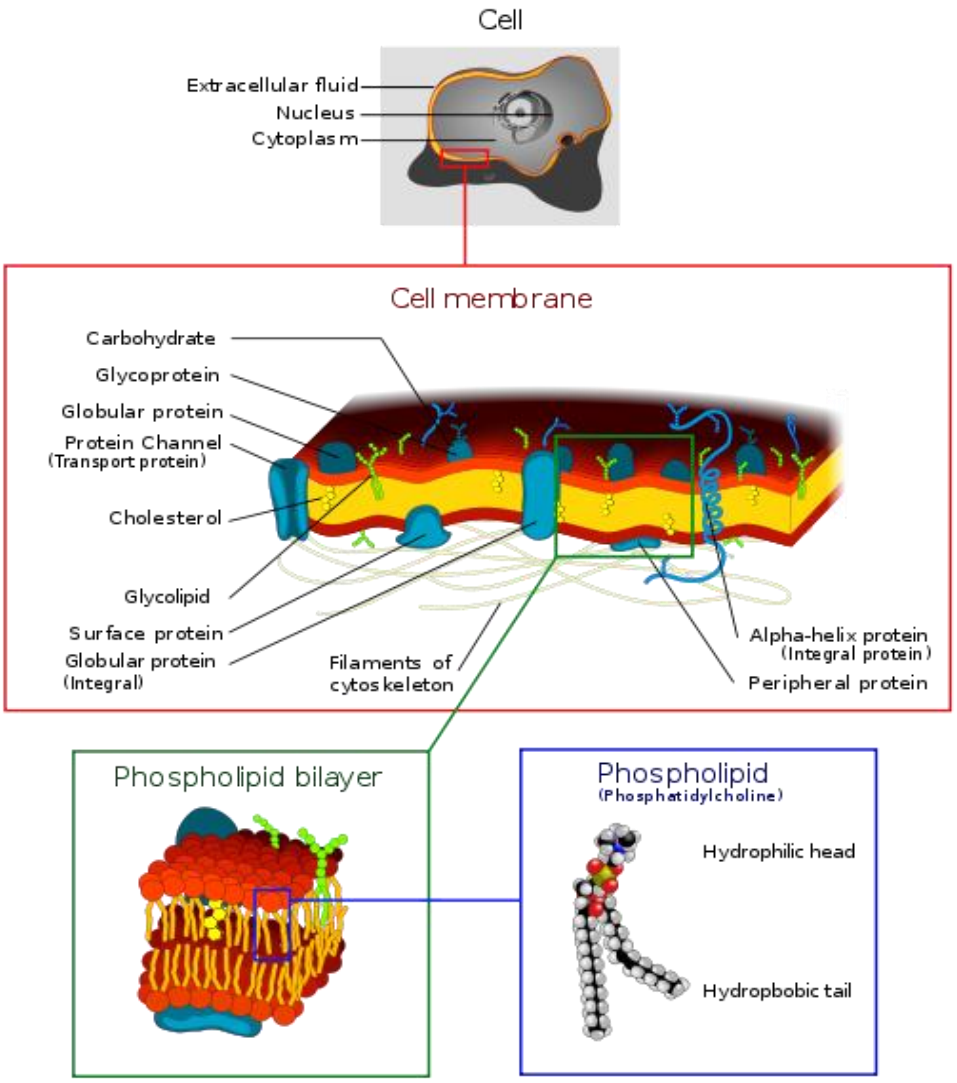

Figure 1.9.: Fluid-mosaic model of a cell membrane (http://users.humboldt.edu/rpaselk/BiochSupp/note_pics/LipidMemb/FluidMosaic_Model.png)

Membranes contain numerous sorts of lipids, all of them are amphipathic in other words they have both hydrophilic and hydrophobic parts. Membranes consists three essential sorts of lipids; phosphoglycerides, sphingolipids, and cholesterol. Mostly

they have a phosphate group making them phospholipids. Most membrane phospholipids are constructed on a glycerol backbone so called phosphoglycerides. Figure 1.10.; show the chemical structure of a phospholipid. Not similar to triglycerides, which consist three fatty acids, membrane glycerides are diglycerides. Two of the hydroxyl groups of their glycerol are esterified to fatty acids, however, the third is esterified to a phosphate group. The molecule, which has the phosphate group and the two fatty acyl chains, is called phosphatidic acid. Nevertheless, phosphatidic acid is almost absent in most membranes. Rather, an extra group added to the phosphate part of membrane phosphoglycerides, mostlt it can be either choline (forming phosphatidylcholine), ethanolamine (forming phosphatidylethanolamine), serine (forming phosphatidylserine), or inositol (forming phosphatidylinositol). Particularly those groups are small and hydrophilic and, collectively with the charged phosphate to which it is binded, establishes a great water-soluble domain at one end of the molecule, so called head group. In contrast the fatty acyl chains are long, unbranched, hydrophobic hydrocarbons. If a membrane fatty acid lacks double bonds then it is fully saturated. If it has one double bond then it is monounsaturated. If it has more than one double bond then it is polyunsaturated. Phosphoglycerides generally contain one unsaturated and one saturated fatty acyl chain (Karp, 1999; (Smith, 1983). 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).

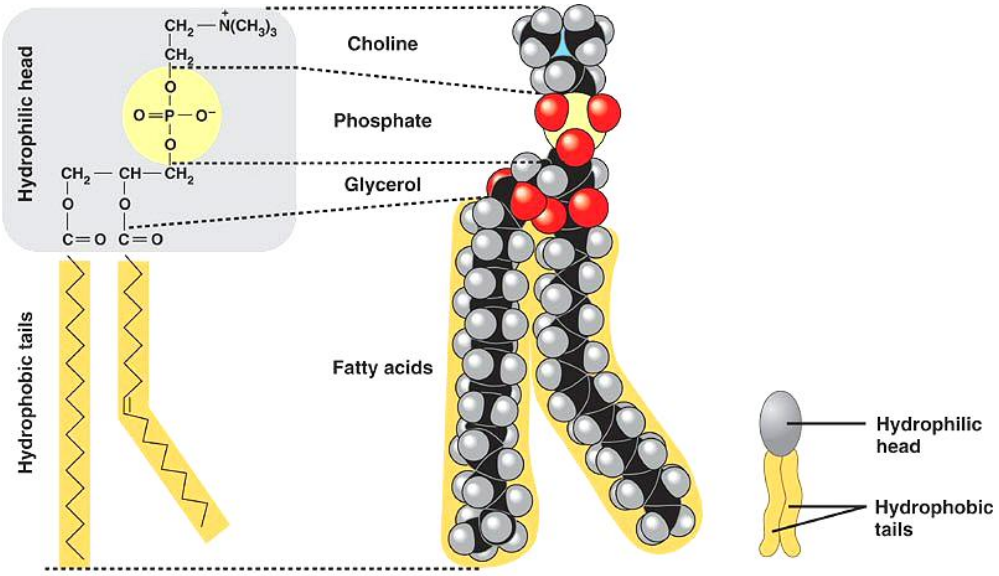

Figure 1.10: Chemical structure of a phospholipid.

1.4. Molecular Motions in Lipid Bilayers

There are various types of molecular motions in lipid bilayers. A phospholipid molecule has three kinds of movements in a membrane. Figure 1.11 shows movements of a phospholipid molecule within membranes. One of these movements is flip-flop motion or transverse diffusion. In this type of motion phospholipids move from one leaflet to another at a very slow rate. The halflife of a phospholipid molecule staying within one layer, as opposed to moving across to the other layer, is measured in hours to days. Such a slow rate is expected because for flip-flop is occuring, the hydrophilic head group of the lipid must cross through the internal hydrophobic sheet of the membrane, which is thermodynamically unfavorable (Karp, 1999; Mathews et al., 2000). In other words, there is a large activation energy barrier for the movement of polar head group through hydrophobic interior (Smith, 1983).

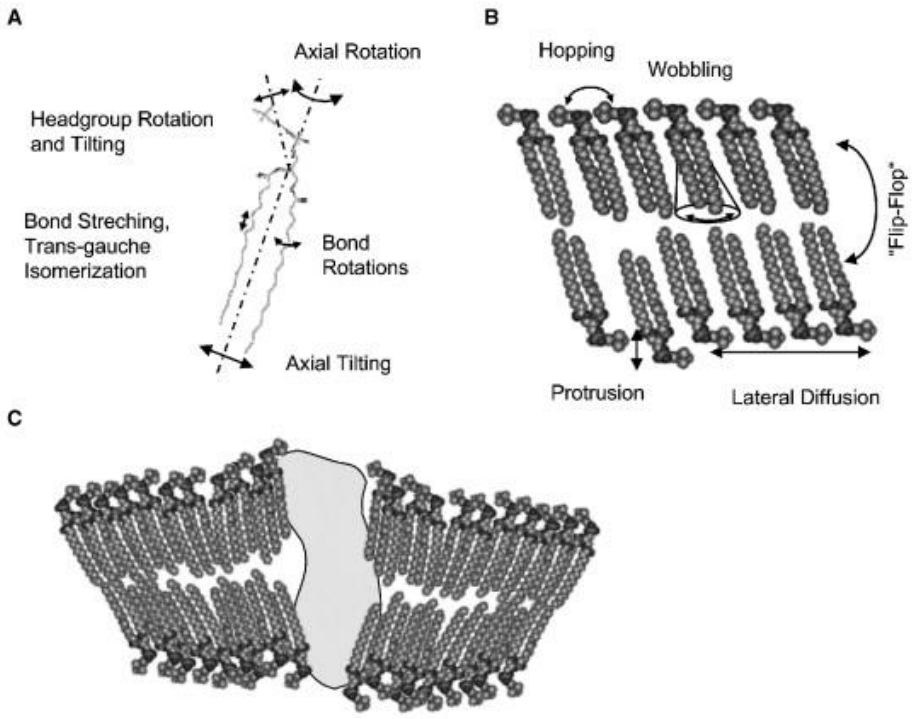

Figure 1.11.: Schematic examples of local bilayer properties including several modes of vibrations and rotations within a single lipid molecule (A), single molecular motions (B) and local membrane perturbation by the inclusion of a membrane protein (C) (Karl Lohner, 2008).

Rotation is another type of motion of phospholipid molecules. Axial rotation of lipids along their long axis perpendicular to the stage of the membrane happen every 0.1-100 ns. In lateral diffusion, phospholipid molecules displace rapidly in the stage of the membrane. Lipid molecules exchange places with neighbouring molecules in the same monolayer. Segmental motion of acyl chains causes an increased disorder towards the center of the membrane (Becker, 1996). Due to the bond rotation potential carbon-carbon bonds can only occur in three rotational states which are trans (t), gauche minus (g-) and gauche plus (g+). Gauche conformers have a higher free energy than trans conformers due to steric hinderence. The average conformation for a free chain is determined by the energy difference between trans and gauche isomers and cooperative interactions between nearest neighbours. All-trans conformation of a poly methylene chain is the lowest potential energy conformation. In this conformation saturated acyl chains are longest, thinnest and

most closely packed. However, a single gauche conformer in an acyl chain causes a big difference in the properties of the neighbouring acyl chains (Seelig & Seelig, 1974) (Jain, 1988).

The lipid bilayer consists of two distinct leaflets, and there is no reason to assume that the lipid composition of the two halves should be identical. In fact, a huge body of substantiation shows that the lipids of the plasma membrane are distributed in a highly asymmetric pattern. The lipid bilayer can be thought of as composed of two more-or-less stable, independent monolayers having different physical and chemical properties (Karp, 1999). Not only individual lipids distributed very asymmetrically, but the distribution also varies considerably among cell types. The consequences of such differences in phospholipid composition are numerous. Fluidity may be different on one side of the membrane or the other. The difference in charged groups on the two surfaces contributes to the membrane potential. Distribution of membrane proteins is also asymmetric. Presence of asymmetry in the distribution of lipids and proteins among the inner and outer leaflets of the bilayer makes the real biological membranes so complex (Mathews et al., 2000).

1.5. Liposomes as Model Membranes

If a little quantity of phosphatidylcholine is suspended in an aqueous solution, which is then subjected to mechanical agitation, the phospholipid molecules become assembled willingly to form the walls of fluid-filled spherical vesicles, called liposomes. The walls of those liposomes have single prepetual lipid bilayer which is organized same as that of the lipid bilayer of a natural membrane. Liposomes have proven invaluable in membrane research (Karp, 1999). Many things can be learned about biomembranes by studying liposomes. In other words, we can have a better knowledge of structural-functional intercourse of proteins, lipids, carbohydrates, pigments, and their complexes, transport mechanisms, energy transduction, signal processing, and our common senses like vision, olfaction, hearing, gustation, and touching senses (Tien and Ottova, 2000). Membrane proteins can be inserted into liposomes and their function studied in a much simpler environment than that of a

natural membrane (Karp, 1999). A liposome may contain one or more lipid bilayer. Most likely, some of those vesicles can form one inside the other in decreasing size, creates a multilamellar structure of concentric phospholipid shells divided by layers of water. Those liposomes can be prepared from a wide range of natural and synthetic lipids among which phospholipids are most commonly used. These phospholipids can be neutral, or positively charged phospholipids (such as phosphatidylcholine, phosphatidylethanolamine, etc.). Cholesterol is frequently used in liposome formulations to increase the stability. Moreover, liposomes can be prepared in sizes varying from 20 nm to 100 μm or more in diameter, according to the desired purpose. Procedures and raw materials for formulating liposomes must be carried out with care to avoid adverse effects on liposome stability (Tien and Ottova, 2000).

Liposomes having more than one bilayer are called multilamellar vesicles (MLV) or multivesicular vesicles (MVV). Figure 1.12 shows the structure of a liposome and the types of liposomes. There are also small unilamellar vesicles (SUV) which have diameters between 20-50 nm. The large unilamellar vesicles (LUV) are prepared from SUVs by applying different techniques like reversephase evaporation method and detergent dialysis (removal) method (Ostro, 1983; Rosoff, 1996; Tien and Ottova, 2000). Real biological membranes are in the form of unilamellar liposomes of approximately 900 nm in diameter. Liposomes can be used besides models of biomembranes, as drug carriers and be loaded with a great diversity of molecules, like proteins, nucleotides, small drug molecules, and even plasmids. Liposomes are highly adaptable, and due to the diversity of their composition, they can be utilized for a large number of applications (Rosoff, 1996; Tien and Ottova, 2000).

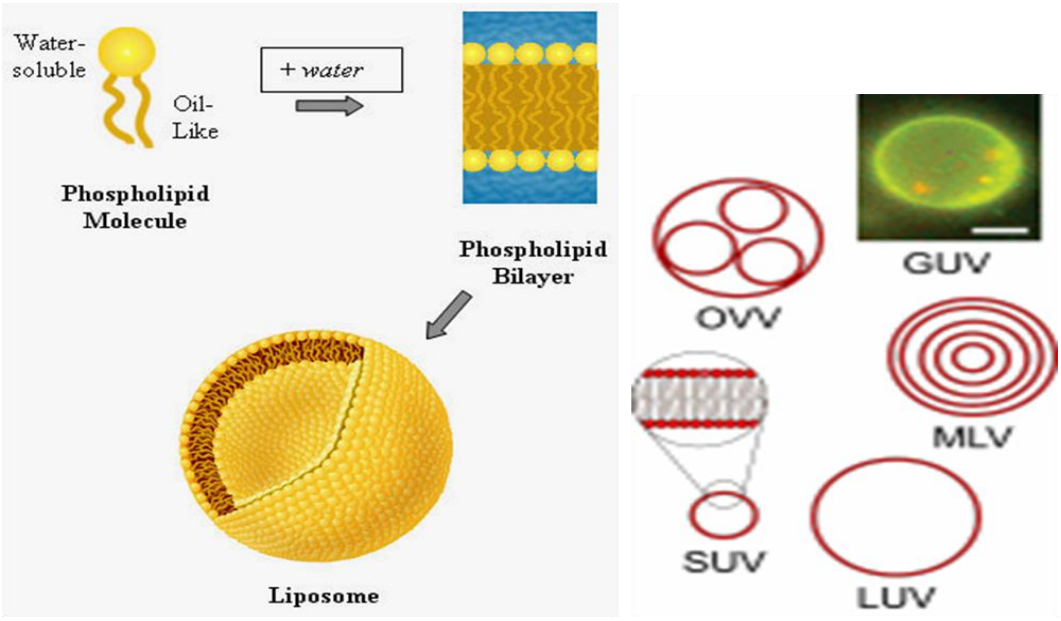

Figure 1.12: The structure of a liposome, small unilamellar vesicle (SUV), large unilamellar vesicle (LUV), multilamellar vesicle (MLV), giant unilamellar vesicle (GUV), oligovesicular vesicle (OVV) (Tresset, 2009).

1.6. Thermotropic Phase Transitions in Lipid Bilayers

The internal temperature of most organisms (other than birds and mammals) fluctuates with the temperature of the external environment. Since it is essential for many activities that the membranes of a cell remain in a fluid state, fluidity allows for interactions to take place within the membrane. For example, membrane fluidity makes it possible for clusters of membrane proteins to assemble at particular sites within the membrane and form specialized structures, such as intercellular junctions, light-capturing complexes, and synapses. Because of membrane fluidity, molecules that interact can come together, carry out the necessary reaction, and move apart. Fluidity also plays a role in membrane assembly. Many of the most basic cellular processes, including cell movement, cell growth, cell division, formation of intercellular junctions, secretion, and endocytosis, depend on the movement of membrane components and would probably not be possible if memranes were rigid, nonfluid structures (Karp, 1999).

The phase transition in lipid bilayers can be described as a cooperative melting of the acyl chains. Shorter chains melt at lower temperatures and longer chains melt at higher temperatures (Phillips et al., 1970). The physical state of the lipid of a membrane is described by its fluidity (or viscosity). As with many other substances, lipids can exist in a crystalline solid phase or a liquid phase of varying viscosity depending on the temperature. For example, a simple artificial bilayer made of phosphatidylcholine and phosphatidylethanolamine, whose fatty acids are largely unsaturated. If the temperature of the bilayer is kept relatively warm (e.g., 37^{o}C), the lipid exists in a relatively fluid state. At this temperature, the lipid bilayer is best described as a two-dimensional liquid crystal. As in a crystal, the molecules still retain a specified orientation; in this case, the long axes of the molecules remain essentially parallel, yet individual phospholipids can rotate around their axes or move laterally within the plane of the bilayer. If the temperature is slowly lowered, a point is reached where the bilayer distinctly changes. The lipid is converted from its normal liquid like state to a frozen crystalline gel in which the movement of the phospholipids is greatly restricted. The temperature at which this change occurs is called the transition temperature (Karp, 1999).

The molecular organization of phospholipids in the bilayer are found in four different states which are; crystalline gel, gel, rippled gel and liquid crystalline. Thus, there are three phase transitions, namely, solid (gel) phase transition, rippled gel phase transition and liquid crystalline phase transition between these four states as the temperature increases. The transition from gel phase to liquid crystalline phase in a phospholipid bilayer is a highly cooperative transition from order to disorder, involving a lateral expansion and a decrease in thickness and density. Within the transition range, two-dimensional domains of liquid and gel phase exist. Below and above this range, the membrane exists in gel and liquid phases, respectively (Jain et al., 1975).

The transition temperature of a membrane or lipid bilayer can be measured calorimetrically. The membrane suspension is placed in a calorimeter, an instrument that measures the energy required to increase the temperature of the specimen. As in

the case of any substance undergoing a transition from a solid to a liquid state, energy is absorbed by the system in order to break the intermolecular restraints without causing a corresponding increase in the temperature of the substance. The transition in phase is seen as a sharp peak in the thermogram (Karp, 1999).

Temperatures, at which the phase transitions of phospholipids occur, are the following: i) The main phase transition temperature (Tm): Transition from rippled gel phase to liquid crystalline phase, ii) The pretransition temperature (Tp): Transition from gel phase to rippled gel phase which occurs 5-10oC below Tm, iii) The sub-transition temperature (Ts): Transition from crystalline gel phase to gel phase (Silver, 1985; Datta, 1987). It is known that the DMPC and DPPC liposomes undergo three following phase transitions (Fuldner, 1981; Ruocco and Shipley, 1982; Janiak et al., 1979; Silver, 1985) where Lc, L, P, and Lcorresponds to crystalline gel, gel, rippled gel and liquid crystalline bilayer structures, respectively (Jain, 1988).

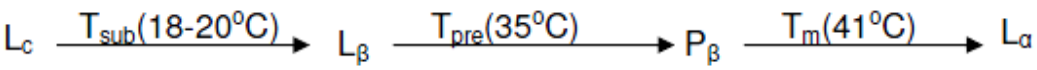

Figure 1.13.1 Lc Tsub(18-20oC) Tpre(35oC) Tm(41oC) shows the lamellar bilayer phases.

Over the main phase transition temperature, the lipids are in liquid-crystalline state. The alkane chains become highly flexible near the middle of the bilayer due to the rotational motions about C-C bonds. If the rotation is 120o, it leads to transient gauche isomer (or kink) formation. The possibility of kink formation increases toward the methyl end of the alkane chains (Heller, 1993).

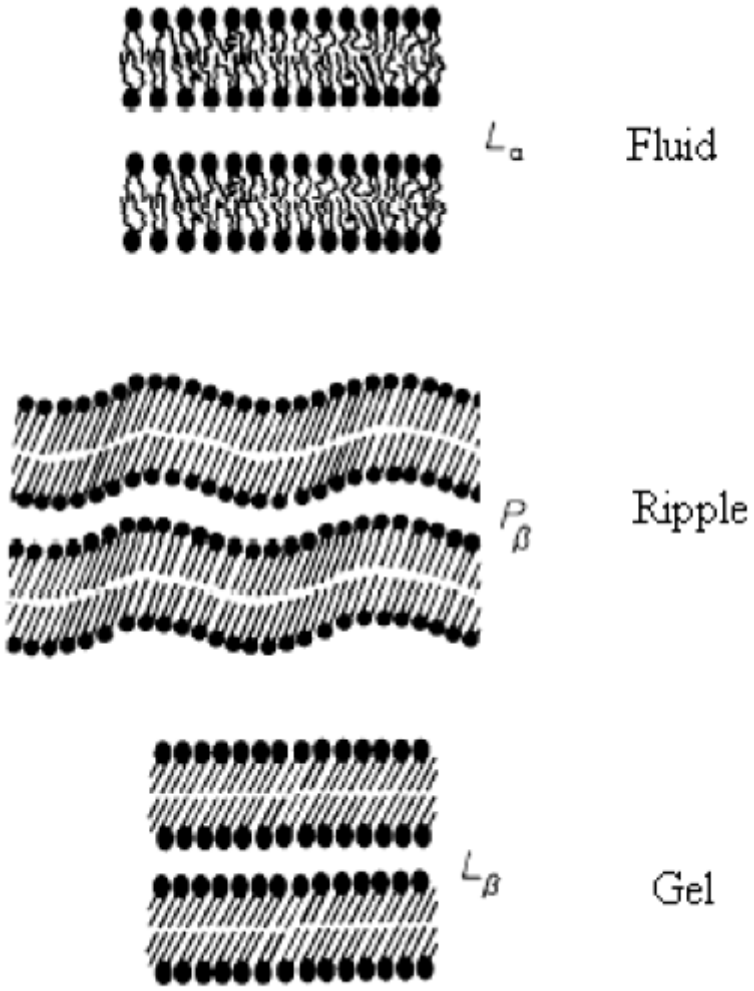

Figure 1.14 Schematic illustration of the lamellar bilayer phases.

In the bilayer, at sufficiently low temperatures, there is a little translational motion of lipid molecules. And their properties resemble those of hydrocarbon crystals in many ways. As the temperature increases, the properties of bilayers demonstrate abrupt changes at one or more temperatures. A small sudden increase in the phase transition curve corresponds to the pretransition peak associated with the tilting of the hydrocarbon chains. And a sudden larger increase corresponds to the main transition peak of the hydrated phospholipids from gel phase to the liquid crystalline phase, which is combined with complete melting of hydrocarbon chains (Severcan & Cannistraro, 1988).

The transition temperature of a particular bilayer depends on the particular lipids of which it is constructed. The most important determinant is the degree to which the fatty acyl chains of the phospholipids are unsaturated, that is, contain double bonds (specifically cis double bonds). The ability of the molecules to be packed together determines transition temperature and fluidity. Another factor that influences bilayer fluidity is fatty acid chain length (Karp, 1999).

1.7. Absorption Spectroscopy

Molecules absorb light. The wavelengths that are absorbed and the efficacy of absorption count upon the structure and the environment of the molecule, whihch makes absorption spectroscopy a helpful tool for characterization of small and large macromolecules (Freifelder, 1982). The total energy of a molecule is given by the sum of energies of every different motion that the molecule has. Then, the formula of total energy:

Etotal = Etransition + Erotation + Evibraion + Eelectronic + Eelectron spin orientation + Enuclear spin orientation

(1.6.1)

In a solution, a molecule can translate, rotate and vibrate. The energies associated with each of these motions are quantized (Campbell, 1984). Light, in its wave aspect, consists of mutually perpendicular electric and magnetic fields, which oscillate sinusoidally as they are propagated through space (Figure 1.15). The energy of the wave is:

$$E = h.c/\lambda = h.\nu$$

(1.6.2)

in which “h” is Planck’s constant, “c” is the velocity of light, “ƛ ” is the wavelength, and “ ν ” is the frequency. When such a wave meets with 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).

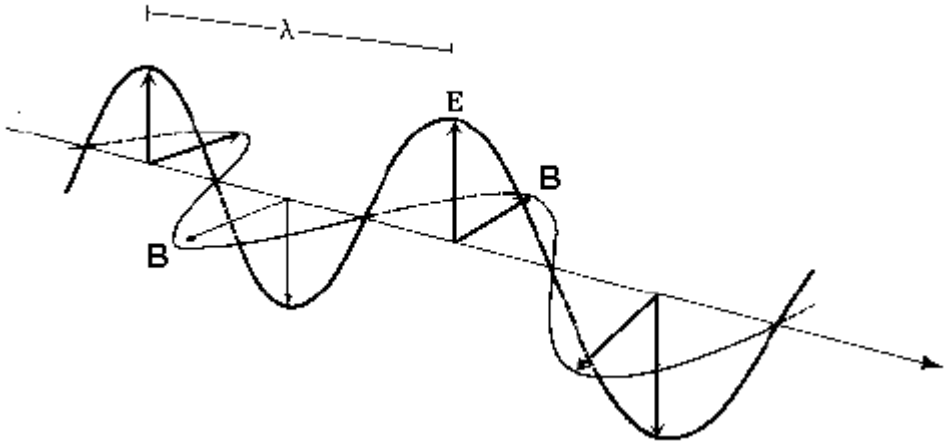

Figure 1.15. Propagation of an electromagnetic wave through space.

The relative likelyhood of the emergence of every single action is a characterization of the certain molecule experienced. If the lights electromagnetic energy is absorbed, the molecule becomes excited or will be in an excited state. A molecule or part of a molecule that can be excited via absorption is called a chromophore. This excitation energy is usually transformed into heat (kinetic energy) by the collision of the excited moecule with another molecule (e.g., a solvent molecule). At several molecules it is reemitted as fluorescence. In all cases, the intensity of the light transmitted by chromophores collection which is less than the intensity of the incident light (Freifelder, 1982).

An excited molecule can have any one of a set of discrete amounts (quanta) of energy explained with the laws of quantum mechanics. Those quantities are named as the energy levels of the molecule. The leader energy levels are defined by the possible spatial distributions of the electrons and are named as electronic energy levels; on these are superimposed vibrational levels, which shows the various modes of vibration of the molecule (e.g., the stretching and bending of various covalent bonds). There are even lower subdivisions called rotational levels, but they are of little importance in absorption spectroscopy.

All these energy levels are usually described by an energy-level diagram (Figure 1.16). The lowest electronic level is called the ground state and all others are excited states (Freifelder, 1982).

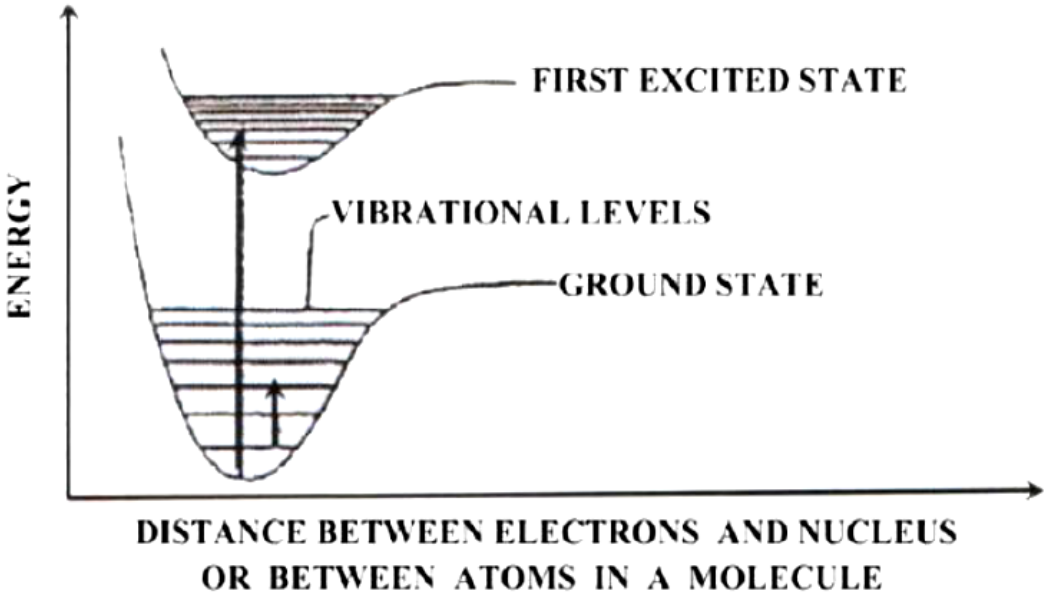

Figure 1.16 Representative energy-level diagram showing the ground state and the first excited state. Vibrational levels are shown as horizontal lines and rotational levels are also shown in between the two vibrational levels in the ground state. A possible electronic transition between the first vibrational level of the ground state and the second 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.

The absorption of energy is most probable only if the absorbed quantity corresponds to the dissimilarities between energy levels. This may be explained by saying that light of wavelength can be absorbed only if

$$\lambda = \frac{hc}{E_2 - \mathrm{E}_1} \qquad (1.6.3)$$

in which E1 is the energy level of the molecule before absorption and E2 is an energy level reached by absorption. An alteration among energy levels is named like transition. Mechanically, a transition among electronic energy states shows the energy needed to replace an electron from one orbit to another. Transitions are demonstrated by vertical arrows in the energy-level diagram. A plot of the likelihood

of absorption vs. wavelength is named as an absorption spectrum and absorption spectroscopy refers to the gathering and analysis of absorption data (Freifelder, 1982).

1.7.1 Infrared Spectroscopy

Infrared spectroscopy is surely one of the most substantial analytical techniques valid to scientists. One of the most important characteristics of IR spectroscopy is that potentially any sample in potentially any state may be examined. Liquids, solutions, pastes, powders, films, fibres, gases and surfaces may all be investigated with a professional choice of sampling technique. Consequently the enhanced instrumentation, and several new sensitive techniques have now been improved to examine formerly insoluble samples (Stuart, 2004). 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 (Figure 1.17). These vibrational levels and, hence, infrared spectra are generated by the characteristic motions (bond stretching, bond bending, and more complex motions) of numerous functional groups (e.g., carbonyl, methyl, amide, etc.). Figure 1.18 shows types of normal vibrations for CO_2 molecule. The importance of infrared spectral analysis is that the mode of vibration of every single group is enormously responsive to changes in chemical structure, environment, and conformation. 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).

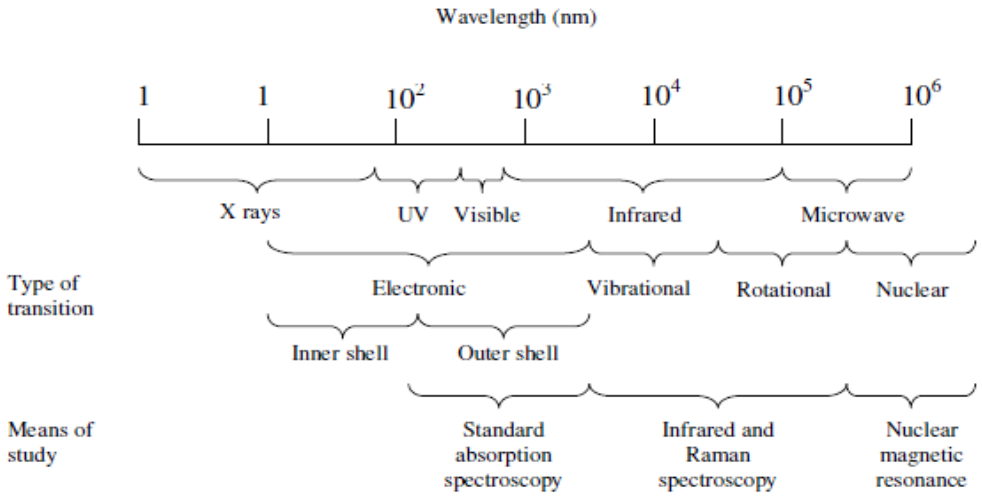

Figure 1.17 The part of the electromagnetic spectrum that is relevant to physical biochemistry.

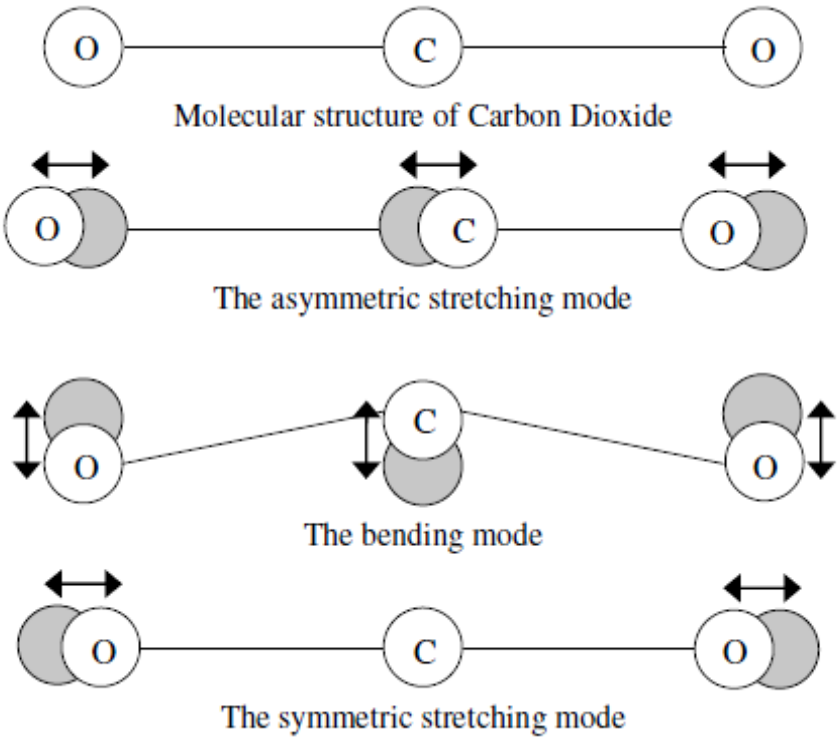

Figure 1.18.: Types of normal vibrations for CO_2 molecule.

Infrared spectroscopy is a technique founded on the vibrations of the atoms of a molecule. An infrared spectrum is mutually acquired by crossing infrared radiation into a sample and defining what part of the incident radiation is absorbed at a

peculiar energy. For a molecule to express infrared absorptions it must have a specific feature, i.e. an electric dipole moment of the molecule must alter at the vibration. The selection rule for infrared spectroscopy is this. For the dissimilarity in electronegativity among carbon and oxygen, the carbonyl group is permanently polarized. If this bond stretches it will raise the dipole moment and, therefrom, C=O stretching is an extreme absorption. A molecule can absorb radiation when the yielding infrared radiation has the equal frequency with one of the fundamental modes of vibration of the molecule. Meaning; the vibrational motion of a little part of the molecule is enhanced while other molecules are not affected (Stuart, 2004).

1.7.2 Instrumentation of Fourier-Transform Infrared Spectrometer

Fourier-transform infrared (FTIR) spectroscopy is grounded on the interaction of radiation among two beams to yield an interferogram (Griffiths and de Haseth, 1986). Produces a signal; as a function of the changing of path length among the two beams. The two domains of distance and frequency are inter-changeable by the mathematical method of Fourier transformation. Main components of an FTIR spectrometer are demonstrated schematically in Figure 1.19. The radiation yielding from the source is crosses through an interferometer to the sample before reaching a detector. Until amplification of the signal, maintained by high-frequency contributions are eliminating via a filter, the data are converted to digital form by an analog to- digital converter and transferred to the computer for Fourier-transformation (Stuart, 2004).

Figure 1.19 Basic components of an FTIR spectrometer.

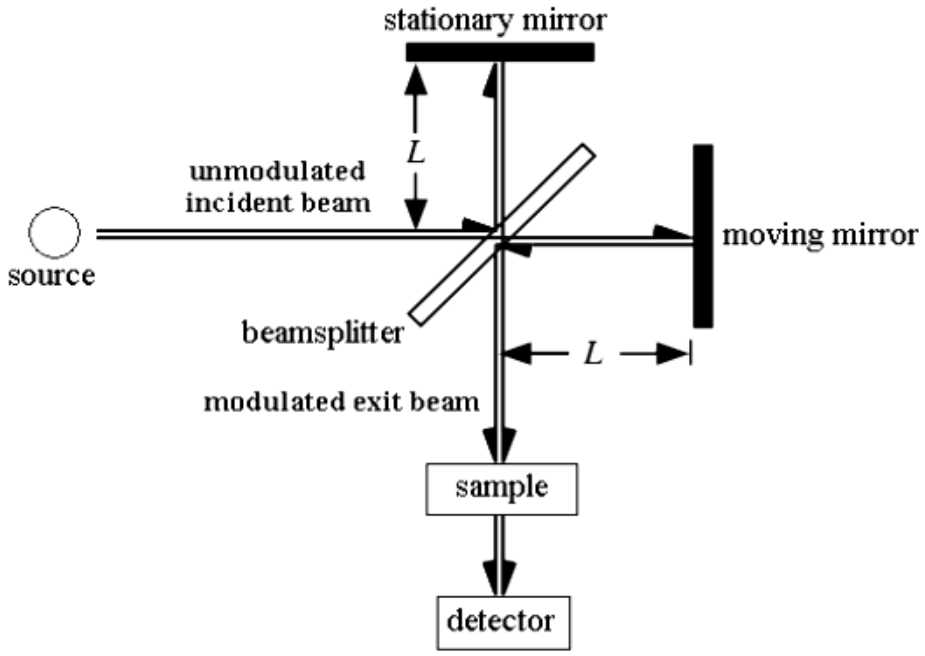

Figure 1.20 Schematic of a Michelson interferometer.

The most general interferometer took part in FTIR spectrometry is a Michelson interferometer (Figure 1.20), consists of two perpendicularly plane mirrors, one them can travel in a direction perpendicular to the ground. A semi-reflecting film, the beam splitter, bisects the planes of these two mirrors. The beam splitter material has to be picked due to the area to be examined. FTIR spectrometers has a Globar or Nernst source for the mid infrared region. If the far-infrared region is to be investigated, a high pressure mercury lamp may be chosen. For the near-infrared, tungsten-halogen lamps are chosen as sources. The moving mirror is an important part of the interferometer. It has to be precisely placed and should be able to scan two distances so that the difference of path can correspond to a familiar value. Numerous factors coupled with the moving mirror needed to be taken in to account when calculating an infrared spectrum. The interferogram is an analogue signal at the detector that has to be digitized to the Fourier-transformation into a traditional spectrum can be formed (Stuart, 2004).

1.7.3 Infrared Spectroscopy in Membrane Research

Infrared spectroscopy can provide valuable structural information about lipids, which are important molecular constituents of membranes. The IR spectra of phospholipids may be seperated into the spectral regions that take origin from the molecular vibrations of the hydrocarbon tail, the intermediate region and the head group (Watts and De Pont, 1986; Lewis and McElhaney, 2002). Acyl chain modes are formed by the hydrocarbon tail. The CH_2 stretching vibrations are the most important vibrations in the infrared spectra of lipid systems and these are the bands in the 3100 to 2800 cm^{-1} region. In some lipid membranes containing unsaturated acyl chains, the typical lamellar liquid crystalline phase converts to a micellar non-lamellar phase while heating (Jackson and Mantsch, 1993). Lipids rearrange during this transation. Temperature controlled research of the phospholipids with FTIR spectroscopy gives sensitive means of studying transitions in lipids.

The head groups and interfacial regions spectral modes also provide important information (Mushayakarara and Levin, 1982). The C=O stretching bands in the 1750-1700 cm-1 region in particular are helpful IR bands to examine the interfacial region of lipid organizations are the ester groups vibrations.

1.8 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is a technique widely utilized to determine the thermal characteristics of numerous of materials including biologically related systems. The principal areas of DSC used studies encompass determination of the effect of structure, pH, hydration, and solvent, on the phase-transition temperatures and enthalpies of biological membranes and pharmaceuticals; thermal characterization of complex procedures, like the denaturation of proteins; and accurate heat measurements in the glass-transition of polymers (Ceckler and Cunningham, 1997). The DSC contains a sample cell and a reference cell that are maintained at the same temperature. As an experiment proceeds, the sample and reference cells are raised in temperature in a controlled manner such that the two

cells always are maintained at the same temperature. The power supplied to heat each cell is monitored during this process. When a phase transition occurs in the sample cell, there is dissimilarity in the amount of energy needed to heat the two cells. The energy required to get both cells to the equal temperature is investigated and converted to get an output of heat capacity vs temperature. The heat capacity vs temperature curve is examined to calculate the transition temperature, (Tm) and the calorimetric enthalpy of transition (ΔHcal) (Ohline et al., 2001).

The preliminary phase transitions like the bilayer gel to liquid-crystalline transition, the Tm, when the heat capacity, Cp, reaches to maximum amount. The amount of the calorimetric enthalpy (ΔHcal) for the phase transition is determined by calculating the area under the peak.

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

(1.7.1)

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

$$\Delta S = \Delta H_{cal} / T_m$$

(1.7.2)

Comparison of ΔHcal, ΔS and Tm gives the effect of a structural alteration (e.g. chain length) about the thermodynamics of the phase transition. Also, not similar to a plain organic compounds gel to liquid melting transition, the phase transition in bilayers includes bigger than only the primary and last states. In fact, intermediary "states" are reformed while the transition occurs, and a "non-twostate" model is needed for phospolipids in liposomes (Mason, 1998; Microcal, Inc., 2002; Sturtevant et al., 1987). These intermediary states are a result of the reformation of domains (e.g. ordered, mobile parts inside the gel phase) before Tm, and are because of lateral motion of the phospholipids inside the bilayer. The asymmetric DSC peak means that there is a non-two-state transition is happening (Ohline et al., 2001).

1.8.1 Instrumentation of Differential Scanning Calorimetry

DSC has two sealed pans: a sample pan and a reference pan. Those pans are usually composed of aluminum, acting like a radiation shield (Dean, 1995). During the heat flow difference between the two is monitored, those pans are heated, or cooled. This process may be done at a perpetual temperature (isothermally), but usually it is done by increasing and decreasing the temperature at a constant rate, also named temperature scanning (Dean, 1995).

While determining, the instrument calculates differences in the heat flow among sample and reference. The data is obtained by an output device, mostly a computer, drawing a plot of the differential heat flow among the reference and sample cell as a function of temperature. If there is not any thermodynamic process, the difference between the sample and reference heat flow varies only mildly with temperature, and a flat, or very shallow base line thermogram is forming. But, an exothermic or endothermic process inside the sample comes up with an important curvature in the difference among heat flows. As a result there is a peak in the DSC curve. Usually, the differential heat flow is imprinted via subtracting the sample heat flow from the reference heat flow. While conventing, exothermic processes occurs as positive peaks (above the baseline) while peaks resulting from endothermic processes are negative (below the baseline) (Dean, 1995). Figure 1.21 shows instrumentation of a DSC.

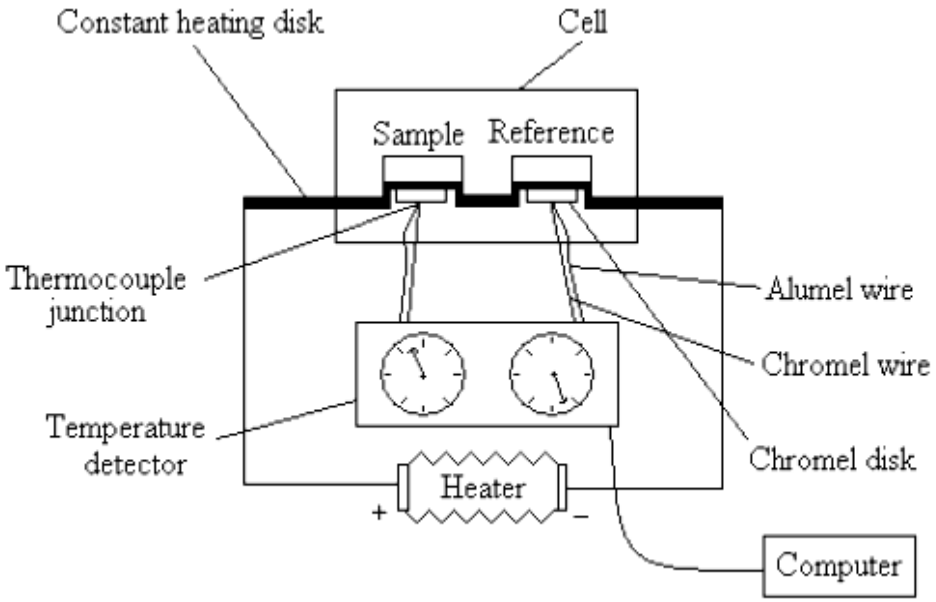

Figure 1.21 Schematic illustration of a DSC.

1.8.2 Differential Scanning Calorimetry in Membrane Research

DSC is a responsive and non-invasive technique (Kazancı et al., 2001). DSC is utilized to characterize phase transitions with respect to thermodynamic parameters like Tm, ΔS, ΔH, and glass transition temperature (Koyama et al., 1999). DSC has a broad usage in drug industry like examining the stability, physical and chemical characteristics of drugs (Schneider et al., 1999). Protein-ligand, protein-protein interactions and protein domain organization research also include utilization of DSC technique (Weber and Salemme, 2003). Investigation of thermal stability and reversibility (Anton et al., 2000), investigation Cp for the measurement of ΔH, purity establishment of chemicals, and chemical half-life calculations (Mayor et al, 2000) are some other practices of DSC. It is a classic method, however it is a novel technique for biological systems. DSC has been utilized to investigate phase transitions in lipid systems and membranes matching the observations with low-angle X-ray diffraction research. The phase transitions investigated in the DSC can correspond to alterations in biological characteristics (Tien and Ottova, 2000). The phase transition from gel to liquid-crystalline is extremely "cooperative." Those kind of transitions, the molecules begin to misorganize and enhance their mobility to mate

each other. By obtaining a different way, molecules cooperate together in absorbing mew mobility; after a molecule obtains motional energy then other molecules around find a easier way to get motional energy. After the temperature reaches to the Tm, the distance diversity of this cooperation increases. Around the Tm, islands of lipids may be observed in increased mobility phase mixed with the lower mobile gel phase. In the DSC of synthetic phospholipids the limited cooperativity of the transition results in a small peak at a lower temperature than the main melting peak, called the pre-transition, as well as a broadening of the main melting transition (Chapman, 1975; Szoka and Papahadjopoulos, 1980; Koyama et al., 1999; Ohline et al., 2001).

1.9. Aim of the study

Psychiatric disorders are prevalently observed nowadays, due to altered living conditions and increased stress factor. Bipolar disorder, anxiety disorders, schizophrenia and depression are the most striking among all psychiatric disorders. As psychiatric drugs are widely used in medical treatments, there is a consistent effect on the development of new drugs with higher efficacy and lesser side effects in comparison to existing drugs for their release to the market. Within this context, a new drug having a Valdoxan trade name with an active component named Agomelatine has been recently developed and released to the market in Europe and started to be used in the treatment of sleep disorders as well as depression. This drug, which has an entirely novel mechanism of action compared to all known psychiatric drugs, promises great hope for effective treatment of depression in near future. Since the drug has been recently developed, there is a significant scarce of information in the literature about the interaction of the drug with the macromolecular components of cells and membranes. In order to provide the efficient use of a drug, it is critically important to know the interactions of the drug with membranes, which are the sites that drugs start to act, at molecular level and depending on drug concentration. In addition, it is also necessary to determine the location and the conformation of the drug within membrane structure.

Plasma membrane or the cell membrane is the structure which separates the cell from the external world. Membranes have various functions. Their one major function is compartmentalization. Because of compartmentalization many chemical activities proceed without any disturbance from the outside and their regulation can be done independently. Also membranes provide a selectively permeable barrier from one side to the other side of the cell.

Biophysical studies dealing with biomembranes are mainly based on the organization, structure and dynamics of the lipid matrix. Order-disorder phase transition or thermotropic mesomorphism is the most frequently studied property of the biological membranes by physical techniques like spectroscopic methods which provide information about molecular motion and molecular moieties. In recent years, there has been a great development in the field of spectroscopic instrumentation. As a result, it becomes possible to apply spectroscopic techniques to the researches of membrane structure and function. Especially non-perturbing techniques, such as Fourier Transform Infrared (FTIR) spectroscopy and Differential Scanning Calorimetry (DSC) provide significant and detailed information about changes in the phase transition behaviour, mobility and structure of individual molecular moieties (Lopez- Garcia et al., 1993; Daniels, 1973).

In this study we report our findings on the effect of agomelatine on lipid organization and fluidity of model membranes which was accomplished by monitoring the thermotropic phase transition profiles of DPPC, DPPG and SM liposomes with different physical techniques. To our knowledge this is the first study to report the interactions of agomelatine with phospholipid membranes.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

Agomelatine (N-[2-(7-Methoxy-1-naphthalenyl)ethyl]-acetamide) (Figure 2.1), Dipalmitoyl phosphatidylcholine (DPPC) (Figure 2.2), Dipalmitoyl phosphatidylglycerol (DPPG), Sphingomyelin (SM) (Figure 2.3) and Phosphate Buffered Saline tablets were purchased from Sigma (St. Louis, MO, USA). All chemicals were obtained from commercial sources at the highest grade of purity available.

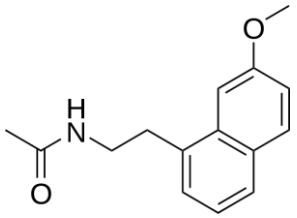

Figure 2.1: Chemical structure of agomelatine.

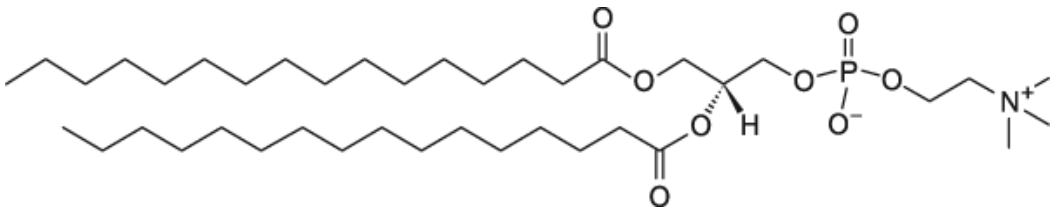

Figure 2.2 : Chemical structure of Dipalmitoyl phosphatidylcholine

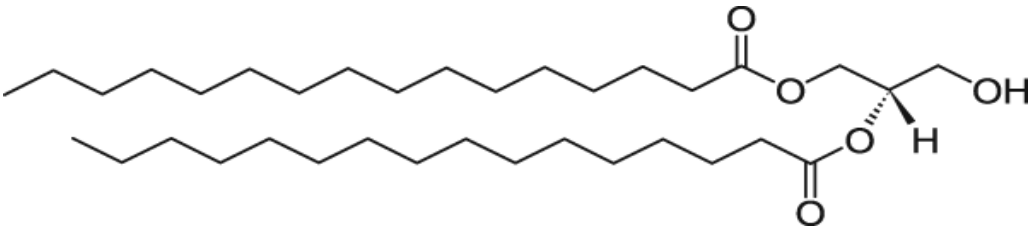

Figure 2.3 : Chemical structure of Dipalmitoyl phosphatidylglycerol

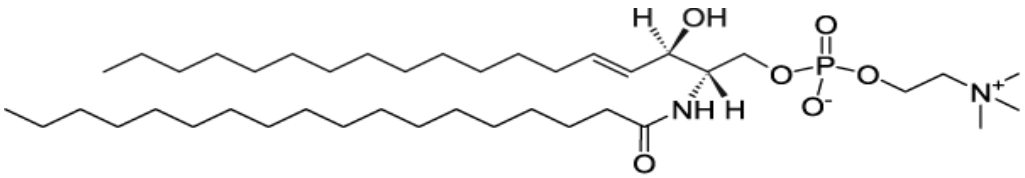

Figure 2.4 : Chemical structure of Sphingomyelin

2.2. Preparation of Phosphate Buffered Saline buffer

Phosphate buffered saline tablets were dissolved in double distilled water and dissolved. Then the pH of the solution was adjusted to 7.4 after dissolving.

2.3. Preperation of Agomelatine Stock Solution

Agomelatine stock solution was prepared by dissolving 2,5 mg agomelatine powder with a molecular weight of 243.30098 g/mol in 1 mL pure ethanol in a glass tube.

2.4. Preparation of MLVs

The samples were prepared according to the procedure reported in (Severcan et al, 2005).

Properties of lipids may alter depending on their structure nevertheless, the same preparation method can be used for all lipid vehicles. Common approach implies two main steps, first step is hydration and the second step is hydration with agitation. Desired amount of DPPC, DPPG, SM individually was dissolved in 150 µl chloroform to obtain a clear lipid solution for complete interference with drug. Then, the solution was subjected to a stream of nitrogen to remove excess chloroform

followed by the vacuum drying for 2 hours. Subsequently, a dry film was obtained. Thin films of lipid were hydrated by adding desired amount of phosphate buffer, (pH 7.4). Multilamellar vesicles (MLVs) were formed by vortexing the mixture for 20 minutes at least 15°C temperature above transition temperature of lipids. MLVs are formed when thin lipid films are hydrated and stacks of liquid crystalline bilayers become fluid and swell. The hydrated lipid sheets detach during agitation and self close in order to form MLVs as seen in Figure 2.5. To prepare agomelatine containing MLVs, appropriate amount of agomelatine from stock solution was initially placed inside the sample tube. Excess ethanol was removed by stream of nitrogen, phospholipid in chloroform was added and the next steps were followed for the preparation of MLVs as described above (Figure 2.6).

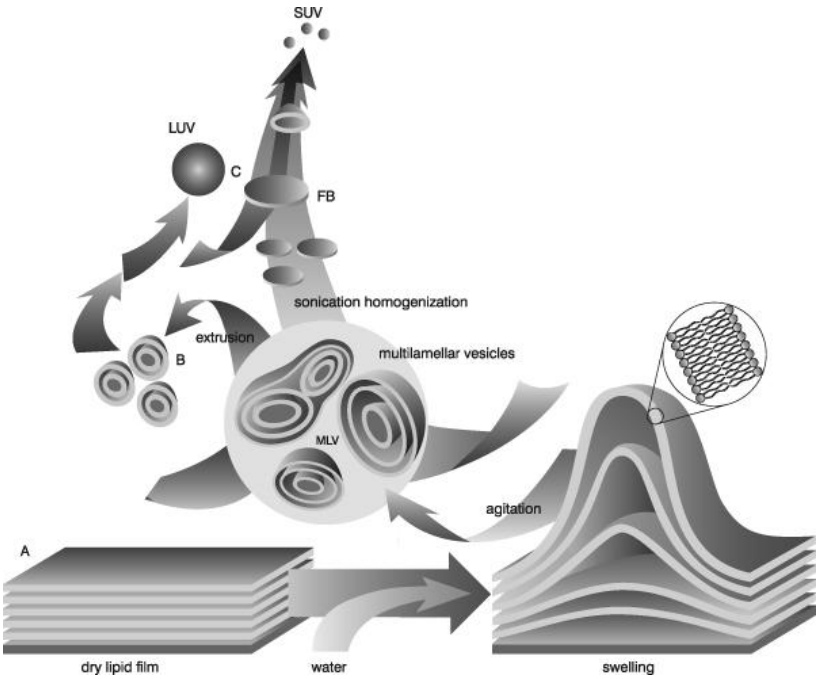

Figure 2.5: Mechanism of Vesicle Formation

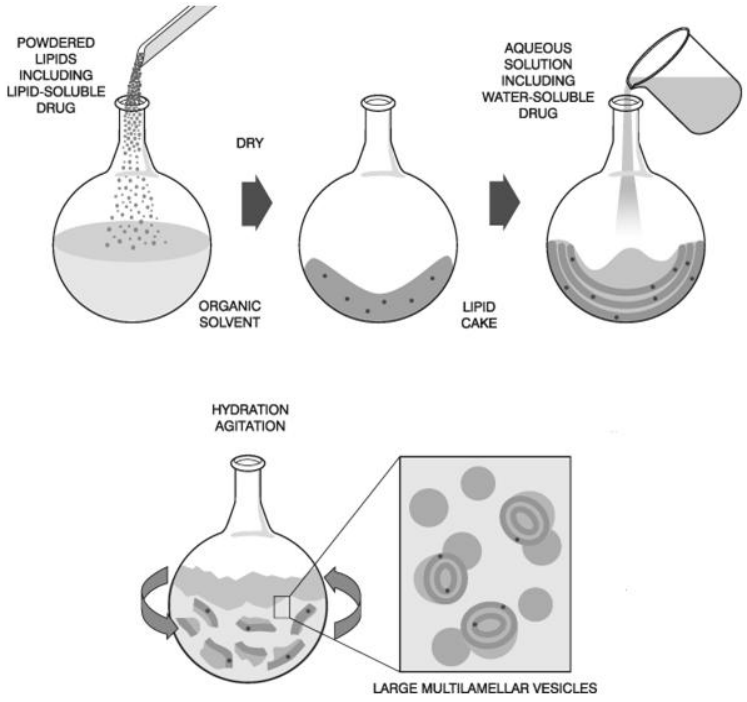

Figure 2.6: Preparation of Lipid for Hydration

2.5. Sample Preparation for FTIR Spectroscopic experiments

For FTIR spectroscopic measurements, 20 µl of liposomes were placed between CaF_2 windows with 12 µl spacer to obtain consistent sample thickness. Spectra were recorded using a Perkin Elmer Spectrum 100 FTIR Spectrometer (Perkin Elmer Inc., Norwalk, CT, USA) equipped with Deuterated Triglycine Sulfate (DTGS) detector, in the temperature range of 20-60 °C. Temperature was controlled digitally by Graseby Specac controller unit (Specac Ltd., Slough, England). Samples were incubated for 5 minutes at each temperature before acquisition of a spectrum. Interferograms were averaged for 100 scans at 2cm $^{-1}$ resolution.

Molecules in the air affect the spectra of samples. To overcome this, spectrum of the air was recorded as a background spectrum and subtracted automatically from the spectra of samples by using appropriate software. The spectrum of air can be seen in Figure 2.7.

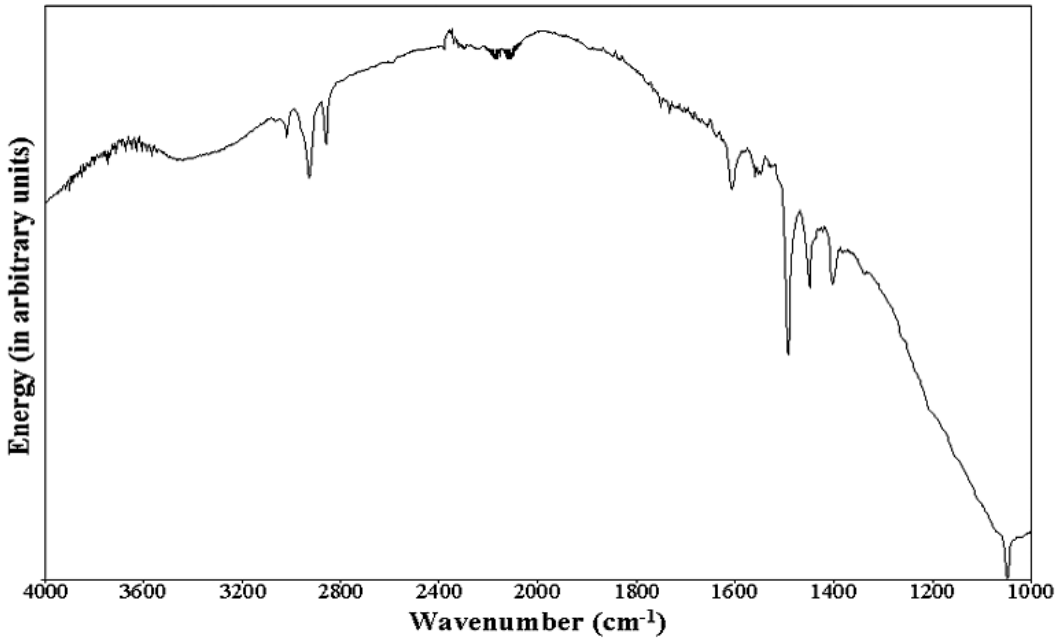

Figure 2.7 : FTIR Spectrum of air

Since the OH stretching bands due to presence of water in buffer appear in the regions of 3400-3200 cm^{-1} and 1800-1500 cm^{-1}, these bands overlap with the other bands of interest which will be analyzed. To improve resolution of the infrared bands, buffer spectra were subtracted from sample spectra at corresponding temperatures. Subtraction process was done by using Perkin Elmer software program by flattening the free water band located around 2300 cm^{-1}. Figures 2.8, 9 and 10 demonstrate the infrared spectra of DPPC,DPPG and SM, respectively, before and after water subtraction.

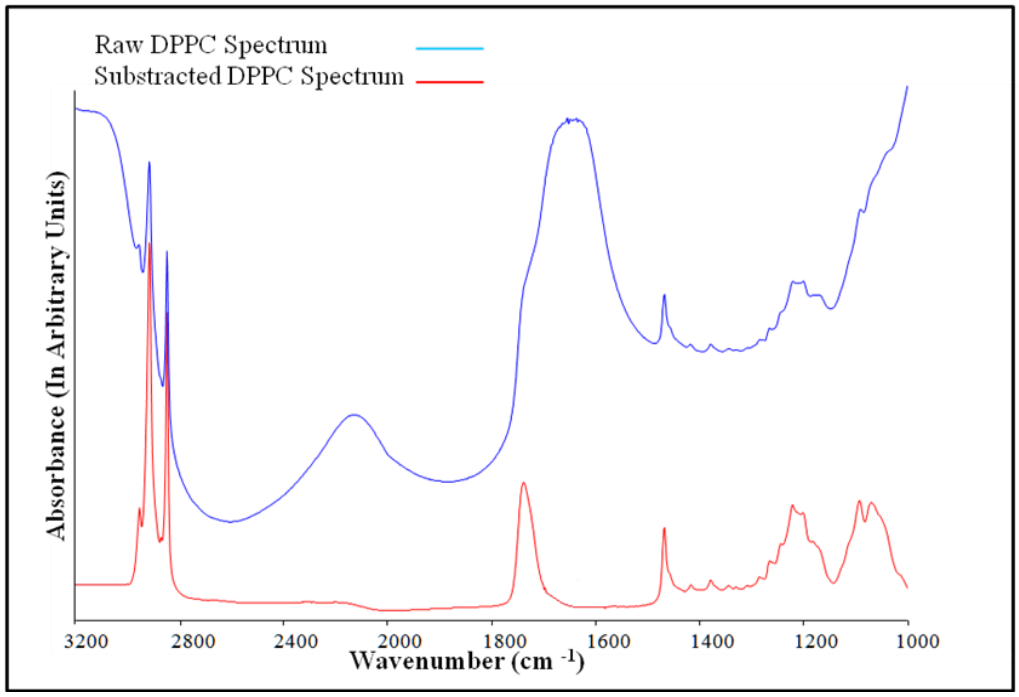

Figure 2.8 : FTIR Spectrum of DPPC liposomes. Blue spectrum represent the raw spectrum of DPPC liposomes before buffer substraction and the red spectrum represents the spectrum of DPPC liposomes after buffer substraction.

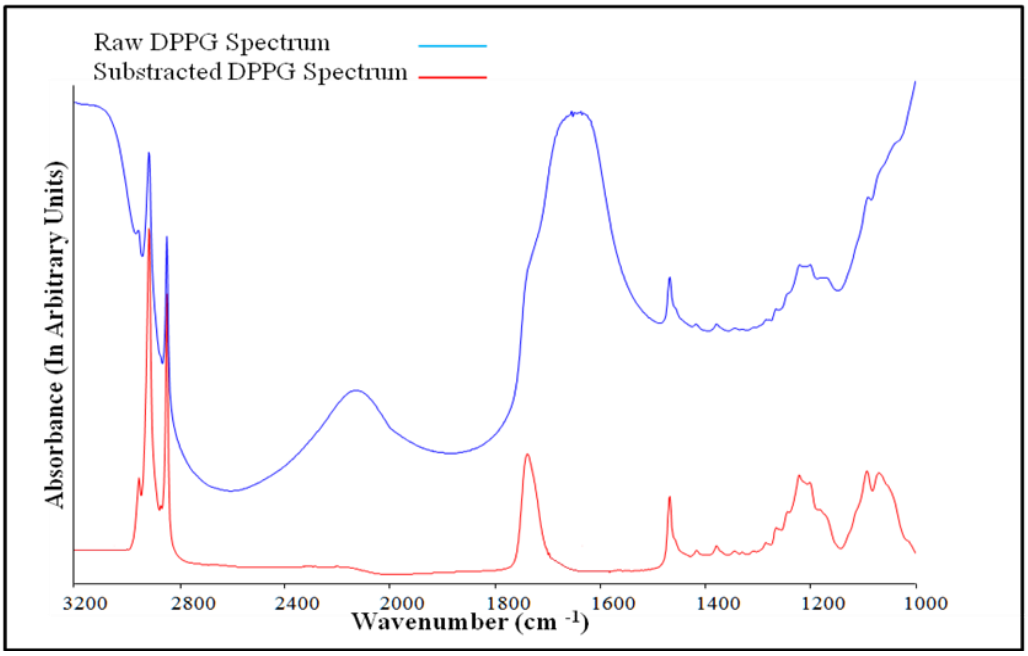

Figure 2.9 : FTIR Spectrum of DPPG liposomes. Blue spectrum represent the raw spectrum of DPPG liposomes before buffer substraction and the red spectrum represents the spectrum of DPPG liposomes after buffer substraction.

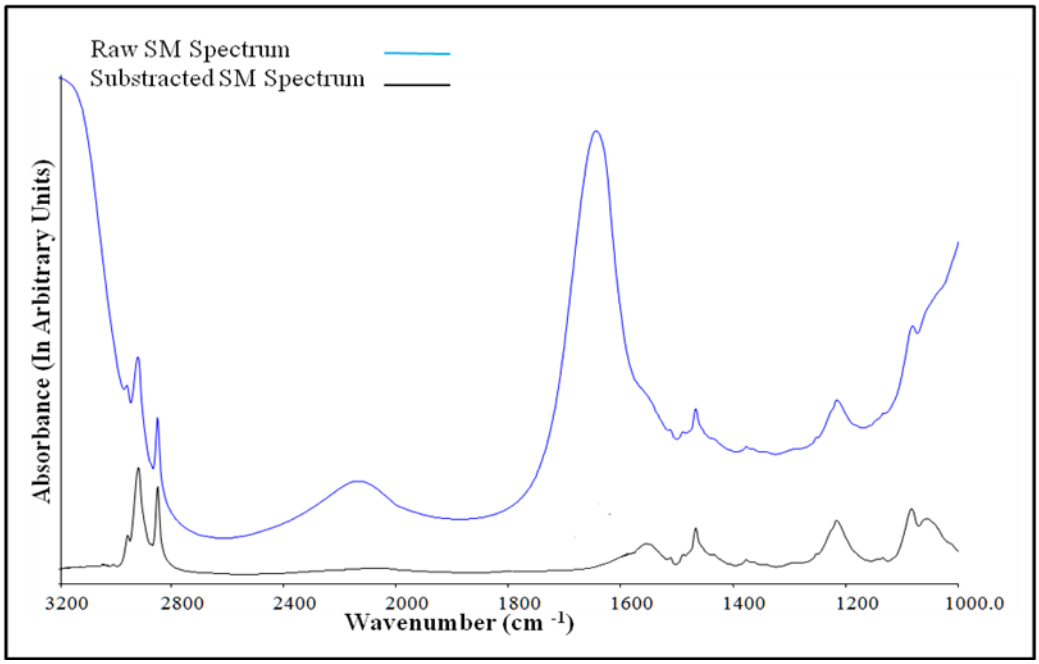

Figure 2.10 : FTIR Spectrum of SM liposomes. Blue spectrum represent the raw spectrum of SM liposomes before buffer substraction and the black spectrum represents the spectrum of SM liposomes after buffer substraction.

For the determination of the variations in peak positions and bandwidths, each original spectrum was analyzed by using the same software. The band positions were measured from the center of weight. The same software was also used for other spectral analyses including normalization. The spectra were normalized with respect to specific bands just for visualization of the spectral differences in the spectra.

2.6. Sample Preparation for Differential Scanning Calorimetry Studies

For calorimetric studies, MLVs were prepared in the absence and the presence of 1, 3, 12, and 18 mol % agomelatine. For the preparation of MLVs thin films containing 2 mg DPPC, DPPG and SM were separetely hydrated by adding 50 µl of phosphate buffer, pH 7.4, and the same procedure mentioned in sample preparation for FTIR spectroscopic experiments section was followed for DSC studies. 50 µl MLVs suspensions were encapsulated in helmitically sealed standard aluminum DSC pans. Indium containing pan was used for reference during analysis.

2.7. Thermogram Analysis for DSC Studies

DSC thermograms were collected using Universal TA DSC Q100 v 6.21 instrument. The scans were performed at 10 °C/min for SM and 1° C/min for DPPC and DPPG. Only heating curves are presented. Cooling curves were essentially identical. Figure 2.11, 12, 13 demonstrate the DSC thermograms of DPPC, DPPG and SM, respectively.

The cooperativity and enthalpy values were calculated by the equations written below (Turker et al, 2011) ;

$$\text{Cooperativity unit} = \Delta H^{o}_{vH} / \Delta H^{o}\ \text{cal}$$

ΔH^{o} cal : area under main transition

ΔH^{o}_{vH} : van`t Hoff enthalpy

$$\Delta H^{o}_{vH} = 6.9\ Tm^{2} / [(T_2 - T_1) / 2]$$

Tm: center point of phase transition (^{o}K)

T_1 ; start temperature for phase transition (^{o}K)

T_2 : end temperature for phase transition (^{o}K)

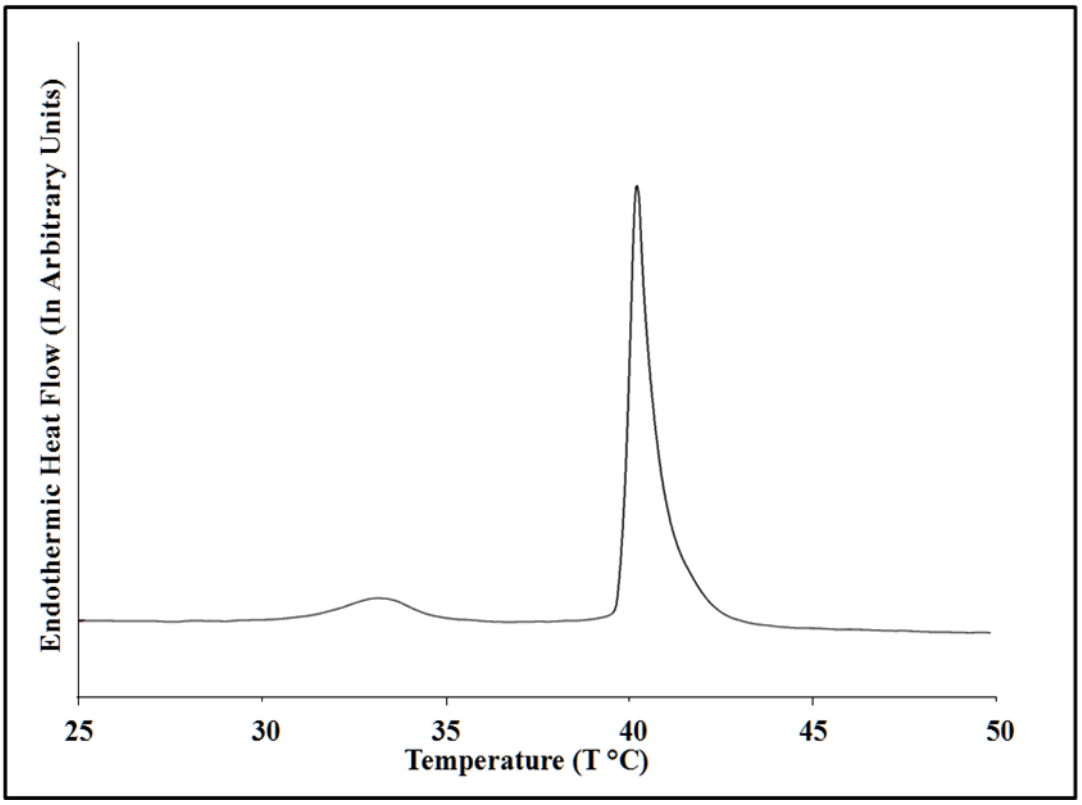

Figure 2.11 : Phase Transition Thermogram of DPPC

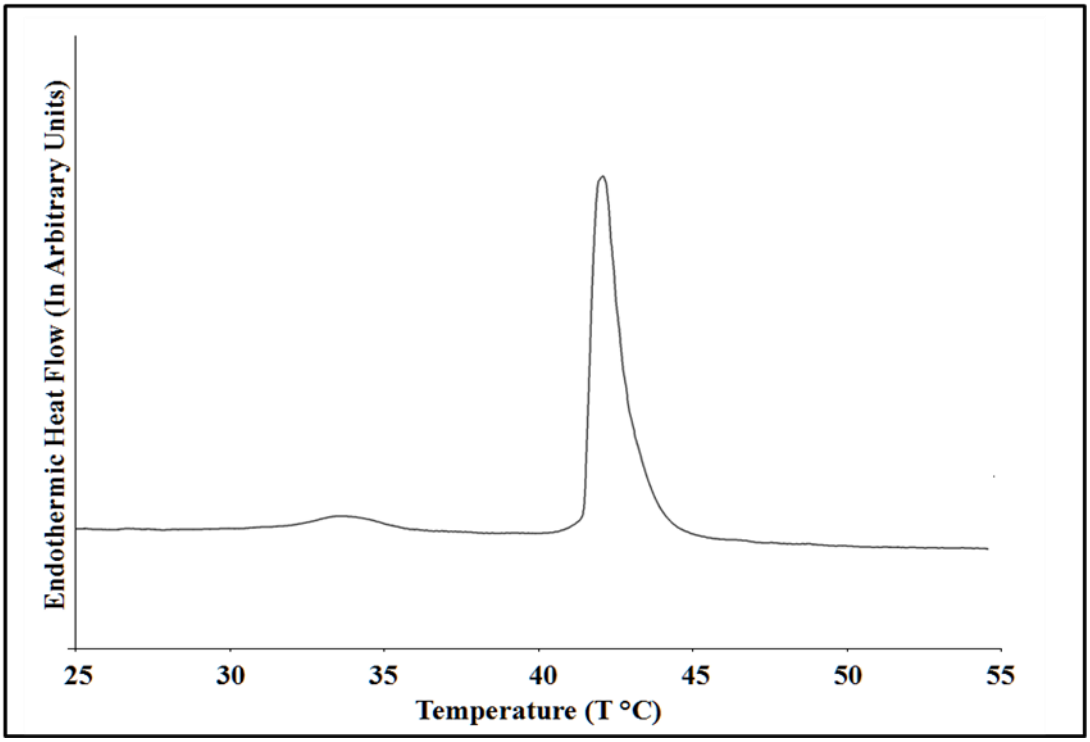

Figure 2.12 : Phase Transition Thermogram of DPPG

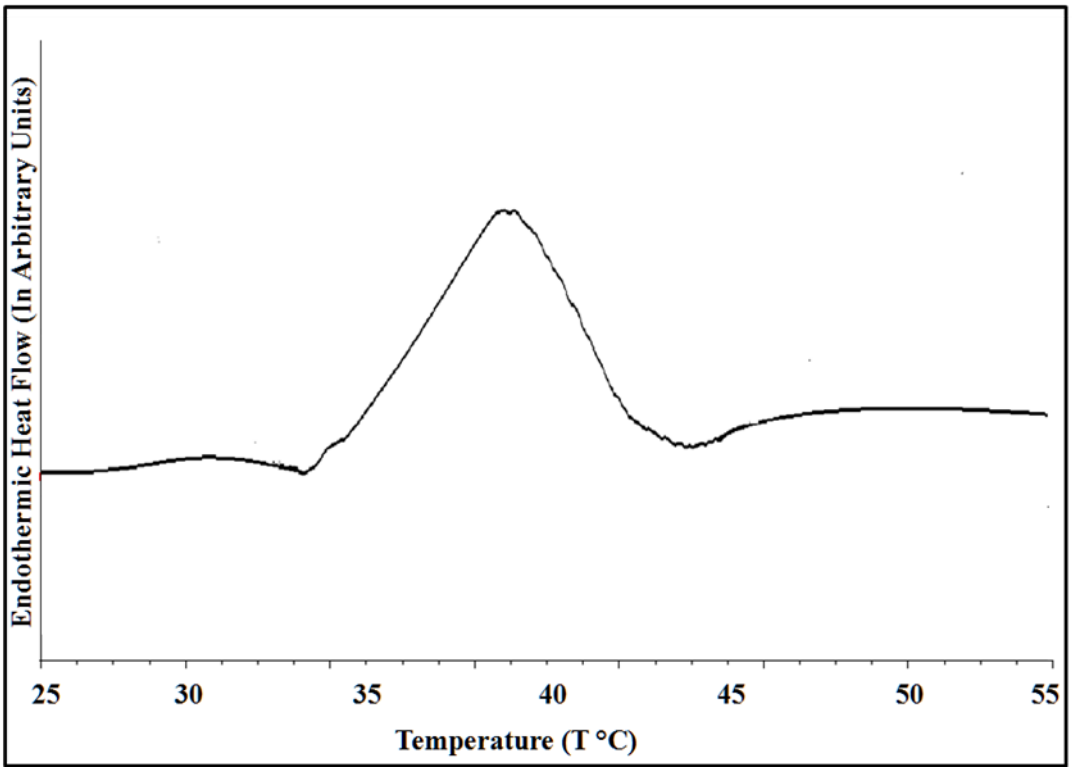

Figure 2.13 : Phase Transition Thermogram of SM

CHAPTER 3

RESULTS

In this thesis study, the effects of agomelatine on DPPC, DPPG and SM liposomes were investigated. For this purpose, 1 mole % and 3 mole % were used as low doses and 12 mole % and 18 mole % were used as high doses of agomelatine. FTIR spectroscopy and Differential Scanning Calorimetry were used to investigate the interaction of agomelatine with lipid MLVs with respect to increasing temperature.

3.1. The effects of agomelatine on DPPC model membrane

3.1.1. FTIR spectroscopic studies

As previously shown in materials and methods FTIR spectrum of lipid membranes has complex structure containing several bands. The band assignment is given in Table 3.1.

Table 3.1. Band assignments of some characteristic infrared absorbtion bands according to their wavenumbers in cm^{-1} (Stuart, 2004).

Wavenumber (cm^{-1})	Assignment	Wavenumber (cm^{-1})	Assignment
3010	=C-H stretching	1085	PO_2^- symmetric stretching
2956	CH_3 asymmetric stretching	1070	CO-O-C symmetric stretching
2920	CH_2 antisymmetric stretching	1047	C-O-P stretching
2870	CH_3 symmetric stretching	972	$(CH_3)_3N^+$ asymmetric stretching
2850	CH_2 symmetric stretching	820	P-O asymmetric stretching
1730	C=O stretching	730,720,718	CH_2 rocking
1485	$(CH_3)_3N^+$ asymmetric bending	1350-1250	O-H bending
1473,1472, 1468,1463	CH_2 scissoring	1560	CO_2^- asymmetric stretching
1460	CH_3 asymmetric bending	1415	CO_2^- symmetric stretching
1405	$(CH_3)_3N^+$ symmetric bending	1615	NH_2 bending
1378	CH_3 symmetric bending	1640-1610, 1550-1485	NH_3^+ bending
1400-1200	CH_2 wagging band progression	1160,1100	NH_3^+ rocking
1228	PO_2^- antisymmetric stretching	1602,1450,760 700	Benzene ring vibrations
1170	CO-O-C asymmetric stretching	1600,1450	Benzene ring vibrations

In the analysis of FTIR spectra, the CH_2 antisymmetric stretching (~2920 cm^{-1}), the CH_2 symmetric stretching (~2850 cm^{-1}), the C=O stretching (~1735 cm^{-1}) and the PO^{2-} antisymmetric stretching (~1080 cm^{-1}) bands were taken into consideration. The alterations at the frequency values of the CH_2 antisymmetric and symmetric stretching vibrations provide information about the membrane phase transition

behavior and membrane order (Korkmaz et al, 2005). The band width of these bands give membrane fluidity information [ref]. Figure 3.1. shows the FTIR spectra of pure DPPC liposomes and DPPC liposomes containing 1mol %, 3 mol %, 12 mol % and 18 mol % agomelatine, recorded at 20˚C. As seen from the figure the frequency value shifts and the changes at the bandwidth of these bands can be observed following the addition of agomelatine.

To obtain further detailed information about C-H stretching bands maximum peak position and bandwidth values were calculated regarding to gel and liquid crystalline phases of the membrane. The order information obtained from the frequency shiftis also used to monitor the average trans/gauche isomerisation of the acyl chains of phospholipids.

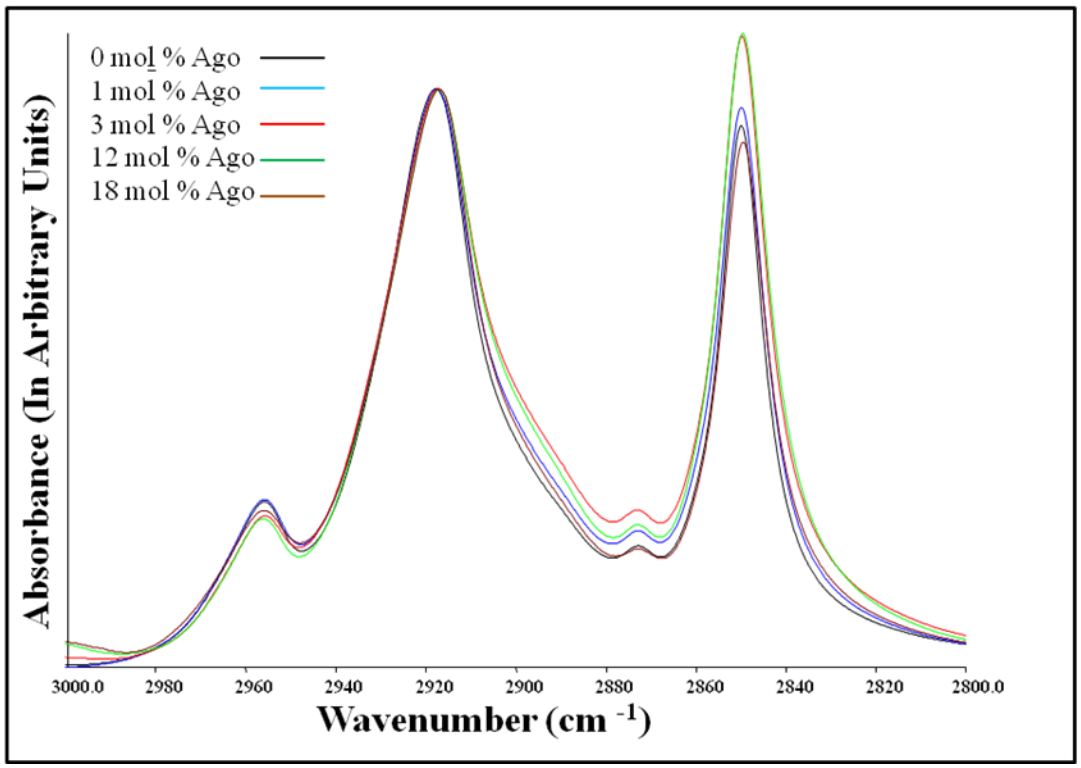

Figure 3.1.: FTIR spectra of DPPC liposomes containing agomelatine of 0 mol% (black), 1 mol% (blue), 3 mol% (red), 12% mol (green), 18% mol (brown) in CH stretching region at 20 ° C (Spectra were normalized with respect to CH_2 asymmetric stretching band)

Figure 3.2 and 3.3 show the temperature dependent frequency changes of the CH_2 antisymmetric and symmetric stretching bands of DPPC liposomes containing diverse concentrations of agomelatine (1-18 mole %). The abrupt frequency change seen around ~ 41 °C in the spectrum of pure DPPC in Figure 3. 2 indicates the transition from gel phase to liquid crystalline phase, and the main phase transition temperature (Tm) value. This observation reveals the change from trans conformations to gauche conformations in the membrane composed of pure DPPC and the Tm of the pure DPPC is around ~ 42 °C (Schultz & Levin, 2011). As it can be inferred from the Figure 3.2. and 3.3., agomelatine decreases the phase transition temperature of DPPC liposomes to the lower degrees. When we take the temperature dependent change in the frequency values of the CH_2 symmetric band into account, we can see that addition of agomelatine results in a shift of the frequency values to lower values in both phases (Figure 3.3). This shift to the lower values indicates the increase in the trans conformers i.e. in order (Toyran & Severcan, 2003) (Schultz & Levin, 2011). The same trend can be inferred from the frequency values of the CH_2 antisymmetric stretching band (Figure 3. 3).

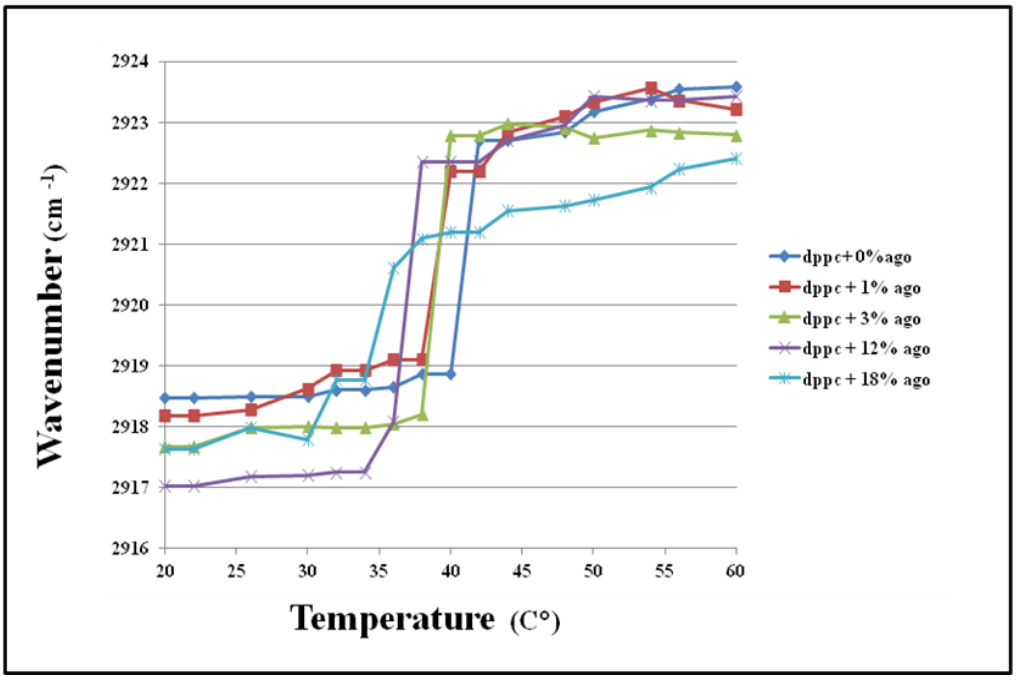

Figure 3.2.: Temperature dependent frequency changes of CH_2 asymmetric stretching mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing DPPC liposomes.

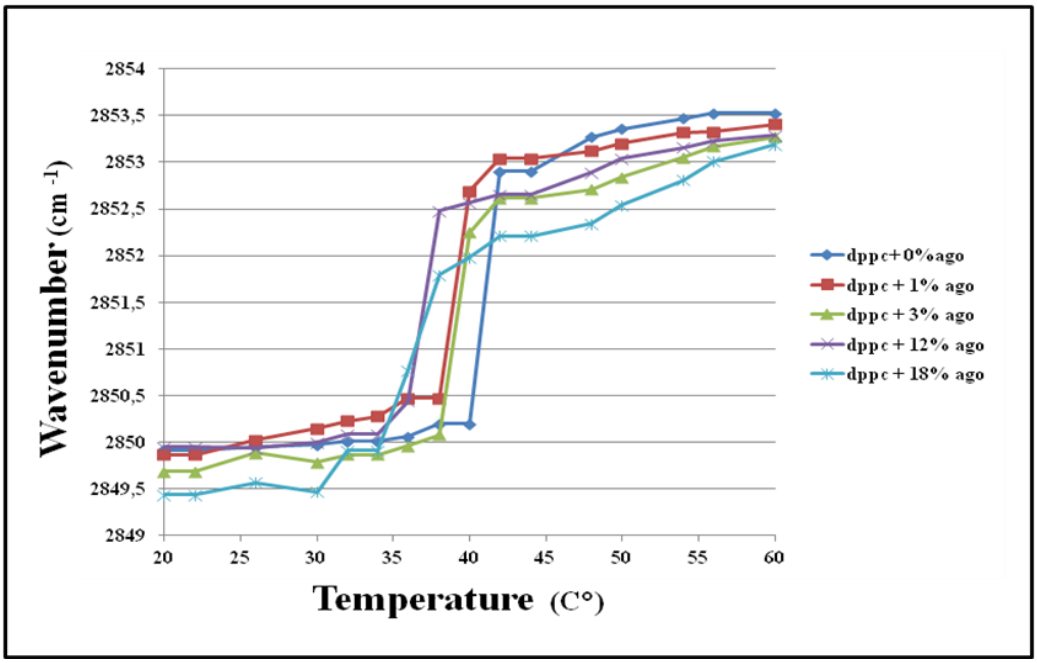

Figure 3.3.: Temperature dependent frequency changes of CH_2 symmetric stretching mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing DPPC liposomes.

Figure .3.4. and 3.5. indicate the temperature dependent bandwidth changes of the CH_2 antisymmetric and symmetric bands of DPPC liposomes in the absence and presence of agomelatine. The alteration in bandwitdth of the C-H stretching bands reflects the changes in the dynamics of the system (Turker et al., 2011). When bandwidth increases it indicates an increase at the dynamics of the system or vice versa. As seen in the figure, both in the gel and liquid crystalline phase, agomelatine increases the bandwidth of DPPC liposomes..

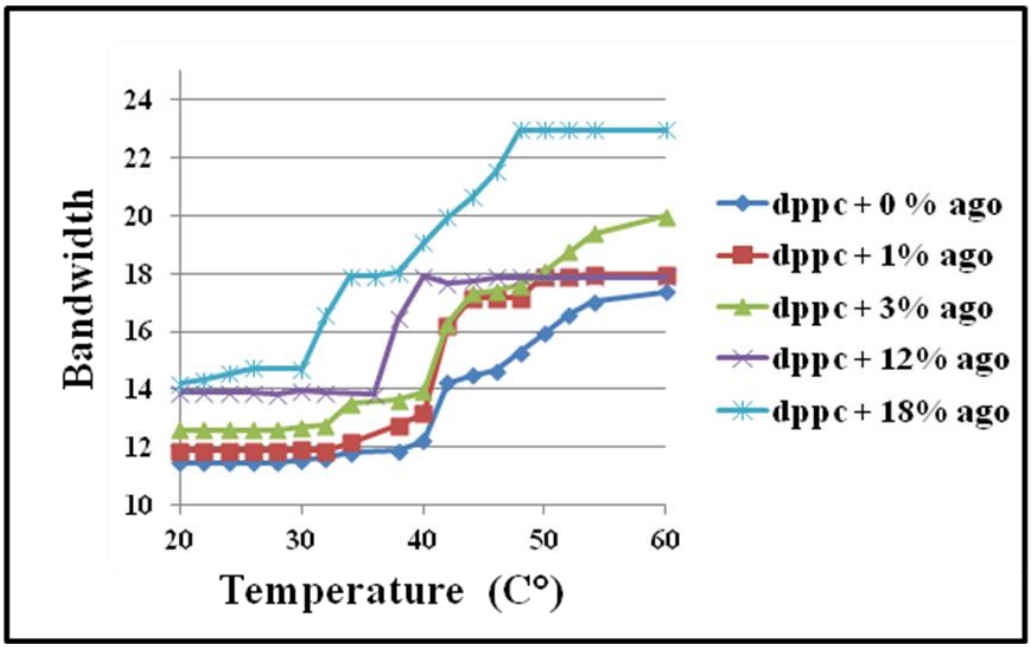

Figure 3.4. : Temperature dependent bandwidth changes of CH_2 asymmetric stretching mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing DPPC liposomes.

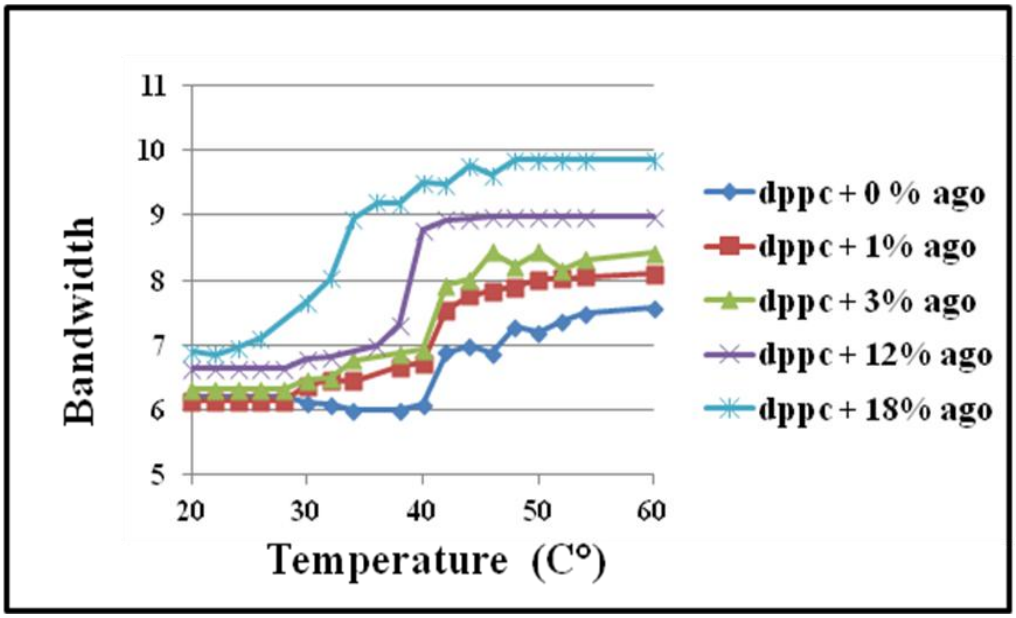

Figure 3.5.: Temperature dependent bandwidth changes of CH_2 symmetric stretching mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing DPPC liposomes.

To obtain information about the interaction of agomelatine and glycerol groups of phospholipids, the frequency values of the C=O band was analyzed. Figure 3.6 shows the 1770–1000 cm^{-1} region of FTIR spectrum of DPPC liposomes in the absence and presence of agomelatine. As seen in the figure, the C=O stretching band is located at 1740 cm^{-1}. This band monitors the glycerol molecules, which functions as a bridge in between the phosphate head and the acly chains of the phospholipids. Figure 3.7 shows the temperature dependent frequency change of the C=O stretching band of DPPC liposomes in the absence and presence of agomelatine. As seen from the figure, at low agomelatine concentrations the increase in the frequency values indicates a decrease in the hydrogen bonding capacity of the glycerol skeleton of phospholipids. In contrast, at high concentrations of agomelatine, a decrease in the frequency values was observed as an indicator of the enhancement of the hydrogen bonding capacity. As a result new H bonds were formed (Toyran et al., 2003).

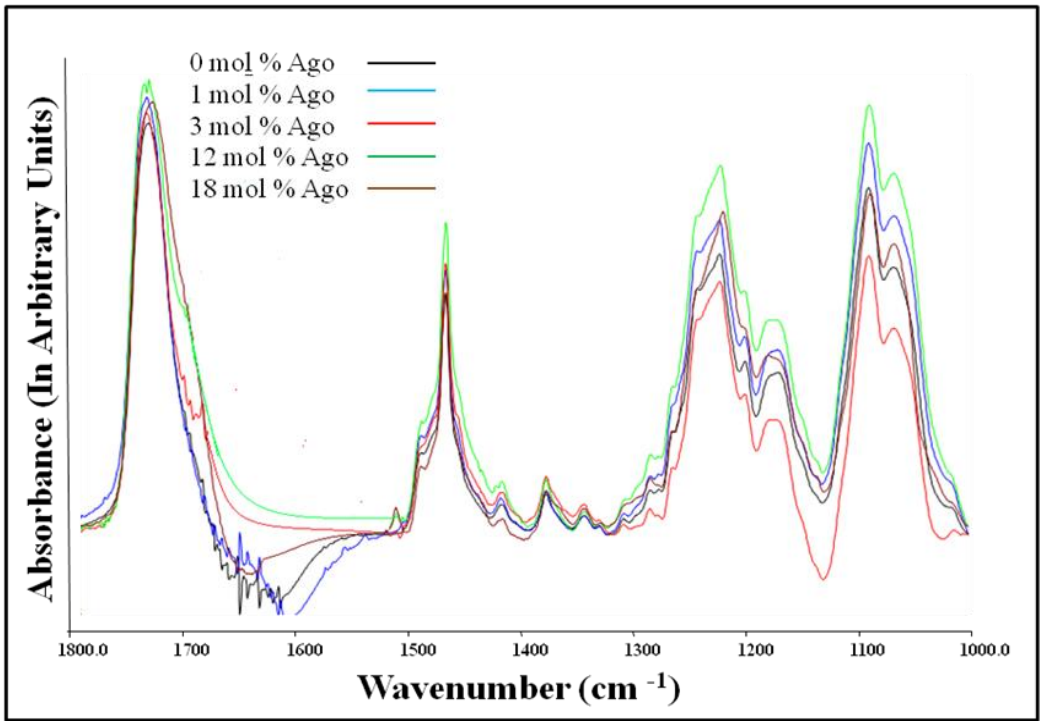

Figure 3.6. : FTIR spectra of DPPC liposomes containing agomelatine of 0 mol% (black), 1 mol% (blue), 3 mol% (red), 12% mol (green), 18% mol (brown) in fingerprint region (1800-1000 cm^{-1}) at 20 ° C (Spectra were normalized with respect to CH_2 asymmetric stretching band)

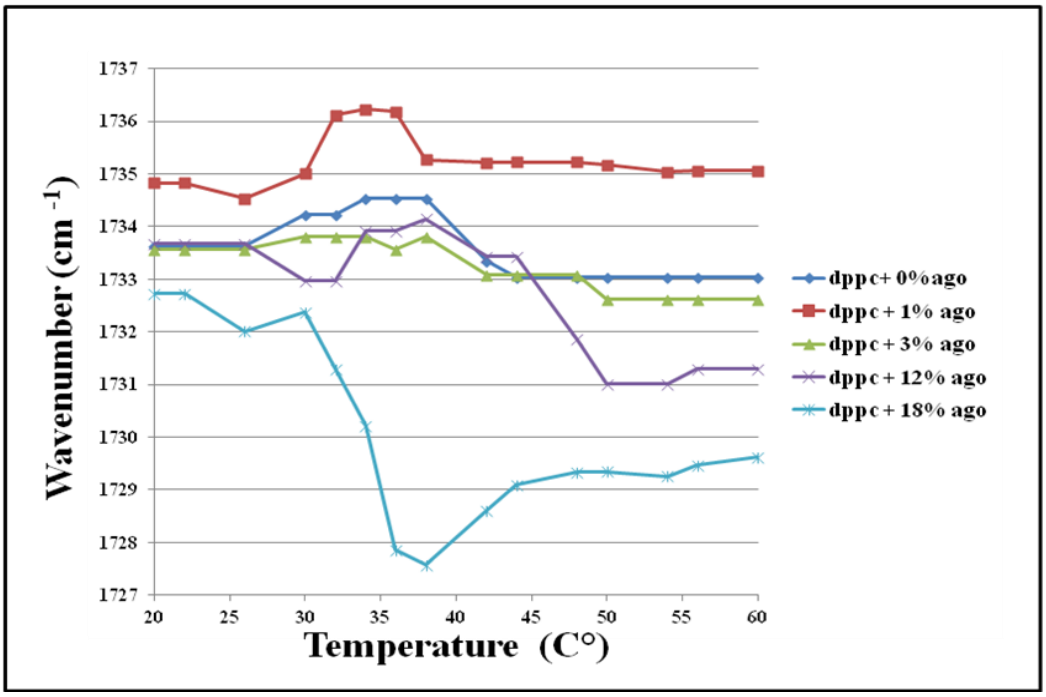

Figure 3.7.: Temperature dependent frequency changes of C=O stretching band mode for mol% 0 **(⧫)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*) agomelatine containing DPPC liposomes.

Figure 3.8 and 3.9. represent the changes in the PO^{2-} asymmetric and symmetric band frequencies with repect to temperature, respectively. This band gives information about the head groups of the phospholipids. The shift in the frequency to the lower degrees of this band reveals that the H-bonding is enhanced in head groups of phospholipids (Goni & Arrondo, 1986) (Toyran et al., 2003). There may be bonds formed with water molecules or with agomelatine. These figures show that the addition of high doses of agomelatine causes a decrease in the frequency values, so agomelatine increases H bonding. At low agomelatine concentration (1 mole %) opposite effect is observed.

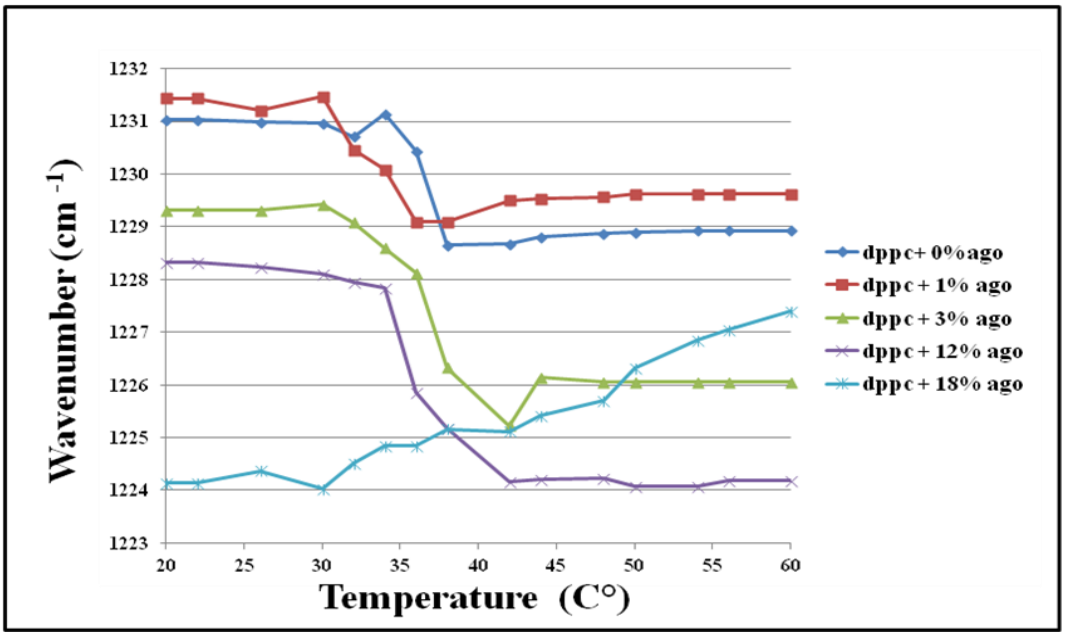

Figure 3.8: Temperature dependent frequency changes of PO^{2-} asymmetric band mode for mol% 0 **(⧫)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*) agomelatine containing DPPC liposomes.

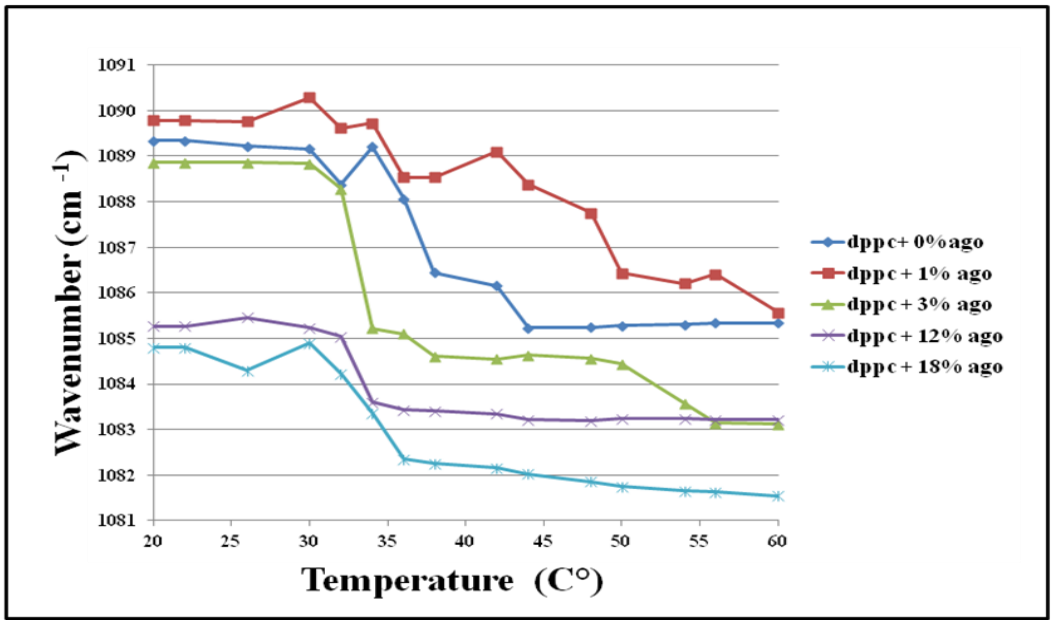

Figure 3.9: Temperature dependent frequency changes of PO^{2-} asymmetric band mode for mol% 0 **(⧫)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*) agomelatine containing DPPC liposomes.

3.1.2. DSC studies

Figure 3.10. shows the DSC thermogram of pure and agomelatine containing (1-18 mole %) liposososmes, and Table 1 shows the phase transition temperatures (Tm), bandwidth and enthalpy changes of DPPC liposomes. The small band around ~ 34 ° C of pure DPPC thermogram is the pre-transition temperature and the great band around 40.84 ° C is the main phase transition temperature. As shown in the figure, with the addition of agomelatine the pre-transition band is disappeared. Furthermore, Figure 3. 11 and Table 3.2. reveal that agomelatine causes an increase in the bandwidth of DSC thermogram of DPPC liposomes.

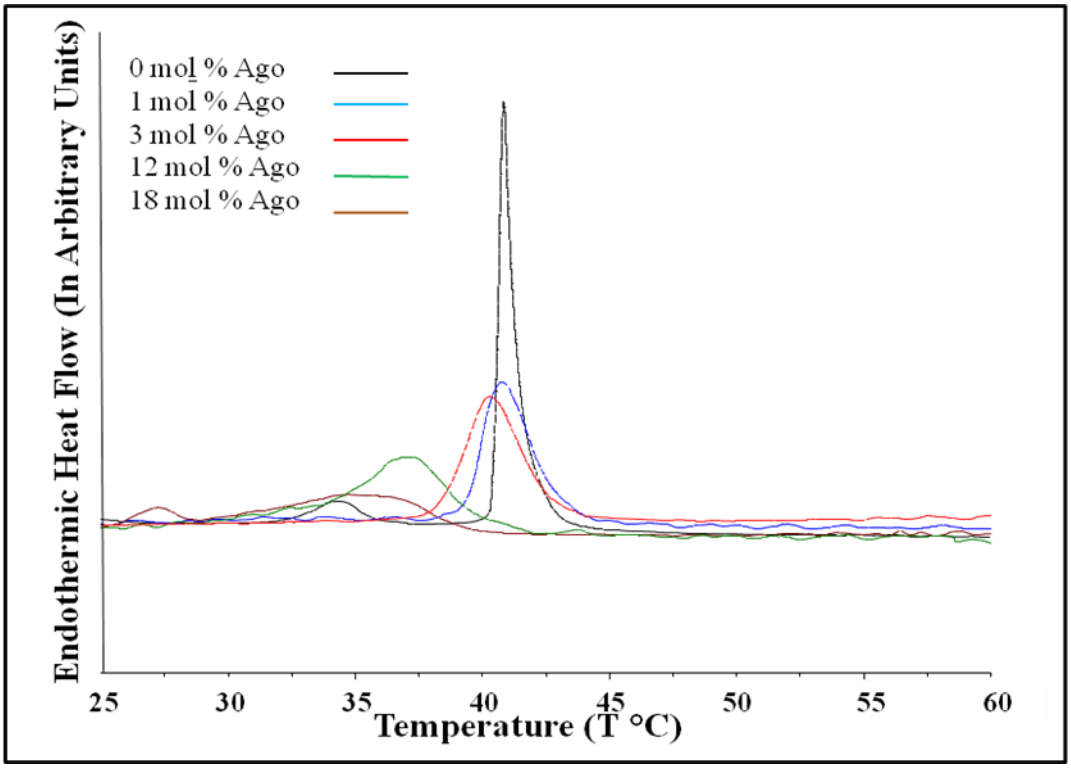

Figure 3.10: DSC thermogram of DPPC liposomes with and without different concentrations of agomelatine.

With the addition of agomelarine, the phase transition temperature shifted to lower values. This increase in bandwidth and the decrease in phase transition temperature, indicate a change in liposome size and packaging; thus, the system becomes more

disordered (Korkmaz & Severcan, 2005). When the transition enthalpy changes of DPPC samples were compared, it was found that the enthalpy change of transition was increased in all agomelatine containing liposomes, except the 18 mole %.

Table 3.2: Transition temperatures (Tm), width at half height (ΔT) and enthalpy of the transitions (ΔH) for DPPC liposomes are given.

Sample	**T_m (^oC)**	**ΔT (^oC)**	**Δ H (cal/g)**
DPPC	41.84	0.62	6.874
DPPC + 1 mole %ago.	40.77	2.14	6.605
DPPC + 3 mole % ago.	40.30	2.52	7.106
DPPC + 12 mole % ago.	37.14	4.14	8.089
DPPC + 18 mole % ago.	34.72	11.25	5.978

3.2. The effects of agomelatine on DPPG model membrane

3.2.1. FTIR spectroscopic studies

This part of the study the interaction of agomelatine and DPPG liposomes were investigated. As mentioned before the changes of the frequency values of CH_2 antisymmetric and symmetric stretching bands provides information about the membrane Tm and order. Figure 3.11. shows the FTIR spectra of pure DPPG liposomes and the DPPG liposomes containing 1 mol%, 3 mol%, 12 mol% and 18 mol% agomelatine in the 3000-2800 cm^{-1} region.

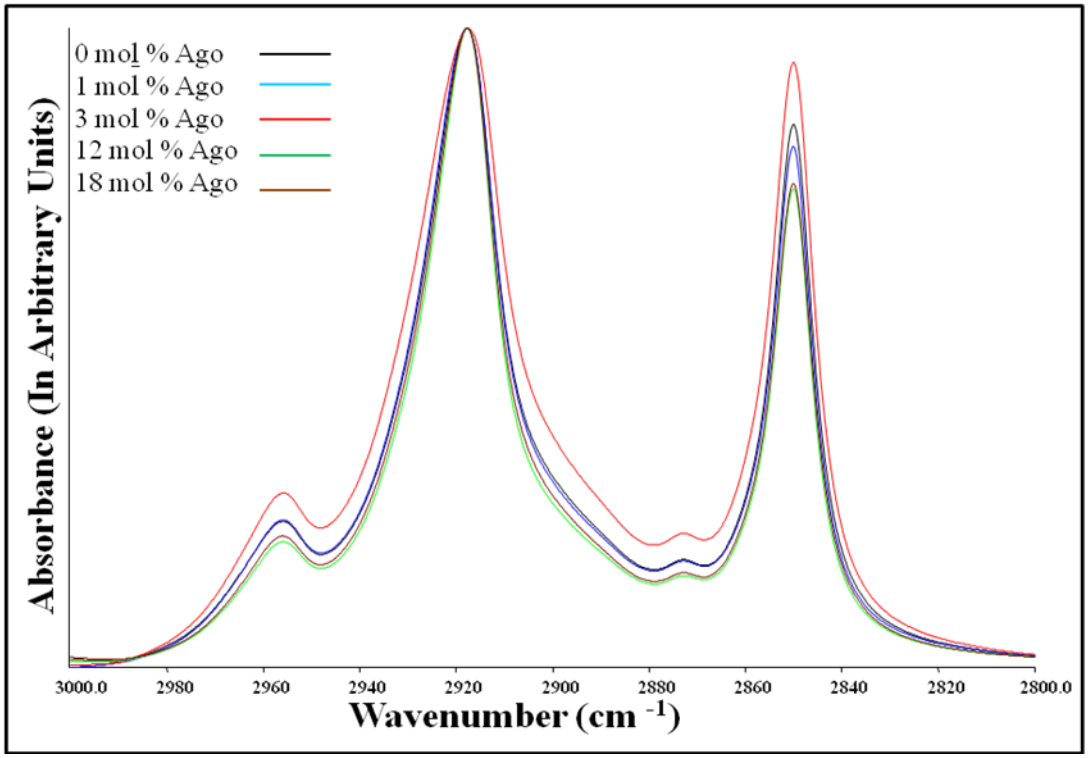

Figure 3.11: FTIR spectra of DPPG liposomes containing agomelatine of 0 mol% (black), 1 mol% (blue), 3 mol% (red), 12% mol (green), 18% mol (brown) in CH stretching region at 20°C (Spectra were normalized with respect to CH2 asymmetric stretching band)

Figure 3.12 and Figure 3.13 represent the temperature dependent frequency changes of the CH_2 antisymmetric and symmetric stretching bands of DPPG liposomes containing different concentrations of agomelatine (1-18 mole %). As can be seen from the figures, agomelatine slightly increases the frequency value in both phases. This result reveals that agomelatine disorders the membranes in DPPG liposomes. Agomelatine also decreases the Tm (~ 41 °C) of DPPG liposomes, which is an indicator of the increased disorder in the system.

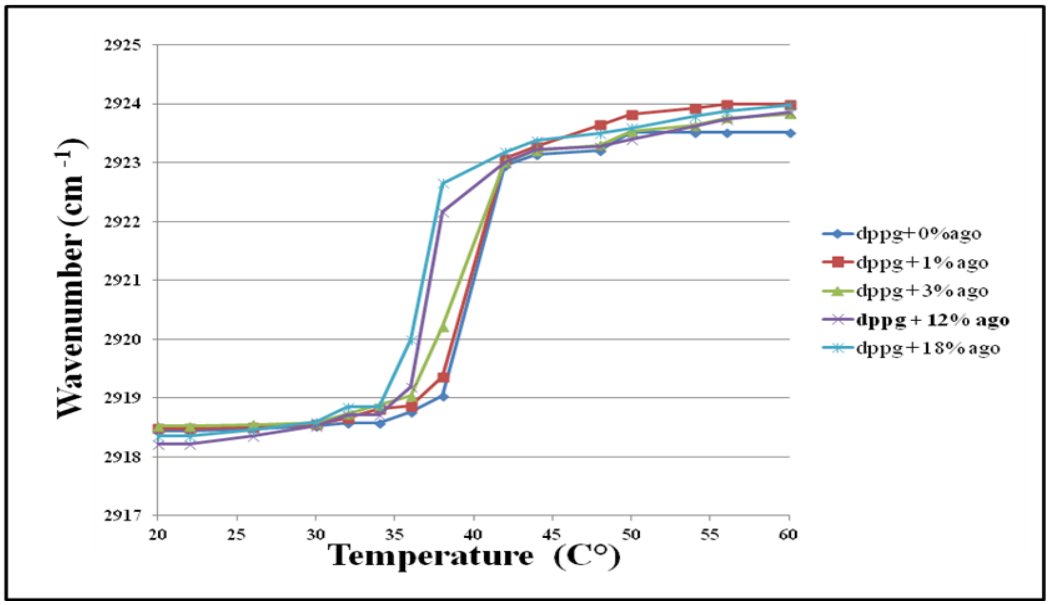

Figure 3.12: Temperature dependent frequency changes of CH_2 asymmetric stretching mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing DPPG liposomes.

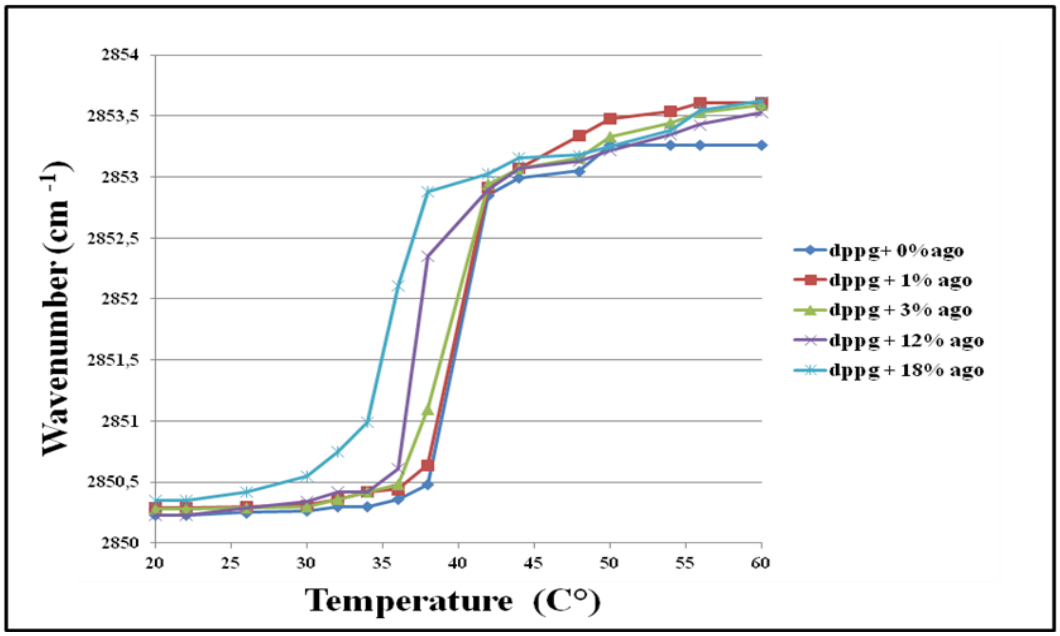

Figure 3.13: Temperature dependent frequency changes of CH_2 symmetric stretching mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing DPPG liposomes.

Moreover, agomelatine increases the bandwidth of both CH_2 antisymmetric and symmetric stretching bands. So DPPG-agomelatine interaction results with the increase of membrane dynamics both in gel and liquid crystalline phases. (Figure 3. 14 & 15)

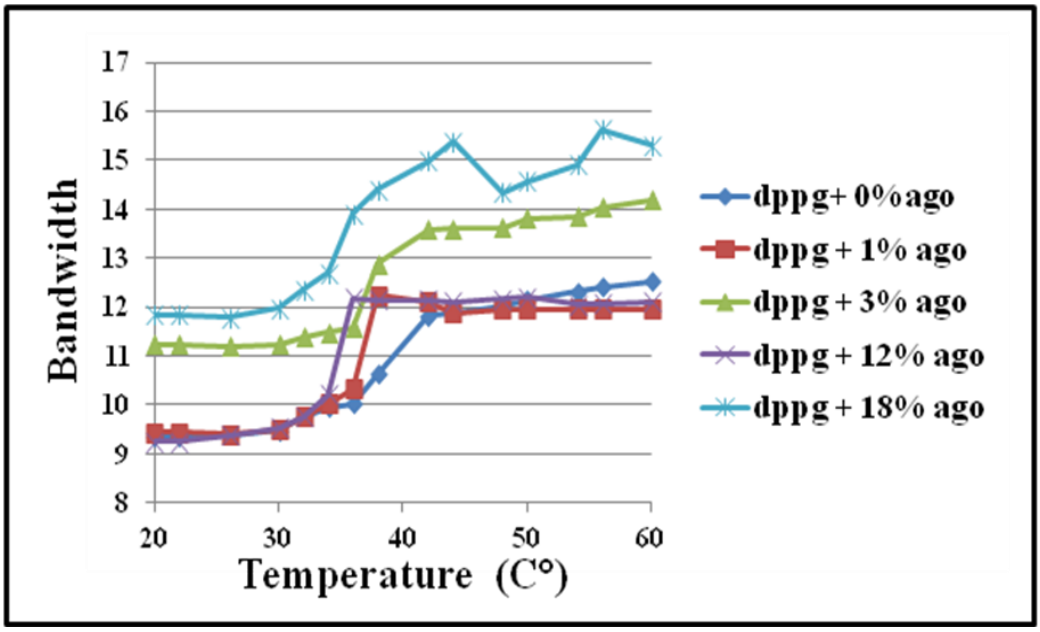

Figure 3.14: Temperature dependent bandwidth changes of CH_2 asymmetric stretching mode for mol% 0 (♦), 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing DPPG liposomes.

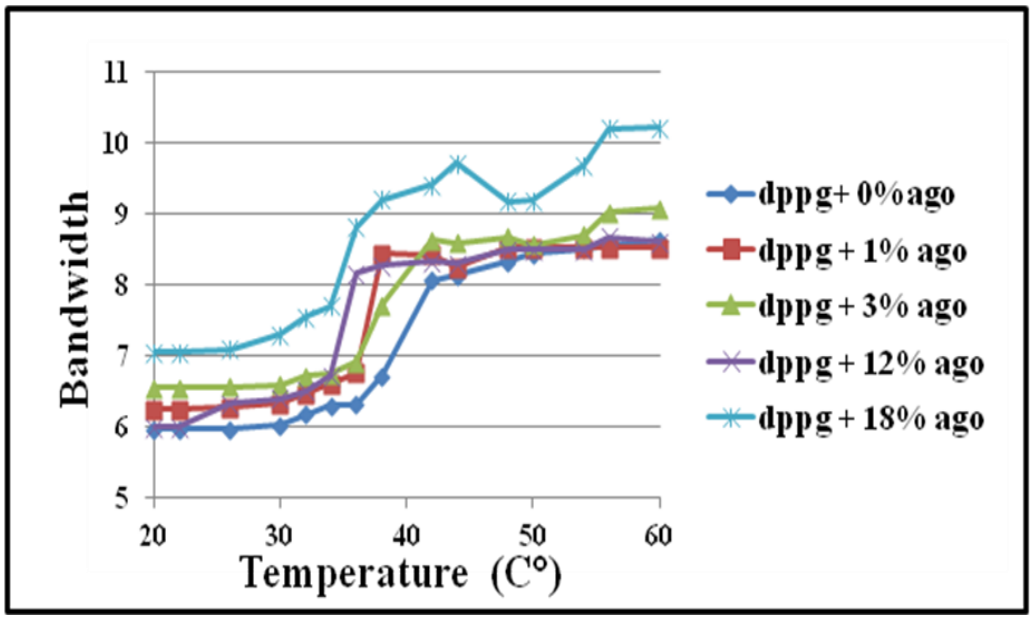

Figure 3.15: Temperature dependent bandwidth changes of CH_2 asymmetric stretching mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing DPPG liposomes.

Figure 3.16 shows the 1770–1000 cm^{-1} region of FTIR spectra of DPPG liposomes in the absence and presence of agomelatine. As seen from the figure, agomelatine increased the C=O stretching band frequency values at gel phases, however, at liquid chrystalline phases agomelatine shifts the frequency of the same band to the lower degrees at low doses (Figure 3.17). Consequently it can be said that agomelatine diminishes the H-bonding capacity of DPPG liposomes in the gel phase although it increases H bonding capacity at low doses, however H-bonding capacity decreases in the liquid crystalline phase of DPPG liposomes.

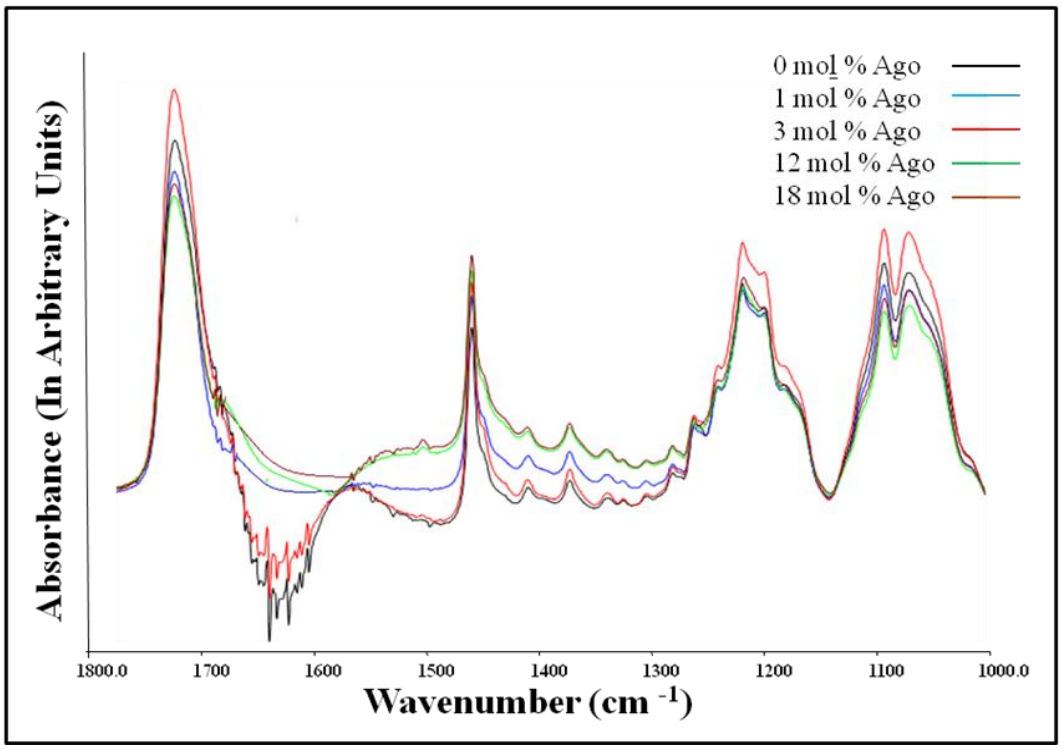

Figure 3.16: FTIR spectra of DPPG liposomes containing agomelatine of 0 mol% (black), 1 mol% (blue), 3 mol% (red), 12% mol (green), 18% mol (brown) in fingerprint region (1800-1000 cm^{-1}) at 20 ° C (Spectra were normalized with respect to CH_2 asymmetric stretching band)

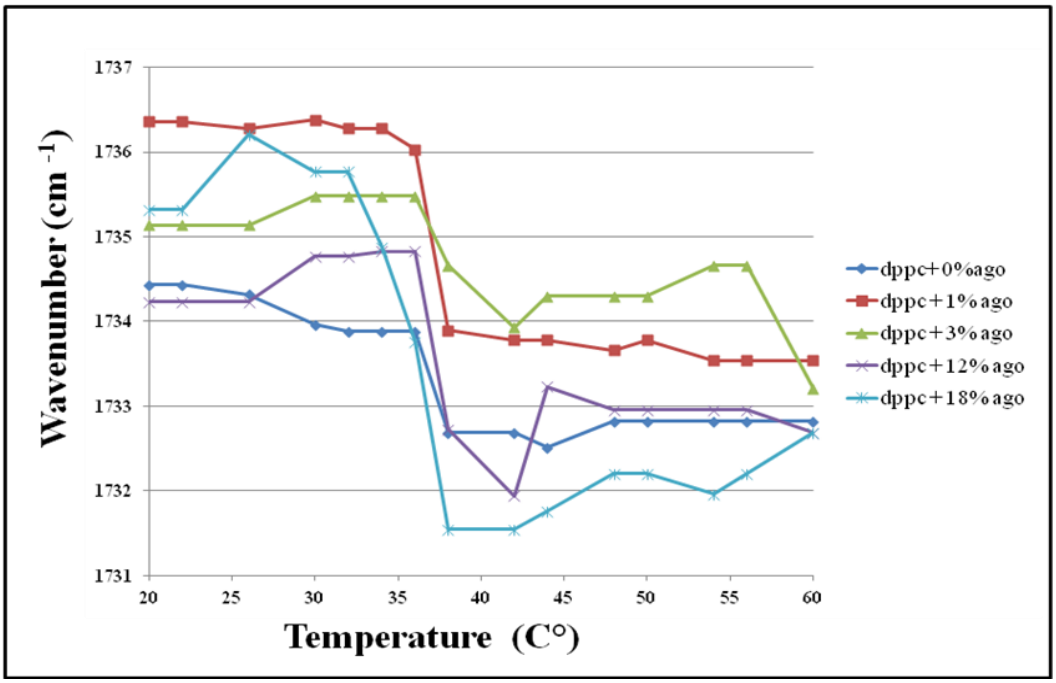

Figure 3.17: Temperature dependent frequency changes of C=O stretching band mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*) agomelatine containing DPPG liposomes.

In addition, agomelatine shifts the the PO^{2-} asymmetric and symmetric band frequencies to the lower values of gel phase but it increases the frequency values of the same bands at liquid crystalline phases (Figure 3.18 & 19). So it enhances H-bonding capacity at gel phase but lowers it at liquid chrystalline phases.

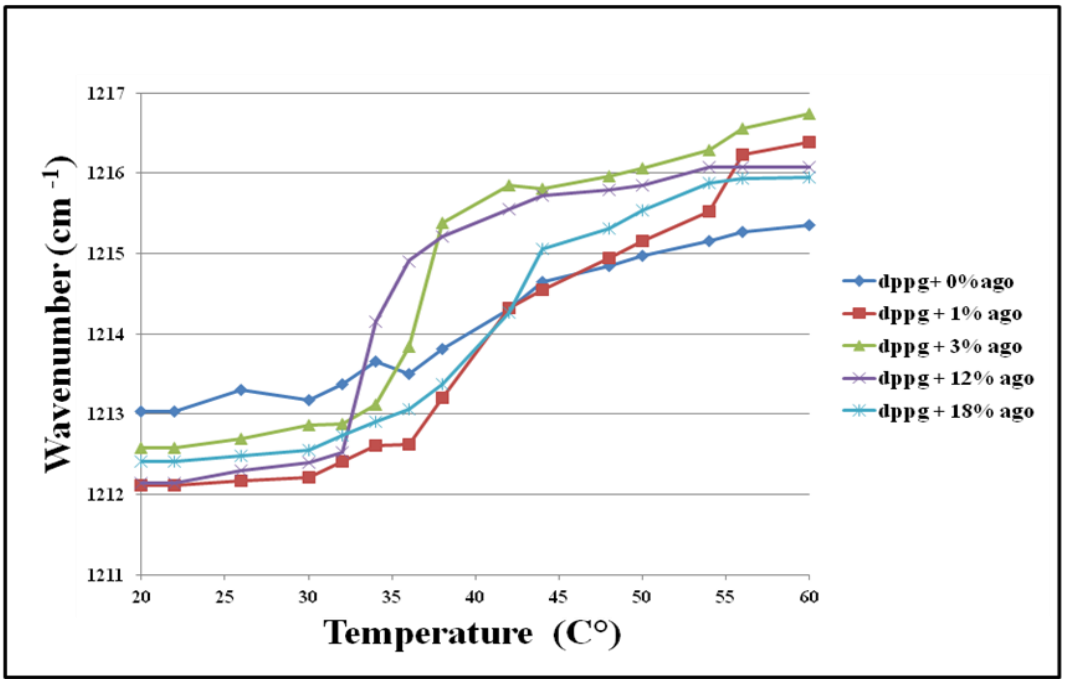

Figure 3.18: Temperature dependent frequency changes of PO^{2-} asymmetric band mode for mol% 0 **(⧫)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*) agomelatine containing DPPG liposomes.

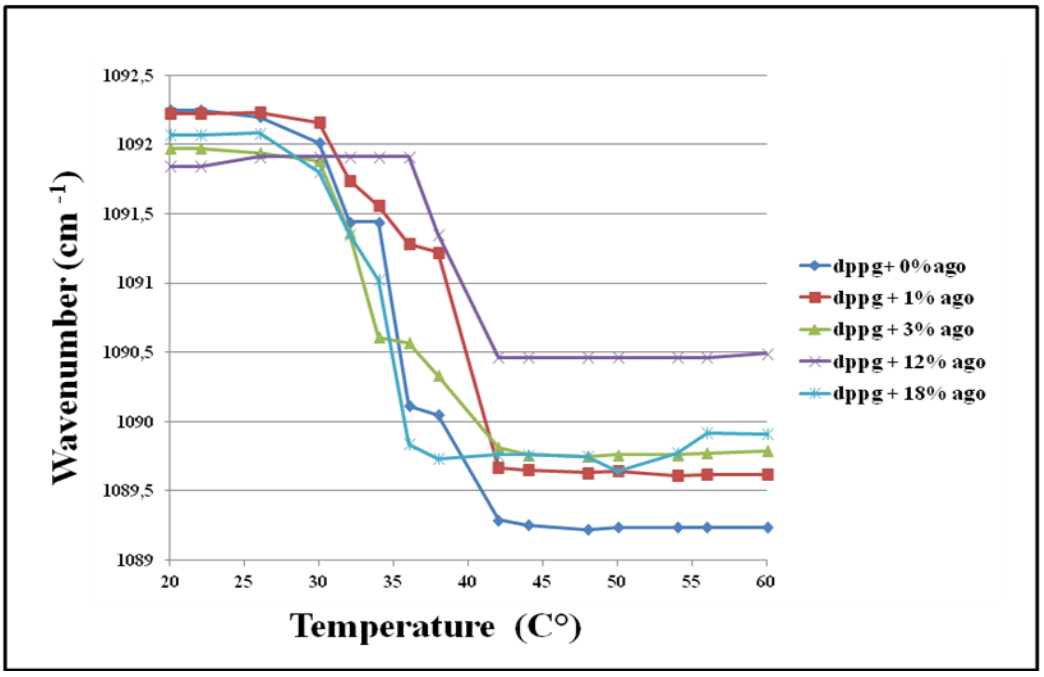

Figure 3. 19: Temperature dependent frequency changes of PO^{2-} symmetric band mode for mol% 0 **(⧫)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*) agomelatine containing DPPG liposomes.

3.2.1. DSC studies

Figure 3. 20 shows the DSC thermogram of pure and agomelatine containing (1-18 mole %) liposososmes, and Table 3.3 shows the phase transition temperatures (Tm), band widths and enthalpy changes of DPPG liposomes. The small band around ~ 33°C of pure DPPG thermogram is related to pre-transition and the larger band around 40.06°C gives the main phase transition temperature. As shown in the figure, with the addition of agomelatine, the pre-transition band is disappeared. Furthermore as can be seen in Figure 3.20 and Table 3.3. agomelatine causes an increase in the bandwidth of DSC thermogram of DPPG liposomes.

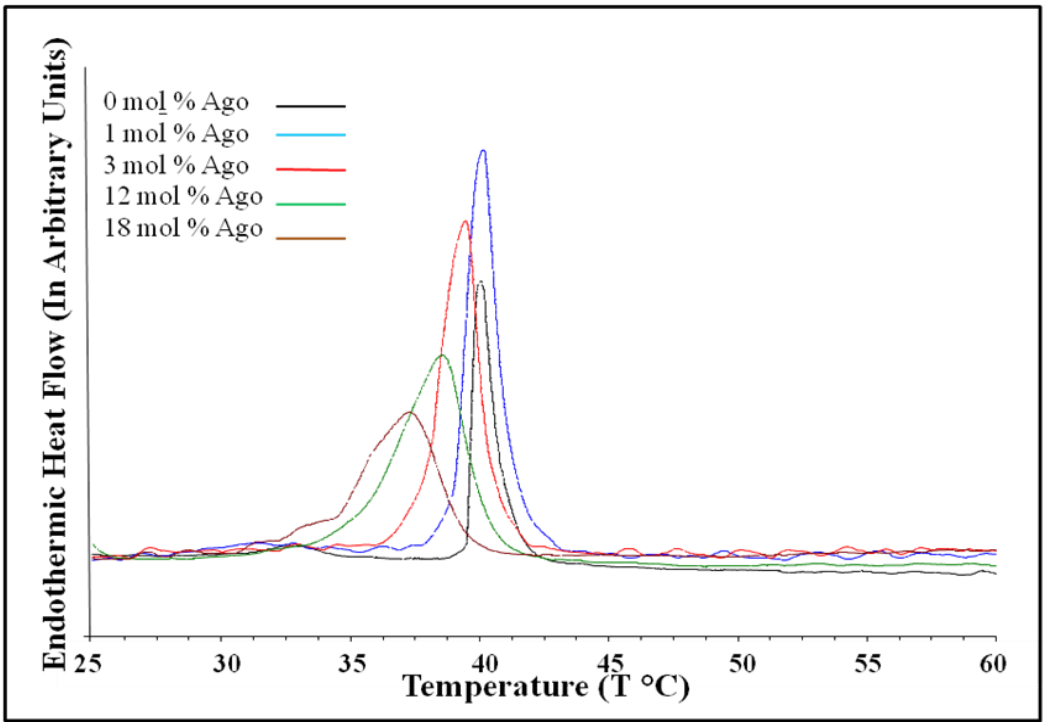

Figure 3.20: DSC thermogram of DPPG liposomes with and without different concentrations of agomelatine.

In the presence of agomelatine the phase transition temperature shifted to lower values. This increase in bandwidth and the decrease in phase transition temperature, indicates a change in liposome size and packaging and the system becomes more disordered (Korkmaz and Severcan, 2005). When the transition enthalpy changes of DPPC samples were compared, it was found that the enthalpy change of transition was increased in all agomelatine containing liposomes, except the 18 mole %. Because the broadening of this band may indicate that at 18 mole % agomelatine containing liposomes there may be lateral phase separation.

Table 3.3.: Transition temperatures (Tm), width at half height (ΔT) and enthalpy of the transitions (ΔH) for DPPG liposomes are given.

Sample	**T_m (^oC)**	**ΔT (^oC)**	**Δ H (cal/g)**
DPPG	40.06	0.91	4.366
DPPG + 1 mole %ago.	40.16	1.33	8.857
DPPG + 3 mole % ago.	39.46	1.65	8.731
DPPG + 12 mole % ago.	38.56	2.97	9.918
DPPG + 18 mole % ago	37.29	3.30	7.59

3.3. The effects of agomelatine on Sphingomyelin model membrane

3.3.1. FTIR spectroscopic studies

The last part of the study examines the temperature dependent effects of agomelatine on SM liposomes. Figure 3. 21 shows the FTIR spectra of pure SM liposomes and SM liposomes containing 1 mol%, 3 mol%, 12 mol% and 18 mol% agomelatine.

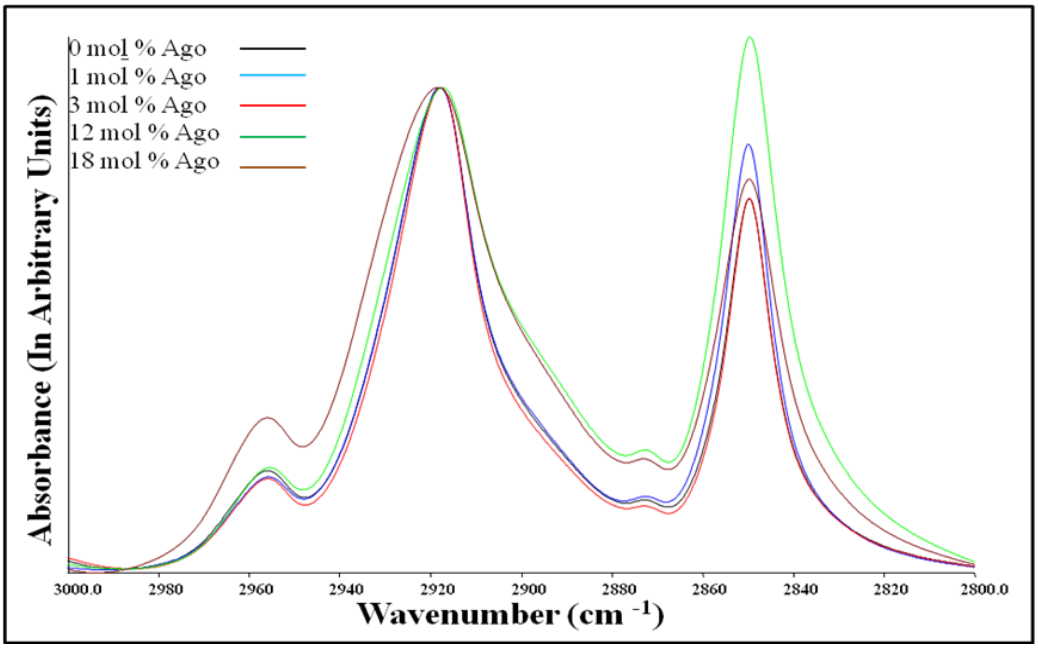

Figure 3.21: FTIR spectra of SM liposomes containing agomelatine of 0 mol% (black), 1 mol% (blue), 3 mol% (red), 12% mol (green), 18% mol (brown) in CH stretching region at 20°C (Spectra were normalized with respect to CH_2 asymmetric stretching band)

When we take band frequency value of CH_2 antisymmetric and symmetric stretching band in to account (Figures 3.22 & 23) it can be seen that agomelatine shifts the frequency values to the lower degrees of both bands at both phases, therefore, agomelatine makes the SM liposomes more ordered. But agomelatine slightly increases the Tm from ~ 40 °C.

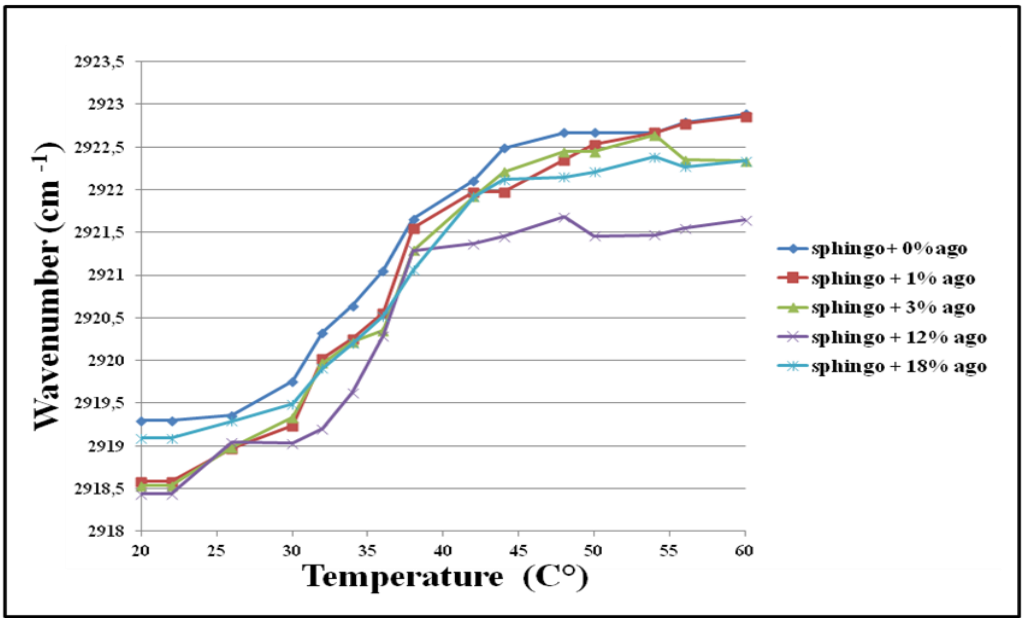

Figure 3.22: Temperature dependent frequency changes of CH_2 asymmetric stretching mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing SM liposomes.

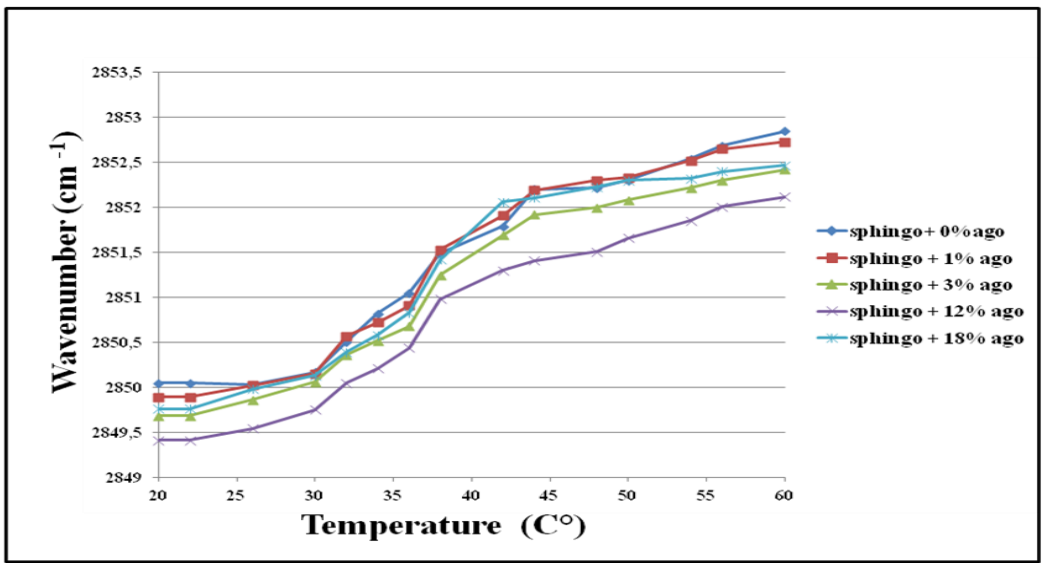

Figure 3.23: Temperature dependent frequency changes of CH_2 symmetric stretching mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing SM liposomes.

Figures 3.24 and 25 represent the bandwidth change with respect to temperature increase of SM liposomes containing diverse concentrations of agomelatine (1-18 mole %). As inferred from the figures agomelatine increases the bandwidth value of those bands so it increases the membrane dynamics.

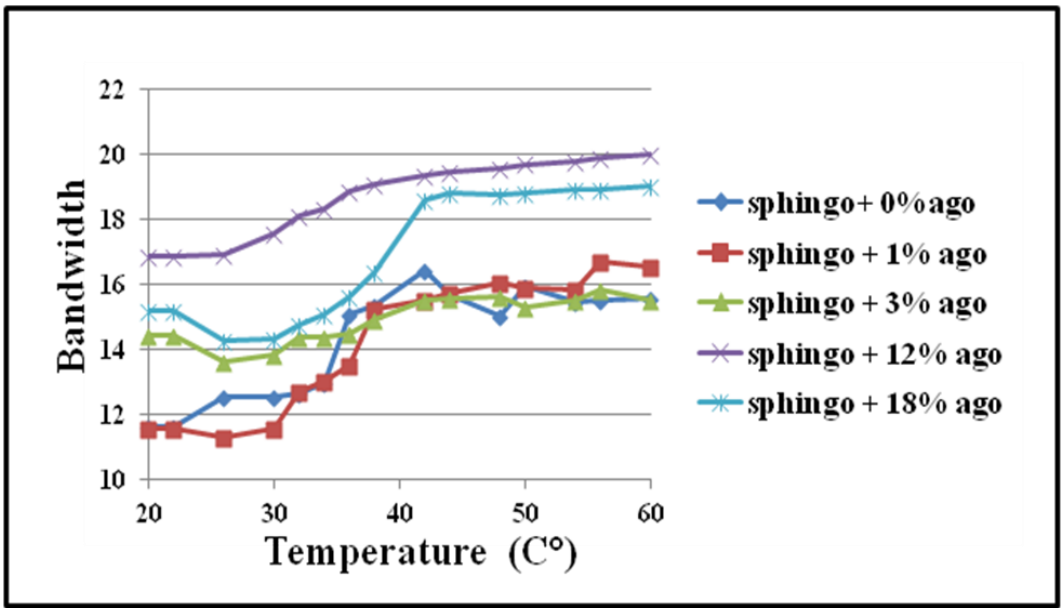

Figure 3.24: Temperature dependent bandwidth changes of CH_2 asymmetric stretching mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing SM liposomes.

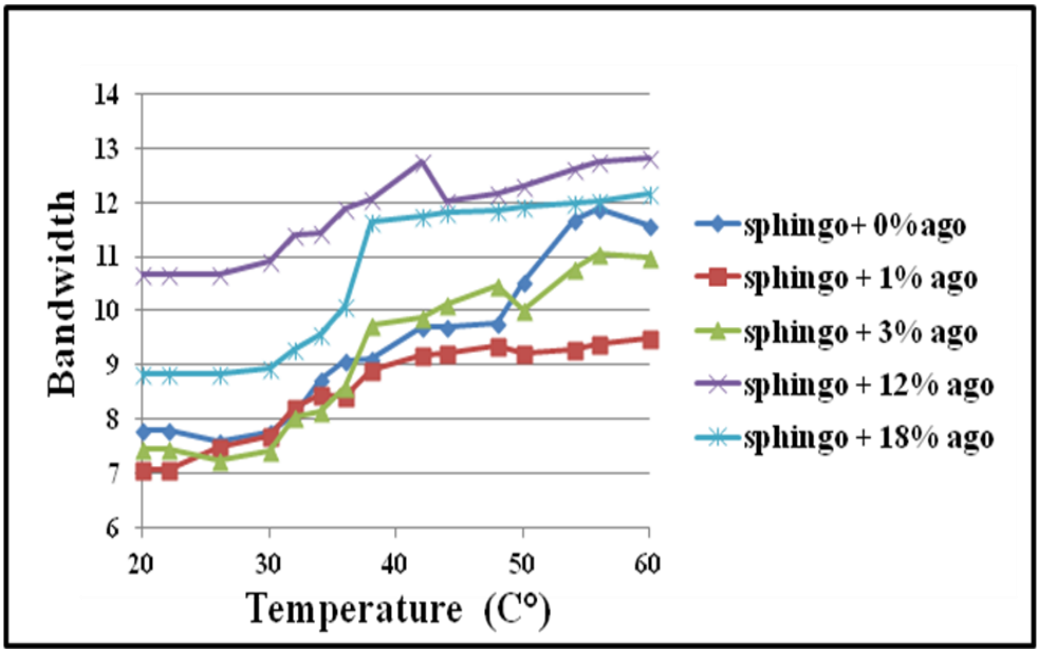

Figure 3.25: Temperature dependent bandwidth changes of CH_2 symmetric stretching mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing SM liposomes.

Since SM is phospholipid not derived from glycerol, there is no C=O band in SM (Figure 3.26). It has the ceramide core (sphingosine bonded to a fatty acid via an N-H linkage).

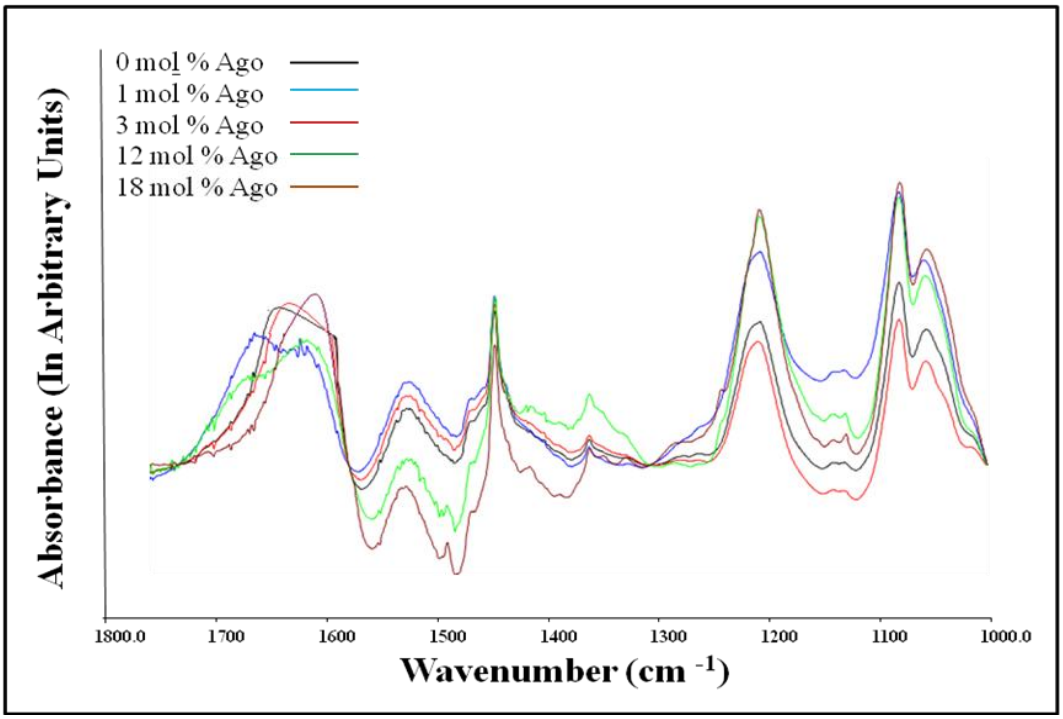

Figure 3.26: FTIR spectra of SM liposomes containing agomelatine of 0 mol% (black), 1 mol% (blue), 3 mol% (red), 12% mol (green), 18% mol (brown) in fingerprint region (1800-1000 cm^{-1}) at 20 ° C (Spectra were normalized with respect to CH_2 asymmetric stretching band)

Agomelatine-head group interactions can be investigated by monitoring the frequencies of the PO^{2-} asymmetric and symmetric bands. Figures 3.27 and 28 reveal that, agomelatine shifts the frequency values of those bands to the lower values. So it can be inferred that agomelatine enhances the H-bonding capacity of SM.

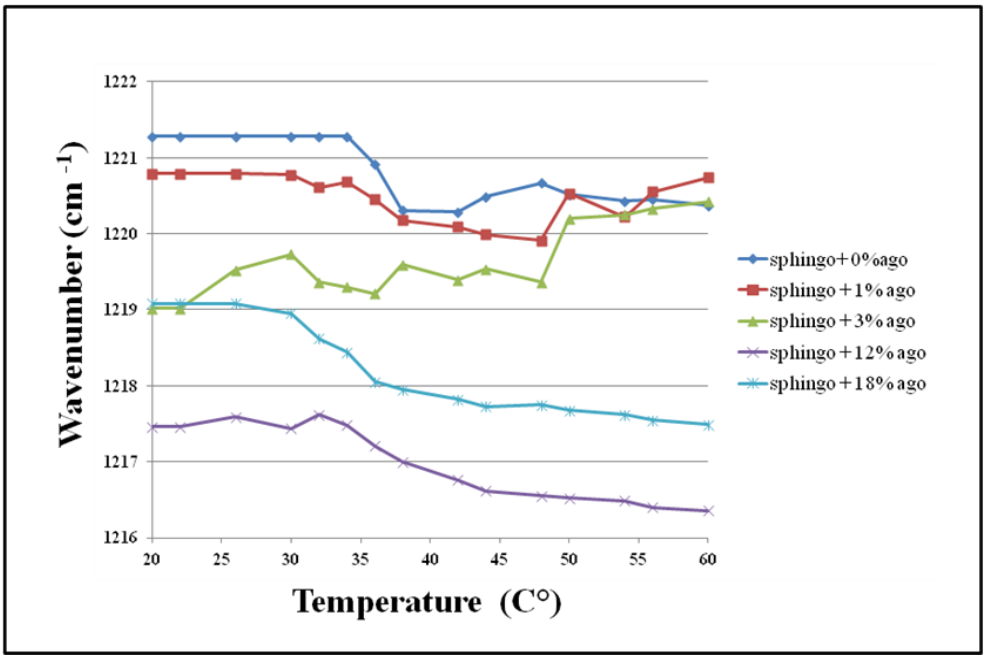

Figure 3.27: Temperature dependent frequency changes of PO2- asymmetric stretching mode for mol% 0 **(♦)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing SM liposomes.

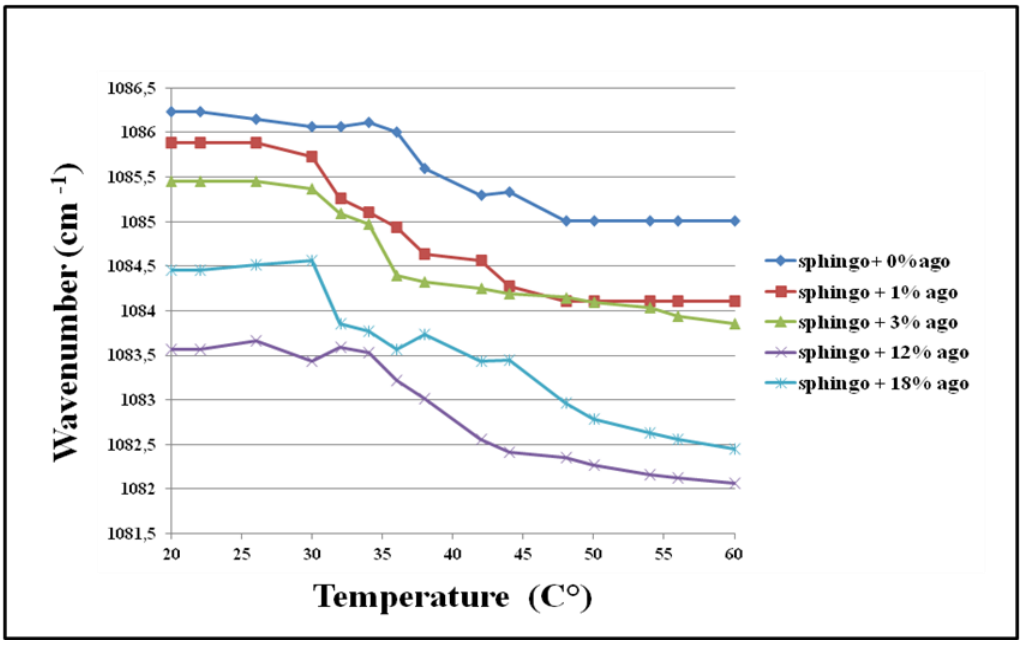

Figure 3.28: Temperature dependent frequency changes of PO2- symmetric stretching mode for mol% 0 **(⧫)**, 1 mole (■), 3 mol% (▲), 12 mol% (X) and 18% mol (*)agomelatine containing SM liposomes.

3.3.2. DSC studies

Figure 3.29, shows the DSC termogram of SM liposome with and without various concentrations of agomelatine. As can be seen from the figure the main phase transition temperature of SM liposoms is around 40.52° (Chiu et al, 2009). A broader phase transition peak observed in SM termogram, comparing to DPPC and DPPG thermograms is due to existence of asymmetric acyl chains in SM. As can be seen in Figure 3.29 and Table 3.4 as agomelatine concentration increases the bandwidth increases and the phase transition temperature of SM liposoms shifts slightly to higher values. Lipid order increases as agomelatin concentration increases. When the transition enthalpy changes of SM samples were compared, it was found that the enthalpy change of transition was increased in all agomelatine containing liposomes.

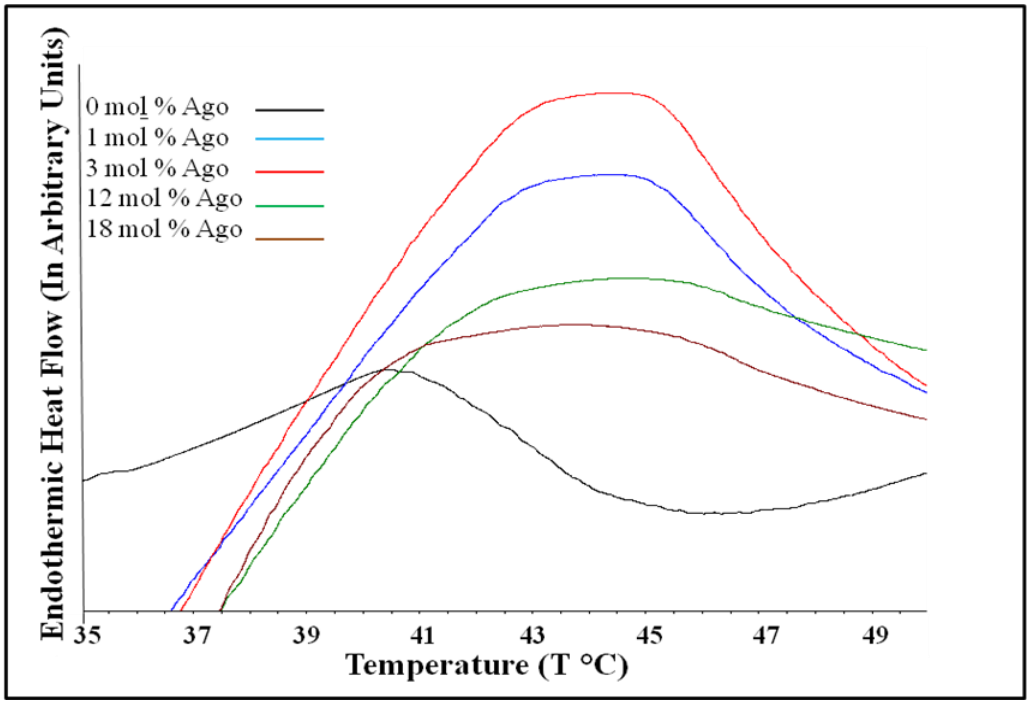

Figure 3.29: DSC thermogram of SM liposomes with and without different concentrations of agomelatine.

Table 3.4: Transition temperatures (Tm), width at half height (ΔT) and enthalpy of the transitions (ΔH) for SM liposomes are given.

Sample	**T_m (^{o}C)**	**ΔT (^{o}C)**	**Δ H (cal/g)**
SM	40,52	10,31	0,530
SM + 1 mole %ago.	44,38	21,65	2,528
SM + 3 mole % ago.	44,55	22,58	3,694
SM + 12 mole % ago.	42,77	17,37	1,890
SM + 18 mole % ago	41,34	14,78	1,677

CHAPTER 4

DISCUSSION

Membranes are main components of cells, which play vital roles in cell viability. For the proper functioning of cells, the appropriate structure of plasma membrane is essential (Jane B. Reece, 2008). The structure, stability and function of membranes are affected by the structure and phase transition behavior of membrane lipids. It has been reported that the bioactivity of drugs may be related to their capacity to alter the physical properties of membrane, such as membrane fluidity or the formation of lipid rafts (Tarahovsky et al, 2008); (Ulrih et al, 2010) as well as to cause altered membrane protein– protein and protein–lipid interactions (Pawlikowska-Pawlega et al, 2003); (Chiou et al, 2010). Therefore, it is important to evaluate or predict drug-membrane interactions in order to comprehend their action mechanisms. In the studies on drug-membrane interactions, since biological membranes are very complex in structure, it is often necessary to study the effects of drugs on artificial membranes, where all experimental conditions can be precisely controlled, before undertaking the study of natural membranes (Casal & Mantsch, 1984). For that reason, in order to clarify the mechanism of action of agomelatine, we evaluated the capability of interaction of agomelatine with cell membrane, which is directly related to the degree of incorporation and the distribution of agomelatine into lipid bilayer (Saija et al, 1995); (Castelli et al, 2003). With this aim, the interaction of the novel antidepressant drug agomelatine with the model membranes by the use of Fourier transform infrared spectroscopy (FTIR) and Differential scanning calorimetry (DSC) was investigated. For this purpose, the effects of agomelatine on MLVs consisting of neutral phospholipids, namely dipalmitoylphosphatidylcholine (DPPC) and sphingomyelin (SM), which are the basic lipids found in the structure of brain cell membrane and a charged phospholipid, namely dipalmitoylphosphatidylglycerol (DPPG) was examined. Although agomelatine was thought to exert its action by

interacting only with receptors on nerve cells, the results of this study demonstrated that agomelatine remarkably affects on the Tm, the acyl chain order, the glycerol backbone and the phosphate head groups and dynamics of all of the investigated MLVs, revealing that the drug also interacts with lipids in membrane structure.

The phase transition temperature (Tm) in membranes is an important factor that determines the structure of membranes. In most of the liposomes, a pre-transition, which indicates the mobility of the polar head, is observed (Yeagle, 2011). The phase transition in lipid bilayers can be described as a cooperative melting of the acyl chains. Shorter chains melt at lower temperatures and longer chains melt at higher temperatures (Phillips et al, 1970). The main transition demonstrating the acyl chain mobility is observed afterwards. Generally, pre-transition is very sensitive to the presence of impurities and it is abolished even at small quantities of impurities (McElhaney, 1986). A decrease in Tm upon drug administration means that the drug imparts a force to the bilayer in order to position itself in the hydrophobic core. The forces holding lipid components together in the structure of the membrane are usually physical in nature, consisting of hydrogen bonds, van der Waals interactions and electrostatic forces.

The results of the current study implicated that the presence of agomelatine led to the shift of Tm to lower temperatures in DPPC and DPPG liposomes, whereas the Tm of SM liposomes shifts to higher temperatures, observed both by DSC and FTIR spectroscopy. As can be seen in the thermograms, all of the lipid systems containing agomelatine undergo a broadened, complex phase transition between the gel and the liquid crystalline phases. The bradening in the phase transition curve implies that drug enters into hydrophobic part of the bilayer and disturbs strong van der waals interaction between the hydrophobic acyl chains so each phospholipid acyl cahins melts slightly at different temperatures. This shows loss in cooparativity and as a result of this a broadening in the phase transition shape is observed. These obrevations also suggest that agomelatine is partially buried in the hydrocarbon core of the bilayer, interacting primarily with the C2-C8 methylene region of the hydrocarbon chains (Yeagle et al., 2004). This may lead to enhancement of the

headgroups resulting with the disturbances of the packing of the system (Goni et al., 1986; (Oszlanczi et al, 2010).

According to the relatively strong intermolecular bonding between groups of the DPPC, DPPG, and SM molecules, the transition temperatures of the liposomes with lower drug amounts appear at higher temperatures. As a result of the high drug concentrations, the transition temperature compared with the previous pure lipid system occurs at a lower temperature value around 34◦C for DPPC and 37◦C for DPPG liposomes. However in SM, the Tm increases with respect to pure SM, where it is 41.34◦C. The reason for this situation may be because of the sphingomyelins unidentical acyl chains. The same transition temperature behaviour were also observed in FTIR spectroscopic studies. At the same time, the transition enthalpy is lower at pure liposomes, indicating that pure liposomes need less energy to melt.

It was previously proposed that an increase in enthalpy most probably indicates a decrease in the contributions in the van der Waals interactions in the system (Nagle & Wilkinson, 1978). The DSC results indicated that the enthalpy change of phase transition of among all agomelatine containing liposomes we used was increased. This also shows that agomelatine most probably decreases the formation of van der Waals interactions in hydrophobic region of membrane structure. Hence, it can be stated that above a certain critical drug concentration, agomelatine not only constitutes a membrane impurity, but instead mediates chain interdigitation.

The significant alterations in Tm generally take place over where protonation/deprotonation of the ionizable groups may occur. This may be reasonable because protonation and/or deprotonation of ionizable groups may change the attractive/repulsive interactions between the polar head groups and change the relative stability of the gel phase by their effects on the close packing interactions of the lipid molecules (Yeagle et al., 2011).The pre-transition peak is abolished in DSC thermograms and the Tm peak becomes broad with increasing concentration of agomelatine both in DSC and FTIR phase transition curves, indicating a larger perturbation of agomelatine to the membrane. These observations show the possible

interaction of agomelatine with the head group as well (Schultz et al., 2011; Mannock et al., 2006). .

There are newly formed shoulders at the DSC thermograms of DPPG and DPPC liposomes including 18 mole % of agomelatine. The shoulder seen at the thermograms of high doses may indicate the lateral phase separation in to drug-rich and drug-poor domains (D'Souza et al, 2009). It is observed that with increasing concentration of agomelatine both the pre-transition peak and the main transition peak become broad and shift to lower temperatures at DPPC and DPPG liposomes. All these results highlight the fact that agomelatine interacts with the head group in such a manner that it destabilizes the membrane architecture to a large extent. The overall DSC and FTIR spectroscopic data indicates that agomelatine induces changes in the structural architecture and physico-chemical characteristics of the liposomes (Pawar et al, 2012) (Goni et al., 1986) (Fang et al, 2001).

The thickness of the membranes is related to the length and average ordering of the hydrocarbon chains, where a decrease in order results in a decrease in membrane thickness or visa versa (Kupiainen et al, 2005). According to the results of FTIR spectroscopy obtained from the analysis of the C-H stretching region, agomelatine increases the lipid order of the DPPC and SM liposome, whereas, it decreases the lipid order of DPPG liposome in both gel and liquid crystalline phases, revealing agomelatine-induced alterations in membrane thickness and structure. In cell membranes, bilayer fluidity and membrane thickness may provide the energetic constraints to select for certain proteins with optimally adapted trans-membrane segments (Lu et al, 2010). Moreover, membrane fluidity is an important concept for membrane fusion and it is required for membrane trafficking, regeneration of various sub-cellular compartments after cell division, and cell growth. Both proteins and lipids take role in the regulation of membrane fluidity (Zhendre et al, 2011) and a controlled membrane fluidity is essential for the proper functioning of transmembrane receptors, such as G coupled receptors (Lee, 2004). Also membrane fusion is a process that is regulated by both lipids and proteins (Zhendre et al., 2011). According to the results of this model membrane study, agomelatine enhances the

membrane fluidity among all types of liposome studied, therefore, membrane fusion mechanism may be affected following the administration of agomelatine in biological membranes.

The increase of lipid order together with the increase of fluidity at DPPC and SM liposomes indicated the formation of drug rich domains upon the application of agomelatine (Vest et al, 2004) (Severcan et al., 2005) (Korkmaz et al., 2005). The formation of lipid bilayer domains in the liquid crystal layer implies the mismatch of the effective lengths of the hydrophobic parts of phospholipid molecules (Lehtonen & Kinnunen, 1997). Generally, biological lipid bilayers are highly organized and interconnected with gel and liquid phase having a relatively small permeability (Barry, 2004; Chen et al, 2010). However, the formation of domains leads to molecular heterogeneity and loose molecular packing (Pawar et al., 2012). These domains have different physical properties, therefore lipid fluidity also is different in different domains (Severcan et al., 1988). The loose packing of lipid bilayers may innervate the intermolecular interactions (Goretta et al, 2012). Also it has been found that the increase of elastic deformability of the membranes and dynamic fluctuation (Pressl et al, 1997) results in enhanced disorder in the overall system (Mavromoustakos et al, 2001). According to the results, agomelatine causes domain formation following the drug addition and results in loosely packed lipid bilayers, where this drug may also cause alterations on the permeability of membranes. Supportingly, our DSC data shows that there is a change in size and packaging of DPPC and DPPG liposomes upon agomelatine addition. Moreover, cooperativity in between phospholipid molecules decreases indicated by the broadened peak among all liposomes. This latter fact and the complexity of the peak indicate the existence of domains in the system with different drug concentrations.

Biologically, microdomains, such as lipid rafts, within the membrane serve as a key organizing principle to cover cellular processes to certain membrane areas. Lipid rafts are characterized by a specific lipid composition, they are rich in SM, cholesterol and certain glycolipids (Simons & Gerl, 2010). Lipid raft theory suggests that specific lipids and proteins partition away from other membrane components to

form functional plasma membrane platforms (Simons & Ikonen, 1997) and implicated in many cellular processes (Hansen and Nichols, 2009; Patel et al., 2008). Despite of specific protein-protein or protein-lipid interactions (Couet et al, 1997) (Lajoie et al, 2007) (Kawashima et al, 2009) which are likely to retain proteins in raft-like domains, the general physicochemical properties of the microdomains appear to play an essential role (Shakor et al, 2011). Moreover, lipid rafts, which are enriched in sphingomyelin, have been proposed to function as platforms, participating in the sorting of receptors, such as G protein-coupled receptors (GPCRs) and tyrosine kinase-coupled receptors, and in the regulation of receptor-mediated signal transduction (Manes et al, 2003) (Simons & Toomre, 2000). Agomelatine caused alterations in the membrane dynamics and order of DPPC, DPPG and SM liposomes, therefore, these changes may also affect the proper functioning of microdomains called lipid rafts in biological membranes. SM is an important lipid that is present in the cellular membranes, myelin sheath, and plasma lipoproteins. A large proportion of SM is found in plasma membranes where SM is enriched on the external leaflet (Auge et al., 2000). Importantly, sphingomyelin deficiency increased responsiveness to signaling of the cognate chemokine ligand CXCL12/CXCR4 pathway, indicating that, in addition to cholesterol, sphingomyelin in lipid rafts modulates the cellular response to cell motility cues. Asano et al., 2012 indicated that SM in lipid rafts modulates signal transduction by interacting with cell surface receptors. Also it is debated that sphingomyelin may be a selective modulator of GPCR signaling, modulating the accumulation and dimerization of these receptors in lipid rafts (Asano et al, 2012). Since agomelatine exerts remarkable alterations in the structure and dynamics of SM liposomes, the administration of the drug in humans may cause structural alterations in the membrane rafts interfering with the proper functioning of the lipid rafts, rich in SM.

The ionization efficiency of the lipids may also affect the structure of the head group (Shanta et al, 2012). Changes in the composition of membrane lipids, topology and spatial organization can affect the biophysical properties of different lipids and their phase behavior which affects the functions of membrane enzymes, receptors and transporters (Benga & Holmes, 1984) (van Meer et al, 2008). Agomelatine, having

one H donor and two H acceptor sites (Kennedy & Eisfeld, 2007), enhances H bonding capacity of all types of liposomes which have been studied in the current study. Electrostatic forces around head group of phospholipids may predominate over the hydrophobic interactions (Perez-Lopez et al, 2009). Agomelatine may be interacting with the head-group of phospholipids, forming hydrogen bonds between oxygen of the carbonyl groups on the lipid head-groups. However it shows a dose dependent effects on glycerol backbone of the phospholipids. At low agomelatine concentrations, a decrease was seen in the hydrogen bonding capacity of the glycerol backbone of DPPC liposomes. In contrast with this, at high concentrations of agomelatine, enhancement of the hydrogen bonding capacity in DPPC liposomes was noticed, which indicates that new H bonds are formed at high agomelatine concentrations. There may be H bonds formed with glycerol backbones of adjacent phospholipids or with agomelatine. Also agomelatine increases H bonding at phosphate headgroup of DPPC and SM liposomes when used at high concentrations. In DPPG liposomes, agomelatine addition causes different H bonding behavior in gel and liquid crystalline phases. In gel phase, agomelatine diminishes the H bonding capacity of glycerol backbone. However, in liquid chrystalline phase, it decreases the H bonding of the glycerol backbone at low doses, whereas increases the H bonding at the high dose. Moreover agomelatine enhances H bonding capacity in gel phase but lowers it in liquid chrystalline phases at the phosphate headgroup of DPPG liposomes. The differences among the gel phase and liquid crystalline phase may be due to the difference of membrane permeability between those two phases. In particular, gel phase bilayers are characterized by tight lipid packing and low permeability, fluid phase analogs are loosely packed and have relatively high permeability (Margin et al., 2006) (Lindner et al, 2004). The highest permeation rate may happen around Tm, where both gel and liquid crystalline phases co-exist; therefore, a mismatch can be suggested in lipid packing between the two phases that produces defects which permeable molecules can pass (Papahadjopoulos et al, 1973) (Petrov et al, 2009). According to the results, the interaction of agomelatine with the charged phospholipid DPPG is different than DPPC and SM when the H bonding of the glycerol backbone and the state of order of acyl chains is considered. One possible reason for this observation can be that the cationic side chains of the drug

may favor electrostatic interactions with the anionic lipid head-group of DPPG, rather than interacting through hydrophobic interactions with the lipid acyl chain region or the glycerol backbone (Clifton et al, 2011).

In addition to temperature changes , gel to liquid crystalline phase transition can also be induced by changes in hydration. Observed progressive decreases in Tm with increasing hydration indicates the adsorption of water molecules or H atoms which decreases the strength of the interactions between neighboring molecules in the lipid bilayer causing a disturbance at the polar head group(Yeagle et al., 2004). Alterations at phosphate headgroup may affect acyl chains (Goni et al., 1986), and the glycerol backbone region modifies most of the polar/non-polar interfacial parts of the bilayer where the chemical structure of the interfacial region can influence the overall conformation of the lipid molecule (Yeagle et al., 2004).

It has been reported that agomelatine binds to melatonin receptors, suppresses cAMP formation and mimics the actions of melatonin by dose dependently inhibiting the firing rate of suprachiasmatic nucleus neurons (Ying et al, 1996). These observations were later substantiated, when it was shown that agomelatine also potently activates cloned human MT1 and MT2 receptors (Audinot et al., 2003) and mimics melatonin (Barden et al., 2005). Regarding the previous studies held on our laboratory; agomelatine exerts the same effects on DPPC liposomes when compared with melatonin (Sahin et al., 2007). Both drugs increase in the number of trans conformers and the dynamics of the membrane. They also increase the H bonding capacity in phosphate group or glycerol backbone of DPPC with chemicals or water molecules. But when it comes to the DPPG liposomes, melatonin causes increase in the order of the membrane both in the gel and liquid crystalline phases at low doses. However, it increases the number of gauche conformers which indicates a decrease in the order of the bilayer at high doses (Sahin et al., 2007). Nonetheless, agomelatine decreases the lipid order of DPPG liposome at all phases. Furthermore, melatonin slightly increases the membrane dynamics both in the gel and liquid crystalline phase at high doses, but in low doses it decreases the membrane dynamics. However, agomelatine enhances the membrane fluidity among all types of liposome studied at all

concentrations. At last, both melatonin and agomelatine increases the strength of H bonding around phosphate head group. It is obvious that agomelatine does not denote the same effects with melatonin on every liposome type. The reason for this dissimilarity may be the difference among their chemical structure. Melatonin has 2 H-bond donors and 2 H-bond acceptors where, agomelatine has 1 H-Bond donor and 2 H-bond acceptors. They might have unknown different action mechanisms, which may be another reason for this difference.

Rodrigues et al. (2002) stated that perturbation of cell membrane structure represents an immediate component of the apoptotic pathway in cells, which results in a rapid disruption of membrane lipid polarity and fluidity, altered protein order, and increased oxidative injury, which precede metabolic and morphologic manifestations of apoptosis. Functionally, the increase in plasma membrane fluidity was found to be associated with apoptosis of nerve cells (Rodrigues et al, 2002). Regarding to our findings of increased membrane fluidity of DPPC, DPPG and SM liposomes with the presence of agomelatine, agomelatine may also play a role in the activation of apoptotic pathways in nerve cells.

Most importantly, in biological membranes agomelatine administration may lead to alterations in the arrangement of lipid-raft associated proteins, such as G protein-coupled receptors (GPCRs) and tyrosine kinase-coupled receptors, and eventually interfere with the regulation of receptor-mediated signal transduction. These alterations in raft arrangement may result in to the reduced intrinsic anisotropy leading to changes in curvature of membrane, which may even cause a dynamic instability leading to the retraction of the connection of membrane with the intercellular cytoskeletal elements. (Lawrence et al, 2003) (Hurtig et al, 2010) (Yang et al, 2009) (Lokar et al, 2012).

The plasma membrane serves as an interface between signaling outside the cell and the cell function. The molecular organization of the membrane landscape plays an extremely important role in a great variety of processes associated with the membrane (Stefl et al., 2012). Phospholipids were mainly recognized as second

messengers and their effect on membrane dynamics and structure was correlated with their role as a host to signaling molecules (Zhendre et al., 2011). Except for protein-protein contacts, the membrane-spanning segments of integral membrane proteins are surrounded by a shell of adjacent boundary lipids that mediates the coupling between the mostly hydrophobic intra-membranous residues of the protein and the lipid bilayer. In addition to electrostatic interactions, specific lipid-protein interactions have also been reported. The alterations in the structure of hydrophobic regions of the membrane may also influences both the structure as well as the function of a number of integral membrane proteins (Lehtonen and Kinnunen, 1997). Since we have observed structural changes in model membranes of all of the phospholipids investigated in this study, these alterations in lipid structure may also affect the protein-lipid interactions in biological membranes, therefore it can be concluded that agomelatine may have an effect on the action mechanisms of integral membrane-spanning proteins in biological membranes.

As a conclusion, cell membrane is extremely vital for an organism, since it has important functions such as ion conductivity and cell signaling and since it serves as the attachment surface for several extracellular structures. Here we shed valuable insights into the molecular mechanism of the interaction of agomelatine with DPPC, DPPG and SM MLVs. Our results revealed that agomelatine causes alterations in the order, packing and dynamics of the model membranes composed of these phospholipids. Although it has been stated that agomelatine only interacts with MT1, MT2 and 5-HT2C receptors in cell membrane, herein this study we have demonstrated that it strongly interacts with phospholipids. It is known that, lipid structure and dynamics may have influence on the structure of membrane bound proteins. Therefore ion conductivity and cell signaling may be affected following the perturbation of membrane bound proteins. However, further studies have to be conducted to specifically address the effect of agomelatine on lipid-protein interactions, thus agomelatine-protein containing MLVs and agomelatine-biological membrane interaction should be investigated.

CHAPTER V

CONCLUSION

The results of this study reveals that agomelatine shows remarkable effects on the Tm, the acyl chain order, dynamics, the glycerol backbone and the phosphate head groups of all the MLVs.

According to the results, agomelatine addition increases the lipid order of the DPPC and SM liposome, whereas, it decreases the lipid order of DPPG liposome. Moreover this drug enhances the membrane fluidity among all types of liposome studied. Since the ordering of the hydrocarbon chains related to the thickness of the membranes and there is the match between the hydrophobic thickness of the lipid bilayer and the length of the trans-membrane domains agomelatine interaction with membrane may cause impairments at the trans membrane sorting. Since agomelatine caused changes in the membrane fluidity membrane fusion mechanism may be affected following the administration of agomelatine. Furthermore if GPCRs activation is related with membrane location, composition and fluidity and agomelatine increases the membrane fluidity, administration of agomelatine may affect the GPCR activation. Since it has been said that agomelatine does not interact with the receptors other than MT1, MT2 and 5-HT2C (Papp et al., 2003) and MT1, MT2 and 5-HT2C are also GPCRs it may not put forward its function through those receptors.

The increase of lipid order and increase of fluidity at DPPC and SM liposome indicates domain formation upon drug addition. Furthermore, increased membrane fluidity and early phase change leads to molecular heterogeneity and loose molecular packing. According to results mentioned before, agomelatine causes domain formation following the drug addition and results with loosely packed lipid bilayers, thus, this drug may cause alterations on the permeability of membranes. Also

alterations in the SM fluidity and order may affect lipid raft formation mechanisms. Since agomelatine increases the order and fluidity of the acyl chains of SM liposomes, it may cause structural alterations in the membrane rafts.

At low agomelatine concentrations, a decrease is seen in the Hydrogen bonding capacity of the glycerol backbone of DPPC liposomes. In contrast with this, at high concentrations of agomelatine, enhancement of the hydrogen bonding capacity is noticed. New H bonds are formed at high agomelatine concentrations. There may be H bonds formed with each other or with agomelatine. So agomelatine increases H bonding at glycerol backbone of DPPC liposomes when used at high concentrations. Also agomelatine increases H bonding at phosphate headgroup of DPPC liposomes when used at high concentrations. Agomelatine enhances the H bonding capacity of SM head group.

Agomelatine diminishes the H bonding capacity in the gel phase of DPPG liposomes although it increases H bonding capacity at high doses but not at low doses in the liquid chrystalline phases of the glycerol backbone. Moreover agomelatine enhances H bonding capacity in gel phase but lowers it in liquid chrystalline phases at the phosphate headgroup. Changes in the composition of membrane lipids, topology and spatial organization can affect the biophysical properties of different lipids and their phase behavior which effects the actions of membrane enzymes, receptors and transporters.

Our data shows that there is a change in DPPC and DPPG liposome size and packaging and system becomes more disordered. Also it was found that the enthalpy change of transition was increased in all agomelatine containing liposomes. In SM liposomes, as the agomelatine concentration increases, the system becomes slightly dis ordered and again there are alterations of the packaging of the system. Those systems undergo a broadened, complex phase transition between the gel and the liquid crystalline phases. Decreasing Tm means the drug imparts fluidity to the bilayer in order to position itself in the hydrophobic core. Observed progressive decreases in Tm with increasing hydration indicates the adsorption of water

molecules or H atoms which decreases the strength of the interactions between neighboring molecules in the lipid bilayer causing a disturbance at the polar head group. Alterations at phosphate headgroup may affect acyl chains, and the glycerol backbone region modifies most of the polar/non-polar interfacial parts of the bilayer where the chemical structure of the interfacial region can influence the overall conformation of the lipid molecule.

Agomelatine caused perturbation on membrane structure and perturbation of cell membrane structure represents an immediate component of the apoptotic pathway which likely accounts for a rapid disruption of membrane lipid polarity and fluidity, altered protein order, and increased oxidative injury, which precede metabolic and morphologic manifestations of apoptosis. Functionally, the increase in plasma membrane fluidity associated with apoptosis of nerve cells. Regarding to our results agomelatine increases membrane fluidity among DPPC, DPPG and SM, so we can say that, agomelatine may induce apoptosis of nerve cells.

Regarding to the enthalpy changes, agomelatine most probably decreases the van der Waals interaction contribution. So it can be said that above a certain critical drug concentration, agomelatine not only constitutes a membrane impurity, but instead mediates chain interdigitation.

Finally it has been told that agomelatine binds to melatonin receptors, suppresses cAmP formation and mimics the actions of melatonin by dose dependently inhibiting the firing rate of suprachiasmatic nucleus neurons. But regarding to previous studies held on our laboratory; agomelatine shows same effects on DPPC liposomes with melatonin. But when it comes to the DPPG liposomes agomelatine does not denote the same effects with melatonin on every liposome type. They might have unknown different action mechanisms, which may be another reason for this difference.

As a conclusion, cell membrane is extremely vital for an organism, since it has important functions such as ion conductivity and cell signaling and since it serves as the attachment surface for several extracellular structures. Here we shed valuable

insights into the molecular mechanism of the interaction of agomelatine with DPPC, DPPG and SM MLVs. Our results revealed that agomelatine causes alterations in the order, packing and dynamics of the model membranes composed of these phospholipids. Although it has been stated that agomelatine only interacts with MT1, MT2 and 5-HT2C receptors in cell membrane, herein this study we have demonstrated that it strongly interacts with phospholipids. It is known that, lipid structure and dynamics may have influence on the structure of membrane bound proteins. Therefore ion conductivity and cell signaling may be affected following the perturbation of membrane bound proteins. However, further studies have to be conducted to specifically address the effect of agomelatine on lipid-protein interactions, thus agomelatine-protein containing MLVs and agomelatine-biological membrane interaction should be investigated.

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