International Journal of New Technology and Research(IJNTR)
ISSN:2454-4116, Volume-3, Issue-3, March 2017 Pages 93-97

Thermo-Induced Dynamics of Model Cell Membrane by Action of Menthol

Pooja Gusain, Naofumi Shimokawa, Masahiro Takagi

***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 hasa significant effect on the model cell membrane. In homogeneous membrane, menthol enhances the fluctuation rate and also changed in the membrane area. We believed that menthol has a direct interaction with the model cell membrane and affects membrane physiochemical properties.**

***Index Terms*-menthol, membrane fluctuation, thermo-responsiveness, molecular area**

I. INTRODUCTION

Menthol, a secondary alcohol, which activates a cold receptor, has a cooling sense to the skin. It has been widely used in the commercial products and has been employed in medicine [1],[2]. Menthol is the oldest traditional Chinese medicine used to relieve minor aches, sunburns, and flavoring agents [3],[4]. It has the ability to trigger brain upon channel activation and eventually responds to cooling sensitization [3]-[5]. Many studies suggested differentbehavior 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 [6]. The mechanism underlying the behavior needs to be explored at the 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 through the channel [7]. The channel opening could be temperature-dependent [8],[9] or voltage-dependent [10] 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 activates TRPV3 and inhibits TRPA1 [11],[12].

Pooja Gusain, **Naofumi Shimokawa**,**Masahiro Takagi***
School of Materials Science, Japan Advanced Institute of Science and Technology, Nomi, Ishikawa 923-1292, Japan.

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 [13]. Liposomes are extensively employed in the drug delivery [14] 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 small Unilamellar vesicles (SUVs), multi lamellar vesicles (MLVs), and giant Unilamellar vesicles (GUVs). Recently, GUVs gained a significant attention as a model membrane to study the interaction of foreign molecules with membrane [15]. The size of the liposome is determined by the balance of curvatures between the inner leaflets and outer leaflets 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. In eukaryotic cells, membrane-protein, ligand-ligand or ligand-protein interaction is needed to regulate cellular processes involved in signal transduction and membrane trafficking. Previous studies provide the role of lipid as foremost platform for recognition of direct interaction of membrane proteins and externally added chemicals. These plant derived signaling molecules interact with the lipids directly or indirectly resulting into sensing. In order to study those interactions with lipids, research on the physical properties change in the membrane induced by such chemical agonist was carried out. Cell-sized liposomes were employed as an artificial membrane to performed further experiments. Taking advantage of cell-sized liposomes, it is possible to observe time-dependent changes in the individual liposomes. Thus, allows real-time observation by optical microscopy.

In this paper, we useda model membrane to investigate the thermo-induced effect of menthol. Neutral lipid DOPC and cholesterol were used to prepare the cell-sized liposomes in the presence of menthol at different concentrations. In model membrane, the physiochemical changes in such as membrane fluctuation, surface area upon menthol addition were demonstrated.

II. MATERIALS AND METHODS

A. *Materials*

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 NacalaiTesque (Japan), respectively. Ultrapure water (specific resistance ≥ 18 MΩ) was obtained from a Millipore Milli-Q purification system.

B. Preparation of Liposomes

Lipid vesicles were prepared by natural swelling method [16]. Lipids, Chol, and menthol were dissolved in a 2:1 vol/vol (chloroform/methanol) solution, making the concentration of 2 mM for lipids, Chol, and menthol. Lipids, Chol, and menthol were mixed at desired concentrations to a final volume of 20 µL. 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 ºC for an hour and was kept overnight at room temperature 21.7 ± 1.7 ºC.

C. Microscopic Observations of membrane fluctuation

The lipid vesicle solution (5 µL) was placed in a silicon well (0.2 mm) on a glass slide and covered with another small cover slip. The silicon well and the coverslip ensured that evaporation of the solution did not occur over the duration of the experiment. The formed GUVs were observed by 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. Lipid vesicles were prepared and observed as above. The stage temperature was carefully changed using a thermal controller (Tokai-Hit MATS-5550RA-BT; Japan), from 22 to 40 °C. The samples were subjected to a temperature increase at a rate of 1.0 °C/min from 21 to 40 °C.

D. Effect of Temperature on Molecular Area of Monolayer Membranes

A Filgen LB-400 (Aichi, Japan) instrument (Kuhn type) was used to measure the π−A isotherms. DOPC/Chol (4:1) was dissolved in chloroform to a final concentration of 2 mM lipids and 5 µL of this solution was added to 100 ml of Milli-Q pure water at each temperature. Temperatures were controlled by attaching the system to the instrument. Waiting for least 10 min, after each temperature was confirmed, the π−A isotherms were measured. Their π−A isotherms were measured using the same procedure and conditions.

III. RESULTS AND DISCUSSION

A. Effect of menthol on membrane fluctuation

Using a natural swelling method to form dry lipid films, two types of the system were prepared, DOPC/Chol and DOPC/Chol/Menthol at various menthol concentrations. The changes in membrane morphology were observed with the help of phase contrast microscopy (Olympus BX-50, Japan) at RT. Fig1. Shows 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 was increased from 21 ºC to 25 ºC. 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 Figure 1 (lower panel). We could find the larger radial fluctuation in the menthol-containing membranes.

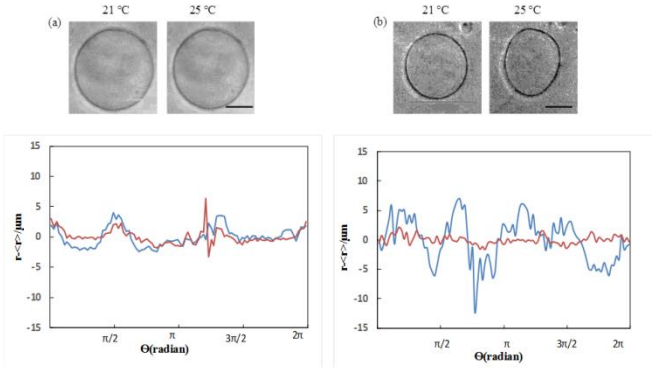

Fig.1 Membrane fluctuation of lipid vesicles.The temperature was increased from RT to 21.0 °C, using a thermo controller. Images of a typical lipid vesicle captured using a phase-contrast microscope. The scale bar is 10 µm. These graph show membrane fluctuation at 21 °C (red), 25 °C (blue) as a function of radius and its distribution. Plotted the value of radius of r–in each θ (θ = ±π/n, n = 1, 2, 3 … 100). (a) DOPC/Chol and (b) DOPC/Chol/Menthol. Scale bar = 10 µm.

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, we couldobserve the clear concentration effect of menthol on the model membrane. The effect of menthol concentration on the thermo-sensitive fluctuation of DOPC/Chol liposomes is depicted in Figure 2. At lower concentration of menthol, the maximum fluctuation can be observed, while at a 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 remained steady. Dynamic real-time observation of membrane dynamics revealed that the menthol containing membrane was more thermo-responsive than without menthol. The increase in the temperature enhanced the number of water molecules near polar head groups, leading to an increase in steric repulsive interaction between them and resulted into the excess surface area of liposomes. As a consequence, liposomes started fluctuating depending on the hydrophilicity of the head group.

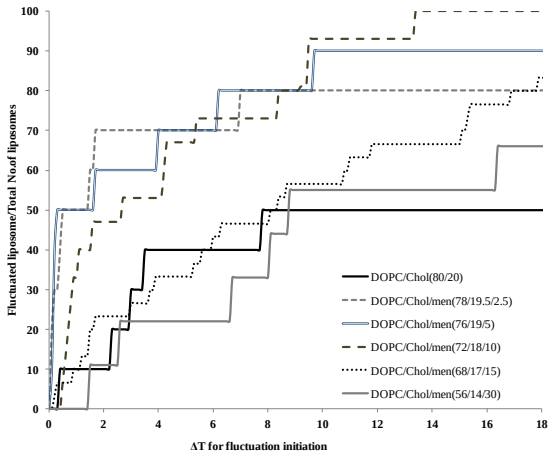

Fig.2 Thermal responsiveness of menthol-containing lipid vesicles.Percentage of lipid vesicles, which started fluctuating at a given level of temperature increase. The vesicles contained DOPC/Chol (black), 2.5 % menthol (light grey dashed), 5% menthol (blue), 10 % menthol (dark grey dashed), 20 % menthol (black dotted), and 30 % (grey solid line). (n=30).

Figure3 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. We 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. We believed that at 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 restricted hence high temperature was required to fluctuate the liposomes.

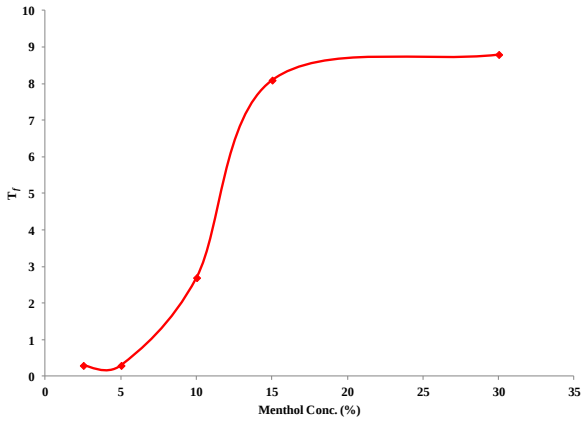

Fig.3 Thermal responsiveness of menthol-containing lipid vesicles.Fluctuating temperature at which 50 % of liposomes started fluctuating at a given level of temperature increase. The graph shows $T_f$against menthol concentration. (n=30).

B. Role of surface tension and pressure on membrane

In order to investigate the area increase by rising temperature, we carried out Langmuir monolayer experiment at different temperature with varying 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

$$\frac{\Delta A}{\Delta T} \sim 1/P$$

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 was reported to be 30-40 mN/m [17]; hence the experiment was carried out at this pressure. It was hypothesized that the membrane dynamics by temperature change, leads to an increase in 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 4 shows the typical P-A curves of DOPC/Chol monolayers at different temperature 20 ºC, 24 ºC, and 28 ºC in the absence and presence of menthol. It was seen that menthol-containing monolayer membranes exhibited a relatively bigger increase in the molecular area upon increasing temperature.

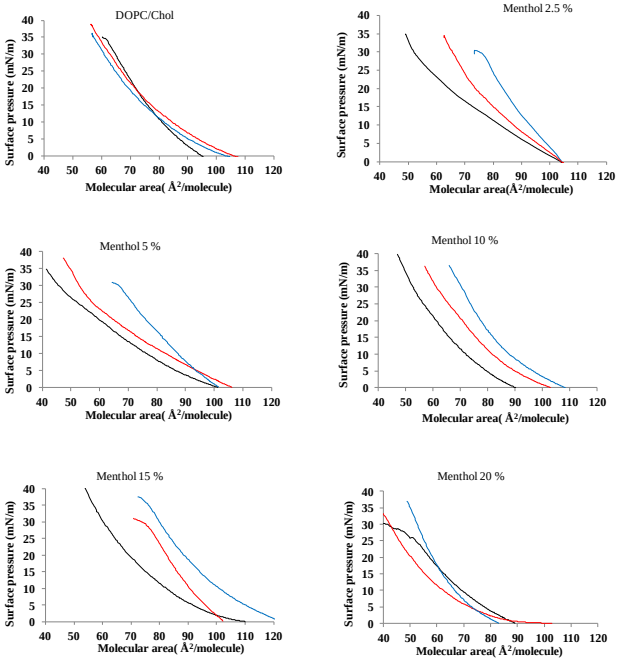

Fig.4Typical thermal responsiveness of lipid monolayer DOPC/Cholesterol and menthol-containing membranes.DOPC/Chol (80/20), DOPC/Chol/Menthol (78/19.5/2.5) DOPC/Chol/Menthol (76/19/5), DOPC/Chol/Menthol (72/18/10), DOPC/Chol/Menthol (68/17/15), and DOPC/Chol/Menthol (64/16/20). Surface pressure (p)–area per molecule (β) 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).

On the other hand, without menthol system i.e., DOPC/Chol system showeda slight increase in area. This result is in good agreement with the fluctuation data where menthol containing lipids are more temperature sensitive. As shown in Figure 5 the changes in the molecular area upon menthol concentration change. The presence of menthol significantly increases the molecular area of DOPC/Chol monolayer at lower concentration till 10 %. This result was in good agreement with our previous studies depicted that

menthol strongly facilitates liposome fluctuation at a 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 the monolayer was higher compared 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.

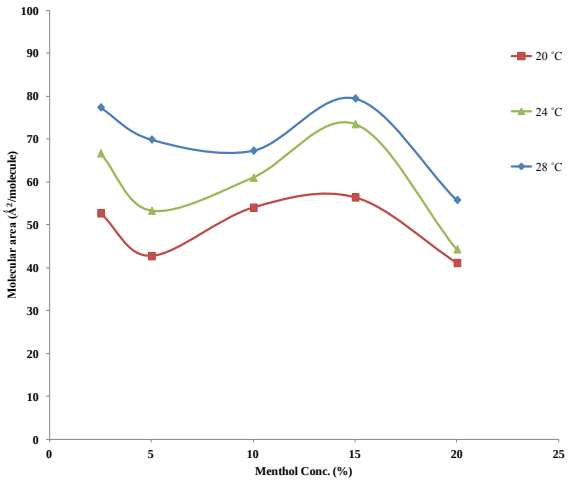

Fig.5Typical thermal responsiveness of lipid monolayer DOPC/Chol and menthol-containing membranes.DOPC/Chol (80/20), DOPC/Chol/Menthol (78/19.5/2.5) DOPC/Chol/Menthol (76/19/5), DOPC/Chol/Menthol (72/18/10), DOPC/Chol/Menthol (68/17/15), and DOPC/Chol/Menthol (64/16/20). Change in the molecular area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

In DOPC/Chol/Menthol system, menthol has a hydroxyl group addition to the cholesterol (OH) and lipid head group. This additional hydrophilic group in the lipid promotes the 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 an increase in temperature resulting into fluctuation of the liposomes. 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.

Menthol-containing monolayer membrane exhibited a bigger increase in the molecular area upon an increase in temperature. It was in agreement with the vesicular fluctuation that membrane containing cholesterol only showsa slight increase in molecular area. Menthol at a concentration of 15 % showed 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 Figure 6 clearly shows the direct effect of menthol on lipid vesicles which cause significant changes in the morphology of the membrane. Menthol-containing lipid vesicles are more thermo-responsive than those cholesterol-containing lipid vesicles. Theincrease in fluctuation occurs as a result of excess membrane area. The presence of hydroxyl group in menthol will interact with 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.6). We also measured the molecular area increase via temperature change by the Langmuir monolayer method. It is very important to consider the hydrophilic interaction among the head group of phospholipids and the 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 the increase in membrane area.

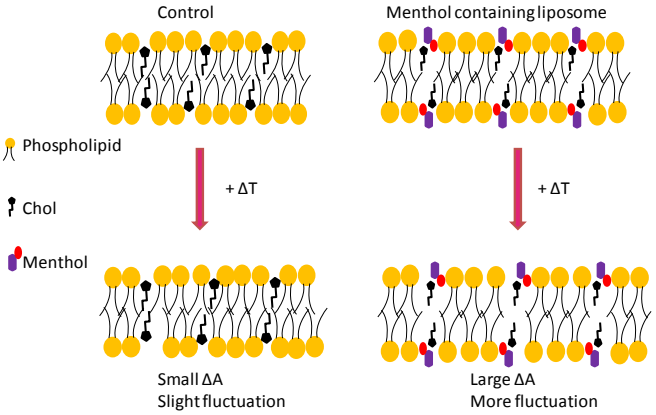

Fig.6 Hypothetic model demonstrating a mechanism of membrane fluctuation in DOPC/Chol system in the presence of menthol.

IV. Conclusion

This is the first attempt to demonstrate direct observation of the dynamic responses of lipid vesicles in real-time. The results clarified that menthol has direct interaction with biomembrane and significantly affects membrane dynamics. In addition, we 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.

Acknowledgment

N. S. acknowledges support from a Grant-in-Aid for Young Scientists (B) (JP26800222) from the Japan Society for the Promotion of Science (JSPS) and a Grant-in-Aid for Scientific Research on Innovative Areas “Molecular Robotics” (JP15H00806) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT). M. T. acknowledges support from a Grant-in-Aid for Scientific Research on Innovative Areas “Thermal Biology” (JP15H05928) from MEXT and a Grant-in-Aid for Scientific Research (B) (JP26289311) from JSPS.

References

[1] N. Galeotti, L. Di Cesare Mannelli, G. Mazzanti, A. Bartolini, C. Ghelardini, Menthol: a natural analgesic compound, Neurosci. Lett. 322 (2002) 145–148. doi:http://dx.doi.org/10.1016/S0304-3940(01)02527-7.

[2] B.G. Green, B.L. McAuliffe, Menthol desensitization of capsaicin

irritation: Evidence of a short-term anti-nociceptive effect, Physiol. Behav. 68 (2000) 631–639. doi:http://dx.doi.org/10.1016/S0031-9384(99)00221-8.

[3] Y.O. Lee, S.A. Glantz, Menthol: putting the pieces together, Tob. Control. 20 (2011) ii1-ii7. doi:10.1136/tc.2011.043604.

[4] R. ECCLES, Menthol and Related Cooling Compounds, J. Pharm. Pharmacol. 46 (1994) 618–630. doi:10.1111/j.2042-7158.1994.tb03871.x.

[5] H. Chuang, W.M. Neuhausser, D. Julius, The Super-Cooling Agent Icilin Reveals a Mechanism of Coincidence Detection by a Temperature-Sensitive TRP Channel, Neuron. 43 (2004) 859–869. doi:http://dx.doi.org/10.1016/j.neuron.2004.08.038.

[6] M.A. Cliff, B.G. Green, Sensory irritation and coolness produced by menthol: Evidence for selective desensitization of irritation, Physiol. Behav. 56 (1994) 1021–1029. doi:http://dx.doi.org/10.1016/0031-9384(94)90338-7.

[7] G. Reid, A. Babes, F. Pluteanu, A cold- and menthol-activated current in rat dorsal root ganglion neurones: properties and role in cold transduction, J. Physiol. 545 (2002) 595–614. doi:10.1113/jphysiol.2002.024331.

[8] D.E. Clapham, C. Miller, A thermodynamic framework for understanding temperature sensing by transient receptor potential (TRP) channels, Proc. Natl. Acad. Sci. . 108 (2011) 19492–19497. http://www.pnas.org/content/108/49/19492.abstract.

[9] S. Chowdhury, B.W. Jarecki, B. Chanda, A Molecular Framework for Temperature-Dependent Gating of Ion Channels, Cell. 158 (2017) 1148–1158. doi:10.1016/j.cell.2014.07.026.

[10] et al. Purves D, Augustine GJ, Fitzpatrick D, Voltage-Gated Ion Channels, in: et al. Purves D, Augustine GJ, Fitzpatrick D (Ed.), Neuroscience, second, Sinauer Associates, Sunderland, 2001. https://www.ncbi.nlm.nih.gov/books/NBK10883/.

[11] Y. Karashima, N. Damann, J. Prenen, K. Talavera, A. Segal, T. Voets, B. Nilius, Bimodal Action of Menthol on the Transient Receptor Potential Channel TRPA1, J. Neurosci. 27 (2007) 9874 LP-9884. http://www.jneurosci.org/content/27/37/9874.abstract.

[12] L.J. Macpherson, S.W. Hwang, T. Miyamoto, A.E. Dubin, A. Patapoutian, G.M. Story, More than cool: Promiscuous relationships of menthol and other sensory compounds, Mol. Cell. Neurosci. 32 (2006) 335–343. doi:http://dx.doi.org/10.1016/j.mcn.2006.05.005.

[13] G. van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipids: where they are and how they behave, Nat Rev Mol Cell Biol. 9 (2008) 112–124. http://dx.doi.org/10.1038/nrm2330.

[14] D.D. Lasic, Novel applications of liposomes, Trends Biotechnol. 16 (1998) 307–321. doi:http://dx.doi.org/10.1016/S0167-7799(98)01220-7.

[15] A. Viallat, J. Dalous, M. Abkarian, Giant Lipid Vesicles Filled with a Gel: Shape Instability Induced by Osmotic Shrinkage, Biophys. J. 86 (2004) 2179–2187. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1304069/.

[16] P. Walde, K. Cosentino, H. Engel, P. Stano, Giant Vesicles: Preparations and Applications, ChemBioChem. 11 (2010) 848–865. doi:10.1002/cbic.201000010.

[17] T. Hamada, Y.T. Sato, K. Yoshikawa, T. Nagasaki, Reversible Photoswitching in a Cell-Sized Vesicle, Langmuir. 21 (2005) 7626–7628. doi:10.1021/la050885y.