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# **Doctoral Dissertation**

**Detailed Membrane Physiochemical Pathways  
Involved In Cold-sensitization Induced by l-menthol**

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# **Detailed Membrane Physicochemical Pathways Involved In Cold-sensitization Induced by l-menthol**

## **Abstract**

Every organism is susceptible to environment change and those too external stimuli like light, pH, temperature and some chemical agonist. One of the interesting phenomena of the human body is to sense a variety of physical and chemical stimuli via a change in the physical properties of the membrane. Our body responds to different signaling molecule in a different manner in order to sense. Menthol, a secondary alcohol from terpenoid family exhibits cooling sensation to the skin. Menthol is also used widely for the treatment of minor aches, as an analgesic, flavoring agent etc. It is well established proven fact that menthol activates TRPM8 ion channel, leading to an opening of ion gates, as a result membrane potential generates and eventually feels cool. There is enough evidence of cold sensitization induced by menthol on a living cell such as transfected HEK cells. However, no validated experimental model is currently known for the studies by which menthol influence and activates signal transduction in various biological membranes. Thus, the mechanisms of these gating behaviors of TRP channels are poorly understood.

In this dissertation, I mainly focused on the effect of menthol and its enantiomers and their interaction with model biomembranes. Moreover, detailed membrane physicochemical pathways involved in cold sensitization induced by l-menthol was discussed. In order to reveal the interaction between menthol and membrane, I employed three different systems, homogeneous model membrane, heterogeneous model membrane and actual cell membrane.

First, studies on the homogeneous membrane were carried out at different concentrations of l-menthol. The change in the morphology such as membrane fluctuation, surface area, and size distribution has been estimated. The results obtained from the homogeneous membrane clearly demonstrated the direct interaction of menthol with a model membrane. It has been reported that

menthol behaves differently depending on its concentration. At lower concentration induces cooling effect while at higher concentration burning/pain sensation induced. By employing the different concentration of menthol, influential interaction with the lipid bilayer and that too dynamics change of the membrane was observed. These findings suggested that menthol has direct interaction with the lipid bilayer and exhibits concentration-dependent manner interaction. In the homogeneous and heterogeneous membrane, the effect of two isoforms of menthol d- and l- in model membrane showed significant differences between them. l-Menthol makes the membrane less temperature sensitive whereas d-menthol have shown thermal responsiveness. Also, d-menthol enhances the membrane fluidity in the absence of cholesterol than l-menthol. Cholesterol presence is very important for altering membrane fluidity, and hence changes the effect of d- and l-menthol with lipid phases depending upon sterol concentration and lipid composition. I further checked the difference in physicochemical properties modulated by d- and l-menthol on biomembrane. In homogeneous membrane, fluctuation profile, thermodynamic analysis by DSC, and membrane fluidity will be discussed. Further, to elaborate deeper understanding, I worked on membrane heterogeneity referred as “lipid raft”. Here, I examined the chirality-dependent interaction of d- and l-menthol with the biomembrane. I successfully provide a clear and significant difference between d- and l-menthol and showed the importance of chirality in biological processes.

Human embryo kidney cell abbreviated as the HEK293 cell, are highly expressed and used in many biological types of research. HEK293 cell was used to assess the effect of d- and l-menthol on cell membrane interaction. I found that d-menthol is toxic to the cell and causes cell death meaning that d-menthol disrupt the membrane dynamics. Finally, to correlate the work done on the model membrane, I chose to work on the actual cell specifically HEK293 cell. The studies on HEK293 cells include cell viability assay, temperature dependent change in raft region, intracellular calcium level and actin.

Keywords: Cold-sensitization, Lipid-raft, Menthol, Membrane dynamics, Membrane fluidity

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**Pooja Gusain**

## LIST OF ABBREVIATION

<i>DOPC</i>	1,2-Dioleoyl- <i>sn</i> -glycero-3-phosphocholine
<i>DPPC</i>	1,2-Dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine
<i>Chol</i>	Cholesterol
<i>d-men</i>	d-Menthol
<i>l-men</i>	l-Menthol
<i>S<sub>o</sub></i>	Solid-ordered
<i>L<sub>o</sub></i>	Liquid-ordered
<i>L<sub>d</sub></i>	Liquid-disordered
<i>T<sub>m</sub></i>	Miscibility Temperature
<i>GP</i>	Generalized Polarization
<i>TRP</i>	Transient Receptor Potential
<i>TRPM8</i>	Transient Receptor Potential Melastatin 8
<i>Rho-DHPE</i>	2-dihexadecanoyl- <i>sn</i> -glycero-3 phosphoethanolamine triethylammonium salt
<i>DLS</i>	Dynamic Light Scattring
<i>DSC</i>	Differential Scanning Calorimetry
<i>CLSM</i>	Confocal Laser Scanning Microscopy
<i>HEK293</i>	Human Embryonic Kidney 293
<i>MβCD</i>	Methyl-β Cyclodextrin
<i>BSA</i>	Bovine Serum Albumin

<i>PBS</i>	Phosphate Buffer Saline
<i>DMEM</i>	Dulbecco's Modified Eagle Medium
<i>CT-B</i>	Cholera toxin subunit B
<i>FBS</i>	Fetal Bovine Serum
<i>v/v</i>	Volume/Volume
<i>Ca<sup>2+</sup></i>	Calcium
<i>RP</i>	Rhodamine Phalloidin



# Table of Contents

<b>CHAPTER 1 GENERAL INTRODUCTION .....</b>	<b>1</b>
<b>1.1 Introduction.....</b>	<b>2</b>
<i>1.1.1 Biological membrane .....</i>	<i>2</i>
<b>1.2 Function of biological membrane .....</b>	<b>4</b>
<i>1.2.1 Barrier function .....</i>	<i>4</i>
<i>1.2.2 Signal transduction .....</i>	<i>5</i>
<b>1.3 Artificial membrane.....</b>	<b>6</b>
<b>1.4 Cholesterol in the Plasma membrane .....</b>	<b>9</b>
<b>1.5 Menthol structure and Biological response .....</b>	<b>11</b>
<i>1.5.1 Menthol structure .....</i>	<i>11</i>
<i>1.5.2 Biological response of menthol.....</i>	<i>11</i>
<b>1.6 Transient Receptor Potential (TRP) .....</b>	<b>13</b>
<i>1.6.1 Effect of Cold and Menthol on TRPM8.....</i>	<i>16</i>
<b>1.7 Objective and Aim of the research .....</b>	<b>18</b>
<b>1.8 Outline of the Thesis .....</b>	<b>19</b>
<b>1.9 References .....</b>	<b>20</b>
<b>CHAPTER 2 THERMO-INDUCED DYNAMICS OF MODEL CELL MEMBRANE BY ACTION OF MENTHOL .....</b>	<b>28</b>
<b>2.1 Introduction.....</b>	<b>29</b>
<b>2.2 Experimental Procedures:.....</b>	<b>31</b>
<i>2.2.1 Materials:.....</i>	<i>31</i>
<i>2.2.2 Preparation of Liposomes: .....</i>	<i>31</i>
<i>2.2.3 Microscopy Observations of membrane fluctuation:.....</i>	<i>32</i>

2.2.4 <i>Effect of Temperature on Molecular Area of Monolayer Membranes:</i> .....	32
2.2.5 <i>Dynamic Light Scattering (DLS) Measurements:</i> .....	33
<b>2.3 Results and discussion</b> .....	<b>33</b>
2.3.1 <i>Effect of menthol on membrane fluctuation</i> .....	33
2.3.2 <i>Role of surface tension and pressure on membrane</i> .....	37
2.3.3 <i>Size distribution profile of lipid bilayer</i> .....	41
<b>2.4 Conclusion</b> .....	<b>43</b>
<b>2.5 References</b> .....	<b>43</b>
<b>CHAPTER 3 EFFECT ON BIOPHYSICAL PROPERTIES OF HOMOGENEOUS MODEL MEMBRANE INDUCED BY d- and l-MENTHOL</b> .....	<b>46</b>
<b>3.1 Introduction</b> .....	<b>47</b>
<b>3.2 Experimental Procedures:</b> .....	<b>49</b>
3.2.1 <i>Materials:</i> .....	49
3.2.2 <i>Preparation of Liposomes:</i> .....	50
3.2.3 <i>Microscopy Observations:</i> .....	50
3.2.4 <i>Measurements of membrane fluidity:</i> .....	51
3.2.5 <i>Differential Scanning Calorimetry (DSC) Measurements:</i> .....	51
<b>3.3 Results and discussion</b> .....	<b>51</b>
3.3.1 <i>Effect of d- and l-menthol on membrane fluctuation</i> .....	51
3.3.2 <i>Influence of d- and l-menthol on fluidity change in homogeneous membrane</i> .....	55
3.3.3 <i>DSC Measurements</i> .....	58
<b>3.4 Conclusion:</b> .....	<b>62</b>
<b>3.5 References</b> .....	<b>63</b>
<b>CHAPTER 4 CHIRALITY-DEPENDENT INTERACTION OF d- and l-MENTHOL WITH MODEL BIOMEMBRANES</b> .....	<b>67</b>
<b>4.1 Introduction</b> .....	<b>68</b>

<b>4.2 Experimental Procedures:</b> .....	71
4.2.1 <i>Materials:</i> .....	71
4.2.2 <i>Preparation of Liposomes:</i> .....	71
4.2.3 <i>Microscopy Observations:</i> .....	72
4.2.4 <i>Membrane fluidity Measurements:</i> .....	72
4.2.5 <i>Miscibility Temperature Measurements:</i> .....	73
<b>4.3 Results and discussion</b> .....	73
4.3.1 <i>Effect of d- and l-menthol on membrane fluidity on heterogeneous membrane</i> .....	73
4.3.2 <i>Effect of cholesterol concentration on membrane heterogeneity</i> .....	79
4.3.3 <i>Distribution of menthol within the lipid bilayer: Concentration effect of menthol</i> .....	86
<b>4.4 Conclusion</b> .....	89
<b>4.5 References</b> .....	90
<b>CHAPTER 5 EFFECT OF MENTHOL ON RAFT STABILIZATION IN BIOLOGICAL MEMBRANES</b> .....	<b>94</b>
<b>5.1 Introduction</b> .....	95
<b>5.2 Experimental Procedures:</b> .....	97
5.2.1 <i>Materials:</i> .....	97
5.2.2 <i>Cell culture:</i> .....	97
5.2.3 <i>Staining and Visualization of lipid rafts:</i> .....	97
5.2.4 <i>Measurement of cell viability:</i> .....	98
5.2.5 <i>Modulation of membrane cholesterol</i> .....	98
5.2.6 <i>Double staining of raft and actin:</i> .....	98
5.2.7 <i>Measurement of intracellular Ca<sup>2+</sup> level:</i> .....	99
<b>5.3 Results and discussion</b> .....	99
5.3.1 <i>Temperature-dependent Raft stabilization induced by menthol</i> .....	99
5.3.2 <i>Effect of d- and l-menthol on the toxicity on HEK293 cells</i> .....	102
5.3.3 <i>Influence of cholesterol on the raft stabilization induced by d- and l-menthol</i> .....	103

5.3.4 <i>Effect of d- and l-menthol induced changes in intracellular Ca<sup>2+</sup> level</i> .....	106
5.3.5 <i>Role of cytoskeleton and F-actin</i> .....	109
<b>5.4 Conclusion</b> .....	<b>112</b>
<b>5.5 References</b> .....	<b>113</b>
<b>CHAPTER 6 GENERAL CONCLUSIONS</b> .....	<b>117</b>
6.1 <b>Prospects of the dissertation</b> .....	119
6.2 <b>References</b> .....	120
<b>ACHIEVEMENTS</b> .....	<b>121</b>

## **List of Tables and Figures**

Table 3.1 Temperature change values at which membrane start fluctuating.....	53
Table 3.2 Temperature change values at which membrane start fluctuating.....	54
Table 3.3 Main transition peak and change in enthalpy values. DOPC, DPPC, DOPC/Chol, and DPPC/Chol-containing d- and l-menthol liposomes.....	62
Table 4.1 Correlation of d- and l-menthol localization in heterogeneous membrane phases and membrane fluidity upon the effect of cholesterol concentration. ....	79
Figure 1.1 Schematic representation of the biological membrane. <sup>5</sup> .....	3
Figure 1.2 Schematic representations of three types of membrane lipids (a) glycerophospholipid, (b) sphingolipids, and (c) cholesterol. <sup>1</sup> .....	4
Figure 1.3 Process involved in signal transduction.....	6
Figure 1.4 Structure of Liposomes and different vesicles formation depending on the size. ....	8
Figure 1.5 Phase behavior of an artificial membrane in three-component system composed of DOPC, DPPC, and Cholesterol. <sup>23</sup> .....	9
Figure 1.6 (a) Chemical structure of cholesterol representing both hydrophilic (OH) and the hydrophobic region (fused ring) and (b) cholesterol localization in the lipid bilayer. <sup>30</sup> .....	10
Figure 1.7 (a) 3D model of menthol and (b) types of a stereoisomer of menthol. <sup>33</sup> .....	12
Figure 1.8 Phylogenetic trees of human TRP channels. <sup>53</sup> .....	15
Figure 2.1 Membrane fluctuation profile of lipid vesicles. Temperature was increased from RT to 40.0 °C, using a thermo controller. An image of a typical lipid vesicle was captured using a phase-contrast microscope. The graph show membrane fluctuation at 21 °C (red), 25 °C (blue) as a function of radius and its distribution. Plotted the value of radius of “r” in each “ $\theta$ ” ( $\theta = \pm \pi/n$ , $n = 1, 2, 3 \dots 100$ ). (a) DOPC/Chol and (b) DOPC/Chol/Menthol. Scale bar = 10 $\mu\text{m}$ .....	34
Figure 2.2 Thermo-sensitive response of menthol-containing lipid vesicles. Percentage of lipid vesicles, which started fluctuating at a given level of temperature increase. The vesicles contained DOPC/Chol (black), 2.5 % menthol (light grey dashed),	

5 % menthol (blue), 10 % menthol (dark grey dashed), 20 % menthol (black dotted), and 30 % (grey solid line). (n=30). .....	35
<b>Figure 2.3 Thermo-sensitive response of menthol-containing lipid vesicles. Fluctuating temperature at which 50 % of liposomes started fluctuating at a given level of temperature increase. The graph shows <math>T_f</math> against menthol concentration. (n=30).</b> .....	36
<b>Figure 2.4 Typical pressure-area curve of lipid monolayer DOPC/Cholesterol and menthol-containing membranes. DOPC/Chol (80/20), DOPC/Chol/Menthol (78/19.5/2.5) DOPC/Chol/Menthol (76/19/5), DOPC/Chol/Menthol (72/18/10), DOPC/Chol/Menthol (68/17/15), and DOPC/Chol/Menthol (64/16/20). Surface pressure (p)–area per molecule (<math>\beta</math>) at 30 mN/m, at each temperature 20, 24, and 28 °C. Shows the typically P-A curves of a membrane at each temperatures 20 (black), 24 (red), and 28 (blue) °C respectively (n = 10).</b> .....	38
<b>Figure 2.5 Typical pressure-area curve of lipid monolayer DOPC/Chol and menthol-containing membranes. DOPC/Chol (80/20), DOPC/Chol/Menthol (78/19.5/2.5) DOPC/Chol/Menthol (76/19/5), DOPC/Chol/Menthol (72/18/10), DOPC/Chol/Menthol (68/17/15), and DOPC/Chol/Menthol (64/16/20). Change in the molecular area upon temperature change.</b> .....	39
<b>Figure 2.6 Model demonstrating the interaction of menthol with lipid bilayer upon fluctuation.</b> .....	40
<b>Figure 3.1 Effect of d- and l-menthol on DOPC lipid vesicles. Percentage of lipid vesicles, which started fluctuating at a given level of temperature raise. The vesicles containing d-menthol (dashed gray), l-menthol (gray) at 10 % molar concentration and control DOPC system (black). (n=30).</b> .....	52
<b>Figure 3.2 Effect of d- and l-menthol on DOPC/Chol lipid vesicles. Percentage of lipid vesicles, which started fluctuating at a given level of temperature increase. The vesicles containing d-menthol (dashed gray), l-menthol (gray) at 10 % molar concentration and control DOPC/Chol system (black). (n=30).</b> .....	54
<b>Figure 3.3 Effect of d- and l-menthol on membrane fluidity of homogeneous model membranes. Typical confocal microscopy images of Laurdan emmission (a), GP values of DOPC membranes (b), DOPC/Chol membranes (c). Black lines for DOPC, DOPC/Chol, red and blue lines indicates d- and l-mentho containing membrane. Scale bar = 10 <math>\mu</math>m.</b> .....	56
<b>Figure 3.4 Effect of d- and l-menthol on membrane fluidity of homogeneous model membranes. Typical confocal microscopy images of Laurdan emmission (a), GP values of DPPC membranes (b), DPPC/Chol membranes (c). Black lines for</b>	

DPPC, DPPC/Chol, red and blue lines indicates result of d- and l-menthol containing membrane. Scale bar = 10 $\mu\text{m}$ . .....	58
<b>Figure 3.5</b> Representative DSC thermographs of (a) DOPC, (b) DOPC/Chol vesicles containing d- and l-menthol during heating at 5 $^{\circ}\text{C min}^{-1}$ . .....	60
<b>Figure 3.6</b> Representative DSC thermographs of (a) DPPC, (b) DPPC/Chol, (c) DOPC, (d) DOPC/Chol vesicles containing d- and l-menthol during heating at 5 $^{\circ}\text{C min}^{-1}$ . .....	61
<b>Figure 4.1</b> Effect of d- and l-menthol on membrane fluidity of $S_0/L_d$ heterogeneous model membranes. Typical confocal microscopy images of Laurdan emission (a), GP values of $S_0/L_d$ phase-separated membranes. Red and black region indicates $L_d$ and $S_0$ domains. Scale bar = 10 $\mu\text{m}$ . .....	75
<b>Figure 4.2</b> Effect of d- and l-menthol on membrane fluidity of $L_0/L_d$ heterogeneous model membranes at Cholesterol 20 %. Typical confocal microscopy images of Laurdan emission (a), GP values of $L_0/L_d$ phase-separated membranes. Red and black region indicates $L_d$ and $L_0$ domains. Scale bar = 10 $\mu\text{m}$ . .....	76
<b>Figure 4.3</b> Effect of d- and l-menthol on membrane fluidity of $L_0/L_d$ heterogeneous model membranes at Cholesterol 30 %. Typical confocal microscopy images of Laurdan emission (a), GP values of $L_0/L_d$ phase-separated membranes. Red and black region indicates $L_d$ and $L_0$ domains. Scale bar = 10 $\mu\text{m}$ . .....	78
<b>Figure 4.4</b> Typical fluorescence microscopic images of $S_0$ domain (a) $L_0$ domain (b), no domain (c). Scale bar = 10 $\mu\text{m}$ . .....	80
<b>Figure 4.5</b> Fraction of phase-separated structures in DOPC/DPPC/Chol/menthol systems at room temperature. We fixed DOPC:DPPC:Chol = 1:1:0 in (a), 2:2:1 in (b), and 7:7:6 in (c). The mole fraction of menthol is 10 %. White, black, and grey bars indicate no domain (homogeneous phase), $S_0$ domain formation, and $L_0$ domain formation, respectively. Number of liposomes counted = 55. ....	80
<b>Figure 4.6</b> Miscibility temperature measurement for DOPC/DPPC/Chol/menthol systems. We fixed DOPC:DPPC:Chol = 1:1:0 in (a), 2:2:1 in (b), and 7:7:6 in (c). The mole fraction of menthol is 10 %. Black lines and black squares indicate DOPC/DPPC/Chol without menthol. Red lines with red circles and blue lines with blue triangles are DOPC/DPPC/Chol with d-menthol and l-menthol, respectively. (d) Change in the miscibility temperature as a function of Chol concentration in lipids defined as $[\text{Chol}]/([\text{DOPC}]+[\text{DPPC}]+[\text{Chol}])$ %.....	82
<b>Figure 4.7</b> Schematic illustration showing mechanism of interaction (upper panel) and localization preference exhibited by d- and l-menthol in three different systems. (a) DOPC/DPPC ( $S_0/L_d$ membrane), (b) DOPC/DPPC/Chol ( $L_0/L_d$ membrane at Chol = 20 %), and (c) DOPC/DPPC/Chol ( $L_0/L_d$ membrane at Chol = 30 %). ....	85

<b>Figure 4.8 Miscibility temperature measurement for DOPC/DPPC/Chol/menthol systems at menthol = 2.5 % in (a), 5 % in (b), 10 % in (c), 15 % in (d), and 30 % in (e). Black lines and black Squares indicate DOPC/DPPC/Chol without menthol. Red lines with red circles and blue lines with blue triangles are DOPC/DPPC/Chol with d-menthol and l-menthol, respectively.....</b>	<b>88</b>
<b>Figure 5.1 Typical HEK293 cells images of an untreated cell (control), after exposure of d- and l-menthol. Menthol concentration was 0.5 mM. Scale bar = 15 <math>\mu</math>m ....</b>	<b>101</b>
<b>Figure 5.2 Typical HEK293 cells images of an untreated cell (control), after exposure of d- and l-menthol with their respective intensity profile. Menthol concentration was 0.1 mM. Scale bar = 15 <math>\mu</math>m .....</b>	<b>102</b>
<b>Figure 5.3 Cell viability in the presence of d- and l-menthol on HEK293 cells at room temperature. ....</b>	<b>103</b>
<b>Figure 5.4 Effect of cholesterol on the interaction of d- and l-menthol with HEK293 cells. Representative confocal microscopy images showing the localization of menthol in cell membranes. Scale bar = 15 <math>\mu</math>m.....</b>	<b>105</b>
<b>Figure 5.5 Cell viability upon a change in membrane cholesterol content in (a) control system, (b) d-menthol loaded HEK293 cells, and (c) l-menthol loaded HEK293 cells. ....</b>	<b>106</b>
<b>Figure 5.6 Change in the intracellular <math>Ca^{2+}</math> level in the absence and presence of d- and l-menthol. The graph of Fluo-3 AM intensity versus component at (a) 17 <math>^{\circ}C</math>, (b) 27 <math>^{\circ}C</math>, and (c) 37 <math>^{\circ}C</math> respectively.....</b>	<b>108</b>
<b>Figure 5.7 Double staining of raft and F-actin in HEK293 cells. Images of HEK293 cells green region (raft), red region (F-actin) and bright region (DIC). (a) HEK 6.6 <math>\mu</math>M RP concentration in EtOH, (b) 126 nM RP concentration in EtOH, and (c) 126 nM RP concentration in PBS. Scale bar = 15 <math>\mu</math>m. ....</b>	<b>110</b>
<b>Figure 5.8 Double staining of raft and F-actin in HEK293 cells. Typical confocal images of HEK293 cells green region (raft), red region (F-actin) in the presence of d- and l-menthol at different temperatures. Scale bar = 10<math>\mu</math>m. ....</b>	<b>111</b>
<b>Figure 5.9 Hypothetical model representing the mechanism of raft dynamics and F-actin on menthol-induced HEK293 cells.....</b>	<b>112</b>



## CHAPTER 1 GENERAL INTRODUCTION

**Abstract:** In this chapter, a detailed background of the whole chapter has been pouring down. Recent studies on the biological membrane, lipid phase behavior, artificial membrane, menthol activity and the mode of a pathway in the cold sensitization are highlighted. The role of menthol as a cooling agent was well established in the sensory TRP channels. However, the related mechanism at molecular/atomic level is still in dark. Current understanding in the field of signal transduction through ion gating will deliver more fundamental approach further. Motivated by the background of these gating mechanisms, I aimed to study deeper and emphasize on basic fundamentals of biological membrane.

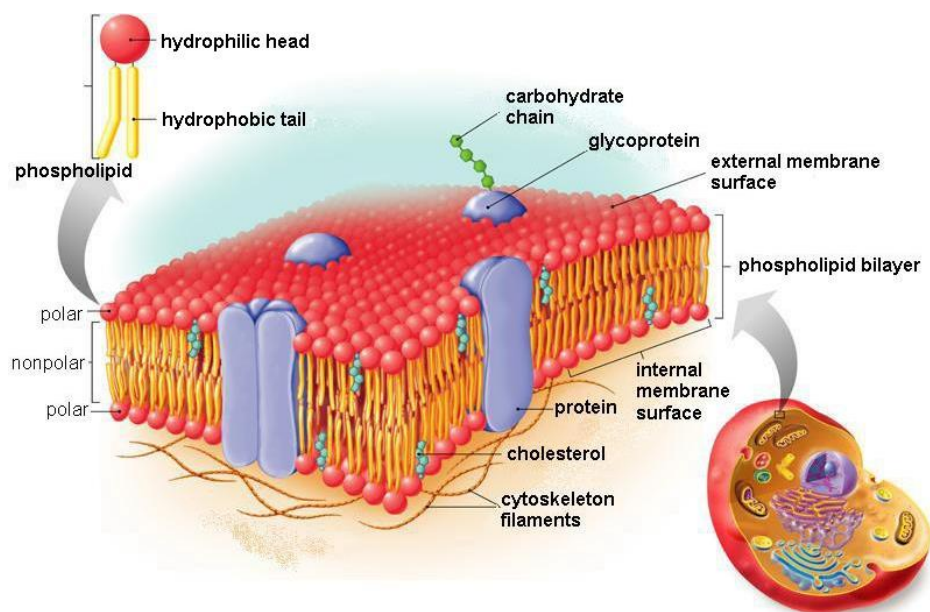
**Keywords:** Lipids, Phase behavior, Lipid raft, Menthol, Cooling sensation, *TRP*, *TRPM8*

## 1.1 Introduction

### 1.1.1 Biological membrane

The biological membrane acts as a selective barrier which enables to separate extracellular matrix from intracellular. Biological membranes are also involved in cell communication facilitated via chemical and electrical signals. The membrane is made up of three major components such as lipids, proteins, and sugar. Phospholipids are the essential component and considered as the backbone of the membrane. The phospholipids have the ability to assemble them to a most stable state.<sup>1</sup> All biological membranes exhibit general structure where proteins are connected with phospholipids bilayer sheet. In 1972, Singer and Nicolson gave us the new eye to the membrane as “The fluid mosaic model” which describes the dynamic and fluid nature of biological membranes.<sup>2,3</sup> It was best describes as two-dimensional model composed of a phospholipids bilayer embedded with various protein molecules within it. Membrane proteins present in the biological membrane are of two types “intrinsic” and “extrinsic” protein. Proteins which are associated into the bilayer and move from one layer to other called intrinsic proteins. On the other hand, extrinsic proteins are those that embedded on the lipid/water interface and are loosely held on a membrane. As the extrinsic proteins are held loose enough the resultant structure becomes highly fluid.

Lipids are amphiphilic in nature and serve as the basic fluid structure to the membrane. Lipid bilayer formed by self-assembly of various amphiphilic lipids.<sup>4</sup> In cell membrane, three major lipids are found namely, glycerophospholipids, cholesterol and sphingolipids (Fig 1.2). These phospholipids have a polar head part and a non-polar tail part which can be saturated or unsaturated in nature. There are two common abundant phospholipids found in the cell membrane name as unsaturated lipid DOPC and saturated lipid DPPC. These two lipids are commonly employed for the preparation of artificial membrane or biomimetic membrane.

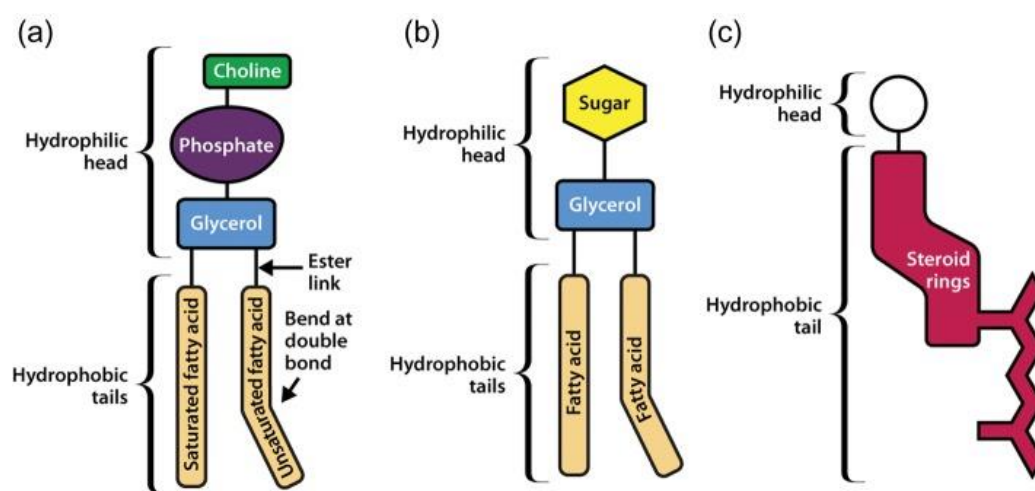


**Figure 1.1 Schematic representation of the biological membrane.<sup>5</sup>**

The head parts of the lipid are hydrophilic in nature and are exposed to the lipid/water interface in the bilayer. On the other hand, non-polar part is buried into the bilayer away from an aqueous phase.

Lipid bilayer possesses another very important physical property i.e “membrane fluidity” and “phase behavior”<sup>4</sup> of individual lipid. The biological membrane is not static rather moves along the surface of the water. The fluidity changes with temperature and the resulting response is known as phase behavior of the lipid bilayer. There are three different types of movement’s in the lipid bilayer as lateral, rotational and flip-flop movement, leads to the change in membrane fluidity. Apart from the membrane fluidity, phase transition temperature ( $T_m$ ) affects the packing of the membrane. At a certain temperature membrane changes from solid gel phase to liquid phase. There are several factors on which membrane fluidity depends:

- Temperature:
- Phase transition temperature
- Unsaturated fatty acids
- Cholesterol
- Protein interactions



*Figure 1.2 Schematic representations of three types of membrane lipids (a) glycerophospholipid, (b) sphingolipids, and (c) cholesterol.<sup>1</sup>*

## 1.2 Function of biological membrane

### 1.2.1 Barrier function

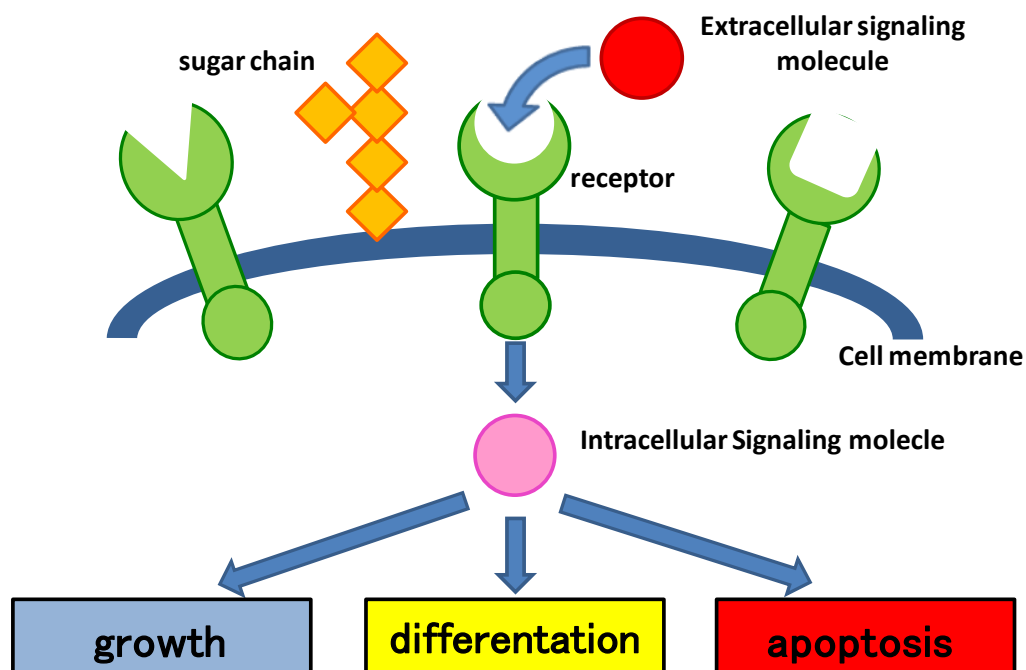
The biological membrane provides a barrier: an essential function of the cell helps to separate the inner environment from its outer environment by a plasma membrane. As described above, lipids are the basic components of biological membranes, small solutes such as water, ethanol, oxygen, etc., can pass through a lipid bilayer by simple diffusion process following the concentration gradient.<sup>6</sup> Similarly, some of the water-soluble ions such as  $\text{Na}^+$ ,  $\text{K}^+$ , sugars and amino acids, however, cannot pass through the membrane.

The plasma membrane has protein barrier, are of two types “transporters” and “channels” for specific molecules in order to pass through the barrier. There are two types of transport across the plasma membrane such as passive transport (simple diffusion, facilitated diffusion and active transport. In the case, passive transport requires no energy to move molecules across the membrane and moves from high concentration gradient to low concentration gradient. On contrary, active transport requires energy in the form ATP and moves molecules from low to high concentration.<sup>7</sup> An example of active transport is a  $\text{Na}^+$ - $\text{K}^+$  pump, where  $\text{Na}^+$  pumps out of the cell and  $\text{K}^+$  in against the concentration gradient. Another path is the channel proteins that are embedded in the plasma membrane and have pore to facilitate the flow of molecules. These carrier proteins can change shape or drag molecule through lipid bilayer to move easily across the membrane.

### *1.2.2 Signal transduction*

Every organism adapts different behaviours in response to the change in the environment. These respond can be induced through various physical and chemical stimuli such as pH, temperature. It has been now years and many researchers gave new frontier to the receptor proteins present on plasma membrane surface. Those molecules that bind specifically to these receptors are called signalling molecules. When such molecules, bind to the receptors, they go through structural changes and hence are activated.<sup>8</sup> As a result of which changes in the physiochemical properties of shape, movement, and functions of the cell happens.<sup>9</sup> Although various physical and chemical stimuli exist in the environment, each organism responds only to particular stimuli. Each receptors protein is activated by selective stimuli and hence, enables the signal detection and communication mechanisms of their cells. Through the analyses of the genes and proteins which are involved in signal transduction provide a better understanding of the basic mechanisms of cellular regulation.

Intercellular signalling molecules include hormones, cytokines lead to growth, differentiation, and apoptosis of the cell (Fig 1.3). However, extracellular signalling molecules act on cell surface receptor as a ligand. They bind to the specific target receptor causes a conformational change in the receptor, thus mediate signal to specific responses.



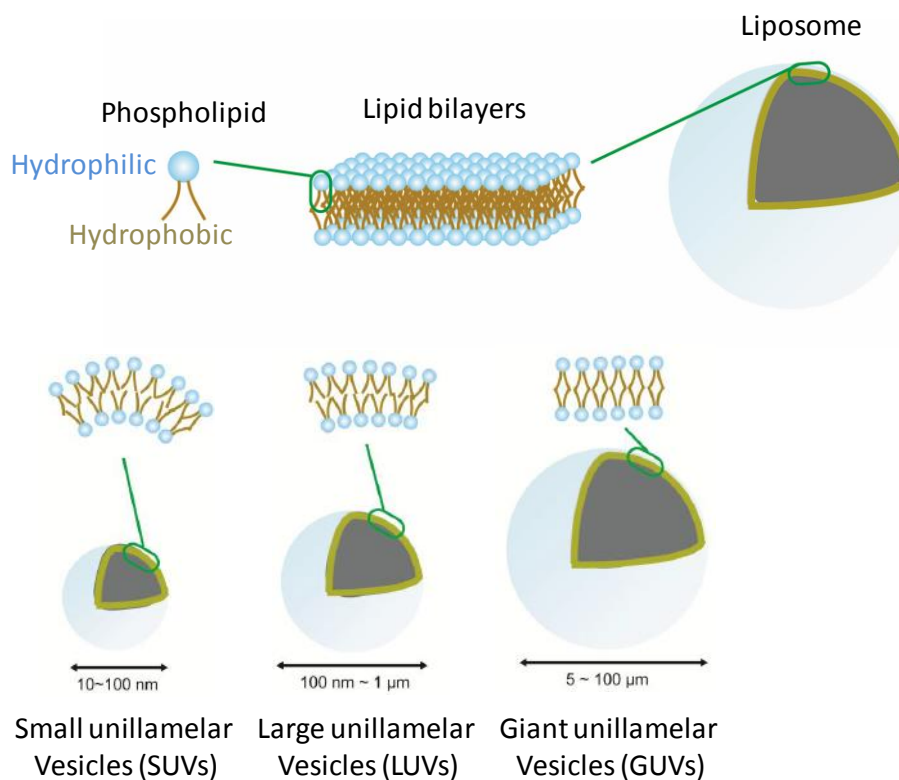
*Figure 1.3 Process involved in signal transduction.*

### 1.3 Artificial membrane

Since biological membrane plays a very crucial role in terms of separating their inner matrix from outer, and vary largely from composition to their structure. Their organization must be dynamic in nature to modulate some of the important function such as signal transduction; membrane trafficking, etc., for cell-cell communication. Due to the huge complexity of biological membrane, alternative model membrane needed with the similar composition and structure to that of an actual membrane. Thus, model membranes (artificial membrane) are dynamic in nature and can be visualized by optical microscopy. Many

researchers made progress on the different model membrane and their utility to solve the problems ranging from phase behavior to sensing pathways.<sup>10</sup> Model membrane systems are classified into four main categories: (1) Langmuir monolayers,<sup>11</sup> (2) hybrid model system,<sup>12</sup> (3) supported bilayers,<sup>12</sup> and (4) lipid vesicles. Cell-sized lipid vesicles<sup>13</sup> commonly referred as liposomes attracts a great attention to mimic actual cell as they exhibit similar structure and composition.<sup>14</sup> These liposomes are composed of either single lipid (homogeneous membrane) or multi lipids (heterogeneous membrane). They can be of three types depending on their size distribution namely, Small Unilamellar vesicles (SUVs), Multi lamellar vesicles (MLVs), and Giant Unilamellar vesicles (GUVs)<sup>15</sup>. There are some disadvantages of SUVs and MLVs such as resolution limitation with optical microscopy. Also, due to their relatively small size, they are not stable hence; it is very hard to trace time-dependent change. Therefore, GUVs attained great importance in order to allow real-time observation using optical microscopy. Liposomes are used as model membranes for different research purpose and used as a vehicle to deliver drugs to particular organs of the body.<sup>16</sup>

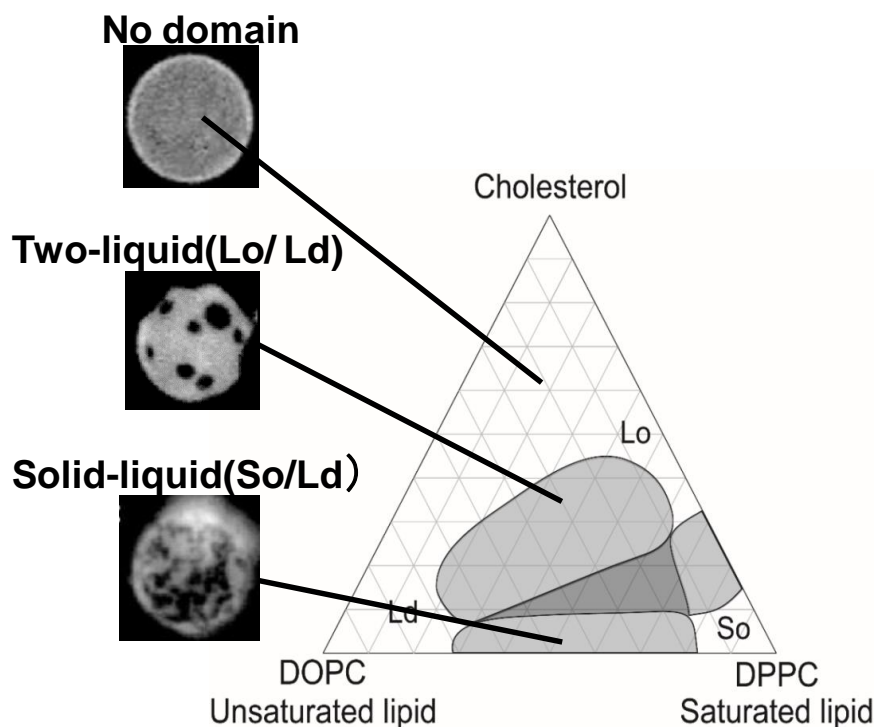
Recently, heterogeneous phase-separated membrane regarded as “raft region” gain significant attention in terms of analyzing different lipid phase behavior. Raft region is highly enriched in sphingolipids, saturated lipid, and cholesterol.<sup>17</sup> The heterogeneous distribution of membrane lipids was suggested to form phase-separated structure, and this structure is called “lipid raft”. The lipid rafts are known to involve various membrane proteins such as channels and receptors. Therefore, rafts are regarded as important platforms for cellular signaling.<sup>18</sup> Indeed, current studies revealed that some sodium channels in the cold/pain pathway are localized in lipid raft. Then property changes of raft structure should affect the functions of these channels. All different types of eukaryotic membranes supposed to exhibits different phase behaviors.<sup>19,20</sup>



**Figure 1.4 Structure of Liposomes and different vesicles formation depending on the size.**

Liposomes with multiple components, we can easily observe heterogeneous phase-separated structures as shown Fig 1.5. In the case of binary components with DOPC/DPPC, Solid order/Liquid disordered ( $S_o/L_d$  in short) phase separation can be observed. On the other hand, ternary components with DOPC/DPPC/Cholesterol, Liquid order/ Liquid disorder phase separation ( $L_o/L_d$  in short) can be observed. This  $L_o/L_d$  phase-separated structure is “membrane raft model” because of similarity in their components.<sup>21</sup> As shown in the figure of the phase diagram, phase-separation is strongly affected by lipid composition. Also, cholesterol plays a very important role in the phase behavior of membrane depending on its concentration. At higher cholesterol concentration than 40 %, no domain structure can be observed.<sup>22</sup>



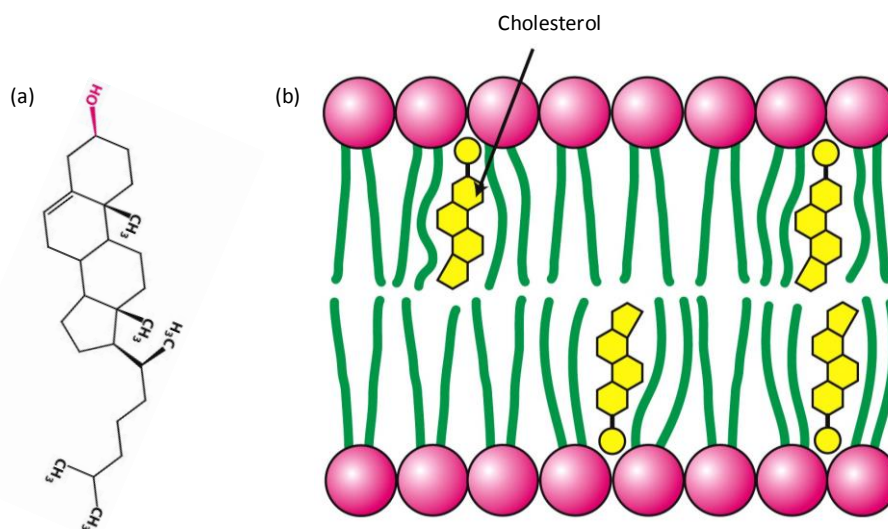


*Figure 1.5 Phase behavior of an artificial membrane in three-component system composed of DOPC, DPPC, and Cholesterol.<sup>23</sup>*

#### 1.4 Cholesterol in the Plasma membrane

Cholesterol is an abundant molecule found in the plasma membrane of every eukaryotic organism. It falls into the class of sterol, consisting of a rigid four fused tetracyclic ring, a polar hydroxyl group, and non-polar side chain. Cholesterol is the one of the crucial components of the biological membrane which helps to maintain the integrity of lipid raft membrane.<sup>24</sup> Another major function of the cholesterol is to modulate physicochemical properties of the membrane. The plasma membrane contains around 30~40 % of cholesterol and varies greatly depending on its lipid composition and cholesterol level.<sup>25</sup> In the lipid bilayer, cholesterol fits into the void created by the double bond of phospholipids.<sup>26</sup> Due to the presence of the rigid fused tetracyclic ring structure, the hydrophobic part (steroid ring) is situated close to the fatty acid chain of the phospholipids. On the other hand, the hydrophilic

part i.e, a polar hydroxyl group of cholesterol is in close proximity to the head group of the phospholipids. It also helps in the immobilization of the neighbouring phospholipids thereby makes it less soluble to small water-soluble molecules. Hence, provide more ordered state /tighten lipid packing and maintains membrane fluidity. Without cholesterol, cell membrane becomes too fluid in nature, hence permeable to some molecules. As discussed in above section 1.3, the biological membrane has raft region which is rich in cholesterol and saturated lipid, where cholesterol plays a very crucial role. It has been reported that cholesterol also plays important role in modulating the activity of the ion channels.<sup>27</sup> Cholesterol is necessary to maintain the functionality of lipid raft such as in signalling capacity. The intracellular lifestyles of the microorganism are affected by the regulation of cholesterol content. Some studies also point another property of cholesterol as a stabilizer of the oxytocin receptor and its binding affinity is strongly dependent on the cholesterol content present in the membrane.<sup>28,29</sup> However the role of cholesterol content in the sensing mechanism and modulating effect on interaction with external stimuli with lipid bilayer are poorly understood.



**Figure 1.6 (a) Chemical structure of cholesterol representing both hydrophilic (OH) and the hydrophobic region (fused ring) and (b) cholesterol localization in the lipid bilayer.<sup>30</sup>**

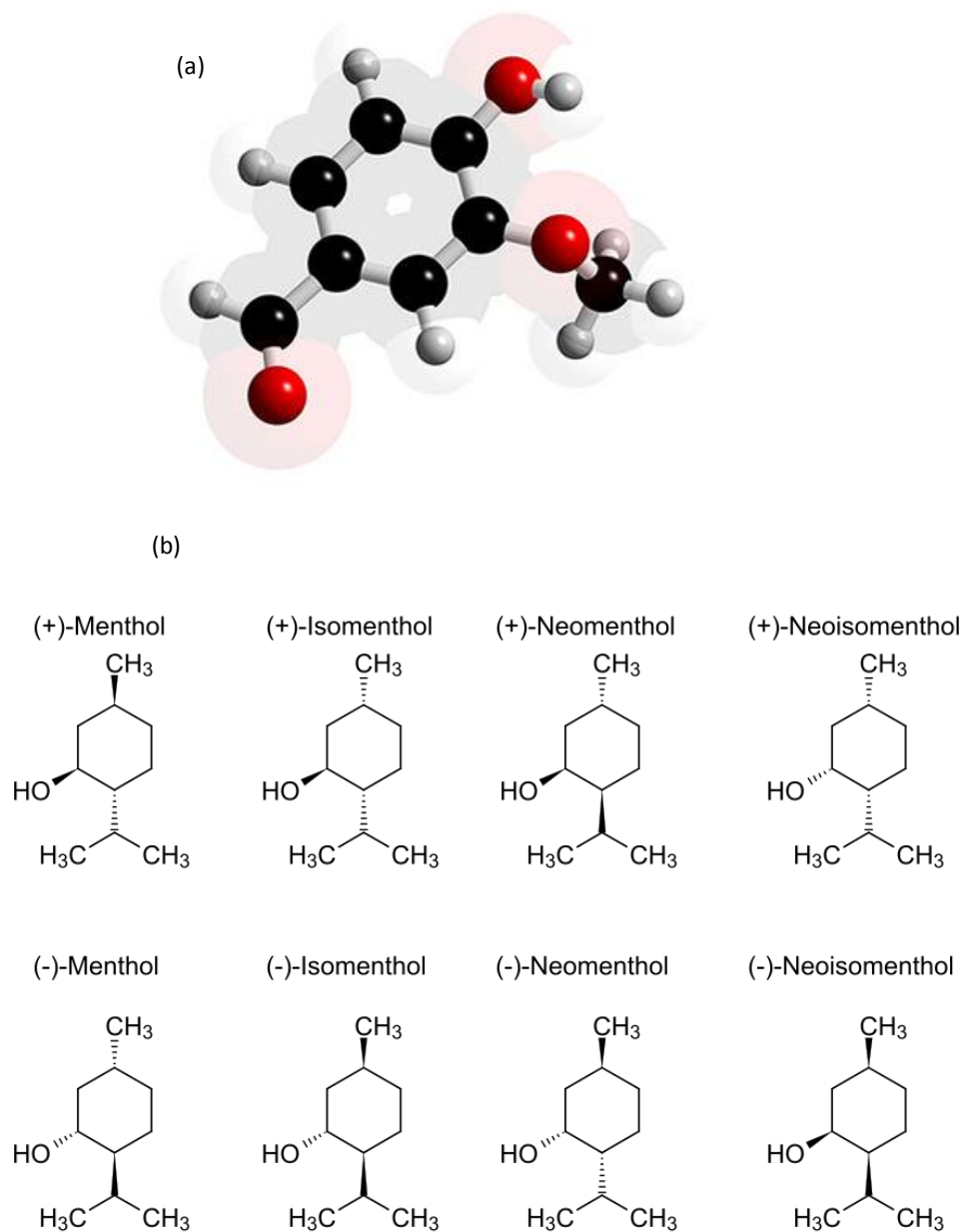
## 1.5 Menthol structure and Biological response

### 1.5.1 *Menthol structure*

Menthol is a six-member monocyclic monoterpenoid, secondary alcohol. It is extracted from various mint oil, and peppermint, or can be made synthetically. Menthol has waxy crystalline, white in colour physical appearance and is solid at room temperature. It exhibits three stereogenic centres ( $n = 3$ ), which gives eight stereoisomers according to  $2^n$  rule, out of all, natural form of menthol is assigned as (1*R*,2*S*,5*R*) configuration. Menthol is widely used in commercial industries as a pain killer for minor throat irritation from gums to toothpaste to vapour rubs.<sup>31</sup> The local anaesthetic qualities have been reported for menthol and other stimulants.<sup>32</sup>

### 1.5.2 *Biological response of menthol*

From past decades, menthol is well known for its cooling sensitization<sup>34</sup> and soothing properties to the body. Menthol has the ability to trigger the cold-sensitive transient receptor potential melastatin (*TRPM8*) ion channel via an electrical signal to the brain in order to sense cold.<sup>35</sup> It is also believed that the analgesic properties of menthol are modulated by activation of kappa opioid receptors.<sup>36</sup> Menthol displays different behaviour in a concentration-dependent manner. It was reported that at low concentration of menthol and low temperature, exerts cooling sense whereas at higher concentration and temperature burning/irritation evoked.<sup>34,37</sup> Generally, menthol activates the *TRPM8* channel, which leads to the opening of the  $\text{Na}^+$  and  $\text{Ca}^{2+}$  ion gate. As a result, there is the generation of an action potential, eventually, sense to the cooling phenomenon.<sup>38</sup> The detailed review of these sensing channels is discussed in later section.



**Figure 1.7 (a) 3D model of menthol and (b) types of a stereoisomer of menthol. <sup>33</sup>**

Due to its soothing properties, it is widely used in food, cosmetics, medicine, and in dental care, etc. Some of the application include as follows:

- Short-term minor throat irritation (balm, Vicks candy).

- Widely used in smoking<sup>39,40,41,42</sup> product inducing cooling relief and flavour.
- Because of certain analgesic properties<sup>43</sup>, it has been used in pain relief such cramps, headache.
- Many perfumery companies used menthol as an additive for fragrance.
- In dental care and oral hygiene, menthol products are extensively used for bad breath. Examples are mouthwash, toothpaste, chewing gum, mouth spray and so on.<sup>44</sup>

## 1.6 Transient Receptor Potential (TRP)

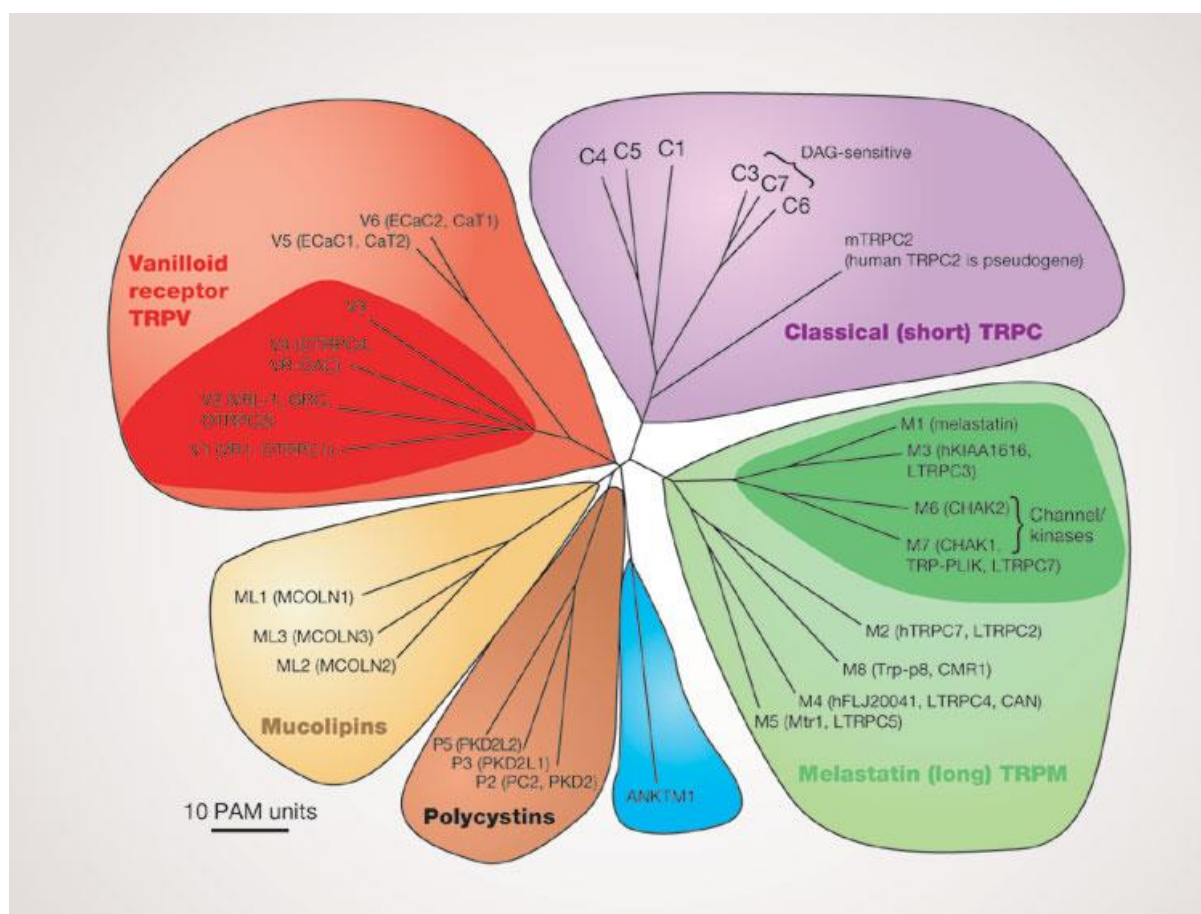
The first discovery of transient receptor potential (TRP) channels was observed in the fruit fly *Drosophila* in 1989. It was found that these channels are able to a response to by light stimuli and temperature change.<sup>45</sup> Recently, elaborate studies on TRP channels gave some of the crucial databases on sensing mechanism. TRP channels in short displays a group of ion channels which act as a platform for cellular sensors. In numerous mammalian cells, these TRP channels are located on the plasma membrane. TRP channel family further classified into seven subfamilies as shown in figure 1.8. The subfamilies are described as transient receptor potential canonical (TRPC)<sup>46</sup>, transient receptor potential vanilloid (TRPV)<sup>47</sup>, transient receptor potential melastatin (TRPM)<sup>48</sup>, transient receptor potential (TRPN), and transient receptor potential (TRPA)<sup>49</sup>, transient receptor potential polycystic (TRPP), and transient receptor potential mucolipin (TRPML).<sup>50</sup> TRP channels are composed of six trans membrane helices S1-S6, where S1-S4 is voltage sensors and S5-S6 are connecting loops, with intracellular N- and C-terminal. These channels induced different types of sensitization on the body such as, warmth, cold, pain and anesthetic.<sup>51</sup> Moreover, TRP channels are believed to act as body's own thermometer to sense cold and hot.

TRP channels are activated and regulated by a wide variety of physical and chemical stimuli and are expressed throughout the body. Some molecules activate specific TRP channels like menthol, chili, mustard, and allow the flow of ion  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  transport in the plasma membrane. Activation of the TRP channels leads to the generation of membrane action potential.<sup>52</sup> This phenomenon helps to translocate important signaling ions, initiation of enzymatic activity, and some process like endocytosis/exocytosis across the cell membrane.

Many mechanism pathways were introduced for the activity of TRP channels including (1) G-protein-coupled receptor-related mechanisms, (2) phosphorylation and (3) ubiquitination. Apart from these processes, some mechanisms of TRP channel gating such as “membrane voltage”, “membrane phospholipids”, and “ligands” are discussed below.

### ***Membrane voltage***

Due to different concentration and charged ion like  $\text{Na}^+$ ,  $\text{K}^+$  present in the cell membrane, generates its own potential know as membrane potential. A typical value of membrane potential inside the cell is around  $-60 \sim -70$  mV. There are two ways for the activation of TRPs can be either induced by temperature-dependent or voltage-dependent.<sup>54</sup> Most of the TRP channels in mammals has voltage dependence and are very sensitive to the external triggers like ligands, pH or change in ambient temperature. TRP channels consist of six transmembrane proteins linked with voltage-gated potassium channels.<sup>52</sup> Among six transmembrane segments, S4 is the one which detects the changes in the electric field and in turn exerts response immediately across the membrane. The shift in the voltage dependence activation results in the opening and closing of the channel. The voltage-dependent sensing to the gating of TRP channels responds to the temperature and chemical agonist stimulus.<sup>55</sup>



**Figure 1.8** Phylogenetic trees of human TRP channels.<sup>53</sup>

### **Membrane phospholipids**

Phospholipids are the major and essential component of the plasma membrane and many studies report an importance of membrane phospholipids in the regulation of TRP channel activity. Phospholipase C (PLC) is activated by the stimulation of receptor present on the plasma membrane. These PLC are one of the important regulators of the TRP channel activity.<sup>53</sup> Phosphatidylinositol 4,5-bisphosphate in short PtdIns(4,5)P<sub>2</sub> is believed to be sensitive to many TRP channels in the plasma membrane. The level of PtdIns(4,5)P<sub>2</sub> in the plasma membrane can change rapidly depending on the action of PLC isoforms and phosphatidylinositol kinases or phosphatases, hence directly or indirectly modulates channel activity of TRP.<sup>56</sup>

Activation of Ca<sup>2+</sup>-dependent PLC is promoted by an increase in intracellular Ca<sup>2+</sup> concentration leading to the reduction of cellular PtdIns(4,5)P<sub>2</sub>, accompanied by decaying of channel activity.<sup>57</sup> This inactivation of channel is caused by a shift in the voltage-dependence to a more positive potential leads to desensitization to such chemical agonist menthol for TRPM8. Other channel like those endolysosome-localized TRPML channels have direct interaction with PtdIns(3,5)P<sub>2</sub>, that results specific activation of channels.<sup>58</sup>

### ***Ligands***

A large number of endogenous ligands are responsible for the regulation of the TRP channel activities. *TRPV1* is a chili receptor and heat sensor (above 42 °C) and is activated by plant-derived pungent chemical such as capsaicin.<sup>59</sup> Another familiar chemical is menthol derived from mint plant responsible for the activation of *TRPM8*<sup>48</sup> channel. Moreover, these two channels *TRPV1* and *TRPM8* can sense a variety of other external stimuli like temperature. Noxious high temperature would directly activate *TRPV1*, on the other hand, cold temperature <25 °C for *TRPM8*.<sup>60</sup> Thus, activation of such sensory TRP channels can be potentiated through the application of both thermal and chemical stimuli. Several TRP channels are receptors for endogenous compounds. TRPCs respond to diacyl glycerol, a lipid product derived from PtdIns(4,5)P<sub>2</sub> breakdown catalyzed by PLC after a G-protein- or tyrosine-kinase-coupled receptor-dependent activation.<sup>61</sup> Arachidonic-acid-related compounds are involved in gating *TRPV1* (arachidonoyl ethanolamide, 12,15-(S)-hydroperoxyeicosatetraenoic acid and leukotriene B<sub>4</sub> ) and TRPV4 (5',6'-epoxieicosatrienoic acid ), and sphingosine, a primary part of sphingolipids, activates *TRPM3*.

#### *1.6.1 Effect of Cold and Menthol on TRPM8*

Transient receptor potential melastatin member 8 (*TRPM8*) is the class of melastatin family, also known as cold and menthol receptor 1 (CMR1).<sup>62</sup> *TRPM8* channel is believed to



act as thermosensors on nerve fibres of somatosensory neurons.<sup>63</sup> TRPM8 is a non-selective  $\text{Ca}^{2+}$  permeable ion channel which can be activated by cold below 25 degree Celsius and various cooling compounds such as menthol and icilin.<sup>31,64</sup> The ion channel is highly permeable sodium, potassium, cesium, and calcium. The TRPM8 channel is modulated by cold and chemical agonist<sup>65</sup> which are directly associated to voltage-dependent gating.<sup>66</sup> As mentioned in above section, activation of a TRPM8 ion channel, by temperature, will lead to depolarization and a shift in voltage-dependent activation curves observed. In a similar way, as a gating modifier, menthol shifts voltage-dependant activation curves towards physiological membrane potentials. l-Menthol has also shown strong inward rectification with high calcium selectivity in prostate cancer cells compared to that normal cells. It has been reported that menthol induces cooling sensation by activation of a TRPM8 ion channel. Tominaga.et.al stated that menthol exhibits an inhibitory effect on TRPM8 channel similar to thermal stimulation.<sup>67</sup> From past decades, menthol is being used in medicinal properties in traditional Chinese medicine. Menthol has been used for its anaesthetic; kappa opioid-mediated anti-nociceptive properties, to relieve minor aches and heartburns.<sup>31</sup> The effect of cold and menthol analgesic in rats and mouse has been extensive studying and showed that TRPM8 helps to mediate such effect.<sup>60</sup> TRPM8 activation further attributed to change in standard state enthalpy ( $\Delta H$ ) and entropy ( $\Delta S$ ) values directly related to channel opening by cooling phenomenon.<sup>68</sup> Different approaches have been speculated for the molecular pathways in cooling sensitization involving different temperature-dependant activation. They might be localized in the specialized domain within the channels. As mention above these specialized domains are known as lipid rafts which play a crucial role in modulating the channel activity. The lipid raft regulates the activity of such TRPM8 channels. Some of the studies revealed TRPM8 response induced by cold and menthol was increased right after the association of the channel with lipid rafts was prevented. Moreover, there is a shift in the

threshold for the activation of TRPM8 to higher temperature upon lipid raft disruption. The direct effect of PI(4,5)P<sub>2</sub> on channel activity has been well characterized for TRPM8.<sup>69</sup> Menthol-evoked currents in TRPM8 inside-out patch clamp recordings run down as consequence of the phosphatases that eliminate PI(4,5)P<sub>2</sub>.<sup>70,71</sup> Other group reported that pH also has the effect on the activation of TRPM8;<sup>72</sup> meaning that in an acidic medium it will inhibit the response of cold and menthol, whereas alkaline pH will induce.<sup>35</sup> Also, menthol may have different mechanism pathway than cold stimuli, as pH does not affect menthol response directly.

Recently, advance research on sensory TRP ion channels showed crucial role in the pain sensation. The effect of external stimuli on the activation of the TRP channels gave some of the important understanding in the signaling pathways. Ranging from a wide spectrum of temperature to chemicals, ion channel activity in the peripheral pain emerged as an important mediation in biological processes. However, there are still many unsolved issues at the molecular level and the behavior of gating mechanism are poorly understood.

## **1.7 Objective and Aim of the research**

Each part of the human body acts for the original roles and every role for each sense is far more crucial for their proper functioning. One of the important functions of our body is the sensing various external stimuli. The different sensitization includes cold, warm, anesthesia, etc. which is provoked by activation of some sensing channels. The activity of these channels is induced by external molecules leads to opening/closing of the channels, thus changes the physiological states of the membrane. Menthol, a chiral molecule, is one of the candidates, inducing cold sensitization to the body by activating a sub family of ion channels, termed as transient receptor potential melastatin 8 (TRPM8). Although many studies have been reported

about the interaction between menthol and membrane, the mechanism at the molecular level is still remains unclear.

This dissertation aimed to scratch physiological changes induced by menthol on the model membrane and related mechanism for its cooling behavior. More, the dissertation focused on the following contents:

1. To study the dynamic response of menthol on the thermo-induced model membrane.
2. Investigation of the influence of chirality on the interaction of d- and l-menthol with the lipid bilayer of a homogeneous model system.
3. To advance the study on chirality dependent interaction of d- and l-menthol localization in the lipid phase of a heterogeneous model system.
4. Elucidation of the effect of menthol and temperature-dependent interaction with the biological membrane (HEK293 cells).

The results of this dissertation provide fruitful information about the interaction of menthol with model biomembrane and biological membrane. Also, clarified the importance of chirality in the biological processes and shows their different mode of interaction on the membrane. For the future research, it will be highly supportive for resolving the unknown mystery of menthol mode of action on the membrane.

## **1.8 Outline of the Thesis**

This dissertation is divided into six chapters and their brief description is summarized below:

Chapter 1 extends a detailed background of the content of the thesis. I have provided brief information of the biological membrane and membrane dynamics including artificial

membrane and phase behavior in an artificial membrane. Recent studies on the TRP channel activation and menthol interaction with the channel were depicted. By understanding the current knowledge about thermo-sensation induced by several factors, the main aim of this study is to unravel mechanism at molecular level.

Chapter 2, I employed homogeneous membrane, cell-sized liposome in order to study whether menthol can directly interact with lipid bilayer. Further, change in the cell-sized liposomes morphology was conducted at a different concentration of menthol. In addition, the different behavior shown by menthol has been reported depending on its concentration.

Chapter 3 displays influence of chirality on physiological properties of the model membrane. I used two isoforms of menthol d- and l-, highlighting the significant difference observed between them. A remarkable change in the fluctuation profile, phase transition temperature and membrane fluidity of lipid bilayer induced by d- and l-menthol were presented. This explains the influence of chirality on menthol-membrane interaction.

Chapter 4 investigates the localization of d- and l-menthol in lipid raft of a heterogeneous model membrane. The importance of cholesterol in modulating the lipid phase behavior induced by d- and l-menthol was also estimated.

Chapter 5 describes how temperature affects the interaction of menthol with the biological membrane (HEK293). Among d- and l-menthol, d-menthol decreases the cell viability suggesting it's toxicity to the cell membrane. The temperature effect paves a clear difference in the association of menthol (d- and l-) with HEK293 cell membrane. The highlight of this chapter is to correlate the studies obtained from the model membrane and provide a better understanding to sensing mechanism.

Chapter 6 summarizes conclusion obtained from the studies.

## **1.9 References**

1. Watson, H. Biological membranes. *Essays Biochem.* **59**, 43–69 (2015).
2. Singer, S. J. & Nicolson, G. L. The Fluid Mosaic Model of the Structure of Cell Membranes. *Science (80-. )*. **175**, 720 LP-731 (1972).
3. Nicolson, G. L. The Fluid—Mosaic Model of Membrane Structure: Still relevant to understanding the structure, function and dynamics of biological membranes after more than 40 years. *Biochim. Biophys. Acta - Biomembr.* **1838**, 1451–1466 (2014).
4. van Meer, G., Voelker, D. R. & Feigenson, G. W. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* **9**, 112–124 (2008).
5. Companies, M.-H. Cell structure.
6. Trimble, W. S. & Grinstein, S. Barriers to the free diffusion of proteins and lipids in the plasma membrane. *J. Cell Biol.* **208**, 259 LP-271 (2015).
7. Skou, J. C. in *Symposium on Biophysics and Physiology of Biological Transport: Frascati, June 15--18, 1965. Proceedings* (eds. Bolis, L., Capraro, V., Porter, K. R. & Robertson, J. D.) 303–308 (Springer Berlin Heidelberg, 1967). doi:10.1007/978-3-662-25134-8\_61
8. Gonzalez-Gaitan, M. Signal dispersal and transduction through the endocytic pathway. *Nat Rev Mol Cell Biol* **4**, 213–224 (2003).
9. Krauss, G. in *Biochemistry of Signal Transduction and Regulation* 119–147 (Wiley-VCH Verlag GmbH, 2001). doi:10.1002/3527600051.ch3
10. Chan, Y.-H. M. & Boxer, S. G. Model Membrane Systems and Their Applications. *Curr. Opin. Chem. Biol.* **11**, 581–587 (2007).
11. Brezesinski, G. & Möhwald, H. Langmuir monolayers to study interactions at model

- membrane surfaces. *Adv. Colloid Interface Sci.* **100–102**, 563–584 (2003).
12. Plant, A. L. Supported Hybrid Bilayer Membranes as Rugged Cell Membrane Mimics. *Langmuir* **15**, 5128–5135 (1999).
  13. Hamada, T. & Yoshikawa, K. Cell-Sized Liposomes and Droplets: Real-World Modeling of Living Cells. *Materials* **5**, (2012).
  14. Akbarzadeh, A. *et al.* Liposome: classification, preparation, and applications. *Nanoscale Res. Lett.* **8**, 102 (2013).
  15. Viallat, A., Dalous, J. & Abkarian, M. Giant Lipid Vesicles Filled with a Gel: Shape Instability Induced by Osmotic Shrinkage. *Biophys. J.* **86**, 2179–2187 (2004).
  16. Lasic, D. D. Novel applications of liposomes. *Trends Biotechnol.* **16**, 307–321 (1998).
  17. Simons, K. & Ikonen, E. Functional rafts in cell membranes. *Nature* **387**, 569–572 (1997).
  18. Simons, K. & Toomre, D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1**, 31–39 (2000).
  19. Heberle, F. A. & Feigenson, G. W. Phase Separation in Lipid Membranes. *Cold Spring Harb. Perspect. Biol.* **3**, a004630 (2011).
  20. Feigenson, G. W. Phase Boundaries and Biological Membranes. *Annu. Rev. Biophys. Biomol. Struct.* **36**, 63–77 (2007).
  21. Ionova, I. V., Livshits, V. A. & Marsh, D. Phase Diagram of Ternary Cholesterol/Palmitoylsphingomyelin/Palmitoyl-oleoyl-Phosphatidylcholine Mixtures: Spin-Label EPR Study of Lipid-Raft Formation. *Biophys. J.* **102**, 1856–1865 (2012).
  22. Feigenson, G. W. Phase behavior of lipid mixtures. *Nat. Chem. Biol.* **2**, 560–563

- (2006).
23. Hamada, T., Kishimoto, Y., Nagasaki, T. & Takagi, M. Lateral phase separation in tense membranes. *Soft Matter* **7**, 9061–9068 (2011).
  24. Krause, M. R. & Regen, S. L. The Structural Role of Cholesterol in Cell Membranes: From Condensed Bilayers to Lipid Rafts. *Acc. Chem. Res.* **47**, 3512–3521 (2014).
  25. McMullen, T. P. W., Lewis, R. N. A. H. & McElhaney, R. N. Cholesterol–phospholipid interactions, the liquid-ordered phase and lipid rafts in model and biological membranes. *Curr. Opin. Colloid Interface Sci.* **8**, 459–468 (2004).
  26. Olkkonen, V. M. & Hynynen, R. Interactions of oxysterols with membranes and proteins. *Mol. Aspects Med.* **30**, 123–133 (2009).
  27. Gimpl, G., Burger, K. & Fahrenholz, F. Cholesterol as Modulator of Receptor Function. *Biochemistry* **36**, 10959–10974 (1997).
  28. Klein, U., Gimpl, G. & Fahrenholz, F. Alteration of the Myometrial Plasma Membrane Cholesterol Content with .beta.-Cyclodextrin Modulates the Binding Affinity of the Oxytocin Receptor. *Biochemistry* **34**, 13784–13793 (1995).
  29. KLEIN, U. & FAHRENHOLZ, F. Reconstitution of the myometrial oxytocin receptor into proteoliposomes. *Eur. J. Biochem.* **220**, 559–567 (1994).
  30. Company, W. H. F. and. *Lipids and cell membrane outline.* (2012).
  31. ECCLES, R. Menthol and Related Cooling Compounds. *J. Pharm. Pharmacol.* **46**, 618–630 (1994).
  32. Lee, Y. O. & Glantz, S. A. Menthol: putting the pieces together. *Tob. Control* **20**, ii1-ii7 (2011).

33. Interent source. Menthol structure.
34. Cliff, M. A. & Green, B. G. Sensory irritation and coolness produced by menthol: Evidence for selective desensitization of irritation. *Physiol. Behav.* **56**, 1021–1029 (1994).
35. Andersson, D. A., Chase, H. W. N. & Bevan, S. TRPM8 Activation by Menthol, Icilin, and Cold Is Differentially Modulated by Intracellular pH. *J. Neurosci.* **24**, 5364 LP-5369 (2004).
36. Galeotti, N., Di Cesare Mannelli, L., Mazzanti, G., Bartolini, A. & Ghelardini, C. Menthol: a natural analgesic compound. *Neurosci. Lett.* **322**, 145–148 (2002).
37. BG, G. Menthol modulates oral sensations of warmth and cold. *Physiol Behav* **35**, 427–34 (1985).
38. Reid, G., Babes, A. & Pluteanu, F. A cold- and menthol-activated current in rat dorsal root ganglion neurones: properties and role in cold transduction. *J. Physiol.* **545**, 595–614 (2002).
39. McCarthy, W. J. *et al.* Menthol vs nonmenthol cigarettes: effects on smoking behavior. *Am. J. Public Health* **85**, 67–72 (1995).
40. Anderson, S. J. Menthol cigarettes and smoking cessation behaviour: a review of tobacco industry documents. *Tob. Control* **20**, ii49 LP-ii56 (2011).
41. Hoffman, A. C. The health effects of menthol cigarettes as compared to non-menthol cigarettes. *Tob. Induc. Dis.* **9**, S7–S7 (2011).
42. Klausner, K. Menthol cigarettes and smoking initiation: a tobacco industry perspective. *Tob. Control* **20**, ii12 LP-ii19 (2011).



43. Green, B. G. & McAuliffe, B. L. Menthol desensitization of capsaicin irritation: Evidence of a short-term anti-nociceptive effect. *Physiol. Behav.* **68**, 631–639 (2000).
44. Dessirier, J.-M., O'Mahony, M. & Carstens, E. Oral irritant properties of menthol: sensitizing and desensitizing effects of repeated application and cross-desensitization to nicotine. *Physiol. Behav.* **73**, 25–36 (2001).
45. Vay, L., Gu, C. & McNaughton, P. A. The thermo-TRP ion channel family: properties and therapeutic implications. *Br. J. Pharmacol.* **165**, 787–801 (2012).
46. Salido, G. M., Sage, S. O. & Rosado, J. A. TRPC channels and store-operated Ca<sup>2+</sup> entry. *Biochim. Biophys. Acta - Mol. Cell Res.* **1793**, 223–230 (2009).
47. Tamara Rosenbaum and Sidney A. Simon. *TRPV1 Receptors and Signal Transduction*. (CRC Press/Taylor & Francis, 2007).
48. McKemy, D. D. in (ed. Heller S)
49. Marilia Z. P. Guimaraes and Sven-Eric Jordt. *TRPA1 : A Sensory Channel of Many Talents*. (CRC Press/Taylor & Francis, 2007).
50. Nilius, B. & Owsianik, G. The transient receptor potential family of ion channels. *Genome Biol.* **12**, 218 (2011).
51. Clapham, D. E., Runnels, L. W. & Strubing, C. The trp ion channel family . *Nat Rev Neurosci* **2**, 387–396 (2001).
52. Zheng, J. Molecular Mechanism of TRP Channels. *Compr. Physiol.* **3**, 221–242 (2013).
53. Clapham, D. E. TRP channels as cellular sensors. *Nature* **426**, 517–524 (2003).
54. Catterall, W. A. Ion Channel Voltage Sensors: Structure, Function, and Pathophysiology. *Neuron* **67**, 915–928 (2010).

55. Bezanilla, F. How membrane proteins sense voltage. *Nat Rev Mol Cell Biol* **9**, 323–332 (2008).
56. Divecha, N. & Irvine, R. F. Phospholipid signaling. *Cell* **80**, 269–278 (1995).
57. Liu, D. & Liman, E. R. Intracellular Ca(2+) and the phospholipid PIP(2) regulate the taste transduction ion channel TRPM5. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 15160–15165 (2003).
58. Tomohiro N, Kiyonaka S, K. K. in *TRP Channels* (ed. MX, Z.) (2011).
59. Cao, E., Cordero-Morales, J. F., Liu, B., Qin, F. & Julius, D. TRPV1 channels are intrinsically heat sensitive and negatively regulated by phosphoinositide lipids. *Neuron* **77**, 667–679 (2013).
60. Chuang, H., Neuhausser, W. M. & Julius, D. The Super-Cooling Agent Icilin Reveals a Mechanism of Coincidence Detection by a Temperature-Sensitive TRP Channel. *Neuron* **43**, 859–869 (2004).
61. Ufret-Vincenty, C. A., Klein, R. M., Hua, L., Angueyra, J. & Gordon, S. E. Localization of the PIP2 Sensor of TRPV1 Ion Channels. *J. Biol. Chem.* **286**, 9688–9698 (2011).
62. McKemy, D. D., Neuhausser, W. M. & Julius, D. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **416**, 52–58 (2002).
63. Bautista, D. M. *et al.* The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature* **448**, 204–208 (2007).
64. Dragoni, I., Guida, E. & McIntyre, P. The Cold and Menthol Receptor TRPM8 Contains a Functionally Important Double Cysteine Motif. *J. Biol. Chem.* **281**, 37353–

- 37360 (2006).
65. Peier, A. M. *et al.* A TRP Channel that Senses Cold Stimuli and Menthol. *Cell* **108**, 705–715 (2002).
  66. Brauchi, S., Orio, P. & Latorre, R. Clues to understanding cold sensation: Thermodynamics and electrophysiological analysis of the cold receptor TRPM8. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 15494–15499 (2004).
  67. Takaishi, M. *et al.* Reciprocal effects of capsaicin and menthol on thermosensation through regulated activities of TRPV1 and TRPM8. *J. Physiol. Sci.* **66**, 143–155 (2016).
  68. Jara-Oseguera, A. & Islas, L. D. The Role of Allosteric Coupling on Thermal Activation of Thermo-TRP Channels. *Biophys. J.* **104**, 2160–2169 (2013).
  69. Jan Benedikt, Jan Teisinger, L. V. and V. V. Ethanol inhibits cold-menthol receptor TRPM8 by modulating its interaction with membrane phosphatidylinositol 4,5-bisphosphate. *Jouranl Neurochem.* **100**, 211–224 (2007).
  70. Liu, B. & Qin, F. Functional Control of Cold- and Menthol-Sensitive TRPM8 Ion Channels by Phosphatidylinositol 4,5-Bisphosphate. *J. Neurosci.* **25**, 1674 LP-1681 (2005).
  71. Mahieu, F. *et al.* Modulation of the cold-activated cation channel TRPM8 by surface charge screening. *J. Physiol.* **588**, 315–324 (2010).
  72. Frank Mahieu, Annelies Janssens, Maarten Gees, Karel Talavera, B. N. and T. V. Modulation of the cold-activated cation channel TRPM8 by surface charge screening. *J. Physiol. Physiol.* **588**, (2010).

## CHAPTER 2 THERMO-INDUCED DYNAMICS OF MODEL CELL MEMBRANE BY ACTION OF MENTHOL

**Abstract:** It is important to understand the physicochemical mechanism that is responsible for the morphological changes in the cell membrane in the presence of various stimuli and sensory compounds. Menthol, popularly known for its cooling sensitization, activates TRPM8- a cold-activated thermo-TRP ion channel. We used cell sized synthetic liposomes that mimic actual cell structure to study the model cell membrane dynamics and the unclear mechanism behind the cooling sensation of menthol on the cell membrane. Hence, we are interested in the direct interaction of menthol with bio-membrane. We observed the effect of menthol on membrane dynamics in artificial membrane. It was also observed that menthol concentration plays an important role and have a significant effect on the model cell membrane. In homogeneous membrane, it has been shown that menthol tends to increase the fluctuation rate and also change in the membrane area also observed. It has been believed that menthol has a direct interaction with the model cell membrane and affects membrane physiochemical properties.

**Keywords:** Menthol, Membrane fluctuation, Surface area, Size

## 2.1 Introduction

Menthol, a secondary alcohol, which activates cold receptor, has cooling sense to the skin. It has been widely used in the commercial products and has been employed in medicine too.<sup>1,2</sup> Menthol is the oldest traditional Chinese medicine used to relieve minor aches, sunburns, flavoring agents.<sup>3,4</sup> It has the ability to trigger the brain upon channel activation and eventually responds to cooling sensitization<sup>3,4,5</sup> Many study still going on the behavior of menthol depending on its concentration. Previously, Green.et.al mentioned that menthol exhibits cooling sensitization at lower concentration and room temperature or below, whereas at higher concentration and temperature it starts producing irritation/pain sensation.<sup>6</sup> Although the mechanism underlying the behavior is still in dark and need to be explore at molecular level. Menthol in general activates the cold TRPM8 ion channel, facilitates the opening of the pore and thereby allows the entry of the ion into the channel.<sup>7</sup> The channel opening could be temperature-dependent<sup>8,9</sup> or voltage-dependent<sup>10</sup> in order to activate TRPs. The temperature sensitivity of these channels is still not clear but may be postulated by three mechanisms. (1) Temperature-dependent change leads to the production of channel-ligand activation. (2) Changes in the temperature may alter the structure of the channel proteins. (3) Lastly, TRPs might sense changes in the membrane tension as a function of temperature change. Menthol apart from TRPM8 activation, also activate TRPV3 and inhibits TRPA1.<sup>11,12</sup>

Cell-sized liposomes have been widely employed as bio-mimic membranes. Although the bilayer organization of cell membranes can be mimicked in artificial liposomes with a simple lipid composition, cell membranes contain thousands of different lipid species, whose cellular distribution is gently controlled. The lipid bilayer has the ability to self-assemble in order to produce desired morphology and physical properties of the membrane.<sup>13</sup> Liposomes are extensively employed in the drug delivery<sup>14</sup> as they are able to encapsulate aqueous and

hydrophobic molecules to deliver into the cells. Cell-sized liposomes can be formed from various phospholipids, among all the most common phospholipids used are: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS). Depending upon the size, cell-sized liposomes are categorized as SUVs, MLVs, and GUVs. Recently, GUVs gained a significant attention as model membrane to study the interaction of foreign molecules with membrane.<sup>15</sup> The size of liposome is determined by the balance of curvatures between the inner leaflet and outer leaflet with negative and positive curvatures, respectively. The membrane curvature strongly depends on the lipid compositions in each leaflet and the interaction between hydrophobic groups across the bilayer membrane. Therefore, it is important to study the effect of lipid composition on nature of liposomes.

Cholesterol contributes to a major part in cell membrane and it has capability of condensing lipids above transition temperature. However, the role of cholesterol in membrane dynamics has not been well understood. The presence of cholesterol can alter the properties of the membrane and governs the cellular signaling processes by regulating membrane proteins.<sup>16</sup> Cholesterol is critically important in the synapse formation as human brain consists of about 25 percent of cholesterol of the body. Cholesterol-containing membranes are less thermo-sensitive than the lipid membrane only. Though, cholesterol has been known to modulate the physical properties of the membrane, but its role in the cellular signaling is still in debate.

In eukaryotic cells, membrane-protein, ligand-ligand or ligand-protein interaction is needed to regulate cellular processes involved in signal transduction and membrane trafficking. Many evidences provide the role of lipid as foremost platform for recognition of direct interaction of membrane protein and externally added chemical. These plant-derived signaling molecules interact with the lipids directly or indirectly resulting into sensing. In

order to study those interactions with lipids, I advance my research on the physical properties change in the membrane induced by such chemical agonist. Cell-sized liposomes were employed as an artificial membrane to performed further research. Taking the advantage of size of these liposomes, it is possible to observe time-dependent changes in the individual liposomes. Thus, allows real-time observation by optical microscopy. Our laboratory extensively used cell sized liposomes to study effects of several kinds of stress and additives (like menthol) on morphological changes.

In this chapter I used model membrane to investigate thermo-induced effect of menthol. Neutral lipid DOPC and cholesterol was used to prepare the cell-sized liposomes in the presence of menthol at different concentration. The physiochemical changes such as membrane fluctuation, surface are, in the membrane upon menthol addition was demonstrated.

## **2.2 Experimental Procedures:**

### *2.2.1 Materials:*

Lipids 1, 2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and Cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, USA). (1*R*, 2*S*, 5*R*)-2-isopropyl-5-methylcyclohexanol (l-menthol) was purchased from Takasago Japan. Chloroform and methanol were from Kanto-Chemical (Japan) and Nacalai Tesque (Japan), respectively. Ultrapure water (specific resistance  $\geq 18 \text{ M}\Omega$ ) was obtained from a Millipore Milli-Q purification system.

### *2.2.2 Preparation of Liposomes:*

Homogeneous lipid vesicles were prepared by the natural swelling method.<sup>17</sup> Lipids, Chol, and menthol were dissolved in a 2:1 vol/vol (chloroform/methanol) solution, to a concentration 2 mM for lipids, Chol and menthol. Lipids, Chol, and menthol were mixed at

desired concentration to final volume 20  $\mu\text{L}$ . The organic solvent was evaporated under a flow of nitrogen gas and the lipids were further dried in a vacuum desiccator for 3 h. The film was hydrated with Milli-Q water at 37  $^{\circ}\text{C}$  for an hour and were kept overnight at room temperature  $21.7 \pm 1.7$   $^{\circ}\text{C}$ .

### *2.2.3 Microscopy Observations of membrane fluctuation:*

5  $\mu\text{L}$  of lipid vesicle solution was placed in a silicon well (0.2 mm) on a glass slide, and cover with cover slip. The use of silicon well and the coverslip ensured that evaporation of the solution did not occur during the entire experiment. The formed GUVs were observed by using phase contrast microscopy (Olympus BX50 Japan). The images were recorded on a hard disc drive at 30 frames/s. GUVs were prepared by unsaturated lipid DOPC, Chol, and menthol to form a homogeneous membrane. The stage temperature was carefully changed using a thermo controller (Tokai-Hit MATS-5550RA-BT; Japan), from 22 to 40  $^{\circ}\text{C}$ . The samples were then subjected to a temperature increase at a rate of 1.0  $^{\circ}\text{C}/\text{min}$  from 21 to 40  $^{\circ}\text{C}$ .

### *2.2.4 Effect of Temperature on Molecular Area of Monolayer Membranes:*

A Filgen LB-400 (Aichi, Japan) instrument (Kuhn type) was used to measure the  $\pi$ -A isotherms. DOPC/Chol (4:1) in the presence and absence of menthol was dissolved in chloroform to a final concentration of 2 mM lipids. 5  $\mu\text{L}$  of this solution was then added to Langmuir trough filled with 100 ml of Milli-Q pure water at each temperature. The temperature was controlled by attaching the thermo controller system to the instrument. Waiting for least 10 min, after each temperature was confirmed, the  $\pi$ -A isotherms were measured. The  $\pi$ -A isotherms were measured using the same procedure and conditions.



### 2.2.5 Dynamic Light Scattering (DLS) Measurements:

Lipid mixtures (DOPC, Chol, and Menthol) were dissolved in chloroform/methanol 2:1 (v/v) in a small brown glass bottles. The organic solvent was evaporated under a flow of nitrogen gas, and the lipids were further dried under vacuum for 3 h to form thin lipid films. The film was then hydrated with deionized water (1000 ml) and subjected to vortex for about 1 min. Finally, solutions were sonicated by ultrasonic cleaning machine at 50 °C for about 30 min, and then transferred to cuvettes for DLS measurements. The size distribution has been done by Zetasizer Nano ZS Malvern instrument.

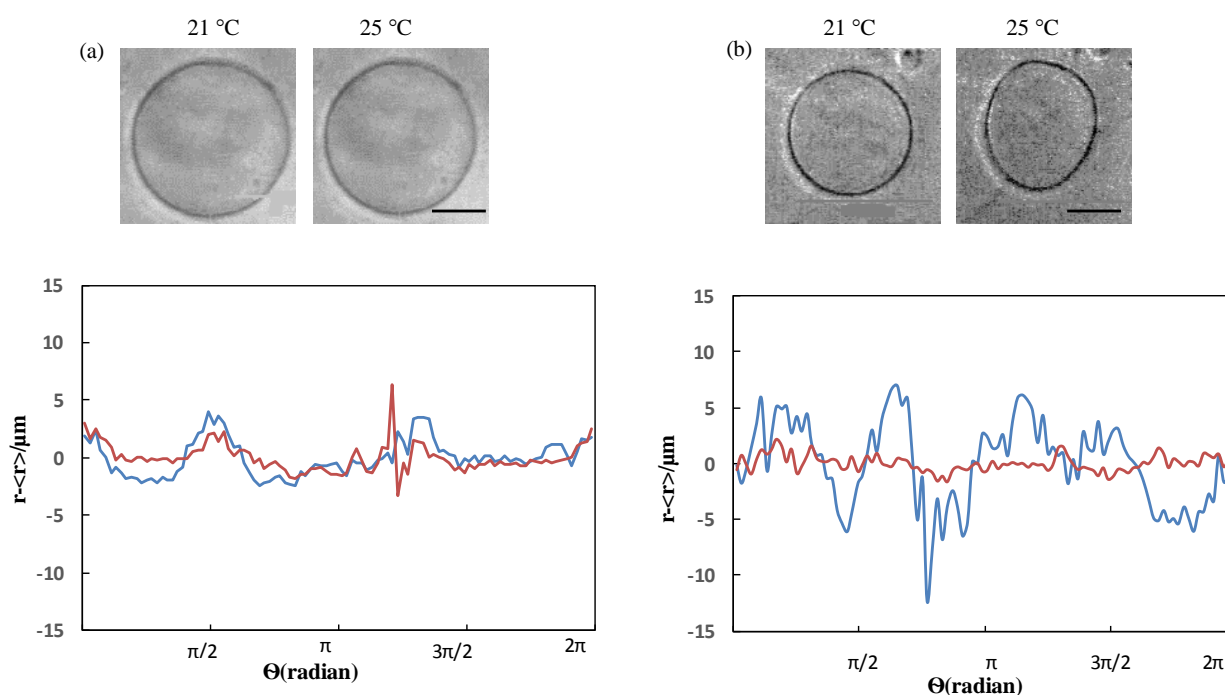
## 2.3 Results and discussion

### 2.3.1 Effect of menthol on membrane fluctuation

Using a natural swelling method to form dry lipid films, two types of system were prepared DOPC/Chol and DOPC/Chol/Menthol at various menthol concentration. The changes in membrane morphology were observed with the help of phase contrast microscopy (Olympus BX-50, Japan) at RT. Figure 2.1 show the typical microscopic images of DOPC/Chol and DOPC/Chol/Menthol along with the degree of membrane fluctuation. The vesicle consisting of DOPC/Chol retained the spherical shape, even if the temperature is increased from 21 °C to 25 °C. On the other hand, the membrane fluctuation was observed in DOPC/Chol/Menthol with same temperature change. In order to clarify this difference, the vesicle radii as a function of polar angle were summarized in Fig 2.1 (lower panel). I could found the larger radial fluctuation in the menthol-containing membranes.

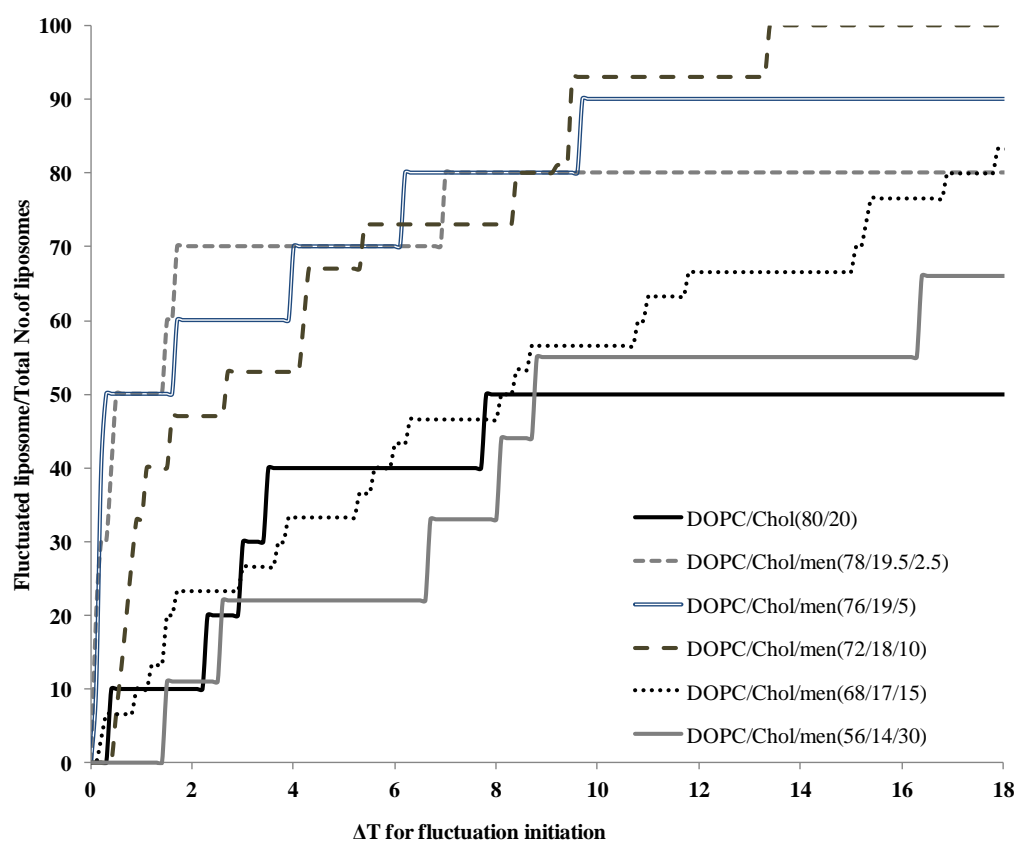
Previous studies showed that menthol has dual behaviors, at higher temperature and concentration it has a tendency to exhibit itching/burning sense while at low temperature and concentration has cool sense. From fluctuation data, I could observe the clear concentration

effect of menthol on the model membrane. The effect of menthol concentration on thermo-sensitive fluctuation of DOPC/Chol liposomes is depicted in Fig 2.2. At lower concentration of menthol, maximum fluctuation could be observed, while at higher concentration rate of fluctuation declined. Generally, the thermo-induced membrane fluctuation was caused by the acquisition of the excess membrane area. The absence of fluctuation in the DOPC/Chol vesicles suggested that the membrane area remain steady. Dynamic real-time observation of membrane dynamics revealed that the menthol-containing membrane was more thermo-sensitive than without menthol. The increase in the temperature enhances the number of water molecules near polar head groups, leading to an increase in steric repulsive interaction between them and results into the excess surface area of liposomes.



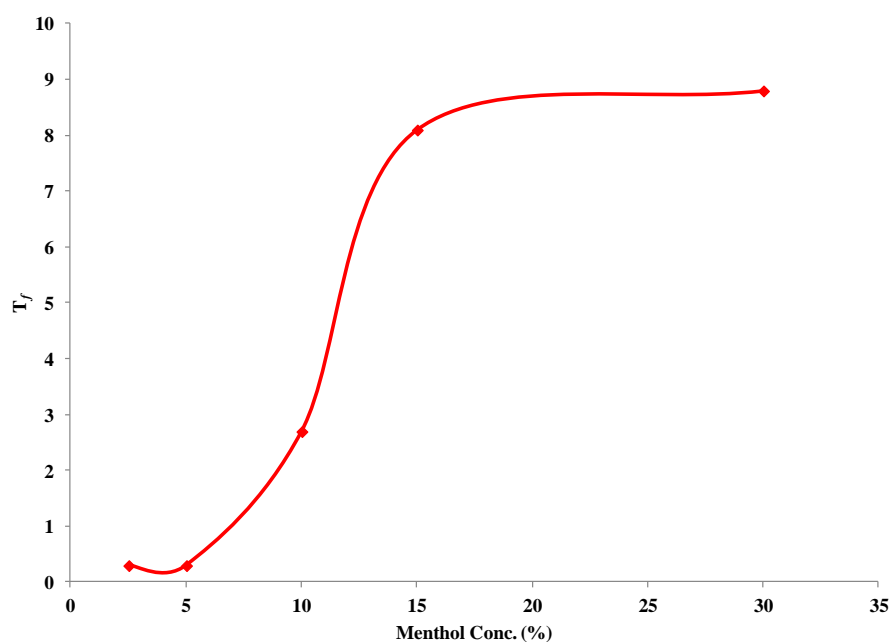
**Figure 2.1** Membrane fluctuation profile of lipid vesicles. Temperature was increased from RT to 40.0 °C, using a thermo controller. An image of a typical lipid vesicle was captured using a phase-contrast microscope. The graph show membrane fluctuation at 21 °C (red), 25 °C (blue) as a function of radius and its distribution. Plotted the value of radius of “r” in each “ $\theta$ ” ( $\theta = \pm \pi/n$ ,  $n = 1, 2, 3 \dots 100$ ). (a) DOPC/Chol and (b) DOPC/Chol/Menthol. Scale bar = 10  $\mu\text{m}$ .

As a consequence, liposomes started fluctuating depending on the hydrophilicity of the head group. DOPC/Chol/Menthol-containing liposomes, menthol has a hydroxyl group addition to the cholesterol (OH) and lipid head group. This additional hydrophilic group in the lipid promotes excess area and resultant fluctuation of menthol-containing liposomes more easily to increase with temperature than DOPC/Chol. The presence of extra-bilayer hydrophilic part of menthol enhances the repulsive interaction among the head part of the lipid with increase in temperature. Thus, exhibit thermo-induced fluctuation of those liposomes.



**Figure 2.2** Thermo-sensitive response of menthol-containing lipid vesicles. Percentage of lipid vesicles, which started fluctuating at a given level of temperature increase. The vesicles contained DOPC/Chol (black), 2.5 % menthol (light grey dashed), 5 % menthol (blue), 10 % menthol (dark grey dashed), 20 % menthol (black dotted), and 30 % (grey solid line). ( $n=30$ ).

In contrast at higher concentration of menthol, as the number of molecules increase, resultant repulsive interaction becomes high enough to create space to insert menthol in the bilayer. Hence the overall fluctuation of liposomes decreased. It was also observed that menthol at 2.5 to 10 % have shown more fluctuation change compare to the system without menthol. Menthol 20 % and 30 % shows very similar behavior to that of DOPC/Chol (control) system.



**Figure 2.3** Thermo-sensitive response of menthol-containing lipid vesicles. Fluctuating temperature at which 50 % of liposomes started fluctuating at a given level of temperature increase. The graph shows  $T_f$  against menthol concentration. ( $n=30$ ).

Figure 2.3 demonstrates the graph of  $T_f$  versus menthol concentration where  $T_f$  is defined as fluctuating temperature at which 50 % of the liposomes started fluctuation. I could clearly observe the higher fluctuating profile at lower concentration as menthol could fit into the head group of DOPC lipid thereby made them more thermo-responsive even at very small temperature increase. On the other hand, less thermo-responsiveness was observed on increasing concentration of menthol. I believed that at a higher concentration, menthol

molecules can penetrate into the lipid bilayer where they can occupy the space near double bond of DOPC lipid. As a consequence, lipid tail movement is restricted hence high temperature is needed to fluctuate the liposomes.

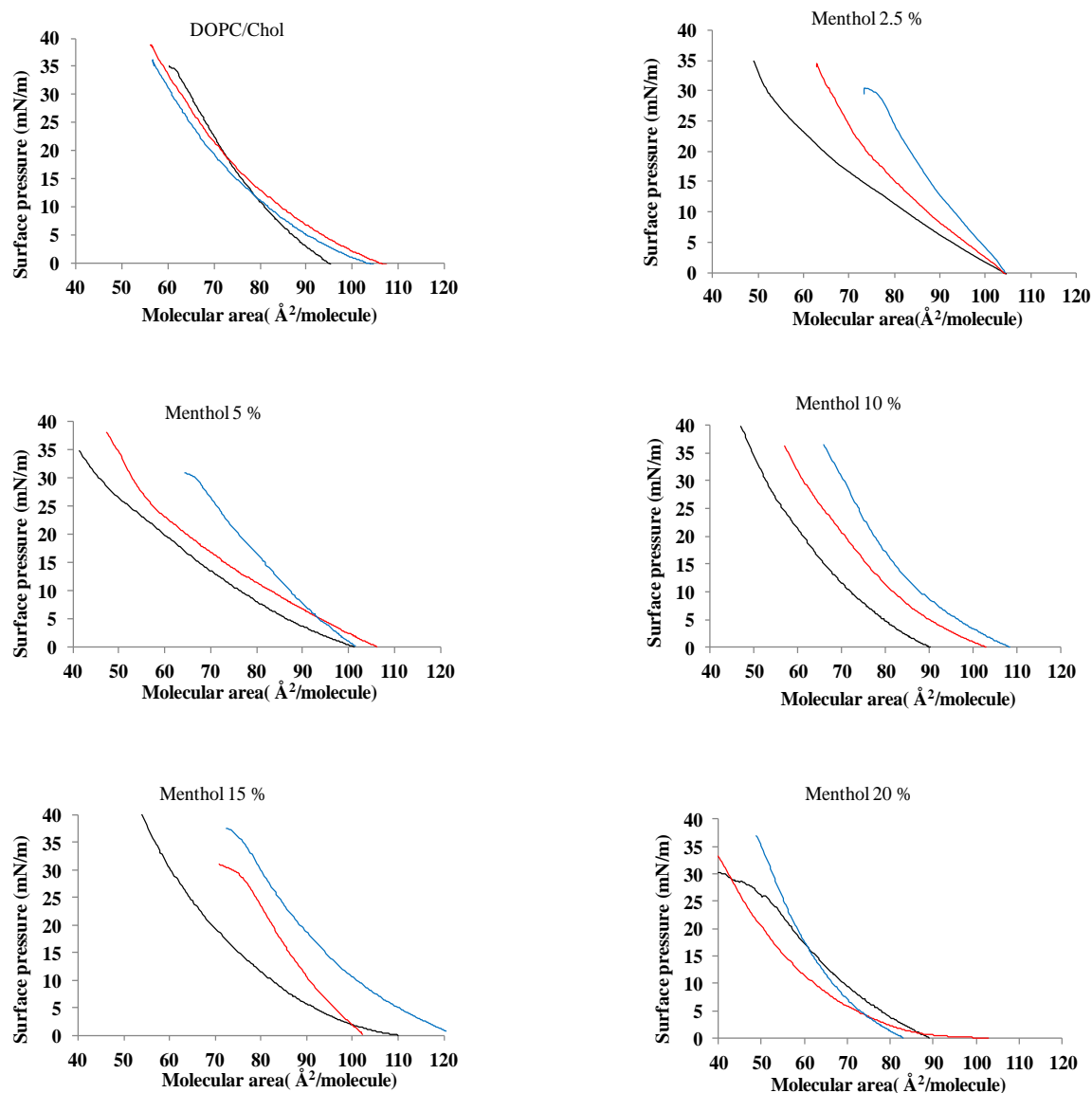
### 2.3.2 Role of surface tension and pressure on membrane

In order to investigate the area increase by rising temperature, I carried out Langmuir monolayer experiment at a different temperature with various concentration of menthol. Surface tension and surface pressure are the two key factors that determine the thermal expansion in the lipid bilayer. Surface area is related to thermal fluctuation directly and is given by the following equation

$$\Delta A / \Delta T \sim 1/P$$

Where **A** is surface area and **T** is temperature

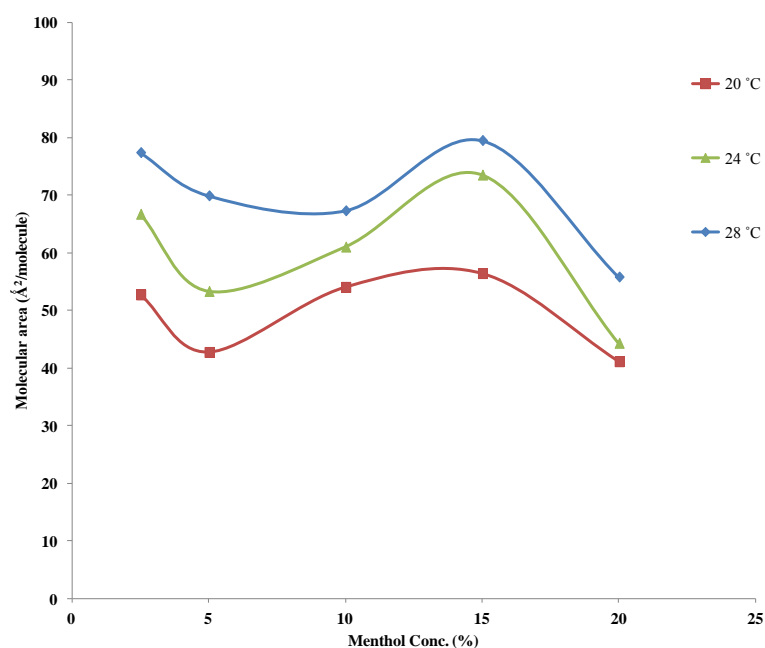
In this experiment two different membrane systems DOPC/Chol and DOPC/Chol/Menthol were prepared by the natural swelling method. The lateral pressure present in the lipid is reported to be 30-40 mN/m<sup>18</sup>; hence the experiment was carried out at this pressure. It was hypothesized that the membrane dynamics upon temperature change, leads to an increase in molecular area. Therefore, the effect of temperature on the molecular area of monolayer membranes using the Langmuir monolayer membrane method was studied. For control DOPC/Chol system was employed whose values closely consistent with previous reports. Figure 2.4 shows the typical P-A curves of DOPC/Chol monolayers at different temperature 20 °C, 24 °C, and 28 °C in the absence and presence of menthol. It was observed that menthol-containing monolayer membranes exhibited a relatively bigger increase in molecular area upon increasing temperature. On the other hand, without menthol system i.e., DOPC/Chol system shows slight increase in area. The result is in good agreement with the fluctuation data where menthol containing lipids are more temperature sensitive.



**Figure 2.4** Typical pressure-area curve of lipid monolayer DOPC/Cholesterol and menthol-containing membranes. DOPC/Chol (80/20), DOPC/Chol/Menthol (78/19.5/2.5) DOPC/Chol/Menthol (76/19/5), DOPC/Chol/Menthol (72/18/10), DOPC/Chol/Menthol (68/17/15), and DOPC/Chol/Menthol (64/16/20). Surface pressure ( $p$ )–area per molecule ( $\beta$ ) at 30 mN/m, at each temperature 20, 24, and 28 °C. Shows the typically P-A curves of a membrane at each temperatures 20 (black), 24 (red), and 28 (blue) °C respectively ( $n = 10$ ).

As shown in Fig 2.5, the change in the molecular area upon temperature changes. The presence of menthol significant increases the molecular area of DOPC/Chol monolayer at lower concentration till 10 %. This result was in good agreement with my previous studies

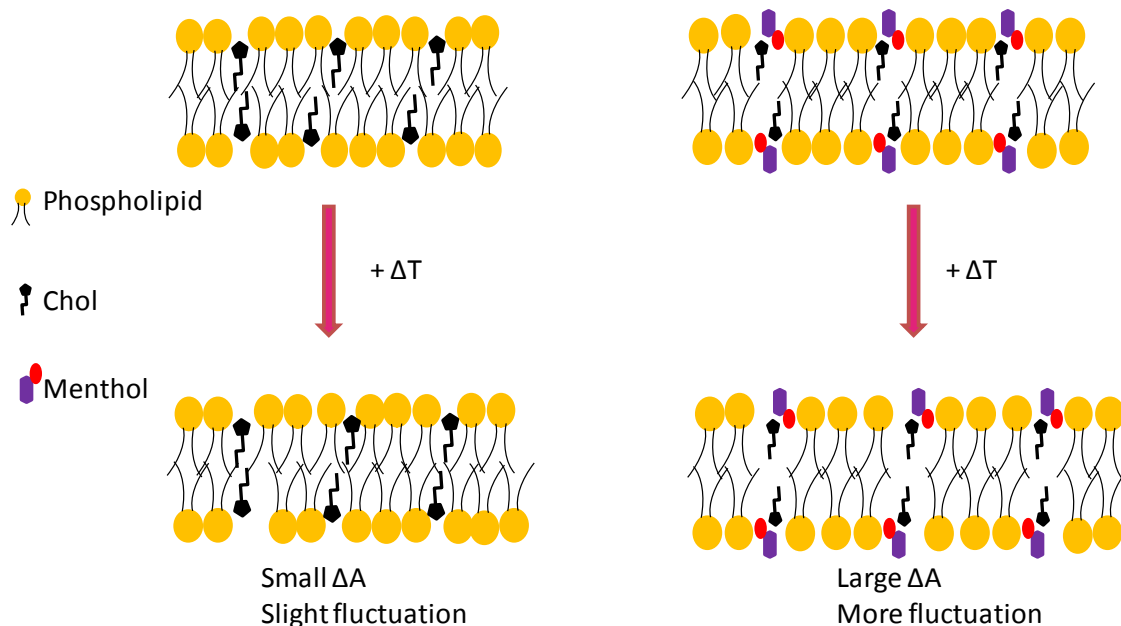
depicted that menthol strongly facilitates liposome fluctuation at lower concentration. It is very obvious to note that on increasing temperature molecular area also increases as consequence of Brownian motion. However, at 15 % menthol the molecular area of monolayer was higher compare to 20 %. The unusual behavior at 20 % shown by menthol may be due to aggregation of menthol molecules at high concentration with distortion of the monolayer.



**Figure 2.5** Typical pressure-area curve of lipid monolayer DOPC/Chol and menthol-containing membranes. DOPC/Chol (80/20), DOPC/Chol/Menthol (78/19.5/2.5) DOPC/Chol/Menthol (76/19/5), DOPC/Chol/Menthol (72/18/10), DOPC/Chol/Menthol (68/17/15), and DOPC/Chol/Menthol (64/16/20). Change in the molecular area upon temperature change.

Menthol-containing monolayer membrane exhibited bigger increase in molecular area upon increase in temperature. It was in agreement with the vesicular fluctuation that membrane containing cholesterol only show slight increase in molecular area. Menthol at concentration of 15 % shows greater surface area followed by 10 %, menthol 20 % showed least surface area. As the temperature increase area also increase for all the system. The

model presented in Fig 2.6 clearly shows the direct effect of menthol on lipid vesicles which bring out significant changes in the morphology of the membrane. Menthol containing lipid vesicles are more thermo responsive than that cholesterol containing lipid vesicles. This increase in fluctuation arises due to excess membrane area. The presence of hydroxyl group in menthol will interact to hydrophilic part of phospholipids thereby resulting into increase in head-head length. It was assumed that menthol might have inserted in between the head groups of phospholipids (Fig. 2.6). We also measured the molecular area increase according to temperature change by the Langmuir monolayer method. It is very important to consider the hydrophilic interaction among head group of phospholipids and hydroxyl group of menthol as well as the hydrophobic interaction among lipids. Higher concentration of menthol rendered the hydrophilic interaction between molecules and inhibited the thermo responsiveness by inhibiting increase in membrane area.



**Figure 2.6** Model demonstrating the interaction of menthol with lipid bilayer upon fluctuation.



### 2.3.3 Size distribution profile of lipid bilayer

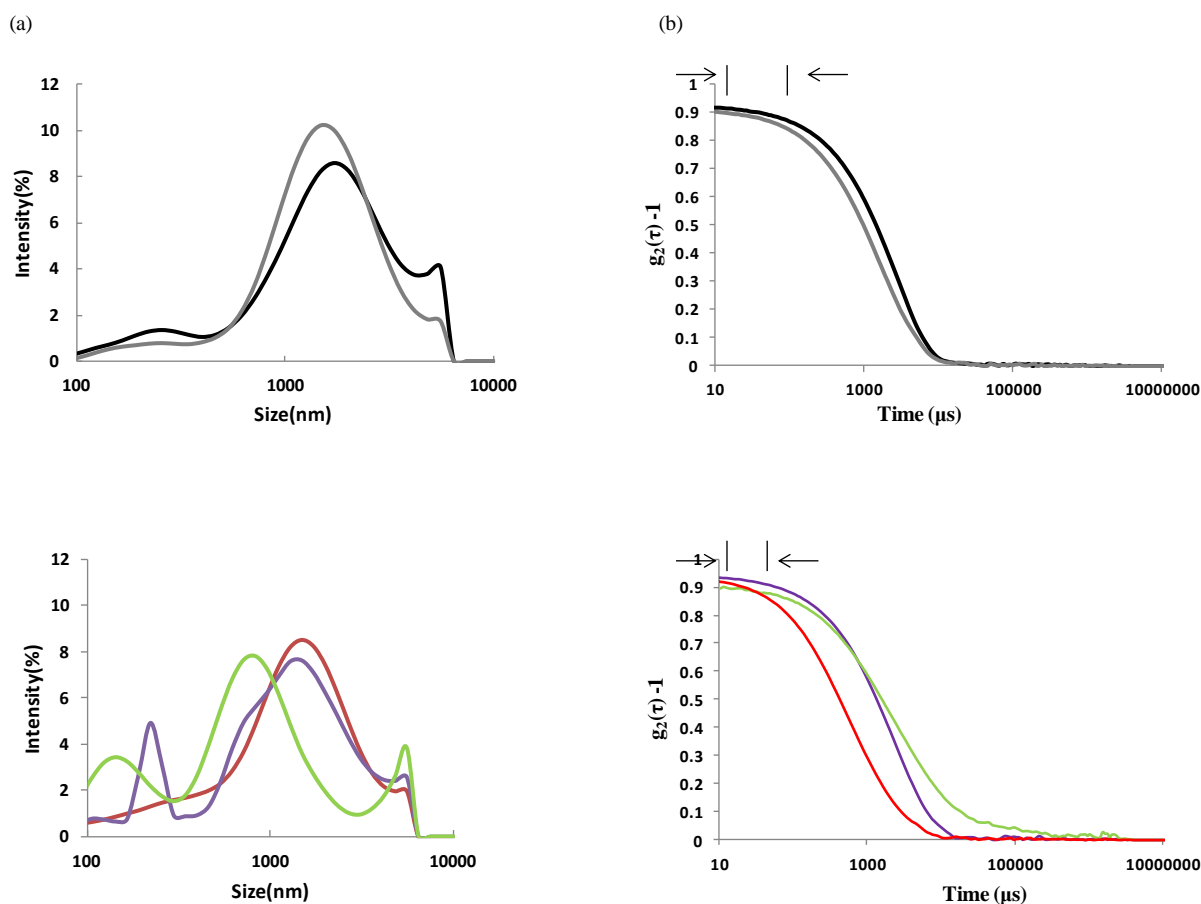
The size of the particles and molecules also give information about their motion in the system. When particles are subjected to laser light, the intensity of scattered light fluctuates and rate of fluctuation will depend on the particle size.<sup>19</sup> These particles undergo Brownian motion and their respective intensity fluctuation was calculated using Stokes-Einstein relationship.

$$d = \frac{kT}{3\pi\eta D}$$

where  $d$  is a hydrodynamic diameter,  $k$  is Boltzmann constant,  $T$  is temperature,  $D$  is diffusion coefficient,  $\eta$  is viscosity.

Due to thermal energy, particles move at very fast velocity, as a result of which the rate of scattered intensity fluctuates from each particle was recorded. The size determination was evaluated by dynamic light scattering (DLS) measurements.<sup>20,21</sup> Another parameter in DLS experiment is correlation function  $g_2(t)$  which provides the diffusion rate of the particles. By understanding the nature of the sample and DLS principle, I further employed size measurements for the menthol-containing membrane. Figure 2.7(a) shows intensity distribution profile in the presence and absence of menthol. Three different concentrations of menthol 2.5, 5, and 10 % were used for analysis. The membrane without menthol was relatively bigger in size as compared to the menthol-containing membrane. On increasing menthol concentration, the size of the liposomes decreases, exhibiting a higher rate of fluctuation at a lower concentration. This size-dependent fluctuation was in agreement with the thermal fluctuation data. In Fig 2.7(b), correlation function with time was plotted for both systems. In the case of DOPC and DOPC/Chol membrane, the signal takes a long time to decay, hence a lag was observed initially. I could say that larger particles move slowly,

thereby correlation decreases slowly. This implies that larger particles takes longer time and thus moves at a slower rate. On the other hand, menthol-containing membrane moves faster, corresponding to smaller particle size, thus correlation decreases quickly. The size distribution data was in good agreement with the fluctuation, showing menthol containing membranes decreases the size.



**Figure 2.7** (a) Size-intensity distribution profile of DOPC (black line), DOPC/Chol (grey line), Menthol-containing 2.5 % (red), 5 % (violet), and 10 % (green) systems. (b) Correlation functions with time for DOPC and menthol-containing systems. Comparison of the intensity of signal at time ( $\mu$ s) versus time lag  $g_2$ .

## 2.4 Conclusion

This is the first attempt to demonstrate direct observation of the dynamic response of lipid vesicles in real-time. The results clarified that menthol has direct interaction with biomembrane and significantly affects membrane dynamics. In addition, I have characterized and discussed the biophysical changes in membrane dynamics of menthol-containing lipid vesicles induced by temperature increase. This interaction of menthol can explain the physicochemical changes in the model cell membrane and may lead to better understanding of the biological membrane dynamics upon such sensing molecules.

## 2.5 References

1. Galeotti, N., Di Cesare Mannelli, L., Mazzanti, G., Bartolini, A. & Ghelardini, C. Menthol: a natural analgesic compound. *Neurosci. Lett.* **322**, 145–148 (2002).
2. Green, B. G. & McAuliffe, B. L. Menthol desensitization of capsaicin irritation: Evidence of a short-term anti-nociceptive effect. *Physiol. Behav.* **68**, 631–639 (2000).
3. Lee, Y. O. & Glantz, S. A. Menthol: putting the pieces together. *Tob. Control* **20**, ii1-ii7 (2011).
4. ECCLES, R. Menthol and Related Cooling Compounds. *J. Pharm. Pharmacol.* **46**, 618–630 (1994).
5. Chuang, H., Neuhauser, W. M. & Julius, D. The Super-Cooling Agent Icilin Reveals a Mechanism of Coincidence Detection by a Temperature-Sensitive TRP Channel. *Neuron* **43**, 859–869 (2004).
6. Cliff, M. A. & Green, B. G. Sensory irritation and coolness produced by menthol: Evidence for selective desensitization of irritation. *Physiol. Behav.* **56**, 1021–1029

- (1994).
7. Reid, G., Babes, A. & Pluteanu, F. A cold- and menthol-activated current in rat dorsal root ganglion neurones: properties and role in cold transduction. *J. Physiol.* **545**, 595–614 (2002).
  8. Clapham, D. E. & Miller, C. A thermodynamic framework for understanding temperature sensing by transient receptor potential (TRP) channels. *Proc. Natl. Acad. Sci.* **108**, 19492–19497 (2011).
  9. Chowdhury, S., Jarecki, B. W. & Chanda, B. A Molecular Framework for Temperature-Dependent Gating of Ion Channels. *Cell* **158**, 1148–1158 (2017).
  10. Purves D, Augustine GJ, Fitzpatrick D, et al. in *Neuroscience* (ed. Purves D, Augustine GJ, Fitzpatrick D, et al.) (Sinauer Associates, 2001).
  11. Karashima, Y. *et al.* Bimodal Action of Menthol on the Transient Receptor Potential Channel TRPA1. *J. Neurosci.* **27**, 9874 LP-9884 (2007).
  12. Macpherson, L. J. *et al.* More than cool: Promiscuous relationships of menthol and other sensory compounds. *Mol. Cell. Neurosci.* **32**, 335–343 (2006).
  13. van Meer, G., Voelker, D. R. & Feigenson, G. W. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* **9**, 112–124 (2008).
  14. Lasic, D. D. Novel applications of liposomes. *Trends Biotechnol.* **16**, 307–321 (1998).
  15. Viallat, A., Dalous, J. & Abkarian, M. Giant Lipid Vesicles Filled with a Gel: Shape Instability Induced by Osmotic Shrinkage. *Biophys. J.* **86**, 2179–2187 (2004).
  16. Goluszko, P. & Nowicki, B. Membrane Cholesterol: a Crucial Molecule Affecting Interactions of Microbial Pathogens with Mammalian Cells. *Infect. Immun.* **73**, 7791–

- 7796 (2005).
17. Walde, P., Cosentino, K., Engel, H. & Stano, P. Giant Vesicles: Preparations and Applications. *ChemBioChem* **11**, 848–865 (2010).
  18. Hamada, T., Sato, Y. T., Yoshikawa, K. & Nagasaki, T. Reversible Photoswitching in a Cell-Sized Vesicle. *Langmuir* **21**, 7626–7628 (2005).
  19. Hoo, C. M., Starostin, N., West, P. & Mecartney, M. L. A comparison of atomic force microscopy (AFM) and dynamic light scattering (DLS) methods to characterize nanoparticle size distributions. *J. Nanoparticle Res.* **10**, 89–96 (2008).
  20. Boyd, R. D., Pichaimuthu, S. K. & Cuenat, A. New approach to inter-technique comparisons for nanoparticle size measurements; using atomic force microscopy, nanoparticle tracking analysis and dynamic light scattering. *Colloids Surfaces A Physicochem. Eng. Asp.* **387**, 35–42 (2011).
  21. Stock, R. S. & Ray, W. H. in *Particle Size Distribution* **332**, 105-114–7 (American Chemical Society, 1987).

## **CHAPTER 3 EFFECT ON BIOPHYSICAL PROPERTIES OF HOMOGENEOUS MODEL MEMBRANE INDUCED BY d- and l-MENTHOL**

**Abstract:** DNA and proteins, lipid membranes, etc. which are biological substances are called “soft matter” due to their essential properties. Soft matter has properties of both liquid viscosity and solid elasticity. Current research using physical properties of lipid membranes has been promoted in the boundary area between organisms and physics. Model membranes provide a platform for many biophysical properties analysis and usage in the drug delivery process. In this chapter single component model membrane with and without cholesterol were used composed of DOPC, and DPPC membrane. It is believed that two isoforms d- and l-menthol, though have same structure showed significant effect on the physical properties of lipid bilayer. At same concentration of menthol, varying temperature from low to high, dramatic changed were observed for both d-/l-menthol containing model membrane. Also, the importance of temperature and cholesterol which believed to alter the properties of membrane induced by d- and l-menthol is demonstrated.

**Keywords:** Phase transition, Membrane fluidity, Fluctuation, Menthol.

### 3.1 Introduction

The cell membrane was formed as a lipid bilayer membrane, and it was considered to be a fluid mosaic model<sup>1,2</sup> in which various lipids and proteins were uniformly distributed in the membrane plane and freely diffused. In recent years, it has been suggested that it has a microdomain structure called raft<sup>3,4,5,6,7</sup>, and raft is considered to be a surfactant unnecessary fraction, and it is formed with many membrane proteins, saturated lipids, cholesterol.<sup>8</sup> The function of cell membranes controls the localization of foreign substances and plays an important role in cellular responses such as membrane dynamics.

Menthol is a cyclic terpene that exhibits three stereogenic centers and therefore exists in eight stereoisomers as shown in figure 6. Among all the eight, (-)-Menthol occurs most widely in nature and generally referred to as “natural” menthol. Only *l*-menthol is believed to exert cooling sensation and exhibits distinct peppermint smell.<sup>9</sup> Prolonged exposure of menthol delivered severe effects on human skin such as irritation, chronic dizziness, vertigo, skin lesions etc.<sup>10</sup> FDA has proved the toxicity of menthol on animals causing cystic spacing in the cerebellar.<sup>11</sup> Apart from its well-known cooling property, menthol display other medicinal properties used as analgesic, muscle cramp reliever, treatment in oral hygiene. The cooling sensitization may arise due to specific interaction with TRPM8 ion channel.<sup>12,13,14</sup> It enhances smooth muscle relaxation, reduces lower esophageal sphincter tone, and reduces the skin barrier by vasodilation. Several adverse effects caused by menthol such as muscle tremors, burning sensation, dizziness, skin rashes, nausea and vomiting has been reported. In human, effect of menthol after exposure to skin was observed and the results show oral ulceration, lichenoid reactions, and burning mouth syndrome. Although, so far there are no such studies made to chronic exposure in humans.

Most stable form of menthol is cyclohexane chair conformation where large equatorial alkyl occupied second position and smaller ones at fifth position.<sup>15</sup> Many quantitative analyses were done on menthol structure and relation in comparison to its other isoforms. However, pure (*l*-) and racemic (*dl*-) had different properties. This difference in properties makes only *l*-menthol to sense cold receptor response whereas other doesn't. Hence, chirality plays a crucial role in the functioning of the molecules in biological processes.

One of the built-in properties of the cell membrane is to undergo structural organization. Such self-organization contributes to biophysical processes such as endocytosis<sup>16</sup> and exocytosis,<sup>17</sup> those are important for cellular function. A small change in the membrane dynamics has an ability to disrupt structural organization. By studying these structural dynamics will provide insight into the physical basis of changes in cellular level which occur during biophysical processes such as membrane invagination. Membrane dynamics and change in the lipid bilayer properties are greatly influenced by various forms of stress including light and heat, and menthol, icilin, capsaicin. As discussed in the introduction lipid bilayer has an important property "fluidity" that alters the structural organization and order of the lipid packing. However, effect of menthol on biophysical properties such as thermal responsiveness and membrane fluidity, has not yet been reported. I was further interested in understanding the effect of temperature on menthol-containing lipid vesicles as to clarify the difference in the property exhibited by *d*- and *l*-menthol on membrane.

DNA and proteins, lipid membranes, etc. which are biological substances are called "soft matter" due to their essential properties. Soft matter has properties of both liquid viscosity and solid elasticity. Current research using physical properties of lipid membranes has been promoted in the boundary area between organisms and physics. Previously in my lab, studies on the membrane localization and membrane dynamics of various substances such as nanoparticles<sup>18</sup> and amyloid  $\beta$  of Alzheimer's disease<sup>19</sup> causing peptide and surfactant



from the viewpoint of soft matter physical properties of membrane had been reported. The research of our lab mainly aims to understand the mechanism of interaction between substances and membranes in a unified way.

I focused on liposomes formed with the same lipid bilayer membrane as the cell membrane. Liposomes can make liposomal GUV of large size of about 5 ~ 100  $\mu\text{m}$  from small liposome SUV with diameter of about 10 ~ 100 nm. Depending on the size, there are various ways to use it, taking the advantages of cell-sized liposome, one can observe its membrane structure in real-time with a microscope, and used it for membrane localization of substances and observation of membrane dynamics.

I employed direct observation of cell-sized liposomes ( $>10 \mu\text{m}$  in diameter) with DOPC and DPPC phospholipid membrane to investigate the effect of *d*- and *l*-menthol on its membrane fluctuation and structure. It was found that two isoforms of menthol *d*- and *l*- showed significant difference in membrane dynamics. The membrane fluctuation data showed that *d*-menthol is more temperature sensitive than *l*-menthol in comparison to the control DOPC liposome. The fluctuation rate is higher in case of *d*-menthol. Also, fluidity measurements using laurdan fluorescent probe, suggested that *d*-menthol makes membrane more fluidic compared to *l*-menthol. Overall I conclude that *d*-menthol has stronger physical effect on membrane than that of *l*-menthol.

## **3.2 Experimental Procedures:**

### *3.2.1 Materials:*

Lipids 1, 2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and Cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, USA). (1*S*, 2*R*, 5*S*)-2-isopropyl-5-methylcyclohexanol (*d*-menthol), (1*R*, 2*S*, 5*R*)-

2-isopropyl-5-methylcyclohexanol (*l*-menthol) were purchased from Wako Japan. Fluorescent dyes, N-(rhodamine red-X)-1, 2-dihexadecanoyl-*sn*-glycero-3 phosphoethanolamine triethylammonium salt (rhodamine DHPE) ( $\lambda_{\text{ex}} = 560$  nm,  $\lambda_{\text{em}} = 580$  nm) obtained from Thermo Fisher Scientific (USA) and 6-Dodecanoyl-2-Dimethylaminonaphthalene (laurdan) was obtained from Funakoshi (Japan). Chloroform and methanol were from Kanto-Chemical (Japan) and Nacalai Tesque (Japan), respectively. Ultrapure water (specific resistance  $\geq 18$  M $\Omega$ ) was obtained from a Millipore Milli-Q purification system.

### 3.2.2 Preparation of Liposomes:

Lipid vesicles were prepared by the natural swelling method.<sup>20</sup> Lipids, Chol, menthol, and fluorescent probe were dissolved in a 2:1 vol/vol (chloroform/methanol) solution, making the concentration 2 mM for lipids, Chol and menthol. Lipids, Chol, and menthol were mixed at desired concentration to a final volume of 20  $\mu\text{L}$ . 2  $\mu\text{L}$  of rhodamine-DHPE and 6  $\mu\text{L}$  of laurdan were further added to the lipid mixture for the observation of membrane fluidity. The organic solvent was evaporated under a flow of nitrogen gas and the lipids were further dried in vacuum desiccators for 3 h. The film was hydrated with Milli-Q water at 37 °C for an hour and were kept overnight at room temperature  $21.7 \pm 1.7$  °C. The final lipid concentration was 0.2 mM and rhodamine-DHPE concentration was 1  $\mu\text{M}$ . The prepared lipid compositions are DOPC (100), DPPC (100), DOPC/*d*- or *l*-menthol (90/10), DPPC/*d*- or *l*-menthol (90/10), DOPC/Chol (80/20), DPPC/Chol (80/20), DOPC/Chol/*d*- or *l*-menthol (72/18/10), and, DPPC/Chol/*d*- or *l*-menthol (72/18/10) for microscopic observation.

### 3.2.3 Microscopy Observations:

5  $\mu\text{L}$  of the lipid vesicle solution was placed in a silicon well (0.2 mm) on a glass slide, and cover with a cover slip. The silicon well and the coverslip ensured that evaporation of the solution did not occur during the entire experiment. The formed GUVs were observed by

phase contrast microscopy (Olympus BX50 Japan) and fluorescence microscopy (Olympus IX71, Japan). The images were recorded on a hard disc drive at 30 frames/s. GUVs were prepared by unsaturated lipid DOPC, saturated lipid DPPC, and Chol to form homogeneous membrane.

#### *3.2.4 Measurements of membrane fluidity:*

For the analysis of membrane fluidity laurdan fluorescent dye has been used as a probe to measure the ordered state of membrane molecules. Laurdan detects changes in membrane fluidity caused by the interaction of determinate surroundings and water molecules by calculating the generalized polarization *i.e.*, GP value Generalized polarization values vary from 1 (no solvent effect) to -1 (complete exposure to bulk water) .

#### *3.2.5 Differential Scanning Calorimetry (DSC) Measurements:*

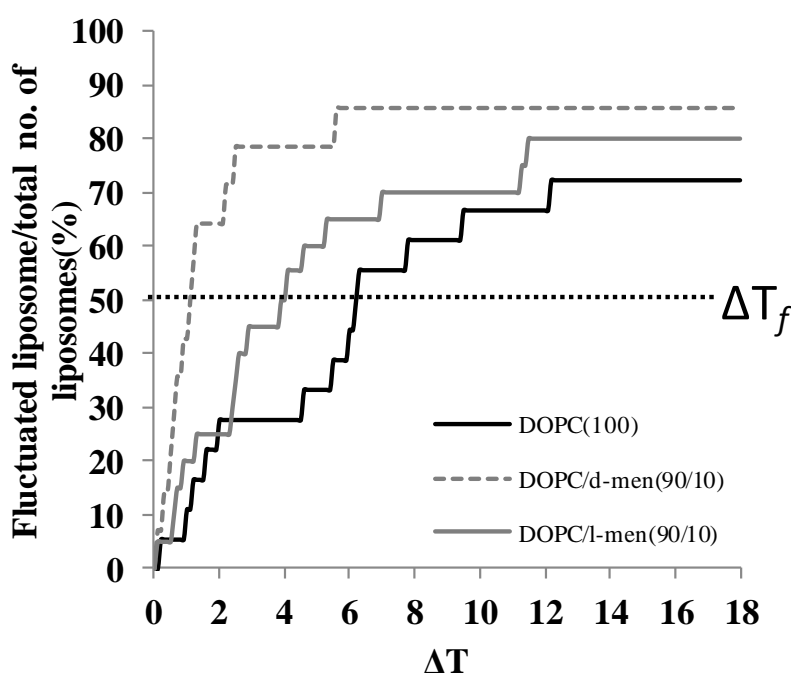
Thermal behavior of lipids in the presence of *d*- and *l*-menthol was studied by DSC. Samples containing *d*- and *l*-menthol were prepared in glass vials. All samples were scanned over two cycles from 20 to 60 °C for minimum error using Mettler DSC with a scanning rate of 5 °C/min. A quantity of around 10-15 µL of samples were used for DSC measurements.

### **3.3 Results and discussion**

#### *3.3.1 Effect of d- and l-menthol on membrane fluctuation*

Previous studies have shown that liposomes made with unsaturated lipid DOPC can observe how dynamics and morphology of spherical shape liposome changes when the temperature is elevated.<sup>21</sup> This is due to the fact that the vibration of the molecules constituting the membrane is intensified by the temperature rise and the area occupied by each molecule increases, so that the surface area with respect to the volume of the entire membrane, that is, "membrane fluctuation" is considered to destabilize membrane. I advance

my study further to investigate the effect of *d*- and *l*-menthol on membrane dynamics and change in the morphology. Thermo-responsiveness characteristic of DOPC only and DOPC/Chol membrane system as a control in the absence and presence of *d*- and *l*-menthol was investigated. On increase of temperature from RT (20.0 °C) to higher temperature, lipid vesicles start to fluctuate. Direct real-time observation of membrane dynamics revealed that the *d*- and *l*-menthol-containing membranes were more thermo-responsive than control lipid vesicles (Fig 3.1 and 3.2). Interestingly, among *d*- and *l*-menthol, *d*-menthol shows higher thermo-responsiveness, meaning that *d*-menthol makes membrane more sensitive to the temperature change.



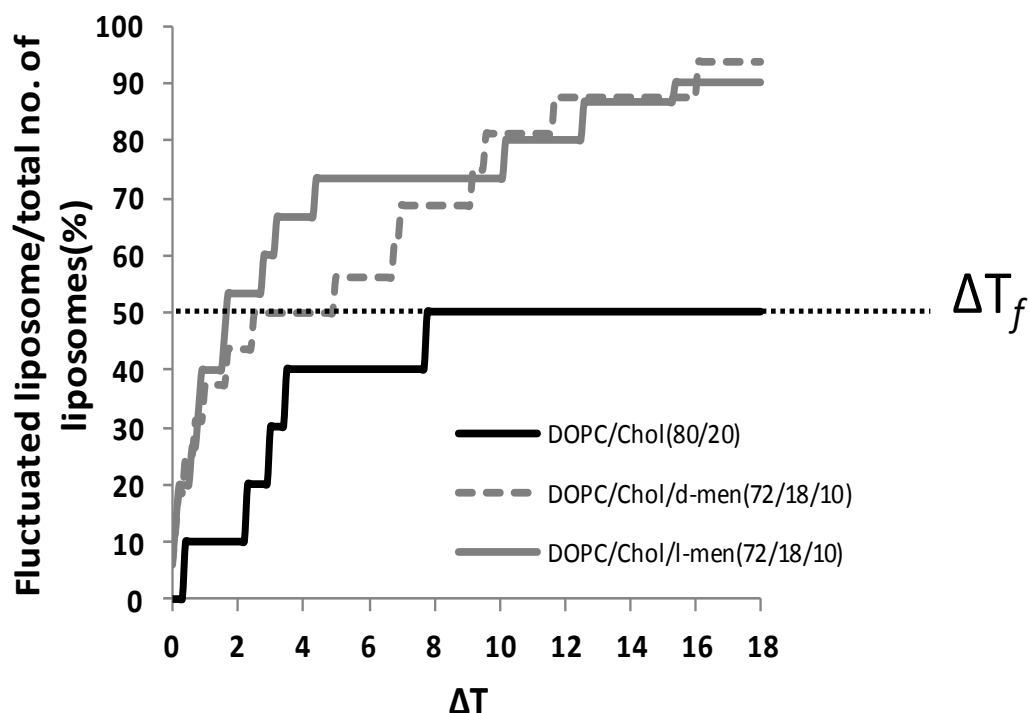
**Figure 3.1** Effect of *d*- and *l*-menthol on DOPC lipid vesicles. Percentage of lipid vesicles, which started fluctuating at a given level of temperature raise. The vesicles containing *d*-menthol (dashed gray), *l*-menthol (gray) at 10 % molar concentration and control DOPC system (black). ( $n=30$ ).

**Table 3.1 Temperature change values at which membrane start fluctuating**

	DOPC	DOPC/ <i>d</i> -men	DOPC/ <i>l</i> -men
$\Delta T_f$ (°C)	6	1.1	3.6

The graph shows the ratio of liposome fluctuated by temperature when given temperature change and  $\Delta T_f$  shown in the table 3.1 shows the temperature necessary for half of the observed samples to start fluctuate. Liposomes containing *d*- and *l*-menthol can be seen to oscillate at a lower temperature, compared to the control ones.

In addition, I observed the state of fluctuation of the membrane composed of DOPC and cholesterol (Fig 3.2). Liposomes containing cholesterol composition showed slightly lower temperature responsiveness than DOPC single composition. When adding the *d*- and *l*-menthol to the DOPC membrane containing cholesterol, it can be seen that the temperature response of the membrane is lower as compared with the case where only *d*- and *l*-menthol was added. It was also found that the effect of lowering the temperature responsiveness is dependent on cholesterol interaction. There was significant difference between DOPC and DOPC/Chol lipid vesicles, indicating that the presence of cholesterol at certain concentration would responsible for the thermo-responsive nature of the *d*- and *l*-menthol containing vesicles. It was stated previously in our lab, there are two parameters that caused enhanced fluctuations in the membrane. This fluctuation can be caused either by (i) decrease in volume (*V*) to area (*A*) ratio or (ii) a decrease of the bending modulus. It is believed that as cell-sized liposomes are spherical in nature; therefore decrease in *V/A* ration likely the reason for membrane fluctuation. Even small change in the temperature was sufficient to trigger fluctuation in menthol-containing membrane.



**Figure 3.2** Effect of d- and l-menthol on DOPC/Chol lipid vesicles. Percentage of lipid vesicles, which started fluctuating at a given level of temperature increase. The vesicles containing d-menthol (dashed gray), l-menthol (gray) at 10 % molar concentration and control DOPC/Chol system (black). ( $n=30$ ).

**Table 3.2** Temperature change values at which membrane start fluctuating.

	DOPC/Chol	DOPC/Chol/d-men	DOPC/Chol/l-men
$\Delta T_f$ (°C)	7.9	2.1	1.6

I propose that the d- and l-menthol has different interaction sites in the lipid bilayer which may cause change in morphology. l-Menthol relatively exhibit hydrophilic interaction at the head group of DOPC, thus slight temperature lead to changes in lipid membrane order. Whereas, d-menthol may interacted with acyl chain of DOPC causes more hindrance results into high thermo-sensitiveness. The results of the thermal fluctuation experiment of the

homogeneous membrane showed that cholesterol involvement suppresses the fluctuation of DOPC membrane by *d*- and *l*-menthol molecule. From this it was suggested that cholesterol inhibits the interaction with the DOPC membrane of the *d*- and *l*-menthol molecule

### 3.3.2 Influence of *d*- and *l*-menthol on fluidity change in homogeneous membrane

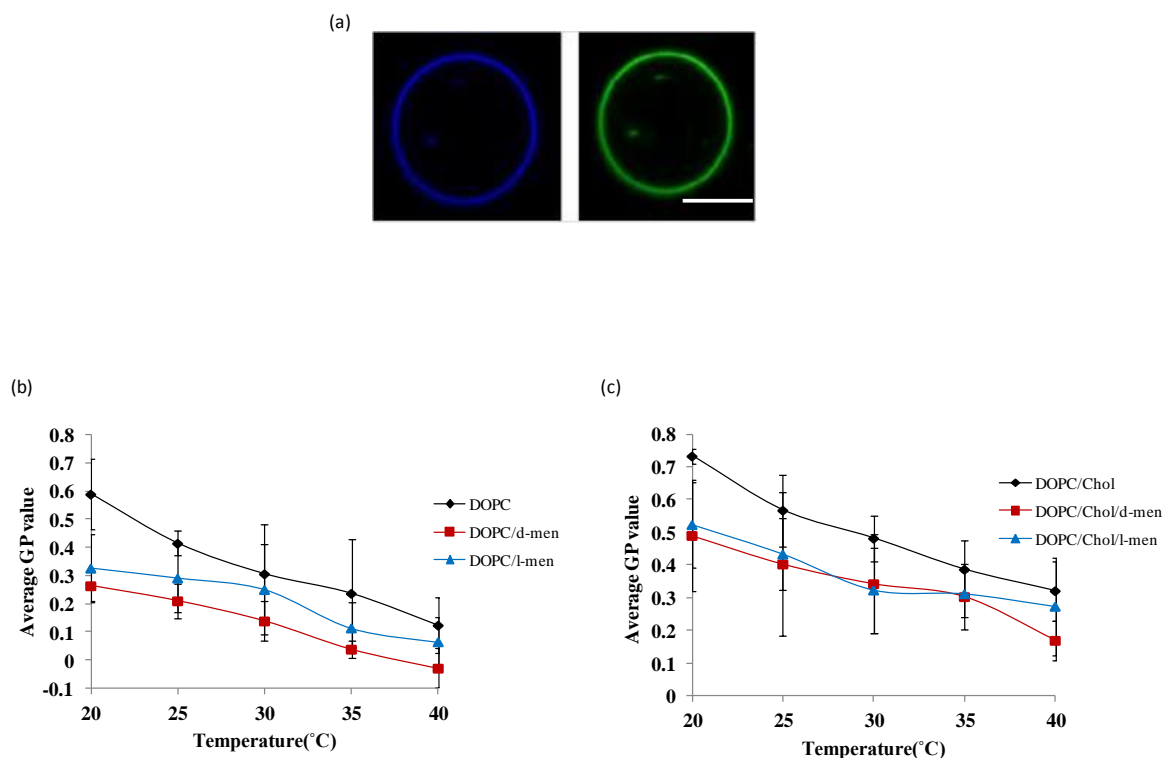
Further the change in the membrane fluidity was determined using laurdan fluorescent probe.<sup>5</sup> These changes are attributed to the spectral shift and can be measure by generalized polarization (GP)<sup>22,23,24,25</sup> value. This spectral shift is due to the dipolar relaxation of laurdan and attributed to water molecules present in the bilayer. The GP value is defined as equation 1.

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad \text{Eq. 1}$$

Where  $I_{440}$  and  $I_{490}$  are the respective emission intensities at 440 and 490 nm

I determined the threshold using the fluorescence intensity at both emission and analyzed the influence of the *d*- and *l*-menthol on the membrane fluidity of the membrane based on average GP value. The graph 3.3 and 3.4 is the average value of GP values calculated based on the fluorescence intensity detected from the 10 Laurdan labeled fluorescence liposomes. Figure 3.3(a) are the typical confocal microscopic images at both emission and Fig 3.3(b) shows average GP values versus temperature change on membrane fluidity in DOPC and DOPC/Chol system. The results of experiment in the DOPC membrane as a control membrane should have liquid-disordered ( $L_d$ ) state in temperature range under transition temperature. According to results, GP value of DOPC membrane had suddenly decreased when the temperature increases. On increasing temperature the mobility of DOPC molecules increases thereby cause the membrane to be more fluidic. On the other hand, fluidity of *d*- and *l*-menthol added membrane was significantly increased as their GP values

becomes lower at higher temperature compared with control. *d*-Menthol made the membrane relatively too fluidic in nature compare to *l*-menthol. Utmost I could argue that *d*-menthol love to go into DOPC-rich  $L_d$  phase hence increases the fluidity.



**Figure 3.3** Effect of *d*- and *l*-menthol on membrane fluidity of homogeneous model membranes. Typical confocal microscopy images of Laurdan emission (a), GP values of DOPC membranes (b), DOPC/Chol membranes (c). Black lines for DOPC, DOPC/Chol, red and blue lines indicates *d*- and *l*-mentho containing membrane. Scale bar = 10  $\mu$ m.

The data was in good agreement to the fluctuation, where *d*-menthol was more temperature sensitive and has higher degree membrane fluctuation. This indicates that presence of *d*- and *l*-menthol destabilized membrane structure against heat, and made them easy to be melting by increasing temperature.

Next, I performed fluidity measurement in the DPPC and DPPC containing *d*- and *l*-menthol. Figure 3.4 shows average GP values versus temperature change on membrane fluidity. This graph shows results of experiment in the DPPC membrane as a control. DPPC

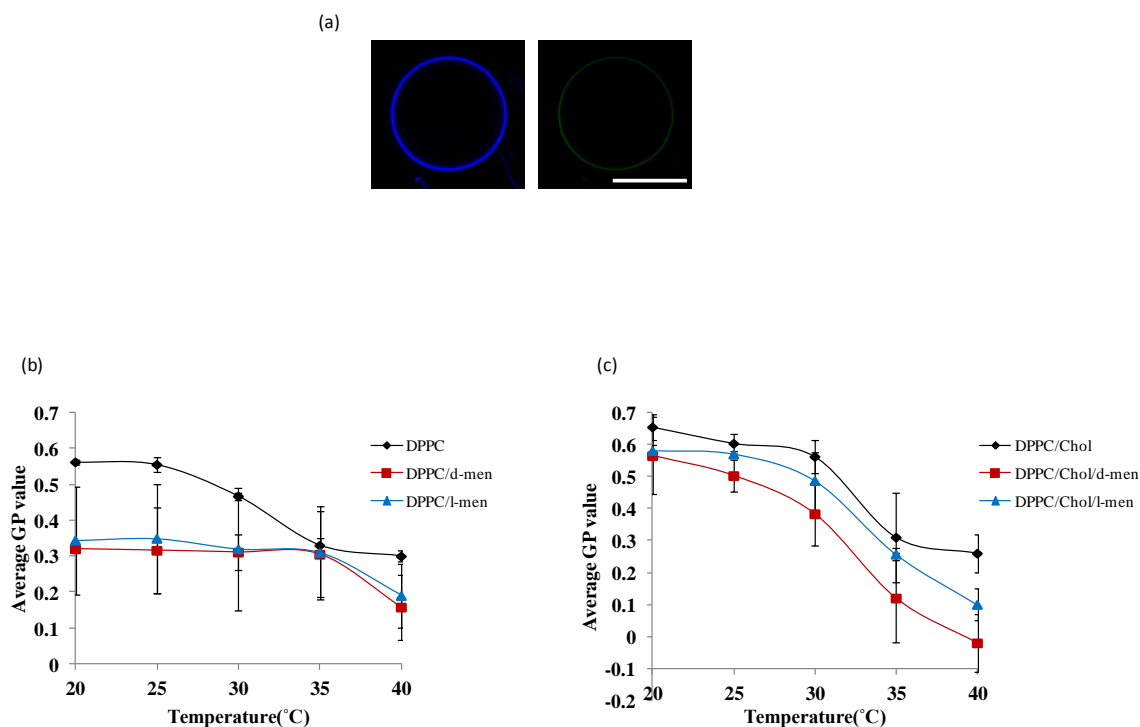


membrane should have solid-like ordered ( $S_o$ ) state in temperature range under transition temperature. According to results, GP value of DPPC membrane had suddenly decreased when the temperature became 42 degree. Melting temperature of DPPC is about 41 degree, so might have said that membrane fluidity of DPPC membrane was suddenly increased by phase transition of membrane due to the temperature increase. On the other hand, fluidity of *d*- and *l*-menthol added membrane was increased at higher temperature compared with control. This phenomenon was similar to that observed in DOPC lipid, indicated that presence of *d*- and *l*-menthol destabilized membrane structure against heat, and made them easy to be melting by increasing temperature.

According to results of GP value, when *d*- and *l*-menthol are involved in DPPC or DPPC/Chol membrane, their structures were fluidic. Therefore, at higher temperature, *d*- and *l*-menthol added membrane was easily disordered than at a lower temperature at both conditions of with and without cholesterol. *d*- and *l*-menthol goes evenly to the DPPC region hence enhances the membrane fluidity in  $S_o$  phase. Moreover there is even distribution of *d*- and *l*-menthol, without preference of interaction as hydrophilic and hydrophobic. *d*-Menthol disturb the packing of acyl chain goes to the  $L_d$ , while *l*-menthol cannot goes easily into acyl chain hence  $S_o$  phase intact. The thermal stability of both DPPC-rich solid-ordered ( $S_o$ ) phase and DPPC/Chol-rich liquid-ordered ( $L_o$ ) phase were decreased in the presence of *d*- and *l*-menthol. Moreover, *d*- and *l*-menthol enhanced the increase in the membrane fluidity in both  $S_o$  and  $L_o$  phase.

Membrane fluidity measurements in homogeneous membrane system composed of DOPC, and DPPC containing *d*- and *l*-menthol in the absence and presence of cholesterol was summarized. These membranes liquid-like ( $L_o$ ) ordered state and liquid-disordered ( $L_d$ ) state, were affected when the temperature was changed. Also in this case, I observed that transition temperature of membrane which containing *d*- and *l*-menthol decreased. So it can

be concluded that, *d*- and *l*-menthol can decrease transition temperature of DPPC rich membrane by them self without involvement of Cholesterol.



**Figure 3.4** Effect of *d*- and *l*-menthol on membrane fluidity of homogeneous model membranes. Typical confocal microscopy images of Laurdan emission (a), GP values of DPPC membranes (b), DPPC/Chol membranes (c). Black lines for DPPC, DPPC/Chol, red and blue lines indicates result of *d*- and *l*-menthol containing membrane. Scale bar = 10  $\mu\text{m}$ .

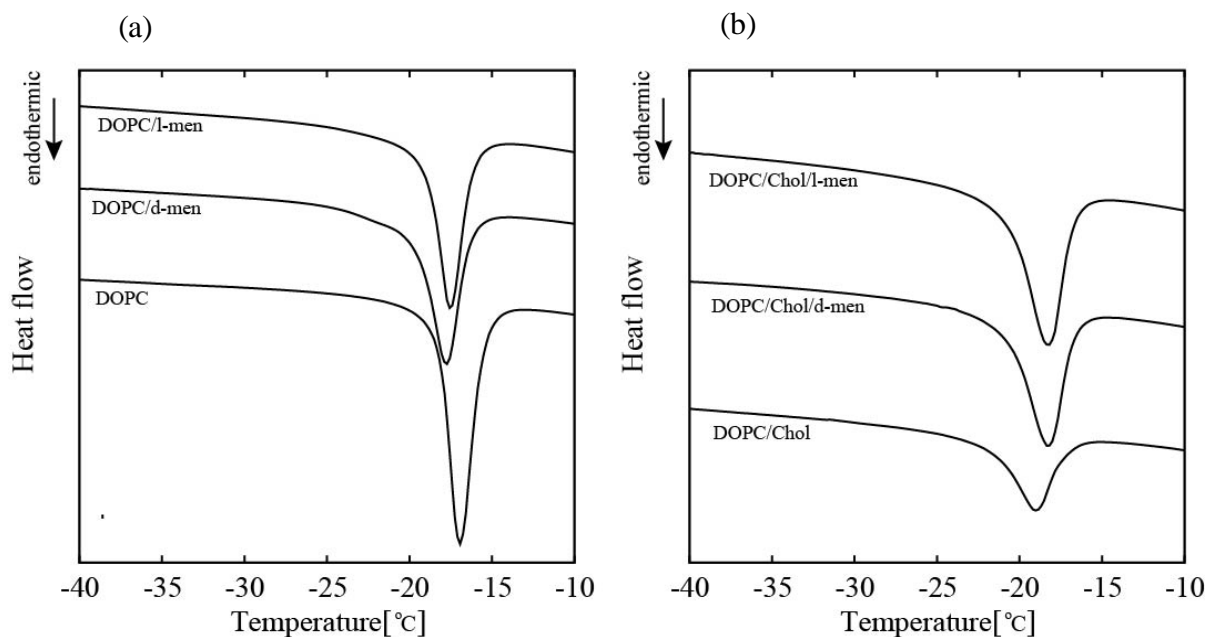
### 3.3.3 DSC Measurements

Liposomes can exist in two different states depending on temperature, at low temperature gel state ( $L\beta$ ) and at high temperature liquid state ( $L\alpha$ ).<sup>26</sup> Gel state corresponds to the more ordered state where lipids are held tightly with each other, while they tend to reduce the ordering at higher temperature. Phospholipids have long chain fatty acids and have specific phase transition temperature ( $T_m$ ) with varying fatty acids composition between these two states.<sup>27</sup> The change in the transition temperature could be due to either expansion of acyl

chain in the hydrophobic region or head group interaction. As a consequence of which, gel to liquid crystalline phase transition will greatly influence by the acyl chain interaction or head group interaction.

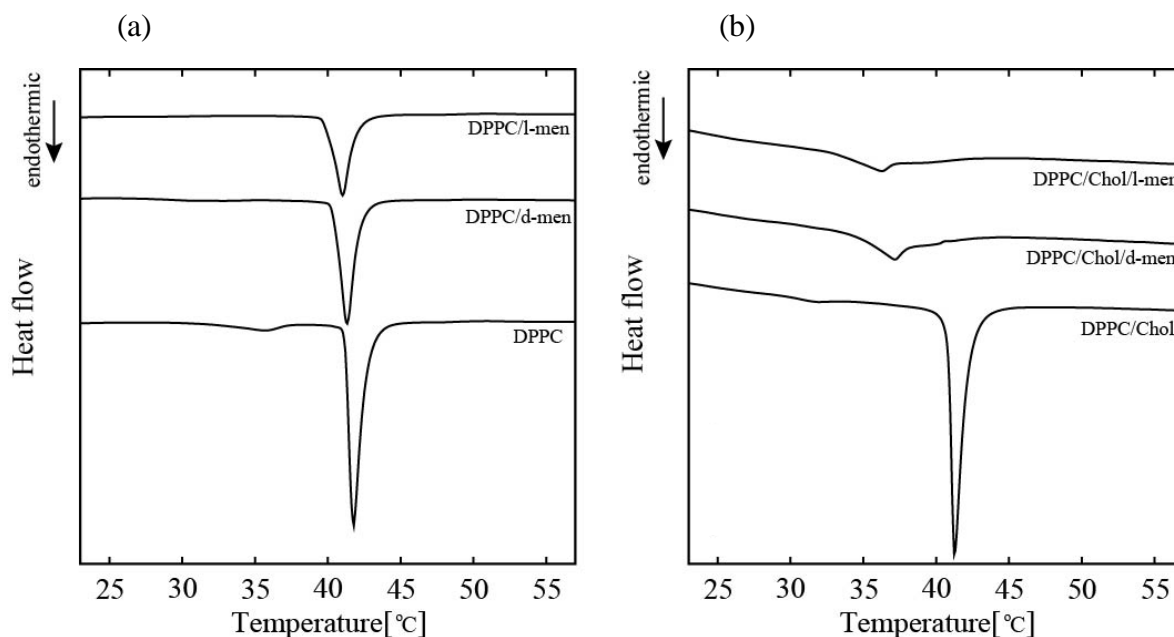
To understand the concept of phase behavior change induced by *d*- and *l*-menthol, thermal analysis has been taken into consideration. Membrane is believed to exert line tension<sup>4</sup> due to mismatch of the area of lipid acyl chain  $L_d$  phase in the  $L_o$  phase. As a consequences, membrane faced loss in its enthalpy or entropy. To overcome line tension exerted, membrane retrieves the area of domain boundary. Thus, lipid domains tend to adopt circular shapes and led to  $L_o$  phase progression. These dynamic processes can be clarified by adopting DSC thermogram measurements. The DSC thermogram measurements for DOPC, DPPC, with and without cholesterol in the presence of *d*- and *l*-menthol are shown in Fig 3.5 and 3.6. On interaction with *d*- and *l*-menthol, shift of the peaks for the main transition melting peak was observed. In the case of DOPC/Chol system there is a shift in  $T_m$  to higher temperature range and increase in enthalpy value. Addition of *d*- and *l*-menthol to DOPC bilayer shifted the main transition peak to a lower temperature. Similarly, in DOPC/Chol system, on addition of both *d*- and *l*-menthol shifts the peak to higher temperature and also broadens the peak. This phenomenon can be explained based on the interaction of *d*- and *l*-menthol with DOPC and Chol. *d*-Menthol exhibits strong perturbing effect to DOPC and Chol hydrophobic part, which supposed to be localized near double bond of lipid tail. Since Chol is situated perpendicular to the plane of bilayer results into more hindrance at hydrophobic region. The decrease in the transition temperature and broadening of the peak is attributed by expansion of acyl chain. Similar trend was observed in the case of *l*-menthol with different interaction site. I believed *l*-menthol have more of hydrophilic interaction with the head group of DOPC and hydrophilic OH part of Chol. This hydrophilic interaction at the surface of lipid will disturb the orderness i.e disordered phase; thereby decrease in the main

transition temperature was observed. The data of DSC shows phenomenal change in DOPC system with and without cholesterol. We could clearly examine that on addition of cholesterol to the system the value of enthalpy ( $\Delta H$ ) decreased drastically. This indicates that cholesterol buried in the bilayer which supposed to increase the mean square diameter, implies that the cholesterol gets precipitate out near the  $T_m$  of DOPC.



**Figure 3.5** Representative DSC thermographs of (a) DOPC, (b) DOPC/Chol vesicles containing *d*- and *l*-menthol during heating at  $5\text{ }^{\circ}\text{C min}^{-1}$ .

From the calculated data, we can predict the interaction of *d*- and *l*- menthol with the bilayer. The DOPC system without cholesterol shows enthalpy due to the separation of the hydrophobes and the polar entity into two separate phases. *l*-Menthol have large decrease in value to the pure DOPC system, signifies the maximum separation from the hydrophobe i.e. acyl chain. While *d*-menthol have enthalpy near to the DOPC system indicating the interaction with hydrophobes too. Similarly, with DPPC bilayer also the effect of *d*- and *l*-menthol was observed with and without Chol Fig 3.6.



**Figure 3.6** Representative DSC thermographs of (a) DPPC, (b) DPPC/Chol, (c) DOPC, (d) DOPC/Chol vesicles containing *d*- and *l*-menthol during heating at  $5\text{ }^{\circ}\text{C min}^{-1}$ .

The pre-transition peak is very sensitive to the change in the membrane by addition or insertion of the additional molecules in bilayer.<sup>28</sup> The pre-transition peak of DPPC bilayer disappears on incorporation of cholesterol and *d*- and *l*-menthol. This indicates that some part of the menthol has interacted with the polar head group of DPPC. Moreover, main transition peak shifted to a lower temperature for both DPPC and DPPC/Chol in both *d*- and *l*-menthol containing system. There was a slight decrease in  $T_m$  for *d*- and *l*-menthol containing DPPC membrane. It implies the fact that both *d*- and *l*-menthol couldn't disturb the tight packing of DPPC membrane, hence  $T_m$  was not affected. Furthermore, incorporation of *d*- and *l*-menthol lowered the main transition of DPPC/Chol membrane, thereby indicating the disordered state of the acyl chain. Moreover, the insertion of the *l*-menthol between the polar heads of DPPC molecule may favor the development of a liquid phase less ordered than the gel phase and slightly decreases the gel-to-liquid phase transition temperature as observed by DSC. The

results of pure DOPC and DPPC bilayer are in good agreement with the previous reported data. The loss of enthalpy was relatively more significant in the DPPC/*l*-men which can be supported by favorable interaction at head part of lipid and may exert line tension on the domain boundary. The data of DSC thermogram is summarized in the table 3.3.

**Table 3.3 Main transition peak and change in enthalpy values. DOPC, DPPC, DOPC/Chol, and DPPC/Chol-containing *d*- and *l*-menthol liposomes.**

Component	$T_m(^{\circ}\text{C})$	$\Delta H(\text{Kj/mol})$
DOPC	-16.93	33.16
DPPC	41.77	33.23
DOPC/ <i>d</i> men	-17.73	27.94
DPPC/ <i>d</i> men	40.74	16.65
DOPC/ <i>l</i> men	-17.57	23.02
DPPC/ <i>l</i> men	40.15	12.58

Component	$T_m(^{\circ}\text{C})$	$\Delta H(\text{Kj/mol})$
DOPC/Chol	-19.02	10.15
DPPC/Chol	41.24	30.12
DOPC/Chol/ <i>d</i> men	-18.52	15.72
DPPC/Chol/ <i>d</i> men	37.13	3.99
DOPC/Chol/ <i>l</i> men	-18.54	17.88
DPPC/Chol/ <i>l</i> men	36.24	3.03

### 3.4 Conclusion:

In conclusion, I have investigated the impact of *d*- and *l*-menthol on the physical properties of the biomembranes. I have revealed that the presence of *d*- and *l*-menthol exhibits different physicochemical properties on the membrane and have different interaction manner with the lipids. Moreover, I have shown the preference of these two *d*- and *l*-menthol on the

association with lipid bilayer at molecular level. d-Menthol was supposed to make membrane more temperature sensitive, further consequences leads to its high fluidity nature. In contrast, l-menthol compare to d-menthol, maintain the membrane ordering of the lipid. I could observe these physical changes due to their difference in the interacting manner with the lipid. d-Menthol might have inserted into the lipid bilayer of DOPC as the voids created by double bonds facilitate this insertion. However, l-menthol has shown preference to saturated DPPC lipid and moreover to the head part of lipid. The thermal stability of both DPPC-rich solid-ordered ( $S_o$ ) phase and DPPC/Chol-rich liquid-ordered ( $L_o$ ) phase were decreased in the presence of d- and l-menthol. Cholesterol plays an important role in modulating the effect of d- and l-menthol on membrane. These findings are crucial to aid in understanding the effect of the lipid composition and cholesterol level on the interaction of d- and l-menthol in membrane respectively.

### 3.5 References

1. Singer, S. J. & Nicolson, G. L. The Fluid Mosaic Model of the Structure of Cell Membranes. *Science* (80-. ). **175**, 720 LP-731 (1972).
2. Nicolson, G. L. The Fluid—Mosaic Model of Membrane Structure: Still relevant to understanding the structure, function and dynamics of biological membranes after more than 40 years. *Biochim. Biophys. Acta - Biomembr.* **1838**, 1451–1466 (2014).
3. Simons, K. & Toomre, D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1**, 31–39 (2000).
4. Hamada, T., Kishimoto, Y., Nagasaki, T. & Takagi, M. Lateral phase separation in tense membranes. *Soft Matter* **7**, 9061–9068 (2011).

5. Klymchenko, A. S. & Kreder, R. Fluorescent Probes for Lipid Rafts: From Model Membranes to Living Cells. *Chem. Biol.* **21**, 97–113 (2014).
6. Simons, K. & Ikonen, E. Functional rafts in cell membranes. *Nature* **387**, 569–572 (1997).
7. Brown, D. A. & London, E. Structure and Function of Sphingolipid- and Cholesterol-rich Membrane Rafts. *J. Biol. Chem.* **275**, 17221–17224 (2000).
8. Ge, M. *et al.* Electron spin resonance characterization of liquid ordered phase of detergent-resistant membranes from RBL-2H3 cells. *Biophys. J.* **77**, 925–933 (1999).
9. Lee, Y. O. & Glantz, S. A. Menthol: putting the pieces together. *Tob. Control* **20**, ii1-ii7 (2011).
10. Hoffman, A. C. The health effects of menthol cigarettes as compared to non-menthol cigarettes. *Tob. Induc. Dis.* **9**, S7–S7 (2011).
11. Motaz Baibars, Simona Eng, Khaldoon Shaheen, Abdul Hamid Alraiyes, and M. C. A. Menthol Toxicity: An Unusual Cause of Coma. *Hindawi , Case Reports Med.* **2012**, 3 (2012).
12. McKemy, D. D. in (ed. Heller S)
13. Macpherson, L. J. *et al.* More than cool: Promiscuous relationships of menthol and other sensory compounds. *Mol. Cell. Neurosci.* **32**, 335–343 (2006).
14. Karashima, Y. *et al.* Bimodal Action of Menthol on the Transient Receptor Potential Channel TRPA1. *J. Neurosci.* **27**, 9874 LP-9884 (2007).
15. Egawa, T., Sakamoto, M., Takeuchi, H. & Konaka, S. Structural Determination of Menthol and Isomenthol, a Minty Compound and Its Nonminty Isomer, by Means of



- Gas Electron Diffraction Augmented by Theoretical Calculations. *J. Phys. Chem. A* **107**, 2757–2762 (2003).
16. Kumari, S., MG, S. & Mayor, S. Endocytosis unplugged: multiple ways to enter the cell. *Cell Res* **20**, 256–275 (2010).
  17. Alberts B, Johnson A, Lewis J, et al. in *Molecular Biology of the Cell* (Garland Science, 2002).
  18. Hamada, T. & Yoshikawa, K. Cell-Sized Liposomes and Droplets: Real-World Modeling of Living Cells. *Materials* **5**, (2012).
  19. Morita, M., Vestergaard, M., Hamada, T. & Takagi, M. Real-time observation of model membrane dynamics induced by Alzheimer's amyloid beta. *Biophys. Chem.* **147**, 81–86 (2010).
  20. Walde, P., Cosentino, K., Engel, H. & Stano, P. Giant Vesicles: Preparations and Applications. *ChemBioChem* **11**, 848–865 (2010).
  21. Yoda, T., Vestergaard, M. C., Hamada, T., Le, P. T. M. & Takagi, M. Thermo-induced Vesicular Dynamics of Membranes Containing Cholesterol Derivatives. *Lipids* **47**, 813–820 (2012).
  22. Yu, W., So, P. T., French, T. & Gratton, E. Fluorescence generalized polarization of cell membranes: a two-photon scanning microscopy approach. *Biophys. J.* **70**, 626–636 (1996).
  23. Harris, F. M., Best, K. B. & Bell, J. D. Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order. *Biochim. Biophys. Acta - Biomembr.* **1565**, 123–128 (2002).

24. Henshaw, J. B., Olsen, C. A., Farnbach, A. R., Nielson, K. H. & Bell, J. D. Definition of the Specific Roles of Lysolecithin and Palmitic Acid in Altering the Susceptibility of Dipalmitoylphosphatidylcholine Bilayers to Phospholipase A2. *Biochemistry* **37**, 10709–10721 (1998).
25. Parasassi, T., Giusti, A. M., Raimondi, M. & Gratton, E. Abrupt modifications of phospholipid bilayer properties at critical cholesterol concentrations. *Biophys. J.* **68**, 1895–1902 (1995).
26. Seeger, H. M., Marino, G., Alessandrini, A. & Facci, P. Effect of Physical Parameters on the Main Phase Transition of Supported Lipid Bilayers. *Biophys. J.* **97**, 1067–1076 (2009).
27. Riske, K. A. *et al.* Lipid bilayer pre-transition as the beginning of the melting process. *Biochim. Biophys. Acta - Biomembr.* **1788**, 954–963 (2009).
28. Zhao, L., Feng, S.-S., Kocherginsky, N. & Kostetski, I. DSC and EPR investigations on effects of cholesterol component on molecular interactions between paclitaxel and phospholipid within lipid bilayer membrane. *Int. J. Pharm.* **338**, 258–266 (2007).

## CHAPTER 4 CHIRALITY-DEPENDENT INTERACTION OF d- and l-MENTHOL WITH MODEL BIOMEMBRANES

**Abstract:** Chirality plays a vital role in biological membranes and has a significant effect depending on type and arrangement of isomer. Menthol has two typical chiral forms, d- and l-isomers adopting different behaviors. l-Menthol is known for its physiological effect on sensitivity (i.e. cooling effect), whereas d-menthol has irritation effect. Menthol molecules may affect not only the thermoreceptors on biomembranes but also the membrane itself. Membrane heterogeneity (lipid rafts, phase separation) depends on the lipid packing and acyl chain ordering. Our interest is to elaborate the chirality dependence of two isomer d- and l-menthol on membrane heterogeneity. To scratch the masked physical differences between the two isoforms of menthol on membrane heterogeneity, we worked on model membrane incorporating microscopic observations.

**Keywords:** Lipid raft, Miscibility temperature, d- and l-menthol, Membrane fluidity, Phase-separation

## 4.1 Introduction

There are certain specialized microdomains enriched in saturated lipid, sphingolipid, and cholesterol content, known as lipid raft.<sup>1,2,3,4,5,6,7,8</sup> These regions are rather packed tightly in liquid ordered phase unlike those disordered phase present in bulk of the biological membrane. Lipid rafts regarded as a platform for the regulation of various biological processes involved in signal transduction and membrane trafficking.<sup>6,9</sup>

Lipid rafts serve to localize some membrane proteins such as ion channel. The activity of a channel is controlled by some specific molecules, and the changes of the activity by molecular binding onto a channel causes generation of sensing e.g. warm, cold,<sup>10</sup> and anesthesia<sup>11</sup>. Such an additive molecule may have effects not only on the channel but also on lipid bilayer, thus alters membrane physical properties. Therefore, channel activity is also being influenced through lipid membrane.<sup>12</sup> Motivated by this mechanism, some researchers revealed the change in the physical property of lipid membranes by some specific sensing molecules. The two important parameters: hydrophobic acyl chain ordering and hydrophilic head group interaction could represent the sensing mechanism leading to channel activation. Hence it becomes very important to perceive these structural changes of lipid on interaction with such sensing molecules.

To investigate the membrane physical properties by external molecule, giant Unilamellar vesicles (GUVs) have attracted considerable attention for the modeling of living cells as they mimic the actual cell structures. GUVs with a diameter of micrometer size<sup>13</sup> are large enough to allow real-time observation by optical microscopy.<sup>14,15</sup> As the lipid compositions of liposomes can be easily changed, liposomes are used to investigate effects of lipid components considerably. Recently, heterogeneous distribution of membrane lipids was suggested to form phase-separated membrane mimicking a raft model.<sup>16</sup> In multicomponent

systems, heterogeneous phase-separated structures can be easily seen as solid-ordered ( $S_o$ ), liquid-ordered ( $L_o$ ) and liquid-disordered ( $L_d$ ) domains, respectively.<sup>17,18</sup>

Almost all organisms can detect surrounding temperature by primary afferent sensory neurons over a long range of temperature from noxious cold to noxious heat. There are two ways to detect these temperatures either through TRP channels present in sensory neurons or TRP channels in skin. TRP channels are not only activated by the temperature but also respond to a variety of chemical agonist. One of the interesting chemicals of such channel activated molecule is menthol, and it plays an important role in sensation pathways. Menthol is a component which triggers the brain into sensing cold. Nerves are the one which carries information in the form of electric currents and hence sense changes in temperature. Transient receptor potential cation channel subfamily M member 8 (TRPM8)<sup>19</sup> is the protein that senses the change in temperature and reveals the cooling sensitization of menthol. Moreover, TRPM8 is also known as the cold and menthol receptor 1 (CMR1) and is present in humans encoded by the TRPM8 gene.<sup>20</sup> TRPM8 ion channel upon activation allows the entry of  $Na^+$  and  $Ca^{2+}$  to the cell that leads to depolarization and eventually generate an action potential. Once the signal is received from sensory neurons (type C- and A-delta) the brain registered in order to reflect the sense of cold and cold pain. Menthol shows dual behavior depending on temperature and concentration: cooling sensations is induced at lower concentration and room temperature, whereas at high temperatures ( $>37\text{ }^\circ\text{C}$ ), it induces the sensation of burning/itching.<sup>21,22,23,24</sup> L-menthol is a versatile compound and interacts with many receptors like TRPM8<sup>25,26,27,28</sup>, transient receptor potential cation channel subfamily V member 3 (TRPV3), transient receptor potential cation channel subfamily V member 1 (TRPV1), and transient receptor potential cation channel subfamily A member 1 (TRPA1).<sup>25</sup> Kappa opioid receptors are also stimulated by l-menthol and enhance the voltage-temperature dependent gate mechanism. It has been also believed that prolonged exposure of l-menthol

will start showing desensitization to cold sensitive fibers similar to the capsaicin self-desensitizing heat sensitive TRPV1.<sup>23</sup> It was also reported that C-terminus of TRPM8 channel contains structural elements which are important in temperature-dependent gating. However, the mechanism behind those temperature-dependent changes is still controversial at the molecular level.

In this diverse world, to understand the importance of chirality becomes a challenging issue. Biological molecules are often chiral such as DNA, proteins, carbohydrates, lipids, steroids, etc. Menthol also has chirality. There are several enantiomers of menthol, and the typical enantiomers are (1*S*, 2*R*, 5*S*)-2-isopropyl-5-methylcyclohexanol (*d*-menthol) and (1*R*, 2*S*, 5*R*)-2-isopropyl-5-methylcyclohexanol (*l*-menthol). *l*-Menthol which is the main form found in nature is widely used in toothpaste, chewing gum, cigarettes<sup>29,30</sup> and so on.<sup>31,22</sup> *l*-Menthol may bind onto TRPM8 and only causes well known desired cooling sensation. On the other hand, *d*-menthol not only does not exist in nature but also does not induce cooling sensation. In this way, although there is a significant difference between *d*- and *l*-menthol in channel activity, the interaction between lipid membrane and *d*- and *l*-menthol are not well understood. To understand the mechanism behind the activity of this *d*- and *l*-menthol, the first step is to elucidate their part of interaction with membranes and where they get located into the membranes.

Even in model membranes, phase separation structures similar to cell membranes can be created by mixing unsaturated lipids, saturated lipids, and cholesterol in various ratios. Uniform membrane liposome like fluid mosaic model<sup>32</sup>, the liquid-liquid phase separation membrane considered as a raft model, further model phase membrane system has another phase separation structure, which is a solid-liquid phase separation membrane which occurs when cholesterol concentration is lowered. The characteristic of each phase state consists of a

soft liquid disordered phase ( $L_d$  phase) formed from unsaturated lipid, a liquid ordered phase ( $L_o$  phase) composed of saturated lipid and cholesterol, saturated lipid.<sup>33</sup> There is no solid order phase ( $S_o$  phase). Traditionally, rafts have been considered DRM fractions and  $L_o$  /  $L_d$  phase separation membranes. However, the DRM fraction cannot distinguish between the  $L_o$  phase and the  $S_o$  phase, and it is possible that the  $S_o$  phase may exist in the cell. Hence, I used GUVs composed of unsaturated lipid (DOPC), saturated lipid (DPPC) and Cholesterol to the study the phase-separated structure in the biomembrane.<sup>13</sup>

## 4.2 Experimental Procedures:

### 4.2.1 Materials:

Lipids 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and Cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, USA). (1*S*, 2*R*, 5*S*)-2-isopropyl-5-methylcyclohexanol (*d*-menthol), (1*R*, 2*S*, 5*R*)-2-isopropyl-5-methylcyclohexanol (*l*-menthol) were purchased from Wako Japan. Fluorescent dyes, N-(rhodamine red-X)-1, 2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (rhodamine DHPE) ( $\lambda_{ex}$ = 560 nm,  $\lambda_{em}$ = 580 nm) obtained from Thermo Fisher Scientific (USA) and 6-Dodecanoyl-2-Dimethylaminonaphthalene (laurdan) was obtained from Funakoshi (Japan). Chloroform and methanol were from Kanto-Chemical (Japan) and Nacalai Tesque (Japan), respectively. Ultrapure water (specific resistance  $\geq 18$  M $\Omega$ ) was obtained from a Millipore Milli-Q purification system.

### 4.2.2 Preparation of Liposomes:

Lipid vesicles were prepared by the natural swelling method.<sup>34</sup> Lipids, Chol, menthol, and fluorescent probe were dissolved in a 2:1 vol/vol (chloroform/methanol) solution, making the

concentration 2 mM for lipids, Chol and menthol and 0.1 mM for a fluorescent probe. Lipids, Chol, and menthol were mixed at desired concentration to be final volume 20  $\mu$ L. 2  $\mu$ L of rhodamine-DHPE was further added to the lipid mixture. The organic solvent was evaporated under a flow of nitrogen gas and the lipids were further dried in a vacuum desiccator for 3h. The film was hydrated with Milli-Q water at 37 °C for an hour and was kept overnight at room temperature  $21.7 \pm 1.7$  °C. The final lipid concentration was 0.2 mM and rhodamine-DHPE concentration was 1  $\mu$ M. The prepared lipid compositions for microscopic observation were DOPC/DPPC/Chol = 50:50:0, 40:40:20, and 35:35:30 as control systems without menthol. When we add 10 mol% menthol to lipid compositions, we fixed DOPC:DPPC/Chol = 1:1:0, 2:2:1, and 7:7:6, that is DOPC/DPPC/Chol/*d*- or *l*-menthol = 45:45:0:10, 36:36:18:10, and 31.5:31.5:27:10.

#### 4.2.3 Microscopy Observations:

5  $\mu$ L of the lipid vesicle solution was placed in a silicon well (0.2 mm) on a glass slide, and cover with cover slip. The silicon well and the coverslip ensured that evaporation of the solution did not occur during the entire experiment. The formed GUVs were observed by fluorescence microscopy (Olympus IX71, Japan) at room temperature  $22.8 \pm 2$  °C. The images were recorded on a hard disc drive at 30 frames/s. GUVs were prepared by unsaturated lipid DOPC, saturated lipid DPPC, and Chol to form two phase membranes with lateral heterogeneity. In order to observe the heterogeneous structure, fluorescent probe Rhodamine-DHPE which is included in DOPC-rich region was used.

#### 4.2.4 Membrane fluidity Measurements:

The fluidity of the membrane was measured by using laurdan as a fluorescent probe. Generalized polarization (GP) value was estimated by the equation

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$



where  $I_{440}$  and  $I_{490}$  are the two emission intensities to detect the shift in the maxima of laurdan.

#### 4.2.5 Miscibility Temperature Measurements:

We counted 55 liposomes for each composition in each temperature between 18 °C and 40 °C for the systems without Chol and between 18 °C and 32 °C for the systems with Chol using thermos-controller, and plotted the fraction of phase-separated liposomes. We calculated miscibility temperature ( $T_m$ ) for the lipid membranes using the obtained experimental plots. The miscibility temperature is defined as the temperature at which the fraction of phase-separated liposome reaches 50 %. To obtain the miscibility temperature, the experimental results were fitted with the sigmoidal Boltzmann function,

$$p = \frac{1}{1 + \exp[(T - T_m)/dt]}, \quad - (1)$$

where  $p$  is the fraction of phase-separated liposomes,  $T$  is the temperature,  $T_m$  is the miscibility temperature, and  $dt$  is the slope of the sigmoidal curve.”

### 4.3 Results and discussion

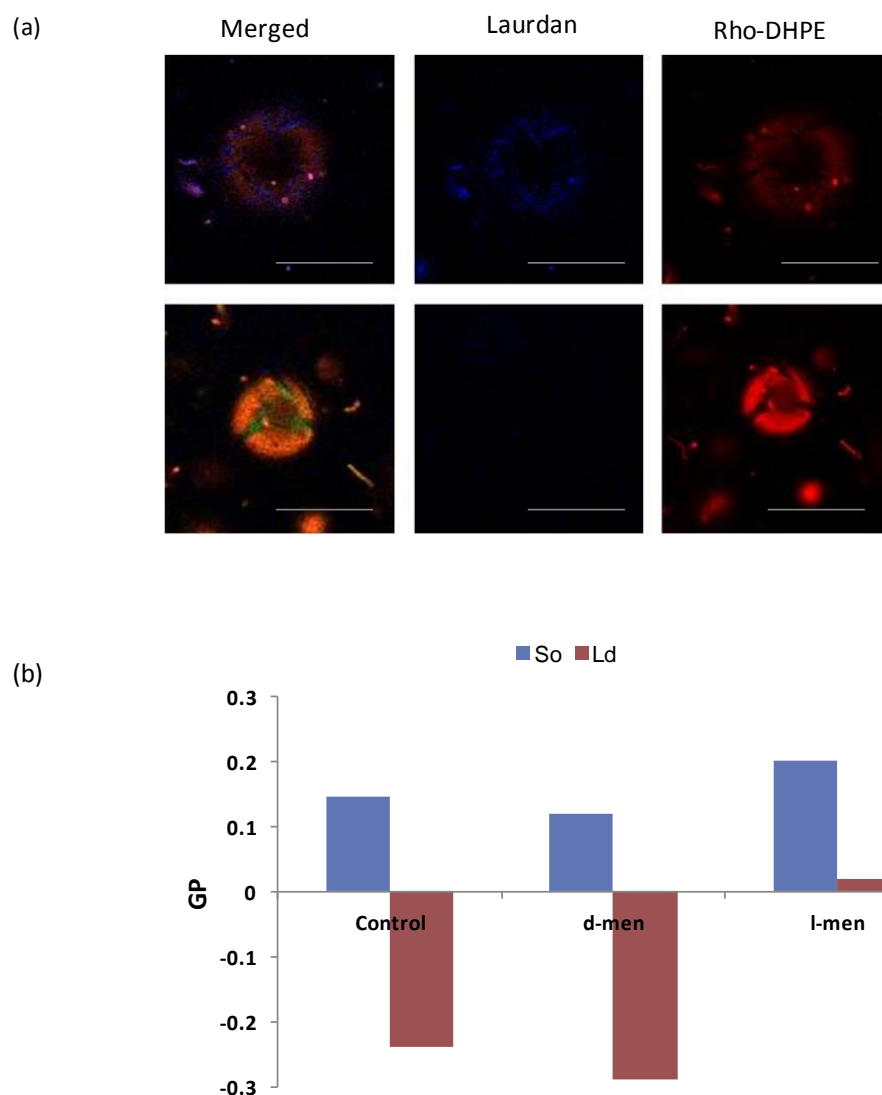
#### 4.3.1 Effect of *d*- and *l*-menthol on membrane fluidity on heterogeneous membrane

Lipids are regarded as an important modulator of many ion channel activity. Lipids can have direct influence as a channel gating modulator or can alter the properties of channel activity by estimating biophysical properties of the membrane-like membrane fluidity. Membrane fluidity greatly depends on the lipid composition and cholesterol content. It is worthwhile to find how *d*- and *l*-menthol induces a change in the membrane fluidity, thus alter the lipid order. Laurdan, a fluorescent probe is used extensively to study membrane fluidity in the model membrane.<sup>35,36</sup>

The probe is sensitive to the water molecules and alters the lipid ordering in the bilayer. Generalized polarization (GP) value tells the order state of the lipid and shows membrane fluidity. The more high GP value, less fluidic will be the membrane, ordered state. On the contrary, low GP value indicates the disordered state of lipid, making membrane more fluid.

In order to evaluate the effect of *d*- and *l*-menthol on membrane fluidity, I choose three different membranes DOPC/DPPC (50/50) and DOPC/DPPC/Chol (40/40/20) and DOPC/DPPC/Chol (35/35/30) labeled with laurdan dye. Liposomes were observed at three excitation wavelengths, including 405 nm, 488 nm, and 545 nm (Rhodamine-DHPE emission). Laurdan GP value was defined as  $GP = (I_{440} - I_{490}) / (I_{440} + I_{490})$ . The temperature was kept constant at 22.4 °C during observation. Figure 4.1(a) show the microscopic images of  $S_o/L_d$  domain formation at room temperature in DOPC/DPPC system.

The distribution of GP values measured in  $S_o/L_d$  membrane system was depicted in Fig 4.1(b). The GP value of  $S_o$  phase was not affected by both *d*- and *l*-menthol addition, whereas GP value of  $L_d$  phase in the case of *d*-menthol, was slightly decreased. This suggested that *d*-menthol could localize in DOPC-rich  $L_d$  phase thereby makes it more fluidic. However, no such change in the GP value of  $S_o$  and  $L_d$  phase was observed for *l*-menthol containing vesicles. I propose that as both DOPC and DPPC have same phosphatidylcholine head group *l*-menthol may interact with each in a similar manner. Some of the DOPC molecules are in gel phase and DPPC molecules in the liquid phase in the binary mixture. *d*-Menthol has preferential for unsaturated DOPC lipid over saturated DPPC lipid. The fluidity of DPPC-rich  $S_o$  phase is low due to the strong packing between DPPC molecules. There is an exclusion of DOPC molecules from  $S_o$  phase, similarly DPPC from  $L_d$  phase, therefore overall  $S_o$  domain becomes rigid.

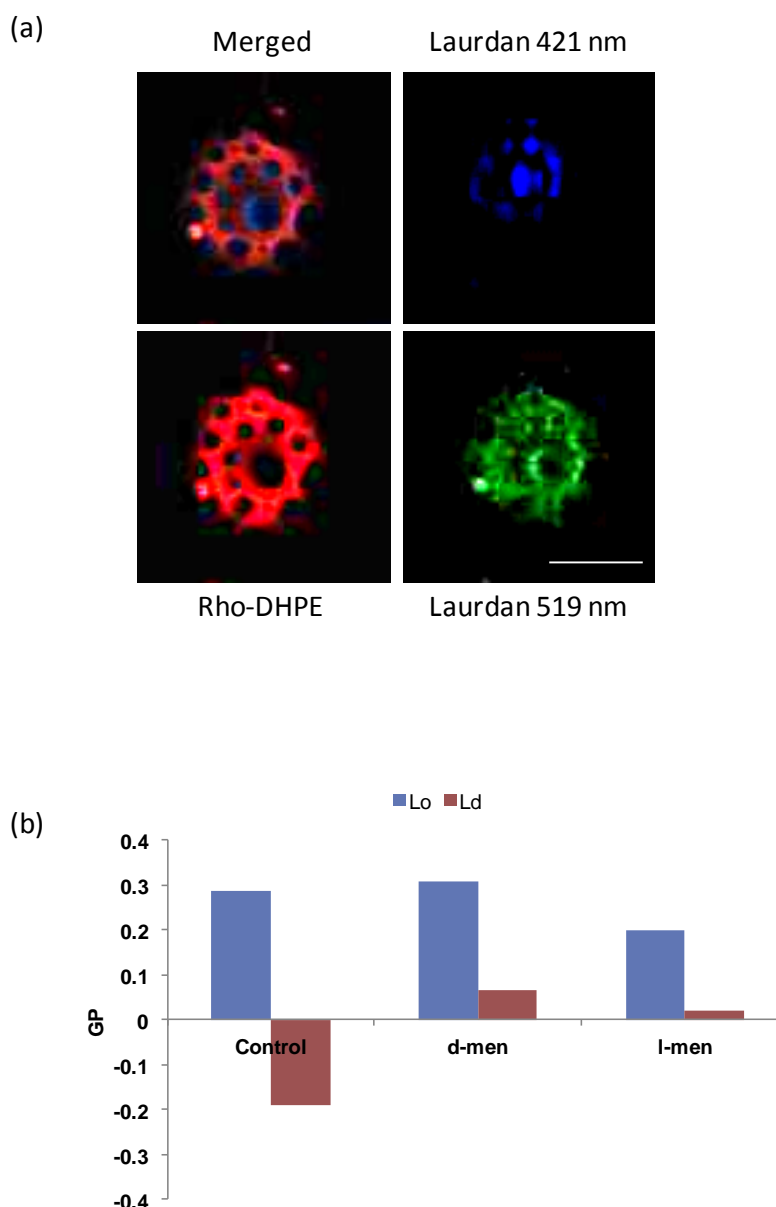


**Figure 4.1** Effect of *d*- and *l*-menthol on membrane fluidity of  $S_o/L_d$  heterogeneous model membranes. Typical confocal microscopy images of Laurdan emission (a), GP values of  $S_o/L_d$  phase-separated membranes. Red and black region indicates  $L_d$  and  $S_o$  domains. Scale bar = 10  $\mu\text{m}$ .

In the case of  $L_o/L_d$  system,  $L_d$  phase has higher GP value on an increase in cholesterol concentration, refers to the more ordered state. On the other hand,  $L_o$  phase becomes more disordered upon an increase in cholesterol concentration, as the GP value becomes lower. This is consistent with the previous reports of cholesterol on the membrane fluidity.

I found that the phase-separated structure so called formed on the surface of the liposome membrane composed of the three components of unsaturated phospholipid DOPC,

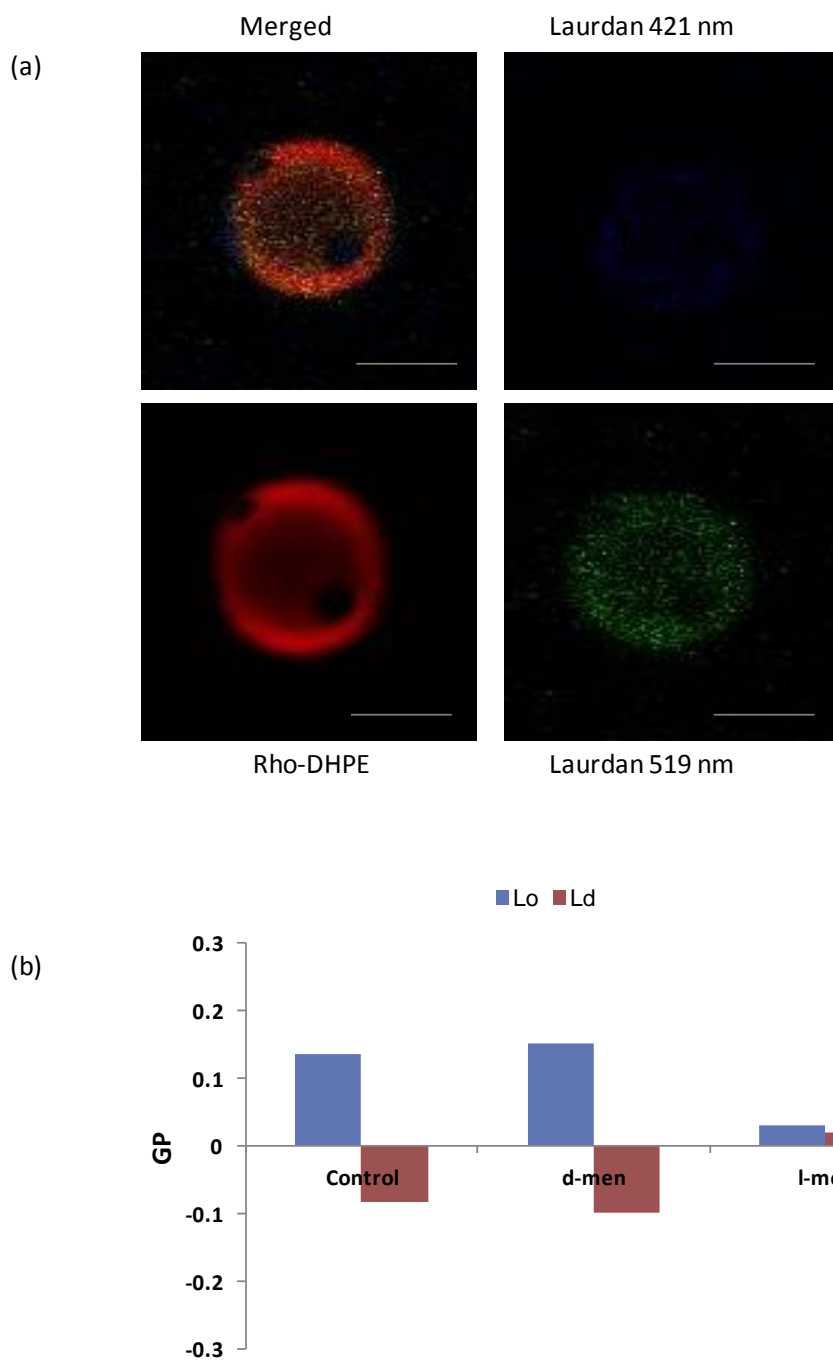
saturated phospholipid DPPC, cholesterol in ternary system lowers due to interaction with the *d*- and *l*-menthol, *i.e.*, it became unstable. At the same time, it was revealed that *d*- and *l*-menthol lowered the fluidity of non-raft region rich in DOPC. In *d*-menthol containing lipid, GP values of  $L_d$  phase were higher than that of control, while  $L_o$  phase remains unchanged as shown in Fig 4.2.



**Figure 4.2** Effect of *d*- and *l*-menthol on membrane fluidity of  $L_o/L_d$  heterogeneous model membranes at Cholesterol 20 %. Typical confocal microscopy images of Laurdan emission (a), GP values of  $L_o/L_d$  phase-separated membranes. Red and black region indicates  $L_d$  and  $L_o$  domains. Scale bar = 10  $\mu\text{m}$ .

This indicated that *d*-menthol mainly affect  $L_d$  phase rendering it fluid in nature and *d*-men did not influence the fluidity of  $L_o$  phase. This could be explained base on the preferential interaction and orientation with lipid. As *d*-menthol tends to interact in the DOPC-rich  $L_d$  phase and possibly occupied the hydrophobic region of the lipid, has the capability to reduce the mobility of the tails and packing. Thus, membrane loses its fluidity by the addition of *d*-menthol. So I concluded that presence of *d*-menthol made loses fluidity of  $L_d$  phase, and this effect made fluidity gaps between  $L_d$  non domain region and  $L_o$  domain region sufficiently smaller. That is the reason why the addition of *d*-menthol destabilized phase-separated structures. On the other hand, *l*-menthol though lowered the GP value of  $L_o$  domain making the DPPC slightly fluidized which could be due to the favorable interaction among the head group of lipid and *l*-menthol.

Lastly, I checked how cholesterol concentration is able to alter the physical property in the biomembrane, so membrane fluidity at higher cholesterol concentration 30 % was investigated. Figure 4.3 shows GP values in ternary mixture DOPC/DPPC/Chol (35/35/30) in the presence of *d*- and *l*-menthol. It was observed that  $L_o$  phase become more disordered in the case of *l*-menthol upon an increase in cholesterol concentration, as the GP value becomes lower. This implies *l*-menthol lose the fluidity of  $L_o$  domain, thereby destabilized the phase-separated structure. In this case amount of cholesterol molecules are very high, hence the there will be more hindrance at the surface caused by the interaction among *l*-menthol, head group of both DOPC and DPPC and cholesterol. This leads to a crowded environment at the surface, thus destabilized phase structure. On the other hand, since *d*-menthol localized into DOPC-rich  $L_d$  region, the  $L_o$  phase remains unaltered whereas a slight decrease in GP value of  $L_d$  phase was observed.



**Figure 4.3** Effect of *d*- and *l*-menthol on membrane fluidity of  $L_0/L_d$  heterogeneous model membranes at Cholesterol 30 %. Typical confocal microscopy images of Laurdan emission (a), GP values of  $L_0/L_d$  phase-separated membranes. Red and black region indicates  $L_d$  and  $L_0$  domains. Scale bar = 10  $\mu\text{m}$ .

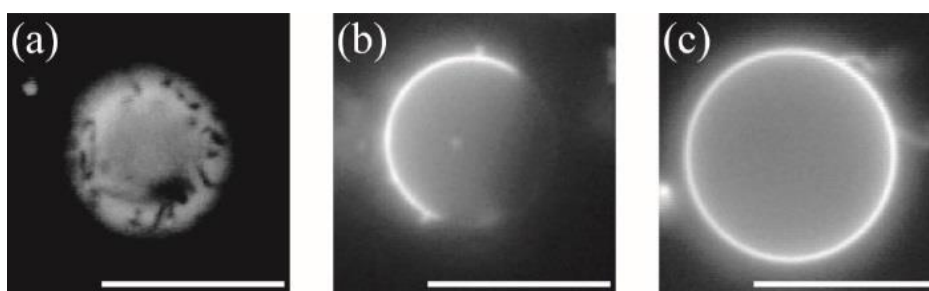
The fluidity measurements were summarized in table 4.1. The detail discussion will be argued in the next section. In contrast, *d*-menthol stabilized the phase-separated structure at cholesterol 30 %, as GP value of  $L_o$  domain remains unaffected. Due to a higher affinity of cholesterol to *d*-menthol, some of the cholesterol molecules makes to DOPC-rich  $L_d$  phase, making DPPC-rich  $L_o$ -phase more stable and ordered.

**Table 4.1 Correlation of *d*- and *l*-menthol localization in heterogeneous membrane phases and membrane fluidity upon the effect of cholesterol concentration.**

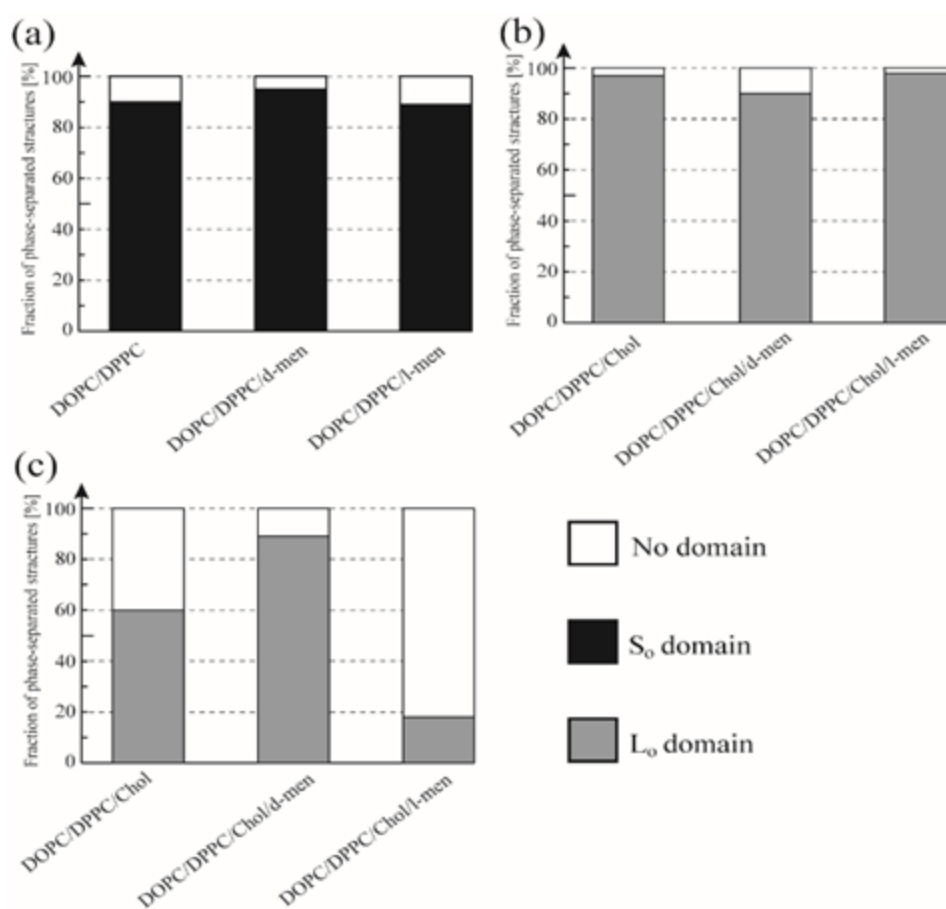
Membrane systems	Phases	Chol = 0%			Chol = 20%			Chol = 30%		
		Fluidity	<i>d</i> -	<i>l</i> -	Fluidity	<i>d</i> -	<i>l</i> -	Fluidity	<i>d</i> -	<i>l</i> -
So/Ld	So	Low	No change		Low	×	×	Low	×	×
	Ld	High	Increase	decrease	High	×	×	High	×	×
Lo/Ld	Lo	Low	×	×	Low	No change	Increase	Low	No change	Increase
	Ld	High	×	×	High	decrease	decrease	High	No change	decrease

#### 4.3.2 Effect of cholesterol concentration on membrane heterogeneity

First, I observed phase separation in lipid membranes containing *d*- and *l*-menthol at room temperature. Figure 4.4 shows the typical fluorescence microscopic images of different types of phase-separated membrane. The typical microscopic image is shown in Fig 4.4(a); DPPC-rich stripe like ( $S_o$ ) domains (dark region) is surrounded by DOPC-rich  $L_d$  phase (bright region) was formed in the binary system. Circular shapes  $L_o$  domains enriched with DPPC and Chol (dark region) surrounded by  $L_d$  phase (bright region), as shown in Fig 4.4(b), were mainly formed in the ternary system.



**Figure 4.4** Typical fluorescence microscopic images of  $S_0$  domain (a)  $L_0$  domain (b), no domain (c). Scale bar = 10  $\mu\text{m}$ .



**Figure 4.5** Fraction of phase-separated structures in DOPC/DPPC/Chol/menthol systems at room temperature. We fixed DOPC:DPPC:Chol = 1:1:0 in (a), 2:2:1 in (b), and 7:7:6 in (c). The mole fraction of menthol is 10 %. White, black, and grey bars indicate no domain (homogeneous phase),  $S_0$  domain formation, and  $L_0$  domain formation, respectively. Number of liposomes counted = 55.

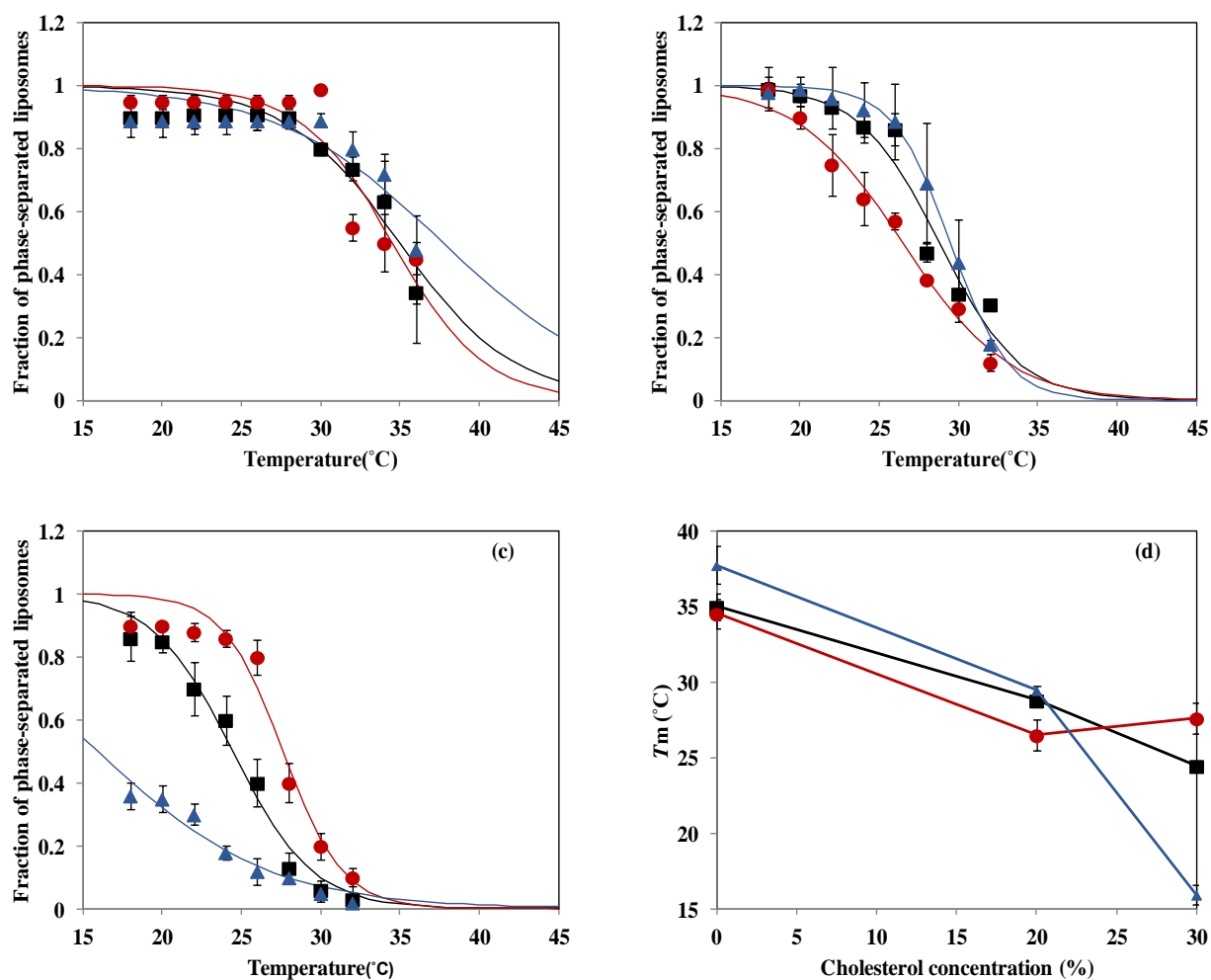


Next, I observed the phase separation of lipid mixtures at room temperature. In the binary system consisting of unsaturated lipid DOPC and saturated lipid DPPC without Chol,  $S_o$  domain is formed predominantly, as shown in Fig 4.5(a).

There was no clear difference in the phase behavior by adding *d*- and *l*-menthol to this binary system as shown in Fig 4.5(a). Similarly, phase separation in DOPC/DPPC/Chol ternary lipid mixture was observed. Also, there was no significant difference between the system without menthol and the *d*- and *l*-menthol containing systems. At Chol = 30% DOPC/DPPC/Chol = 35/35/30 system, I found significant effects by *d*- and *l*-menthol on phase behavior in Fig 4.5(c). The phase-separated structure was stabilized by *d*-menthol, whereas *l*-menthol significantly suppresses the phase separation.

In order to understand the effects of *d*- and *l*-menthol on the phase separation more clearly, the miscibility temperature was observed over the temperature range from 18 °C to 38 °C using a thermo controller. The miscibility temperature  $T_m$  is defined as the temperature at which the fraction of heterogeneous GUVs becomes 50 % as mentioned in Materials and Methods. Figure 4.6(a) shows fractions of liposomes which formed phase-separated structures on DOPC/DPPC liposomes in each temperature range. The black filled square and a solid line show the result of control systems, and others red and blue are for *d*- and *l*-menthol containing liposomes, respectively. Symbols (square, circle, and triangle) denote the experimental data, and lines are obtained from Eq. (2) to fit the experimental results. In the case of DOPC/DPPC binary system and DOPC/DPPC/Chol ternary system at Chol 20 %, phase-separated structures were not strongly affected by the addition of *d*- and *l*-menthol molecules as shown in Fig 4.6(a) and (b). These results are essentially consistent with the phase behavior at room temperature, as shown in Fig 4.5(a) and (b).

At Chol = 30 %, however, I could observe the clear and significant phase behavior differences induced by *d*- and *l*-menthol, as shown in Fig 4.6(c) and (d). Notably, *d*-menthol stabilized phase separation, whereas *l*-menthol dramatically lowers the fraction of phase separation. The same tendency is also seen in Figure 4.5(c). In Fig. 4.6(d), we showed the change of the miscibility temperature as a function of Chol concentration.



**Figure 4.6** Miscibility temperature measurement for DOPC/DPPC/Chol/menthol systems. We fixed DOPC:DPPC:Chol = 1:1:0 in (a), 2:2:1 in (b), and 7:7:6 in (c). The mole fraction of menthol is 10 %. Black lines and black squares indicate DOPC/DPPC/Chol without menthol. Red lines with red circles and blue lines with blue triangles are DOPC/DPPC/Chol with *d*-menthol and *l*-menthol, respectively. (d) Change in the miscibility temperature as a function of Chol concentration in lipids defined as  $[Chol]/([DOPC]+[DPPC]+[Chol])$  %.

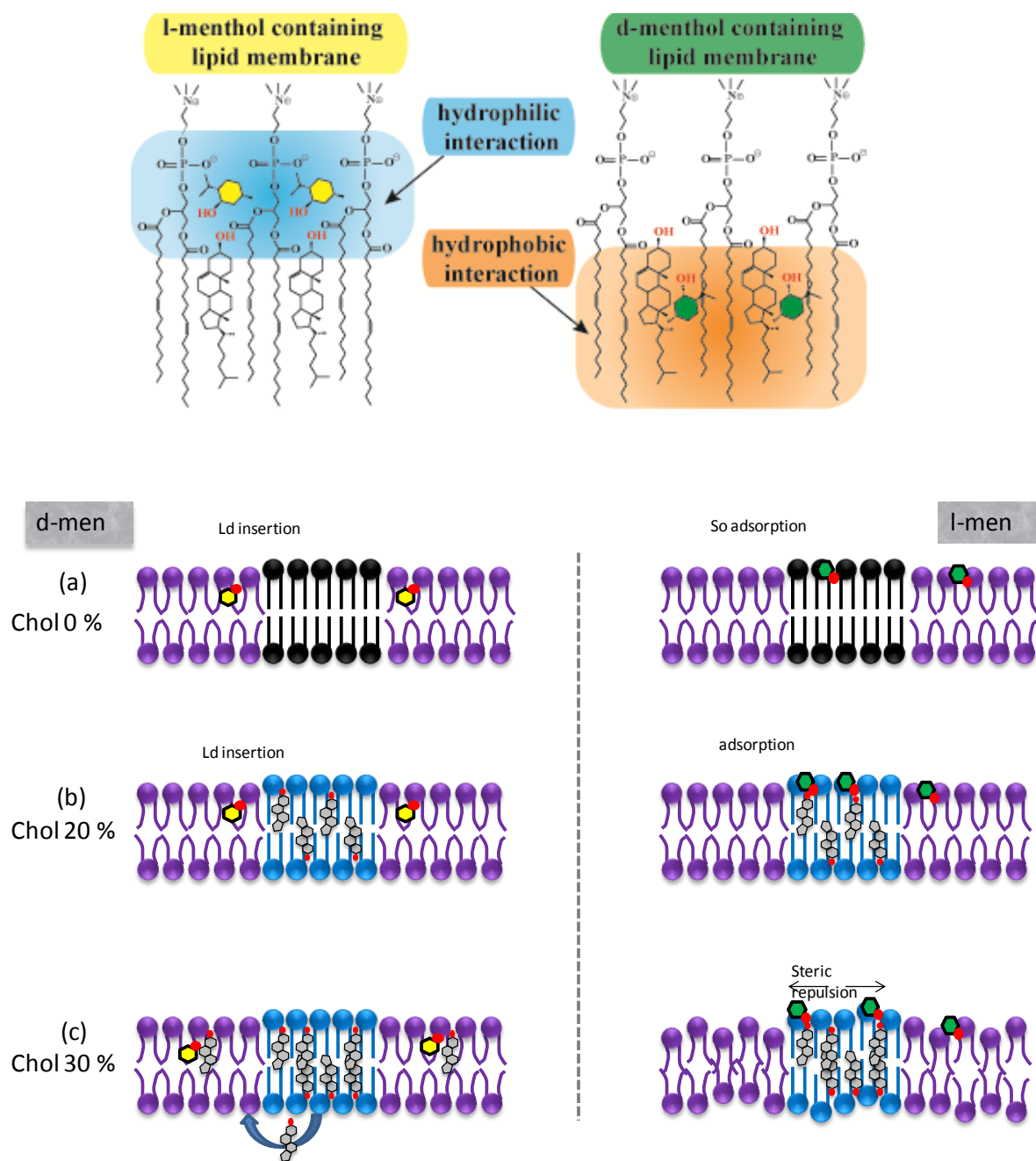
I could see the clear trend that the miscibility temperature is higher for *d*-menthol and that is lower for *l*-menthol at Chol = 30 %. During microscopic observation, the transformation from homogeneous to  $L_d$  domains surrounded by  $L_o$  phase (reverse domains) in *l*-menthol-containing membranes at Chol = 30 % occurs just after excitation light irradiation. This result implies that the homogeneous phase may be a metastable phase. However, we ignore such a transformation, since artifact including lipid oxidation by light irradiation should be eliminated.

In the case where Chol concentration is 0 %, the addition of *d*/*l*-menthol does not affect the phase behavior. NMR measurement revealed that the main interaction site between *d*-menthol and lipid is hydrophobic, however, *l*-menthol may situate at the interface of a polar head group of PC and hydrophobic part (data not shown). *l*-Menthol is believed to have cooperative interaction among PC (C=O), cholesterol (OH) influenced by temperature. This temperature sensitive interaction at the interface may lead to a change in the channel activity, thus may involve in the cold/burning sense induced by *l*-menthol. *d*-Menthol is localized in the DOPC-rich  $L_d$  phase because *d*-menthol could not be included in tightly packed DPPC-rich  $S_o$  phase. Therefore, *d*-menthol does not affect the  $S_o$  domain formation. On the other hand, *l*-menthol mainly interacts with the hydrophilic part (phosphocholine: PC), *l*-menthol may interact with DOPC and DPPC in the same manner. Since the number density of lipid molecules in  $L_o$  phase is larger than that in the  $L_d$  phase, *l*-menthol prefers to be localized in  $L_o$  phase. As a result, hydrophilic region of the  $L_o$  phase becomes crowded and the steric repulsion may arise.

However, since the attraction between DPPC molecules is strong enough, the addition of *l*-menthol does not disturb the domain formation in DOPC/DPPC mixtures (depicted in Fig 4.7(a), (b)).

This tendency is almost same as the case of DOPC/DPPC/Chol mixture at Chol = 20 %. At Chol = 30 %, however, *d*-menthol stabilizes the  $L_o$  domain formation. We image that there is a competitive nature between *d*-menthol and Chol as both localized in the hydrophobic environment. As mentioned above, *d*-menthol may partition into  $L_d$  phase mainly. Some Chol molecules in the  $L_o$  phase migrate to the  $L_d$  phase due to the attraction between *d*-menthol and Chol. As a result, the amount of Chol in the  $L_o$  phase is decreased, stabilizing  $L_o$  domains, because the homogeneous phase exhibits at higher Chol concentration (e.g. DOPC/DPPC/Chol (35/35/30)) (figure 4.6(c)). Because both *d*-menthol and Chol exist in the hydrophobic region, the direct attraction between *d*-menthol and Chol may be present. On the other hand, the phase separation was strongly suppressed by the addition of *l*-menthol in DOPC/DPPC/Chol at Chol = 30 %. As stated above, *l*-menthol may be localized in the densely packed DPPC-rich  $L_o$  phase due to hydrophilic interaction. Since Chol concentration is high enough (Chol = 30 %), the lipid packing in the  $L_o$  phase is relatively weak compared with the case of Chol = 20 %. In addition, *l*-menthol may partition largely into the  $L_o$  phase including the large amount of Chol due to hydrophilic interaction among PC, the hydroxy group of Chol and *l*-menthol. Therefore, the steric repulsion in the crowded hydrophilic region by *l*-menthol disturbs  $L_o$  domain formation significantly. Figure 4.7 show schematic illustration depicting mechanism of localization preference exhibited by *d*- and *l*-menthol in three different systems.

These findings can be explained by considering the change in the lipid bilayer physical state near the phase transition. From the results obtained I proposed a model showing a possible interaction of *d*/*l*-menthol with lipid bilayer which could affect the cold sensing properties of TRPM8. We also believe that TRP channels can sense the temperature-dependent changes in the lipid bilayer.



**Figure 4.7** Schematic illustration showing mechanism of interaction (upper panel) and localization preference exhibited by *d*- and *l*-menthol in three different systems. (a) DOPC/DPPC (*So/Ld* membrane), (b) DOPC/DPPC/Chol (*Lo/La* membrane at Chol = 20 %), and (c) DOPC/DPPC/Chol (*Lo/La* membrane at Chol = 30 %).

Briefly, rise in the temperature and cholesterol concentration could alter the lipid packing, indeed leads to change in membrane protein function such as involved in the

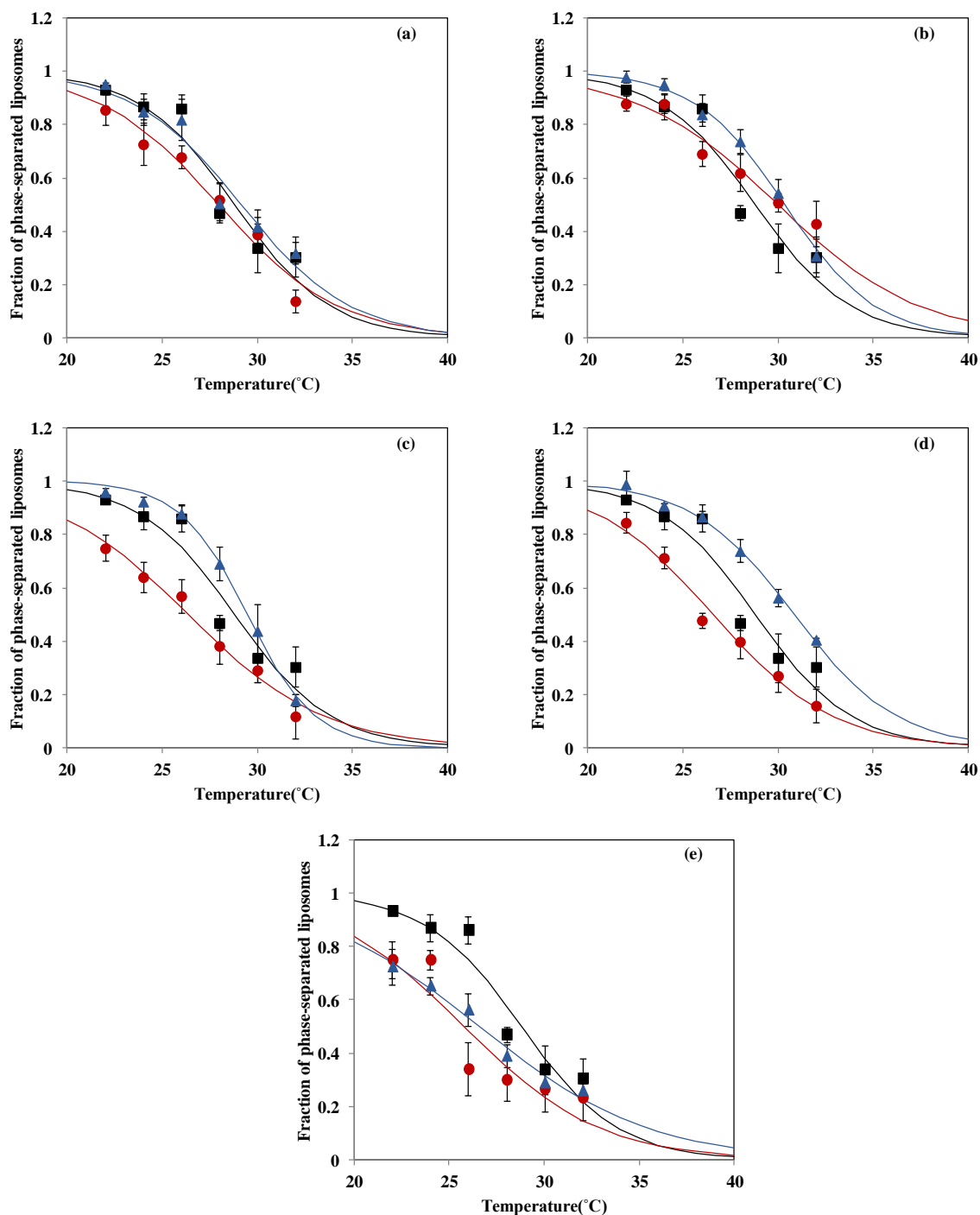
function of the channel. It is interesting to speculate that since *l*-menthol have shown hydrophilic interaction predicted by  $^{13}\text{C}$ -NMR at g2 and C1(C=O) position of lipid, this interaction could be influenced by temperature. Previous studies<sup>33</sup> supports our data obtained from  $^{13}\text{C}$ -NMR about the hydrogen bond between ester group of PC (C=O) and the hydroxyl group of Chol. At a lower temperature, Chol movement is restricted in the bilayer; thereby stabilized the membrane structure that corresponds to cold sensitization at cold temperature. The phase behaviour data demonstrated at lower Chol concentration, *l*-menthol displays strong hydrophilic interaction stabilizing the head group interaction which could relate to cooling phenomena even at low temperature. On increasing temperature as well as cholesterol concentration the same interaction now becomes more hindered and mobile to cause head group expansion, thus destabilized the lipid structure. On the other hand *d*-menthol exhibit hydrophobic interaction localized in the hydrophobic region with Chol causing strong hindrance, disturbing the packing *i.e.*, destabilized the membrane. It was reported that some of the TRP channels are sensitive to the cholesterol content and is important in channel functioning. I strongly believed that the hydrophilic interaction around g2 and C1 (C=O) position of lipid plays an important role in modulation of TRPM8 channel activity. Some amino acid residue of TRPM8 channel may involve to those temperature sensitive interactions at hydrophilic surface incriminate in the cold sense by *l*-menthol. Furthermore, these dynamic changes in the membrane properties enable to picture the related mechanism behind the cooling sensation. These experiments persuade further attention to the regulation of membrane protein channel activity by the lipid environment.

#### 4.3.3 *Distribution of menthol within the lipid bilayer: Concentration effect of menthol*

The activity and distribution of menthol in the bilayer is still controversial and many on-going types of research gave different sight of its interaction with the bilayer. Menthol is a

monocyclic monoterpenoid organic compound, used as a flavoring agent and to relief minor aches. It has been reported that menthol cooling property is concentration dependent manner, and its effect varies significantly upon a change in the concentration. Previous reports reflect cooling sensitization of *l*-menthol at low concentration and also lower temperature will enhance cooling sensation. To the same context, higher concentration/ prolonged exposure of *l*-menthol exerts pain/burning sensation.<sup>21</sup> It has been also believed that menthol act as a penetration enhancer and has been widely used in medication. Menthol shows high efficiency as penetration enhancer. Taking this fact into consideration, an interaction of menthol with the lipid bilayer was evaluated. The two important factor concentration and temperature effect were focused in order to unveil molecular interaction. Thus, I worked on different concentration (2.5, 5, 10, 15, and 30 %) of menthol, and investigate the effect of *d*- and *l*-menthol on miscibility temperature of phase-separated membrane. In Fig 4.8, miscibility temperature was measured when 50 % of the phase-separated membrane was formed. The measurement was made from temperature range 22 to 32 °C at different concentration of *d*- and *l*-menthol. There is no significant difference was observed in both *d*- and *l*-menthol at a lower concentration 2.5 %.

However, *d*-menthol still destabilized the phase separation at 2.5 and 5 % compare to *l*-menthol. On increasing concentration further 10 % and 15 %, a similar trend was seen between *d*- and *l*-menthol as in the lower concentration. Interestingly at very high concentration 30 %, *l*-menthol showed dramatically destabilized phase-separated structure, meaning that lipid will be highly disordered. This model at menthol 30 % corresponds to the case similar what I observed in Chol 30 %.



**Figure 4.8** Miscibility temperature measurement for DOPC/DPPC/Chol/menthol systems at menthol = 2.5 % in (a), 5 % in (b), 10 % in (c), 15 % in (d), and 30 % in (e). Black lines and black Squares indicate DOPC/DPPC/Chol without menthol. Red lines with red circles and blue lines with blue triangles are DOPC/DPPC/Chol with *d*-menthol and *l*-menthol, respectively.

Previous reports provide information that at each and every concentration, menthol can penetrate into the lipid bilayer.<sup>37</sup> As discussed above that *d*-menthol prefer to localize in the



DOPC-rich  $L_d$  phase, and tends to destabilized the phase-separated membrane, I could observe a slight decrease in the miscibility temperature at a lower temperature. On the other hand, *l*-menthol has a preferential liking to both head group of the lipid and might distribute between two different lipid hydrophilic parts.

As a result packing among the two neighboring lipids remains unaffected by the addition of *l*-menthol. In the introduction part, it was clearly sighted the behavior of menthol is concentration dependent. At higher concentration (~30 % and above), *l*-menthol disturbed the bilayer property and renders pain effect to the body. I believed that at higher concentration, because of a significant number of menthol molecules, the interaction between the lipid head group becomes more significant, leads to loosening of lipid head. The expansion of lipid head group, in turn, increases the flexibility of the overall membrane. Hence, *l*-menthol can able to penetrate within the bilayer, responsible for the disorderness of the bilayer.

#### **4.4 Conclusion**

I successfully investigated the chirality-dependent interaction of these two isoforms *d*- and *l*-menthol on the stability of membrane heterogeneity. The phase behavior in DOPC/DPPC/Chol system at different cholesterol concentration was studied thoroughly in the presence of *d*- and *l*-menthol. All the findings give a strong evidence to prove the core importance of Chol in the biomembrane. Furthermore, different concentrations of *d*- and *l*-menthol strongly alter the phase behavior and membrane integrity. *l*-Menthol is likely stabilized the raft like structure at membrane cholesterol concentration, which can be related to a model for cooling sensitization. However, high cholesterol and menthol concentration may represent the model for pain sensation as it could incur the perturbation in the lipid bilayer. These findings may aid future to understand of the cooling phenomenon caused by menthol and cold compounds more precisely.

## 4.5 References

1. Simons, K. & Toomre, D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1**, 31–39 (2000).
2. Pralle, A., Keller, P., Florin, E.-L., Simons, K. & Hörber, J. K. H. Sphingolipid–Cholesterol Rafts Diffuse as Small Entities in the Plasma Membrane of Mammalian Cells. *J. Cell Biol.* **148**, 997 LP-1008 (2000).
3. Simons, K. & Ikonen, E. Functional rafts in cell membranes. *Nature* **387**, 569–572 (1997).
4. Lingwood, D. & Simons, K. Lipid Rafts As a Membrane-Organizing Principle. *Science (80-. )*. **327**, 46 LP-50 (2009).
5. Klymchenko, A. S. & Kreder, R. Fluorescent Probes for Lipid Rafts: From Model Membranes to Living Cells. *Chem. Biol.* **21**, 97–113 (2014).
6. Allen, J. A., Halverson-Tamboli, R. A. & Rasenick, M. M. Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci* **8**, 128–140 (2007).
7. Harder, T. & Simons, K. Caveolae, DIGs, and the dynamics of sphingolipid—cholesterol microdomains. *Curr. Opin. Cell Biol.* **9**, 534–542 (1997).
8. van Meer, G., Voelker, D. R. & Feigenson, G. W. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* **9**, 112–124 (2008).
9. Pike, L. J. The challenge of lipid rafts. *J. Lipid Res.* **50**, S323–S328 (2009).
10. Clapham, D. E. TRP channels as cellular sensors. *Nature* **426**, 517–524 (2003).
11. Sugahara, K., Shimokawa, N. & Takagi, M. Destabilization of Phase-separated

- Structures in Local Anesthetic-containing Model Biomembranes. *Chem. Lett.* **44**, 1604–1606 (2015).
12. Kopeć, W., Telenius, J. & Khandelia, H. Molecular dynamics simulations of the interactions of medicinal plant extracts and drugs with lipid bilayer membranes. *FEBS J.* **280**, 2785–2805 (2013).
  13. Wesołowska, O., Michalak, K., Maniewska, J. & Hendrich, A. B. Giant unilamellar vesicles - a perfect tool to visualize phase separation and lipid rafts in model systems. *Acta Biochim. Pol.* **56**, 33–39 (2009).
  14. Blosser, M. C., Horst, B. G. & Keller, S. L. cDICE method produces giant lipid vesicles under physiological conditions of charged lipids and ionic solutions. *Soft Matter* **12**, 7364–7371 (2016).
  15. Morita, M., Vestergaard, M., Hamada, T. & Takagi, M. Real-time observation of model membrane dynamics induced by Alzheimer's amyloid beta. *Biophys. Chem.* **147**, 81–86 (2010).
  16. Hanzal-Bayer, M. F. & Hancock, J. F. Lipid rafts and membrane traffic. *FEBS Lett.* **581**, 2098–2104 (2007).
  17. Hamada, T., Kishimoto, Y., Nagasaki, T. & Takagi, M. Lateral phase separation in tense membranes. *Soft Matter* **7**, 9061–9068 (2011).
  18. Heberle, F. A. & Feigenson, G. W. Phase Separation in Lipid Membranes. *Cold Spring Harb. Perspect. Biol.* **3**, a004630 (2011).
  19. McKemy, D. D. in (ed. Heller S)
  20. Olsen RV, Andersen HH, Møller HG, Eskelund PW, A.-N. L. Somatosensory and

- vasomotor manifestations of individual and combined stimulation of TRPM8 and TRPA1 using topical L-menthol and trans-cinnamaldehyde in healthy volunteers. *Eur J Pain* **18**, 1333–42 (2014).
21. BG, G. Menthol modulates oral sensations of warmth and cold. *Physiol Behav* **35**, 427–34 (1985).
  22. ECCLES, R. Menthol and Related Cooling Compounds. *J. Pharm. Pharmacol.* **46**, 618–630 (1994).
  23. Green, B. G. & McAuliffe, B. L. Menthol desensitization of capsaicin irritation: Evidence of a short-term anti-nociceptive effect. *Physiol. Behav.* **68**, 631–639 (2000).
  24. Cliff, M. A. & Green, B. G. Sensory irritation and coolness produced by menthol: Evidence for selective desensitization of irritation. *Physiol. Behav.* **56**, 1021–1029 (1994).
  25. Macpherson, L. J. *et al.* More than cool: Promiscuous relationships of menthol and other sensory compounds. *Mol. Cell. Neurosci.* **32**, 335–343 (2006).
  26. Frank Mahieu, Annelies Janssens, Maarten Gees, Karel Talavera, B. N. and T. V. Modulation of the cold-activated cation channel TRPM8 by surface charge screening. *J. Physiol. Physiol.* **588**, (2010).
  27. Reid, G., Babes, A. & Pluteanu, F. A cold- and menthol-activated current in rat dorsal root ganglion neurones: properties and role in cold transduction. *J. Physiol.* **545**, 595–614 (2002).
  28. Bautista, D. M. *et al.* The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature* **448**, 204–208 (2007).

29. Klausner, K. Menthol cigarettes and smoking initiation: a tobacco industry perspective. *Tob. Control* **20**, ii12 LP-ii19 (2011).
30. Anderson, S. J. Menthol cigarettes and smoking cessation behaviour: a review of tobacco industry documents. *Tob. Control* **20**, ii49 LP-ii56 (2011).
31. Salgado, M. V. & Glantz, S. A. Direct disease-inducing effects of menthol through the eyes of tobacco companies. *Tob. Control* **20**, ii44 LP-ii48 (2011).
32. Singer, S. J. & Nicolson, G. L. The Fluid Mosaic Model of the Structure of Cell Membranes. *Science (80-. )*. **175**, 720 LP-731 (1972).
33. Veatch, S. L. & Keller, S. L. Separation of Liquid Phases in Giant Vesicles of Ternary Mixtures of Phospholipids and Cholesterol. *Biophys. J.* **85**, 3074–3083 (2003).
34. Walde, P., Cosentino, K., Engel, H. & Stano, P. Giant Vesicles: Preparations and Applications. *ChemBioChem* **11**, 848–865 (2010).
35. Yu, W., So, P. T., French, T. & Gratton, E. Fluorescence generalized polarization of cell membranes: a two-photon scanning microscopy approach. *Biophys. J.* **70**, 626–636 (1996).
36. Harris, F. M., Best, K. B. & Bell, J. D. Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order. *Biochim. Biophys. Acta - Biomembr.* **1565**, 123–128 (2002).
37. Wan, G., Dai, X., Yin, Q., Shi, X. & Qiao, Y. Interaction of menthol with mixed-lipid bilayer of stratum corneum: A coarse-grained simulation study. *J. Mol. Graph. Model.* **60**, 98–107 (2015).

## CHAPTER 5 EFFECT OF MENTHOL ON RAFT STABILIZATION IN BIOLOGICAL MEMBRANES

**Abstract:** From the nature insight raft selectively localizes receptor proteins and the like, three-dimensional membrane structure change such as two-dimensional membrane structure change (clustering of rafts) to raft-mediated signal transduction and vesicular transport, It is believed to play an important role through membrane dynamics in response to stimuli such as endocytosis. Understanding the adsorption mechanism of substances on the cell membrane surface and understanding membrane dynamics responding by adsorption not only leads to elucidation and understanding of mechanisms of pathogenesis such as diseases but also to the design of substances such as drug discovery, becomes very important. Here, the effect of d- and l-menthol on raft stabilization was discussed. With the understanding gained from biomembrane model system, I further extend my study on the effect of d- and l-menthol on association and stabilization with raft using HEK293 cells.

**Keywords:** Raft, menthol, Cooling sensation, Cholesterol, F-actin, Intracellular Ca<sup>2+</sup>, Cell viability

## 5.1 Introduction

The cell membrane is one where intercellular matrix and the external materials come into contact. In addition to separating the living body from the outside,<sup>1</sup> the membrane changes the structure dynamically and helps to take in and out of the substances. Specifically, it delivers a crucial role in the life process by dynamic membrane deformation such as phagocytosis, pinocytosis, endocytosis involving receptors, and so on.<sup>2,3</sup> In recent years many studies emphasize the role of membrane dynamics and its involvement in signal transduction. The fluid mosaic model suggested that protein molecules are floating in the lipid bilayer and are associated dynamically.<sup>4,5</sup> The molecules bound to receptors on the cell membrane through endocytosis process including a wide variety of cell functions. However, it is noteworthy to pay attention to those physicochemical changes that are governed by “raft.”<sup>6,7,8,9</sup> In the endocytosis, invagination of the cell membrane occurs while forming a bag-like structure that is separated from the cell membrane to form vesicles. This vesicle plays a role in delivering encapsulated molecules to various places in the cell. In addition, endocytosis plays a very important role as a biological defense mechanism, and one of them is neutrophil. There is a mechanism to eliminate pathogens such as bacteria and microorganism invaded into the cell by the process known as phagocytosis. In recent years, lipid raft evolved as a platform for many cellular processes and as a mediator in signal transduction.<sup>8</sup> Therefore, it was suggested that lipid raft via endocytosis is used for such virus invasion. In general, activation of the protein receptors present in the cell upon binding with target molecules generates signals, which in turn generate intracellular signaling. The intracellular signaling molecule evokes the activation of the pore thus transferred from one molecule to other signaling molecules. As a result, cell gives a response to various cellular functions like cell death, growth, differentiation. Research on intracellular signal transduction has been actively carried out and many of the transmission pathways have been elucidated.

However, there are few kinds of research focusing on membrane dynamics involved in the initiation of the signal transduction.

Menthol an organic compound derived from peppermint is believed to exert cooling sensitization dependent on the concentration used.<sup>10,11</sup> It has also shown to activate cold TRPM8<sup>12,13,14,15</sup> ion channel and modulate voltage-gated ion channel. Due to its ability to exhibit chirality, some studies revealed that menthol somehow acts stereoselectively in some systems. Hall et al showed the stereoselectivity of l-menthol on GABA<sub>A</sub> currents compared to d-menthol.<sup>16</sup> But the similar effect was not observed in the case of where menthol did not show stereospecificity in the modulation of glycine receptors. On the other hand, Abrar et al was not able to detect a stereo-selectivity of menthol actions on the  $\alpha$ 7-nACh receptor.<sup>17</sup> Several debates are going on the action of menthol on the membrane and their specificity depending on concentration is still remaining debate. Although Meckemy et al described menthol sensation exhibited by mammalian primary neurons to TRPM8 at a molecular level,<sup>18</sup> but a detailed mechanism is under discussion. Yuji et al found that lower concentration of menthol able to cause TRPA1 channel activation, whereas higher concentration leads to a reversible channel blocking.<sup>19</sup> Macpherson demonstrated, even at 1 mM menthol concentration is enough to inhibit the cold or pungent activated TRPV1, leading to fast inhibition of outward and inward currents.<sup>20</sup>

Since TRPM8 channel is localized in Chol-rich specialized membrane domains called “raft”. TRP channels might sense temperature-mediated changes in lipid rafts that facilitate the assembly of intracellular signaling cascade.<sup>21,22</sup> Also, TRP channel activity may sensitive to the membrane cholesterol content. The change in the lipid membrane environment modulates the properties of TRPM8 channel and may suggest a role of lipid raft in cold transduction. By understanding the role of the raft and its importance in the signaling pathways, I further pursue to elaborate the study on raft structure. In this chapter I worked on



biological membrane specifically employing HEK293 cells to elucidate temperature effect on the raft stabilization induced by d- and l-menthol paves a clear difference in the association of menthol (d- and l-) with HEK293 cell membrane.

## 5.2 Experimental Procedures:

### 5.2.1 Materials:

Human embryonic kidney 293 cell (HEK293) was purchased from ATCC cell bank (Japan). Water-soluble cholesterol, methyl- $\beta$ -cyclodextrin (M $\beta$ CD), bovine serum albumin (BSA), and trypan blue were obtained from Sigma-Aldrich (USA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), Pluronic F-127, Fluo-3 AM, Alexa Fluor 488 conjugated cholera toxin subunit B (CT-B) ( $\lambda_{\text{ex}} = 560 \text{ nm}$ ,  $\lambda_{\text{em}} = 580 \text{ nm}$ ), Alexa Fluor 594 conjugated cholera toxin subunit B, Rhodamine phalloidin were from Invitrogen (USA). Phosphate buffer salt (PBS) was purchased from Takara Bio, respectively. (1*S*, 2*R*, 5*S*)-2-isopropyl-5-methylcyclohexanol (d-menthol), (1*R*, 2*S*, 5*R*)-2-isopropyl-5-methylcyclohexanol (l-menthol) were purchased from Wako Japan.

### 5.2.2 Cell culture:

HEK293 cells, the human embryo kidney cell were derived from human embryonic kidney cells grown in tissue culture. HEK cells is a rounded cell that grows in cell culture in DMEM, high glucose (Dulbecco's modified eagle) medium supplement with 10 % (v/v) FBS, 200 mM Glutamex (Gibco 35050-06) and penicillin/streptomycin (Gibco 15140-122), incubated at 37 °C in 5 % CO<sub>2</sub> environment.

### 5.2.3 Staining and Visualization of lipid rafts:

Lipid rafts were labeled by treating cells with 1  $\mu\text{g/ml}$  CT-B Alexa Fluor 488 and 0.02 % (v/v) BSA in PBS followed by incubation at 37 °C for 10 min. Then cells were

washed with DMEM (non-serum). The cells were observed after addition of d- and l-menthol with confocal laser scanning microscopy at different temperatures.

#### *5.2.4 Measurement of cell viability:*

Trypan blue exclusion was used to estimate HEK293 cell viability upon the effect of d- and l-menthol. Untreated, cholesterol-depleted, cholesterol added cells in the presence of d- and l-menthol were investigated. 5  $\mu$ l of cell suspension was added to 5  $\mu$ l of 0.4 % trypan blue stain to an Eppendorf tube and mixed well. Then 10 $\mu$ l of the sample mixture was placed into sample slide and let the sample settle for 30 seconds. Finally, the number of live cells and dead cells were counted using countess II FL automated cell counter (Invitrogen).

#### *5.2.5 Modulation of membrane cholesterol*

Membrane cholesterol of HEK293 cells was depleted by treatment of cells with 2 mM methyl- $\beta$  cyclodextrin (M $\beta$ CD) in non-serum DMEM medium for 10 min at 37 °C. M $\beta$ CD is considered to remove cholesterol from the plasma membrane.<sup>23</sup> Further to increase cholesterol content (excess of cholesterol); HEK293 cells were treated with 1 mM soluble cholesterol in PBS for 10 min at 37 °C. Membrane cholesterol level was measured by using cell-base detection assay kit (Cayman, USA). d- and l-Menthol-containing cells were then added to the HEK293 cells for 10 min at 37 °C.

#### *5.2.6 Double staining of raft and actin:*

HEK293 cells were washed twice with non-serum DMEM, and then lipid rafts were labeled by treating cells with 1  $\mu$ g/mL CT-B Alexa Fluor 488 and 0.02 % (v/v) BSA in PBS followed by incubation at 37 °C for 10 min. The supernatant was discarded and stained the cells with 2.5  $\mu$ L Rhodamine phalloidin (126nM) followed by 0.02 % (v/v) BSA in PBS; incubate at 37 °C for 5 min. Then cells were washed with DMEM (non-serum).

### 5.2.7 Measurement of intracellular $Ca^{2+}$ level:

The intracellular  $Ca^{2+}$  level of cells was measured using Fluo3-AM, a visible light excitation  $Ca^{2+}$  chelating fluorescence label.<sup>24</sup> Untreated cells first washed two times with serum-free DMEM medium, the cells then loaded with 10 $\mu$ g/ml Fluo3-AM in the presence of 0.14 mg/ml Pluronic F-127 and HEPES at 37 °C for 15 min. The cells were then diluted with HEPES and were incubated at 37 °C for 25 min. Fluo-3AM loaded cells then washed two times with serum free DMEM medium and one time with HEPES. Finally, cells were resuspended in HEPES buffer and used for measurements. Prior to the measurement, d- and l-menthol were added to the cells respectively. The intracellular  $Ca^{2+}$  level was measured by analyzing emitted Fluo3-AM fluorescence intensity by Varioskan Microplate reader.

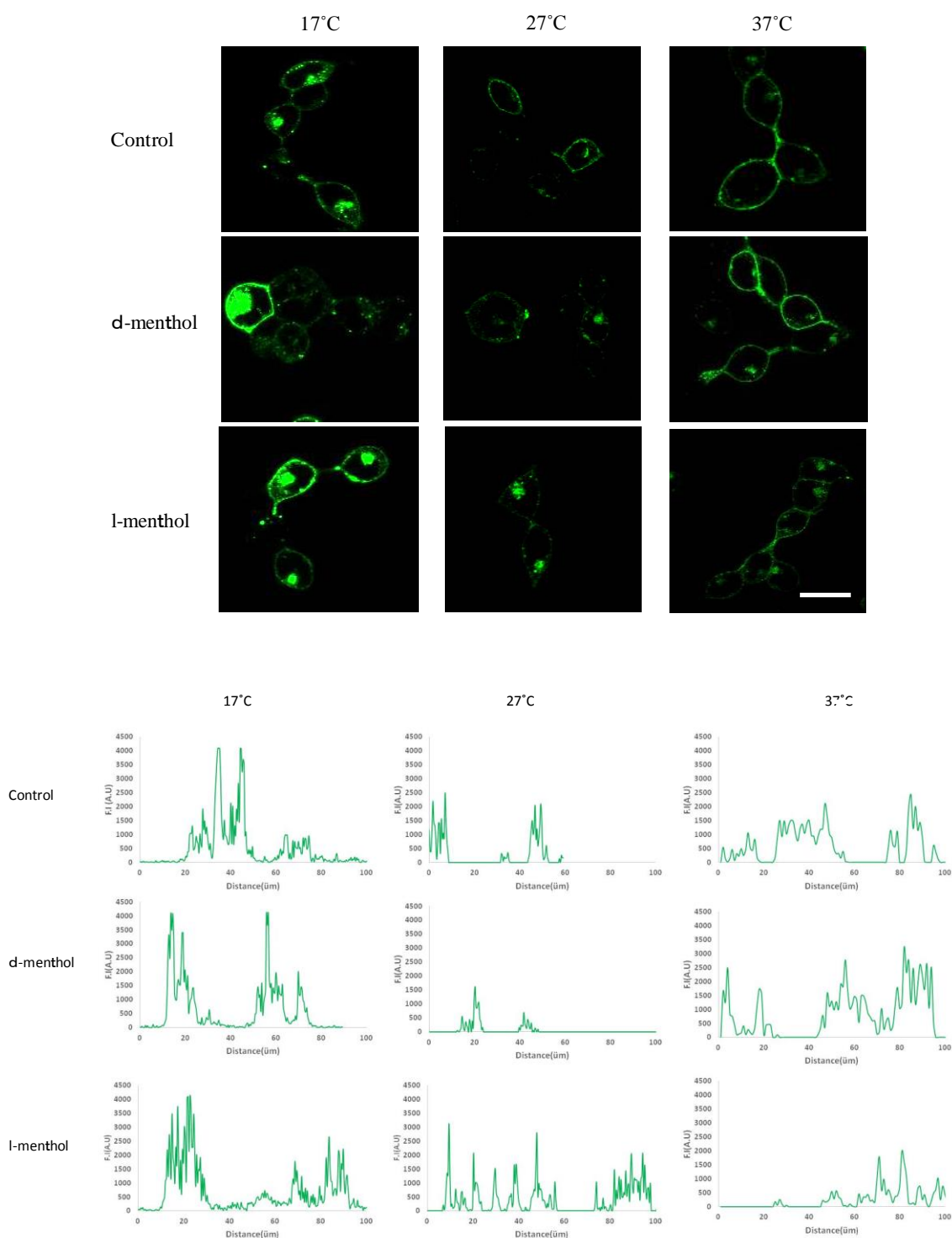
## 5.3 Results and discussion

### 5.3.1 Temperature-dependent Raft stabilization induced by menthol

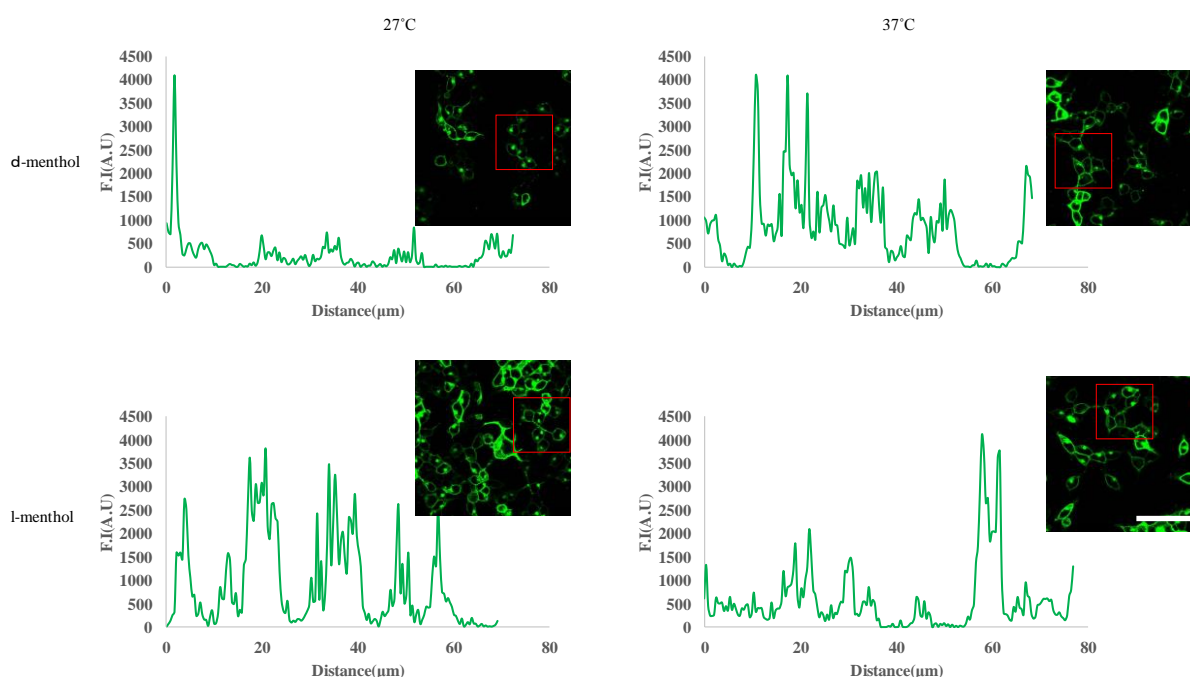
TRPM8, a sub-family of an ion channel which is responsible for cold sensing in the body is activated by a cooling compound such as menthol. l-menthol believe in exerting cooling sensation by activating TRPM8 ion channel<sup>15</sup> whereas d-menthol doesn't do so. Some reports reveal that dl-menthol has some degree of cooling sensation to the body very less compare to l-menthol. Although the channel activity is induced by addition of external stimuli, their mechanism at the molecular level still remains elusive. Hence, we speculate that menthol has direct interaction with the membrane rather than directly to TRPM8. That is why: I started working on the actual cell to clarify the different interaction possibilities by d- and l-menthol. Figure 5.1; show the staining and visualization of the raft at different temperature 17, 27, and 37 °C induced by d- and l-menthol on HEK293 cells. l-Menthol exhibited higher intensity profile suggesting an association with membrane *i.e.*, stabilized raft than d-menthol which can be related to the cooling phenomenon induced by l-menthol only.

A lot of evidence shown the cooling sensitization of l-menthol at very concentration and temperature, whereas relatively high concentration and at higher temperature induces pain sensation. In contrary, d-menthol does not have cooling sensitization and believed to destabilize the membrane. From the raft experiment, I predicted that at 27 °C, l-menthol stabilized raft structure and serves as a model for cooling sensitization. In contrast, at 37 °C, l-menthol tends to destabilized raft, suggesting it pain/irritation effect on the membrane. The results obtained from the raft experiment, I can suggest the satisfactory explanation why l-menthol destabilized raft at higher temperature although our body sense cold after the application of menthol to the skin at body temperature (37 °C). On the application of menthol (higher concentration) to the skin, initially gives a cooling sensitization, but gradually induces pain sensation just like the effect observed in raft experiment at 37 °C. It might be true that very low concentration of menthol can make raft stabilized at 37 °C just like our body response after menthol application. Hence, further, I carried out a similar experiment at a lower concentration of menthol (0.1 mM) to observe this phenomenon (Fig 5.2). At a lower concentration (0.1 mM), l-menthol relatively stabilized raft at 37 °C and it is believed even at a very low concentration l-menthol could stabilize the raft structure corresponds to a similar condition of cooling at body temperature. Also in the case of l-menthol at 17 °C, clustering could be observed in the raft region compared to d-menthol. The profile of control at 17 °C and l-menthol at 27 °C looks very similar and may indicate that 27 °C temperatures was crucial for cooling effect. I clarified that raft clustering and intracellular signal transduction occur by adding l-menthol to HEK293 cells significantly compare to d-menthol. Also, until now, only rafts have been thought of as two-dimensional movement that moves across the cell membrane surface, from my study, rafts revealed that cluster formation is in the interior of the cell, not only on the membrane surface, three-dimensional movement of the raft is

occurring. I believed that this cluster can function as a transient platform and has potential to deliver biochemical signals.



**Figure 5.1** Typical HEK293 cells images of an untreated cell (control), after exposure of d- and l-menthol. Menthol concentration was 0.5 mM. Scale bar = 15 μm

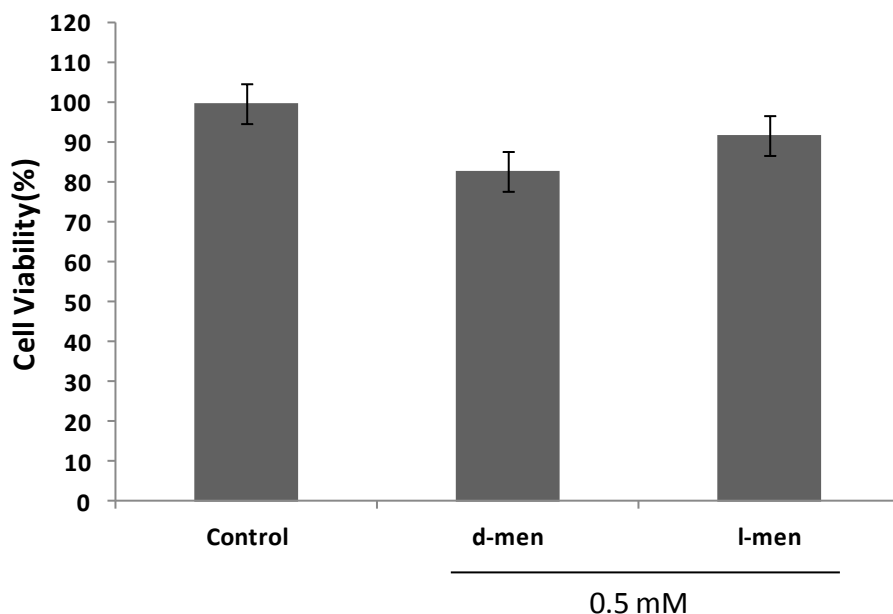


**Figure 5.2** Typical HEK293 cells images of an untreated cell (control), after exposure of *d*- and *l*-menthol with their respective intensity profile. Menthol concentration was 0.1 mM. Scale bar = 15 μm

### 5.3.2 Effect of *d*- and *l*-menthol on the toxicity on HEK293 cells

Menthol is cyclic monoterpene compound widely used in food and commercial products as a cooling compound.<sup>25,26</sup> It also induces  $\text{Ca}^{2+}$  influx due to activation of *TRPM8*, a  $\text{Ca}^{2+}$  permeable cold activated member of TRP channels. Menthol is a chiral compound, both isoforms *d*- and *l*- believes in showing different activity on membrane. One form has therapeutic effect while other has a toxic effect. So, it's important to analyze the toxicity induced by the different isoform of menthol in HEK293 cells. To assess the effect of menthol on HEK cell membrane, the viability of cells was estimated using trypan blue assay. Figure 5.3 shows the decrease in cell viability on the addition of *d*-menthol whereas *l*-menthol makes no significant effect on HEK293 cell. It can be concluded that among two isoforms of menthol, *d*-menthol is toxic to the cell compare to *l*-menthol leading to cell death. These data

are in good agreement with the studies on Jurkat T cells showing the toxic effect of d-menthol on the membrane.



**Figure 5.3** Cell viability in the presence of d- and l-menthol on HEK293 cells at room temperature.

### 5.3.3 Influence of cholesterol on the raft stabilization induced by d- and l-menthol

Many studies have demonstrated a modulating role of cholesterol in the biological membrane. But the effect of d- and l-menthol and association to the membrane has not been investigated. Therefore, I focused on the initial interaction and cytotoxicity induced by d- and l-menthol to HEK293 cells upon a change in the membrane cholesterol level.

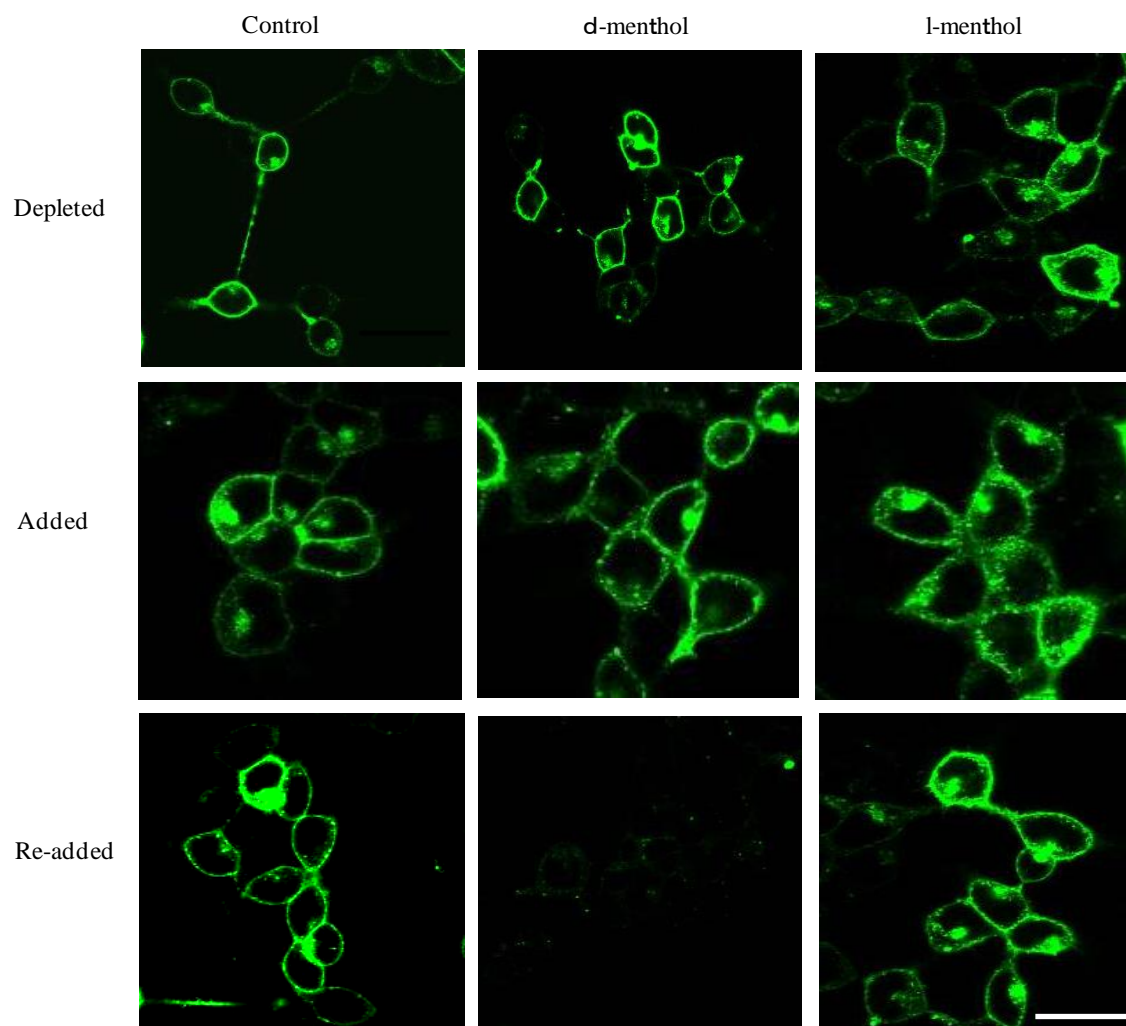
I found that HEK293 cells with the basal cholesterol content, *i.e.*, control system were able to interact with menthol (Fig 5.4). The green color in the microscopic images represents the lipid rafts of the cell membrane. Microscopic observation revealed that the d- and l-menthol association with membranes is different from each other.

Amount of membrane cholesterol remarkably influenced the intensity of d-/l-menthol localization in HEK293 cells. When cholesterol was depleted by approximately 40 % of the initial content, the amount of d- and l-menthol associating with the cells was lower than untreated cells. This implies that loss of membrane cholesterol affects the localization of the d- and l-menthol in cells. On the other hand, when the cells are enriched in cholesterol (more than 40 % of the basal content, an excess of cholesterol) amount of menthol partitioning in cells were significantly different in both d- and l-menthol. It was suggested that d- and l-menthol interacts with lipid rafts of the cell membrane in a different manner and the interaction was influenced by cholesterol level. l-Menthol has a specific interaction with lipid and cholesterol and raft stability is dependent on cholesterol content. Whereas d-menthol due to its preferential localization into the hydrophobic region similar to cholesterol the repulsive force is stronger leading to more destabilization of the raft. This result was in good agreement with the previous results obtained from model membrane where I found that cholesterol content strongly affects the interaction of d- and l-menthol with the membrane (chapter 4).

Further to investigate the effect of membrane cholesterol on d- and l-menthol induced cytotoxicity to HEK293 cells, cell viability at different cholesterol levels in the absence and presence of d-/l-menthol was estimated using trypan blue assay. Use of this protocol is relatively fast and easy method to assess cell viability. The basic principle is that membrane of viable/live cells has a selective permeability, so it does not absorb certain dyes such as trypan blue, while dead cells are exposed to such dye.

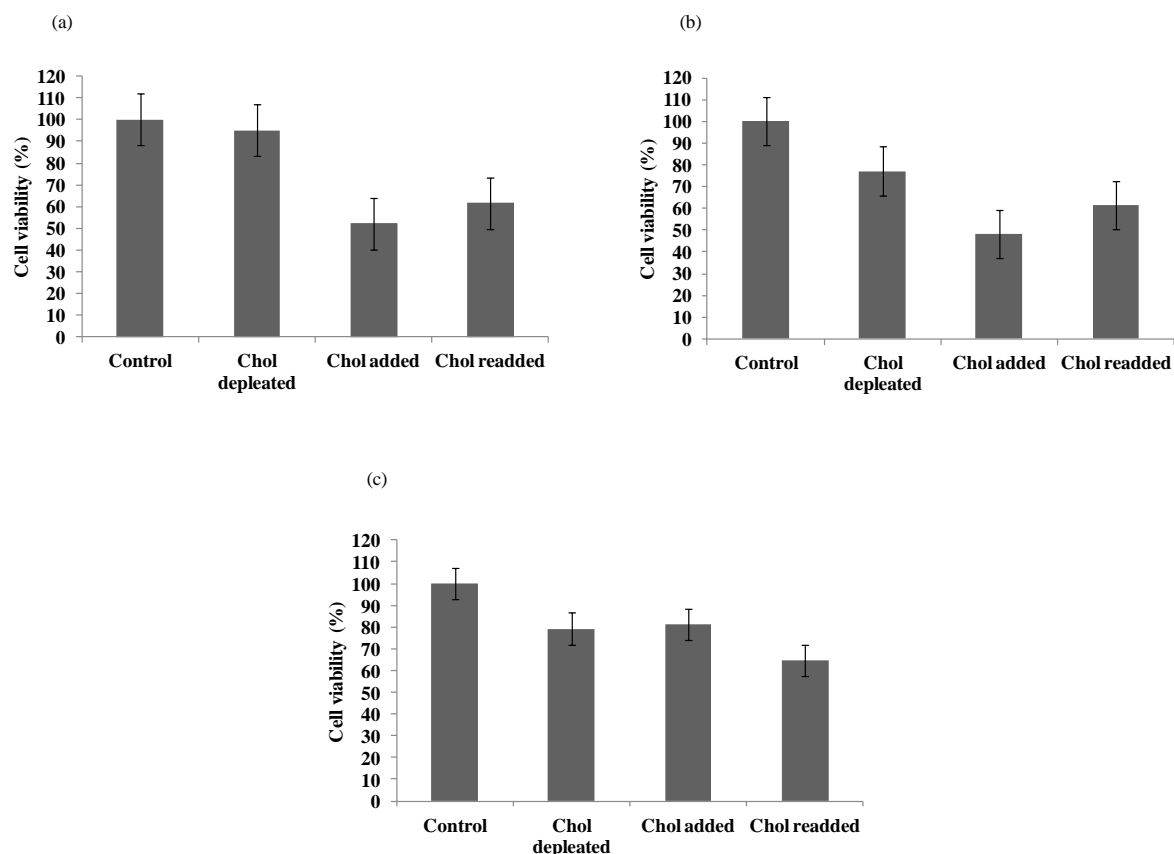
When membrane cholesterol was depleted some percent of cells died in the presence of d- and l-menthol in comparison to the control. This suggests the insignificant effect of d- and l-menthol when membrane loss its cholesterol content as mention earlier menthol interaction is not enough to disturbed the strong packing of lipid membrane.





**Figure 5.4** Effect of cholesterol on the interaction of *d*- and *l*-menthol with HEK293 cells. Representative confocal microscopy images showing the localization of menthol in cell membranes. Scale bar = 15  $\mu\text{m}$

The excess of cholesterol induces cell death in the presence of *d*-menthol, whereas a slight increase in the cell viability was observed for *l*-menthol. This implies that high cholesterol content in the membrane did affect *d*-menthol cytotoxicity to HEK293 cells. The enrichment of cholesterol may induce an inhibitory effect on the toxicity to HEK293 cells.



**Figure 5.5** Cell viability upon a change in membrane cholesterol content in (a) control system, (b) *d*-menthol loaded HEK293 cells, and (c) *l*-menthol loaded HEK293 cells.

In contrary, the cell viability was not affected by *l*-menthol comparatively, suggesting the crucial interaction of menthol and cholesterol in the membrane. It was reported that on increasing the *l*-menthol concentration, the viable cell was gradually decreased. Though I haven't performed at different concentration but it was in consistent with previously reported data showing the cell proliferation or cell death upon menthol action.

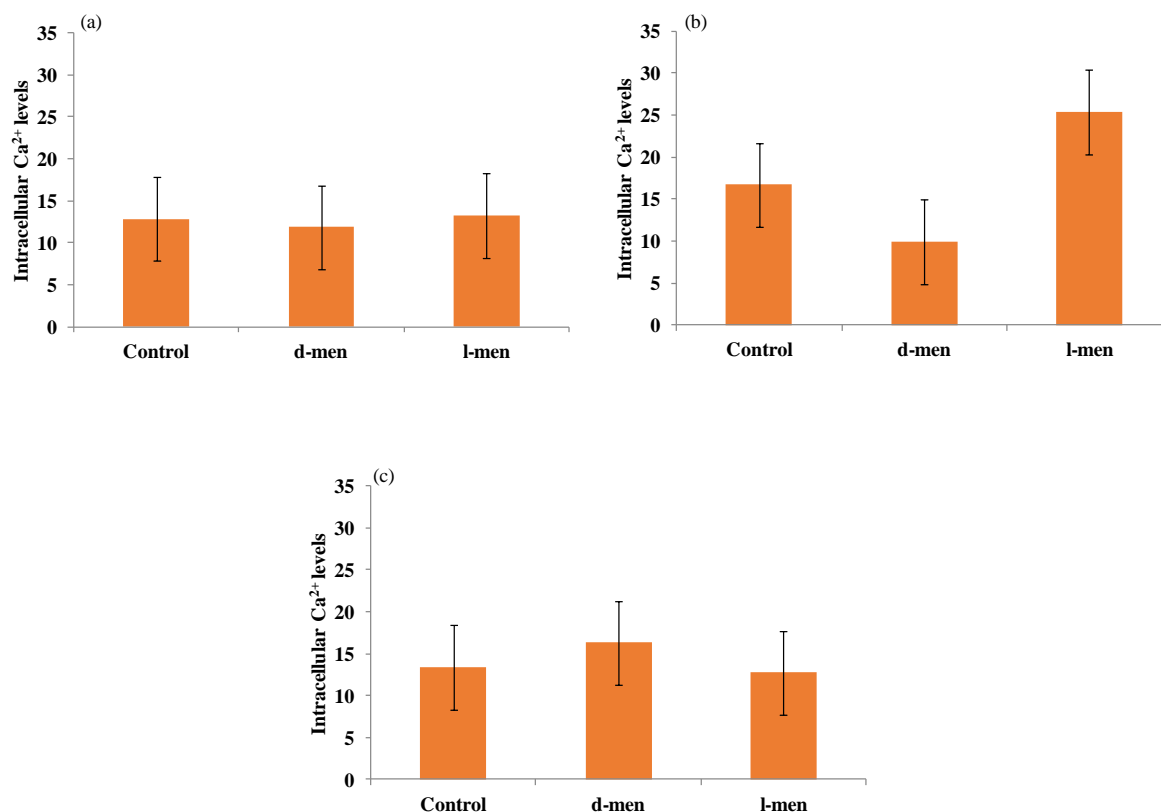
#### 5.3.4 Effect of *d*- and *l*-menthol induced changes in intracellular $Ca^{2+}$ level

In general, calcium plays very important role in signal transduction in the plasma membrane. Calcium is believed to act as the second messenger for cell-cell communication.<sup>24</sup> Calcium is stored in intracellular organelles such as mitochondria and endoplasmic reticulum (ER). The concentration of calcium in the cell varies in different organelles. The calcium

concentration outside the cell is about 2 mM, whereas inside the cell in the cytosol (100 nM) and endoplasmic reticulum (600  $\mu$ M).<sup>27</sup> If the concentration is high enough inside the cell leads to muscle contraction, synaptic plasticity, substance release etc. Thus needs to maintain the low concentration level of calcium in the cell. Hence,  $\text{Ca}^{2+}$  gradient plays an important role in the signaling pathways through  $\text{Ca}^{2+}$  pumps. Also, change in the extracellular  $\text{Ca}^{2+}$  concentration can influence the resting potential of the membrane. Ringer and his co-workers<sup>28,29,30</sup> demonstrated the requirement of  $\text{Ca}^{2+}$  containing perfusate for contraction of frog heart and later found that  $\text{Ca}^{2+}$  induces muscle contraction while another ion  $\text{K}^{+}$  and  $\text{Na}^{+}$  did not.

To study the intracellular calcium level, many  $\text{Ca}^{2+}$  indicators fluorescent dyes has been used due to their ability to produce quite large signals. One of the commonly used  $\text{Ca}^{2+}$  indicator is Fluo-3 AM, is synthesized from BAPTA combine with fluorescein-like structure. Fluo-3 AM is a long range wavelength calcium probe and can be excited by using argon laser and has an absorption maximum at 506 nm. Alone it does not respond to calcium; while once enter to the cells readily undergoes to hydrolysis and convert into the Fluo-3 free acid.<sup>24</sup>

In order to understand whether d- and l-menthol change the membrane properties, I assessed menthol-induced  $\text{Ca}^{2+}$  influx into the cytosol (Fig 5.6). The effect of d- and l-menthol on intracellular  $\text{Ca}^{2+}$  was not seen at 17 °C, indicating that interaction of d- and l-menthol is independent of calcium at a lower temperature. In another way, it was assumed that cooling did not evoke  $[\text{Ca}^{2+}]_i$  response in both the case. Peier.et.al revealed that when the temperature was decreased from 25 to 15 °C an increase in intracellular  $\text{Ca}^{2+}$  was observed in TRPM8 expressing cells.



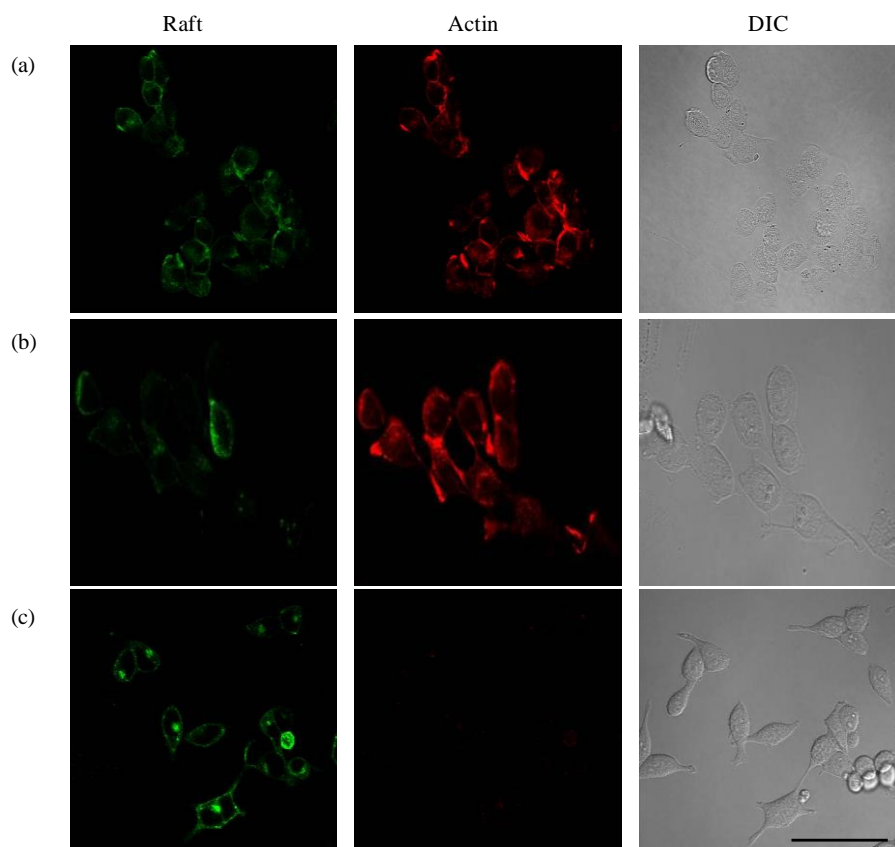
**Figure 5.6** Change in the intracellular  $Ca^{2+}$  level in the absence and presence of d- and l-menthol. The graph of Fluo-3 AM intensity versus component at (a) 17 °C, (b) 27 °C, and (c) 37 °C respectively.

This phenomenon was not observed in non-transfected cells. The result at 17 °C is consistent with what Peier.et.al observed. On increasing temperature to 27 °C, there is a robust increase in the  $[Ca^{2+}]_i$  upon l-menthol addition, whereas d-menthol tends to reduced  $[Ca^{2+}]_i$ , as represented by lower cytosolic ion concentration. The increase in the  $Ca^{2+}$  was due to the ability of l-Menthol to promote association with the cell membrane. I supposed that l-menthol could activate calcium permeable channel opening at 27 °C which may signify the cooling sensitization exhibited by l-menthol only. In contrast, d-menthol might not associate to the channel leading to more loss of calcium content. Similarly, further increase temperature to 37 °C, l-menthol lowers the  $[Ca^{2+}]_i$  indicates the reversible effect on a channel which can be related to desensitization.

### 5.3.5 Role of cytoskeleton and F-actin

In all eukaryotic cells, there are filamentous structures which are responsible for various kinds of movements including cell opening, cell-to-cell junction and deeply contributes to intracellular signal transduction. Actin has the property to generate and maintain the integrity of the membrane morphology and polarity in the process of endocytosis and intracellular trafficking. Stabilization and destabilization of actin in the cells or self-organization to higher order meshwork are regulated by actin-protein binding.<sup>31</sup> As a consequence, these activities are under control of signaling pathways. Polard et.al depicts that cytoskeleton networks are important to regulate the change in the shape of the cells and driven the cell migration.<sup>32</sup> Recent studies have demonstrated protein-protein or protein-RNA mediated interaction in the generation of phase separated domains localized in the subcellular regions leads to activation/trigger of signaling pathways.<sup>33</sup> The cytoskeleton assists the organization of signaling pathways in 2D through the partition into cells and provides the docking sites for proteins to recognize the changes.<sup>34</sup> It has been reported in T-cell signaling that actin filaments dynamics tune clustering of membrane proteins.<sup>35</sup> Recent ongoing works describe various roles on the teamwork between F-actin function at the membrane and signaling pathways. However, the general mechanisms how cytoskeleton actin filaments help in to coordinate signaling pathways induced by external stimuli are still unanswered. On the basis of these understanding, I proposed a model to demonstrate the role of actin in relation to the raft stabilization and how it can be related to sensing mechanism. In order to achieve this hypothesis, I checked the effect of d- and l-menthol on actin stability together with raft. F-actin is visualized by staining HEK293 cells with Rhodamine phalloidin (RP) marker. RP belongs to a phalloidin toxic family and is harmful to HEK293 cells, so it is very important to optimize the general condition for F-actin staining. Some of the researchers reported that high concentration of RP leads to cell death as a result of a significant rise in the RP intensity was

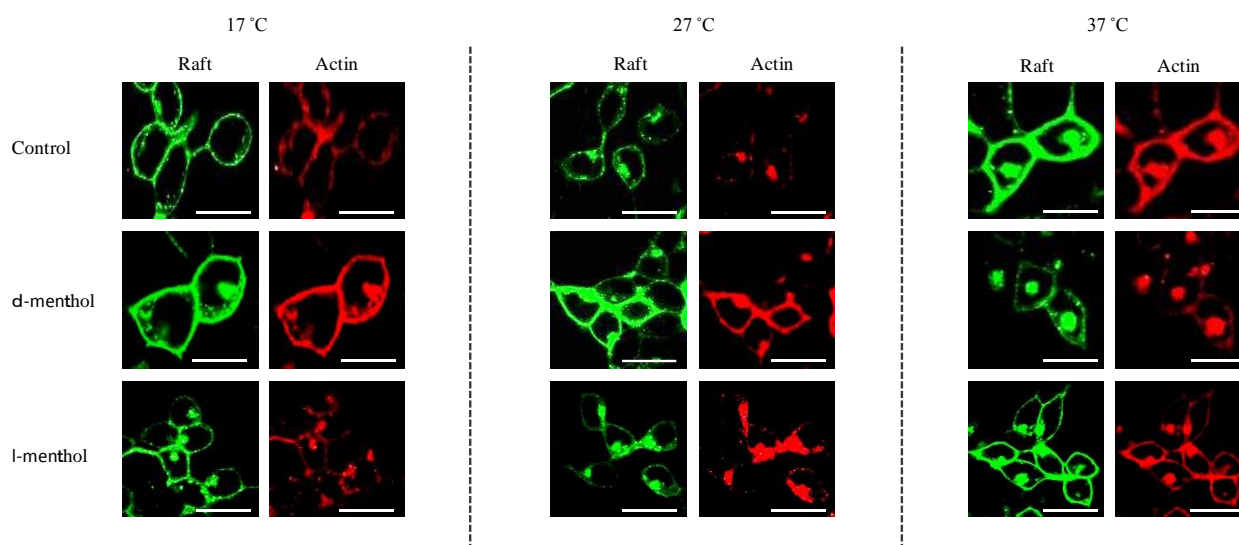
observed. In my laboratory for Jurkat T-cell, RP (6.6  $\mu\text{M}$ ) concentration dissolved in ethanol was used to stain F-actin. On contrary at this concentration HEK293 cells immediately dies as shown in Fig 5.7(a).



**Figure 5.7** Double staining of raft and F-actin in HEK293 cells. Images of HEK293 cells green region (raft), red region (F-actin) and bright region (DIC). (a) HEK 6.6  $\mu\text{M}$  RP concentration in EtOH, (b) 126 nM RP concentration in EtOH, and (c) 126 nM RP concentration in PBS. Scale bar = 15  $\mu\text{m}$ .

Further, I reduced the concentration to 126 nM and also change medium from ethanol to PBS to observe the HEK293 cells morphology (Fig 5.7(b) and (c)). I have successfully optimized the conditions and concentration of RP to observed HEK293 cells. Further experiment will be conducted using 126 nM RP for HEK293 cells staining in future. The

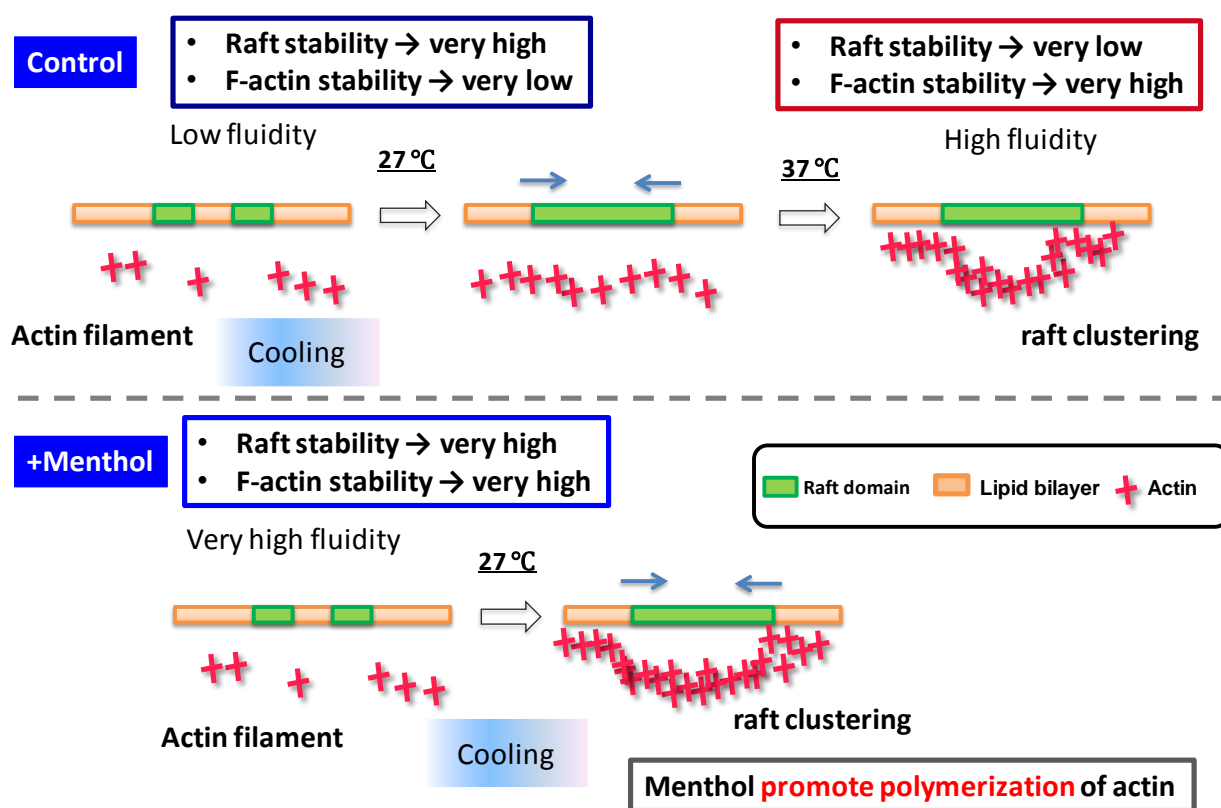
effect of d- and l-menthol on F-actin stabilization or destabilization at different temperature may give some approach to justify the cooling sensitization by l-menthol and temperature-dependent behavior. Figure 5.8 shows the temperature-dependent raft clustering and F-actin polymerization on HEK293 cells at different temperatures with and without menthol. I could observe raft clustering at higher ( $< 27\text{ }^{\circ}\text{C}$ ) temperature in untreated HEK293 cells. The observation indicates that raft clustering is actin-dependent at higher temperature whereas no raft cluster was observed at lower temperature ( $17\text{ }^{\circ}\text{C}$ ). There could be two possible mechanisms for raft clustering (1) actin-independent at lower temperature and (2) actin-dependent at higher temperature.



**Figure 5.8** Double staining of raft and F-actin in HEK293 cells. Typical confocal images of HEK293 cells green region (raft), red region (F-actin) in the presence of d- and l-menthol at different temperatures. Scale bar =  $10\mu\text{m}$ .

Furthermore, effect of d- and l-menthol was also observed on actin-dependent raft clustering as shown in figure 5.8. In the case, when d-menthol was added to the HEK293 cells raft cluster was prominent at higher temperature ( $37\text{ }^{\circ}\text{C}$ ). On the other hand, in the presence of l-menthol even at lower temperature raft cluster could be observed. The data suggested that in

menthol-induced HEK293 cells raft clustering needs polymerization of F-actin. I believed that there is fluidity sensors molecule present at the junction of actin and raft membrane which could detect the biophysical changes like membrane fluidity. These detectors can response to those changes leading to either stabilization or destabilization of actin filaments. The hypothetical model shown in figure 5.9 demonstrates the possible mechanism for the raft stability via actin polymerization on HEK293 cells.



*Figure 5.9 Hypothetical model representing the mechanism of raft dynamics and F-actin on menthol-induced HEK293 cells.*

## 5.4 Conclusion

In this chapter, I have demonstrated the temperature-dependent interaction of menthol with biological membrane specifically HEK293 cells. Based on the findings from raft analysis, 1-



menthol showed a relative mechanism of cooling sensitization at a temperature near 27 °C, while higher temperature could destabilize the raft structure. TRPM8 channel activation is evoked either by cold or menthol (lower concentration). By lowering the concentration, we observed stabilization of raft similar to that cold response. d-Menthol on the other hand destabilized raft structure at all conditions implying its disruptive nature. Cholesterol importance in the regulation of raft properties and stability was demonstrated too. The effect of d-menthol was shown to be negative to cell viability demonstrate the toxic effect of d-menthol on the cell. Effect of l-menthol interaction on the permeability of HEK293 cells to Ca<sup>2+</sup> was promoted at threshold temperature 27 °C. The results of biological cell and model membrane correlate with each other, which further aid to more the clear picture of sensing mechanism at a molecular level.

## 5.5 References

1. Liu, A. P. & Fletcher, D. A. Biology under construction: in vitro reconstitution of cellular function. *Nat. Rev. Mol. Cell Biol.* **10**, 644–650 (2009).
2. Alberts B, Johnson A, Lewis J, et al. in *Molecular Biology of the Cell* (Garland Science, 2002).
3. Kumari, S., MG, S. & Mayor, S. Endocytosis unplugged: multiple ways to enter the cell. *Cell Res* **20**, 256–275 (2010).
4. Nicolson, G. L. The Fluid—Mosaic Model of Membrane Structure: Still relevant to understanding the structure, function and dynamics of biological membranes after more than 40 years. *Biochim. Biophys. Acta - Biomembr.* **1838**, 1451–1466 (2014).
5. Singer, S. J. & Nicolson, G. L. The Fluid Mosaic Model of the Structure of Cell Membranes. *Science* (80-. ). **175**, 720 LP-731 (1972).

6. Pike, L. J. The challenge of lipid rafts. *J. Lipid Res.* **50**, S323–S328 (2009).
7. Simons, K. & Ikonen, E. Functional rafts in cell membranes. *Nature* **387**, 569–572 (1997).
8. Simons, K. & Toomre, D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1**, 31–39 (2000).
9. Hanzal-Bayer, M. F. & Hancock, J. F. Lipid rafts and membrane traffic. *FEBS Lett.* **581**, 2098–2104 (2007).
10. BG, G. Menthol modulates oral sensations of warmth and cold. *Physiol Behav* **35**, 427–34 (1985).
11. Green, B. G. & McAuliffe, B. L. Menthol desensitization of capsaicin irritation: Evidence of a short-term anti-nociceptive effect. *Physiol. Behav.* **68**, 631–639 (2000).
12. Reid, G., Babes, A. & Pluteanu, F. A cold- and menthol-activated current in rat dorsal root ganglion neurones: properties and role in cold transduction. *J. Physiol.* **545**, 595–614 (2002).
13. Dragoni, I., Guida, E. & McIntyre, P. The Cold and Menthol Receptor TRPM8 Contains a Functionally Important Double Cysteine Motif. *J. Biol. Chem.* **281**, 37353–37360 (2006).
14. Frank Mahieu, Annelies Janssens, Maarten Gees, Karel Talavera, B. N. and T. V. Modulation of the cold-activated cation channel TRPM8 by surface charge screening. *J. Physiol. Physiol.* **588**, (2010).
15. Andersson, D. A., Chase, H. W. N. & Bevan, S. TRPM8 Activation by Menthol, Icilin, and Cold Is Differentially Modulated by Intracellular pH. *J. Neurosci.* **24**, 5364 LP-

- 5369 (2004).
16. Hall, A. C. *et al.* Modulation of human GABAA and glycine receptor currents by menthol and related monoterpenoids. *Eur. J. Pharmacol.* **506**, 9–16 (2004).
  17. Ashoor, A. *et al.* Menthol Binding and Inhibition of  $\alpha$ 7-Nicotinic Acetylcholine Receptors. *PLoS One* **8**, e67674 (2013).
  18. McKemy, D. D. in (ed. Heller S)
  19. Karashima, Y. *et al.* Bimodal Action of Menthol on the Transient Receptor Potential Channel TRPA1. *J. Neurosci.* **27**, 9874 LP-9884 (2007).
  20. Macpherson, L. J. *et al.* More than cool: Promiscuous relationships of menthol and other sensory compounds. *Mol. Cell. Neurosci.* **32**, 335–343 (2006).
  21. Clapham, D. E., Runnels, L. W. & Strubing, C. The trp ion channel family . *Nat Rev Neurosci* **2**, 387–396 (2001).
  22. Clapham, D. E. TRP channels as cellular sensors. *Nature* **426**, 517–524 (2003).
  23. Mahammad, S., Dinic, J., Adler, J. & Parmryd, I. Limited cholesterol depletion causes aggregation of plasma membrane lipid rafts inducing T cell activation. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **1801**, 625–634 (2010).
  24. Takahashi, A., Camacho, P., Lechleiter, J. D. & Herman, B. Measurement of Intracellular Calcium. *Physiol. Rev.* **79**, 1089 LP-1125 (1999).
  25. Lee, Y. O. & Glantz, S. A. Menthol: putting the pieces together. *Tob. Control* **20**, ii1-ii7 (2011).
  26. ECCLES, R. Menthol and Related Cooling Compounds. *J. Pharm. Pharmacol.* **46**, 618–630 (1994).

27. Boyle, J. Molecular biology of the cell, 5th edition by B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter. *Biochem. Mol. Biol. Educ.* **36**, 317–318 (2008).
28. Ringer, S. Concerning the Influence exerted by each of the Constituents of the Blood on the Contraction of the Ventricle. *J. Physiol.* **3**, 380–393 (1882).
29. Ringer, S. A further Contribution regarding the influence of the different Constituents of the Blood on the Contraction of the Heart. *J. Physiol.* **4**, 29–42.3 (1883).
30. Ringer, S. A third contribution regarding the Influence of the Inorganic Constituents of the Blood on the Ventricular Contraction. *J. Physiol.* **4**, 222–225 (1883).
31. Colin, A., Bonnemay, L., Gayrard, C., Gautier, J. & Gueroui, Z. Triggering signaling pathways using F-actin self-organization. *Sci. Rep.* **6**, 34657 (2016).
32. Pollard, T. D. Cytoskeletal functions of cytoplasmic contractile proteins. *J. Supramol. Struct.* **5**, 317–334 (1976).
33. Li, P. *et al.* Phase Transitions in the Assembly of Multi-Valent Signaling Proteins. *Nature* **483**, 336–340 (2012).
34. Forgacs, G., Yook, S. H., Janmey, P. A., Jeong, H. & Burd, C. G. Role of the cytoskeleton in signaling networks. *J. Cell Sci.* **117**, 2769 LP-2775 (2004).
35. Chichili, G. R., Westmuckett, A. D. & Rodgers, W. T Cell Signal Regulation by the Actin Cytoskeleton. *J. Biol. Chem.* **285**, 14737–14746 (2010).

## CHAPTER 6 GENERAL CONCLUSIONS

In this dissertation, I have explored deep understanding of the membrane interaction and physiochemical changes upon external stimuli *i.e.*, menthol at the molecular level. Moreover, the mechanical pathway involved in the cold-sensitization induced by l-menthol was clarified. The structure of biomembrane is mainly consists of lipid bilayer made up of various lipid molecules. Recently, biomembranes exhibit assorted membrane structure characterizes by membrane heterogeneity (lateral phase behaviour) and membrane morphological changes. Membrane heterogeneity also refereed as “lipid raft”<sup>1,2</sup> are important in the cellular processes such as signal transduction, membrane trafficking,<sup>1,3</sup> membrane fluidity. However, very rare studies have been reported on the phase behavior and membrane morphology changes upon external added stimuli. Thus, the effect of additives on membrane is poorly understood and need to be elucidated in more fundamental way. In the present study, I have investigated the effect of menthol on model and biological membrane morphology in the presence of cholesterol. Furthermore, the role of cholesterol in the modulation of the membrane properties in the absence and presence of menthol was demonstrated.

In chapter 1, detailed information and basic knowledge about membrane structure, their phase behavior change depending on lipid composition and cholesterol content was described. Additionally, recent studies on menthol cooling property to its related mechanism on TRP channels<sup>4</sup> were discussed in brief. Through these understanding I advance my study further to reveal the basic mechanism pathway involved in cooling sensitization by working on model membrane and proposed the objective of my dissertation.

In chapter 2, I have shown the effect of menthol on thermo-induced membrane dynamics. I worked on model membrane that mimics the biological membrane with similar lipid

composition. The results obtained from this chapter demonstrate that presence of menthol induces membrane fluctuation, meaning that changes the thermo-sensitiveness of DOPC/Chol liposomes and made them more sensitive to temperature change. This change occurred as a result of excess surface area determined by using Langmuir monolayer experiment. It was shown that lower concentration of menthol exhibited higher fluctuation profile and an increase in surface area. I proposed the head group interaction facilitated by menthol may responsible to cause change in the membrane morphology. The association of menthol to the head group facilitates these dynamics.

In chapter 3, effect of d- and l-menthol on biophysical properties on homogeneous model membrane was provided. d-Menthol showed stronger effect on membrane properties compared to l-menthol. The ability to increase membrane fluidity and decrease in the main transition temperature shown by d-menthol indicates its preference to DOPC-rich  $L_d$  phase. In contrary, l-menthol did not change the fluidity and transition temperature significantly in the DOPC and DPPC system. This implies that l-menthol may prefer to be localized at head part of lipid. Thus, did not affect the strong packing among DPPC molecules.

In chapter 4, in order to reveal the preferential localization of d- and l-menthol and their interaction with biomembrane was depicted. Heterogeneous membrane system composed of DOPC, DPPC, Chol was taken into consideration. It was observed that d-menthol at Chol = 0%, the addition of d- and l-menthol does not affect the phase behavior. Similar trend was observed at Chol = 20 %. At Chol = 30 %, d-menthol stabilizes the  $L_o$  domain formation, while the phase separation was strongly suppressed by the addition of l-menthol. The study revealed that cholesterol plays a crucial part in the alteration of channel functioning. I proposed a possible mechanism showing different interaction sites shown by d- and l-menthol in the biomembrane. The temperature-sensitive hydrophilic interaction exhibited by l-

menthol may alter the TRPM8 channel functioning subsequently leading to the activation of TRPM8.

In chapter 5, HEK293 cells were employed to assess the effect of d- and l-menthol on raft stabilization and menthol-cell membrane interaction. The results found from this chapter demonstrated influence of temperature and menthol concentration in the stabilization of raft. At lower temperature, l-menthol could stabilize the raft structure, whereas destabilized raft structure at higher temperature. On the other hand, d-menthol destabilized raft structure independent of temperature. Stabilization of raft was observed at further lowering the concentration of l-menthol, which depicts the cooling sensitization mode at two conditions (1) lower concentration of l-menthol and (2) lower temperature. Also loss of membrane cholesterol causes cell death by d- and l-menthol, suggesting the importance of cholesterol in the regulation of cellular processes. On the other hand, cytosolic  $Ca^{2+}$  content was promoted at lower temperature in the case l-menthol enhances the association to the membrane.

By using both model membrane and biological membrane, I have shown the different localization and interaction of d- and l-menthol. In contrast, the role of cholesterol to alter the physicochemical properties of membrane was demonstrated. I have shown the dominant hydrophilic interaction exhibited by l-menthol and its cooperative effect together with cholesterol and lipid make the membrane stabilized. While d-menthol is moreover display hydrophobic interaction, localized in hydrophobic region of the lipid along with cholesterol. The findings of the dissertation are useful to clarify the cooling mechanism induced by l-menthol at molecular level.

## **6.1 Prospects of the dissertation**

This dissertation successfully demonstrated first attempt to assess the interaction of signaling molecule “menthol” with model cell membrane. Different approaches had been

attempted to clarify physiological changes and morphology change induced by menthol along with the importance of chirality in the biological processes. These dynamic changes in the membrane properties enable to picture the related mechanism behind sensation such as warmth, cold, and anesthesia. Therefore, studies on lipid raft and membrane lipids could offer more information to unravel the mechanism involved in the signal transduction.

## 6.2 References

1. Hanzal-Bayer, M. F. & Hancock, J. F. Lipid rafts and membrane traffic. *FEBS Lett.* **581**, 2098–2104 (2007).
2. Simons, K. & Ikonen, E. Functional rafts in cell membranes. *Nature* **387**, 569–572 (1997).
3. Simons, K. & Toomre, D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1**, 31–39 (2000).
4. Vay, L., Gu, C. & McNaughton, P. A. The thermo-TRP ion channel family: properties and therapeutic implications. *Br. J. Pharmacol.* **165**, 787–801 (2012).



# ACHIEVEMENTS

## List of Publications

### Journals

1. **Pooja Gusain**, Naofumi Shimokawa, and Masahiro Takagi. “Thermo-induced dynamics of model cell membrane by action of menthol”, International Journal of New Technology and Research, Vol 3, pp 93-97 2017.
2. **Pooja Gusain**, Shinya Ohki, Kunihide Hoshino, Yoshio Tsujino, Naofumi Shimokawa, and Masahiro Takagi. “Chirality-dependent interaction of d- and l-menthol with model biomembranes”, BBA Biomembrane (in press).
3. Pooja Gusain, Naofumi Shimokawa, Masahiro Takagi “Effect on biophysical properties of homogeneous model membrane induced by d- and l-menthol”, to be submitted.
4. **Pooja Gusain**, KeangOk Baek, Naofumi Shimokawa, and Masahiro Takagi. Role of lipid rafts in modulation of ion channel functioning via temperature-dependent mechanism”, (In preparation)

### Presentations

#### International Conferences

1. **Pooja Gusain**, Kunihide Hoshino, Yoshio Tsujino, Naofumi Shimokawa, Masahiro Takagi. Chirality dependant interaction and stabilization of d-&l-menthol in lipid rafts model. *57<sup>th</sup> International Conference on Bioscience of Lipids, 4-8<sup>th</sup> Sep 2106, Mont Blanc, France. (Oral)*

2. **Pooja Gusain**, Kunihide Hoshino, Yoshio Tsujino, Naofumi Shimokawa, Masahiro Takagi. Differences in Membrane Dynamics Induced by d- and l-Menthol. *Physics of Cells: From Molecule of Systems (PhysCell 2015)*, 30-4<sup>th</sup> Sep 2015, Bad Staffelstein, Germany. (Poster)
3. **Pooja Gusain**, Naofumi Shimokawa, Masahiro Takagi. Effect of Menthol's Chirality on Membrane Dynamics. *International Symposium on Fluctuation and Structure out of Equilibrium 2015*, 20-22 Aug 2015. Kyoto, Japan. (Poster)
4. **Pooja Gusain**, Tsuyoshi Yoda, Naofumi Shimokawa, Masahiro Takagi. Effect of Menthol on Thermo-Induced Cell Membrane. *LSBE*, 7-9 Nov 2013, Osaka, Japan. (Poster)
5. **Pooja Gusain**, Tsuyoshi Yoda, Naofumi Shimokawa, Masahiro Takagi. Thermo-sensation of Menthol on Cell-Sized Model Membrane. *International symposium on advanced materials*, 11 Oct 2013, JAIST, Japan. (Poster)

#### Domestic Conferences

1. **Pooja Gusain**, Naofumi Shimokawa, Masahiro Takagi. Interaction of d- and l-menthol on lateral organization of lipid raft. *JISMS2017*, 6-7<sup>th</sup> Mar 2017, JAIST, Japan. (Poster)
2. **Pooja Gusain**, Kunihide Hoshino, Yoshio Tsujino, Naofumi Shimokawa, Masahiro Takagi. Effect of d- and l-menthol on stability of membrane heterogeneity. *68<sup>th</sup> Annual meeting of the Society for Biotechnology of Japan (SBJ)*, 28-30<sup>th</sup> Sep 2016, Toyama, Japan. (Poster)
3. **Pooja Gusain**, Kunihide Hoshino, Yoshio Tsujino, Naofumi Shimokawa, Masahiro Takagi. Membrane dynamics Induced by Physical differences between d- and l-isoforms of menthol. *JAIST-SAST 2015*, JAIST, Japan. (Poster)

4. **Pooja Gusain**, Kunihide Hoshino, Yoshio Tsujino, Naofumi Shimokawa, Masahiro Takagi. Physical changes induced by optical isomers of menthol on membrane dynamics. *67<sup>th</sup> SBJ Annual Meeting 2015, 26-28<sup>th</sup> Oct 2015, Kagoshima, Japan. (Poster)*
5. **Pooja Gusain**, Tsuyoshi Yoda, Naofumi Shimokawa, Masahiro Takagi. Effect of menthol on cell-sized liposome. *CSJ Regional Meeting in Hokuriku Area, 22 November 2013, JAIST, Japan. (Poster)*
6. **Pooja Gusain**, Masahiro Takagi. Dynamic Response of Menthol on Thermo-Induced Cell Membrane: More than Receptors. *Biophysical society of japan, 28-30 Oct 2013, Kyoto, Japan. (Poster)*
7. **Pooja Gusain**, Tsuyoshi Yoda, Naofumi Shimokawa, Masahiro Takagi. Dynamic Response on Thermo-Induced Cell Membrane Containing Menthol. *JAIST Poster Challenge 2013, JAIST, Japan. (Poster)*