Effect of cholesterol and its oxidized derivatives on membrane interaction and aggregation of Alzheimer's amyloid beta

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Doctoral Dissertation

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Abstract

Cholesterol is the most abundant animal steroid, consisting of four fused, rigid rings, a polar hydroxyl group at C3, and a branched, nonpolar iso-octyl side chain at C17 of ring structure. The most important function of cholesterol is to be a vital structural constituent and physicochemical property modulator of cell membranes. In addition, the sterol serves as the metabolic precursor of steroid hormones and bile acids, and it is essential for neuronal activities and functions. Cholesterol is a susceptible target of cellular oxidation induced by enzymes and reactive oxygen species (ROS), generating two main categories of oxidized derivatives (oxysterols) which are (i) those oxygenated on the side-chain and (ii) those oxygenated on the sterol ring. Compared to cholesterol, oxysterols have an additional oxygen group that renders oxysterols relatively more hydrophilic and different from cholesterol in the orientation in membranes. Oxysterols exhibit both positive and negative biological roles. Some of them at physiological concentrations play an important impact in cholesterol homeostasis, sterol biosynthesis, and cell signalling, while others have harmful effects and contribute to some human diseases. Both cholesterol and oxysterols have been widely implicated in Alzheimer's disease, the most common neurodegenerative disorders in humans. Accumulating evidences have demonstrated that the accumulation, aggregation and cytotoxicity of amyloid beta (Aβ) peptide in the brain are key processes in the pathogenesis of the disease. The interaction of Aβ with cell membranes plays crucial roles in these processes. Cholesterol, with the function as an essential component and property modulator of cell membranes, remarkably alters Aβ/membrane interaction. However, the role of cholesterol as a protective factor or a deleterious agent in Aβ/membrane interaction remains controversial. In addition, the impact of oxysterols in this interaction is not fully understood although these compounds are reported to have high abilities to change cell membrane properties.

This dissertation aimed to investigate the effect of cholesterol and oxysterols on the interaction of Aβ with the lipid bilayer of membranes and membrane-mediated aggregation of the peptide. Three membrane systems different in the level of complexity, including homogeneous, heterogeneous model membranes and actual cell membranes, were used. First, I clarified how cholesterol and two commonly occurring and reportedly harmful oxysterols, 7-ketocholesterol (7keto) and 25-hydroxycholesterol (25OH), influence the interaction of Aβ-40 and Aβ-42 aggregated species with the lipid bilayer of a homogenous membrane system and associated dynamics of the membrane. Second, the localization of Aβ-42 protofibrils, which is widely reported to be a harmful species, in lipid lateral compartments of a heterogeneous model membrane in presence of cholesterol and 7keto was unravelled. Third, I further advanced my study on the link between these compounds and Aβ/membrane interaction by using living cells. Last, the influence of cholesterol and 7keto on kinetics of Aβ aggregation mediated by model membranes, and the morphology and biological membrane interaction of some Aβ aggregates were investigated.

The studies on homogeneous model membranes clearly show that oxysterols mediated localization of Aβ in membranes and the peptides-induced membrane dynamics, in contrast to role of cholesterol in inhibiting Aβ/membrane interaction. The effect of 7keto and 25OH are different due to distinct positions of the additional oxygen group in their structures. The former induced a high propensity of membrane toward association with Aβ, while the latter made membrane more capable of morphological changes in response to the peptide. Comparing two common Aβ isoforms, Aβ-42 protofibrils were more interactive with homogeneous membranes than Aβ-40 species. These findings suggest the inhibitory effect of cholesterol and enhancing influence of oxysterols on the interaction of Aβ with the lipid bilayer of membranes.

In heterogeneous model membranes, which retain the lateral lipid organization of cell membranes, cholesterol decreased the localization of Aβ-42 protofibrils in solid-ordered domains and increased that in liquid-ordered domains. The sterol changed the amount of Aβ associating with liquid-disordered (Ld) phase in different tendencies depending on the composition of heterogeneous membrane systems. These effects were attributed to cholesterol's capability of altering the fluidity of lipid phases. On the other hand, 7keto mainly enhanced the fluidity and interaction of protofibrillar Aβ-42 with Ld phase. These results demonstrate the impact of cholesterol in directly modulating Aβ

interaction with lipid domains of membranes in addition to its effect on Aβ/GM1 binding as reported previously. They also indicate the harmful impact of cholesterol oxidized derivatives which promotes Aβ association with heterogeneous membranes.

Jurkat T cell, a kind of white blood cell and a target of Aβ, was used to assess the effect of cholesterol and 7keto on protofibrillar Aβ-42/cell-membrane interaction. I found that the loss of membrane cholesterol strongly enhanced the interaction of Aβ-42 protofibrils with Jurkat T cells and decreased the viability the cells exposed to the protofibrils compared to cells with basal cholesterol content. Conversely, the increase in cholesterol content did not significantly change these processes. On the other hand, 7keto had a high ability to enhance the localization of Aβ-42 protofibrils in Jurkat T cell membranes and increase the effects of the peptide which reduce cell viability and increase cytosolic Ca^{2+} content of the cells. These influences of cholesterol and 7keto were discussed based on their ability to change membrane fluidity as indicated by studies on model membranes. The results suggest that cholesterol has the beneficial role in Aβ-induced toxicity to Jurkat T cells, in agreement with previous studies on neuronal cells, while 7keto may be a harmful factor in this process.

Regarding to Aβ-42 aggregation mediated by model membranes, the strikingly different effects of cholesterol and 7keto were demonstrated. The presence of cholesterol in DOPC vesicles moderately inhibited the kinetics of nuclei formation and considerably accelerated fibrillar Aβ-42 growth. However, the formation of nuclei from monomers was slightly increased and fibril elongation was remarkably inhibited by the partial substitution of membrane cholesterol with 7keto. Moreover, cholesterol-containing vesicles induced a faster formation of fibrils which has a low propensity to cells, while 7keto-containing vesicles inhibited the formation of fibrils, maintain the peptide in protofibrillar aggregates which were highly able to localize in cells. Since the cytotoxicity of Aβ remarkably depends on the aggregated state, these results suggested that cholesterol hinders Aβ cytotoxicity to cells by accelerating the formation of fibrils, while 7keto mediates Aβ cytotoxicity by inhibiting the conversion of protofibrils to mature fibrils.

In conclusion, I have shown that cholesterol has a protective role and oxysterols, in particular 7keto, are risk factors in Aβ-induced cytotoxicity. The effect of cholesterol and oxysterols is associated with to their abilities to alter interaction of Aβ with membranes and fibrillation of the peptide mediated by membranes. In general, cholesterol inhibited Aβ/membrane interaction and accelerated the formation of Aβ fibrils which are less harmful to cells than other aggregate species. Conversely, oxysterols enhanced the interaction and hindered Aβ fibrillation, thereby maintaining the existence of Aβ protofibrils, widely reported to be a harmful species. As far as I am aware, this dissertation is the first systematic study about the effect of cholesterol oxidative derivatives on Aβ/membrane interaction. The findings of this dissertation are important to clarify the impact of oxidative stress in Aβ-induced cytotoxicity and neroinflammation in the pathogenesis of Alzheimer's disease. They also suggest that prevention and/or repair of oxidative stress by antioxidants and reduction of ROS generation may be a potential approach in the treatment of Alzheimer's disease.

Keywords: Cholesterol, Oxysterols, Amyloid-beta/membrane interaction, Amyloid-beta aggregation, Alzheimer's disease

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Phan Thi Thanh Huong

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Chapter 1

General introduction

Abstract

In this chapter, the background of the study was introduced by reviewing related studies. A general overview of structure, metabolism, and function of cholesterol and its oxidized derivatives (oxysterols) was provided, indicating that these compounds play essential biological roles including the ability to modulate properties of cell membranes. In addition, the impact of amyloid beta (Aβ) in pathogenesis of Alzheimer's disease, the most common neurodegenerative disease, was shown. Current understandings suggest that Aβ/cell-membrane interaction is a key event in Aβ aggregation and cytotoxicity, and this interaction significantly depends on membrane structure and properties. However, the role of cholesterol in Aβ/cell-membrane interaction is controversial, while the impact of oxysterols is not fully investigated. Based on this background, objectives of the study were proposed.

Keywords: Cholesterol, Oxysterols, Cell membranes, Alzheimer's disease, Amyloid beta-induced toxicity, Amyloid beta/membrane interaction

1.1. Cholesterol and its oxidized derivatives (oxysterols)

1.1.1. Cholesterol

1.1.1.1. Structure of cholesterol

Cholesterol is the most abundant animal steroid, a class of lipids derived from cyclopentanoperhydrophenanthrene compound which features four fused, nonplanar rings [1]. Cholesterol consists of a rigid tetracyclic ring structure (sterol nucleus), a polar hydroxyl group attaching to sterol nucleus at C3, and a branched, nonpolar iso-octyl side chain at C17 of sterol nucleus (Figure 1.1) [2]. This structure enables cholesterol to have a weak amphiphilic property [2] with a small hydrophilic headgroup at one end of the molecule combining with hydrophobic rings and hydrocarbon tail at the other end.

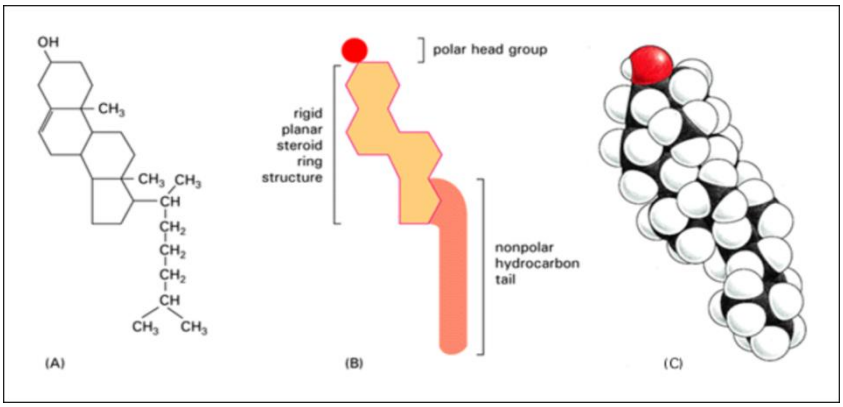

Figure 1.1. Structure of cholesterol. (A) Chemical formula, (B) Schematic drawing, and (C) Space-filling model of cholesterol [3].

1.1.1.2. Cholesterol homeostasis

Cholesterol homeostasis in humans is a well-balanced combination of biosynthesis, uptake, esterification, and metabolism [4]. Cholesterol is synthesized from acetate through a series of different reactions involving in a multi-enzyme cascade [2,5]. Hydroxymethylglutaryl-CoA (HMG-CoA) is considered as the key flux-controlling enzyme of cholesterol biosynthesis. This process takes place in the liver, which synthesizes the sterol for its usage in digestion as well as for the demand of other tissues, with the exception of the brain. Cholesterol is delivered in the bloodstream in the form of cholesteryl esters by very low density lipoproteins (VLDL). During their transportation in capillary, VLDLs convert to low density lipoproteins (LDL) which are subsequently taken

up by peripheral cells using LDL receptor-mediated endocytosis [2]. Cholesterol is also reversely transported from extrahepatic cells to the liver by high density lipoproteins (HLD) [6]. In addition to synthesized cholesterol, liver cells can obtain dietary cholesterol via receptor-mediated endocytosis [2]. The elimination of cholesterol from the body is mainly performed by bile acid excretion in the liver [7].

The brain has its endogenous cholesterol biosynthesis [8]. The main source of central nervous system (CNS) is astrocyte cells from which the sterol is secreted as a component of apoliprotein E (apoE), a kind of small HDL-like particle [9]. ApoE plays a central role in cholesterol transport among cells of the brain, especially from astrocytes to neuronal cells. CNS cholesterol is distinct from that of other tissues because of the blood-brain-barrier (BBB) that prevents plasma lipoproteins from entering into CNS and inhibits the significant export of CNS lipoproteins to peripheral circulation [10]. However, some studies have recently shown the relation between plasma cholesterol and brain cholesterol metabolisms [4]. Circulation HLDs are able to cross the BBB, thus provide the brain with a small cholesterol amount from the periphery [11]. In addition, there is the delivery of excess CNC cholesterol to the circulation by the conversion of the sterol to 24S-hydroxycholesterol (24S-OH) which is capable of traversing the BBB [12]. The brain is the most cholesterol-enriched organ of the mammalian body. This organ comprises approximately 25% of total body cholesterol although it contributes less than 10% of body mass [8].

1.1.1.3. Function of cholesterol

The major function of cholesterol is to be a vital structural constituent and physicochemical property modulator of cell membranes. The concentration of cholesterol in plasma membrane is about 30%-40% of the total membrane's lipid composition [13]. The sterol also appears in the membrane of cellular organelles at lower levels [2]. In the lipid bilayer of membranes (discussed in section 1.2), cholesterol orients perpendicular to the bilayer plane [14]. The polar hydroxyl group of cholesterol is close to headgroup of adjacent phospholipids, whereas its hydrophobic sterol nucleus and side chain partition in nonpolar hydrocarbon tail regions of the bilayer (Figure 1.2). The fused ring structure of cholesterol having a greater rigidity is able to interact with and partially immobilize the upper part of hydrocarbon side chains of neighboring phospholipids, thus decreasing their mobility and tightening lipid packing. On other hand, cholesterol prevents the cluster and

crystallization of these side chains [3]. Thereby, the sterol determines the fluidity, permeability, and phase separation of membranes [3,15].

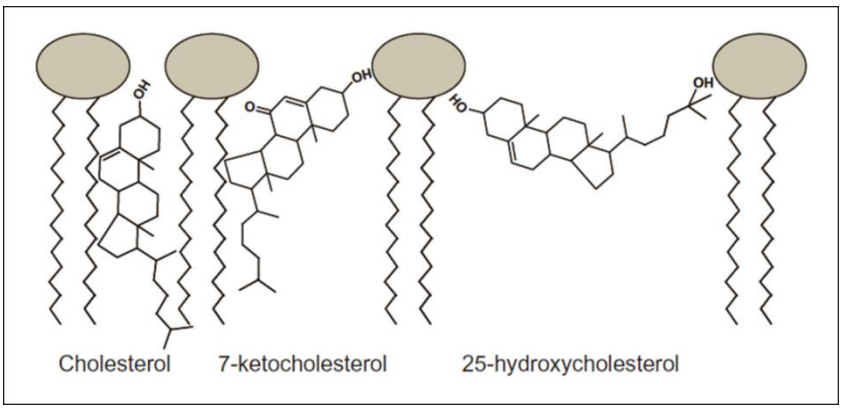

Figure 1.2. Orientation of cholesterol and its oxidized derivatives, 7-ketocholesterol and 25-hydroxycholesterol, in the lipid bilayer of cell membranes [14].

Another function of cholesterol is to act as the metabolic precursor of steroid hormones and bile acids. Steroid hormones such as estrogens and progestogens regulate many biological processes, including sexual development, immune functions, and carbohydrate metabolism [2]. Bile acids are important compounds, which accelerate the digestion and absorption of fats and fat-soluble molecules in the intestine [16]. Therefore, cholesterol plays essential roles in metabolism and development of the body.

Moreover, cholesterol is important to neuronal activities and functions [9], as indicated by the high cholesterol content in the brain. CNC cholesterol is majorly contained in myelin sheaths of oligodendroglia [17], a kind of glial cells that support, insulate the axon and enhance the speed of electrochemical impulse transmission [18]. The lower concentration of CNC cholesterol is detected in plasma membranes of neuronal and astrocyte cells. It has been reported that cholesterol is required for synapse generation, synaptic plasticity, neurotransmission, and brain morphology during embryonic development [19].

Due to these important impacts of cholesterol, dyshomeostasis of this sterol is harmful and associated with some human diseases. Accumulating evidences have demonstrated that cholesterol deposition in arteries contributes to cardiovascular diseases and stroke, two leading causes of death in humans [2]. Disturbances in CNS

cholesterol metabolism are linked to some neurodegenerative illnesses such as Alzheimer's and Huntingtion's diseases [9].

1.1.2. Cholesterol oxidized derivatives (oxysterols)

1.1.2.1. Cholesterol oxidation

Cholesterol is a susceptible target of cellular oxidation induced by enzymes and reactive oxygen species (ROS), generating many kinds of oxysterols [20] (Figure 1.3). Enzymatic oxidation of cholesterol is a part of cholesterol homeostasis [21]. The enzymes, many of which are hydroxylases, oxygenate the side chain of the sterol, leading to the formation of oxysterols with an additional hydroxyl group. 24-hydroxycholesterol (24OH), 25-hydroxycholesterol (25OH), and 27-hydroxylcholesterol (27OH) are abundant products of this oxidation pathway [20]. On the other hand, ROS-induced oxidation (autooxidation) of cholesterol is a consequence of oxidative stress [22], which arises from the imbalance between the production of ROS and antioxidant defenses of living systems caused by aging and/or influences of external factor such as oxidants and radiation [23]. The target of cholesterol autooxidation is the sterol nucleus, mainly at C7, producing a variety of ring-oxygenated sterols with extra oxygen groups [20]. Recently, amyloid beta (Aβ), the key peptide of Alzheimer's disease (AD) pathogenesis, has been shown to have cholesterol-oxidizing activity in combination with redox-active metals including Fe^{2+} and Cu^{2+}. The major oxidative products of Aβ/Cu^{2+} complex are 4-cholesten-3-one [24] and 7β-hydroxycholesterol (7βOH) [25]. It has been shown that the level of some important oxysterols in fresh normal human plasma is in a range from 0.010 to 0.100 μM [26].

1.1.2.2. Structure and function of oxysterols

Compared to cholesterol, oxysterols have an additional oxygen group, which is hydroxyl, carbonyl, hydroperoxy, carbonxyl, or epoxy. This group renders oxysterols relatively more hydrophilic and different from cholesterol in dimensional structure. Many oxysterols are confined in cell membranes and they adopt a different orientation relative to cholesterol [14]. Oxysterols tend to expose both hydroxyl group at C3 of sterol ring and the extra oxygen group to the polar interface of membranes. Therefore, ring-oxygenated oxysterols produce a remarkable tilt with respect to the interface, while the side chain-oxygenated are likely to orient horizontally (Figure 1.2) [14,27]. The orientation of

oxysterols in membranes is affected by some factors such as membrane surface pressure and lipid phase. It has been reported that 7keto can position nearly perpendicular to the bilayer plane like cholesterol in tightly packed phase [28] and oxysterols with the additional oxygen group in the side chain adopt an inverted orientation under high surface pressure [29].

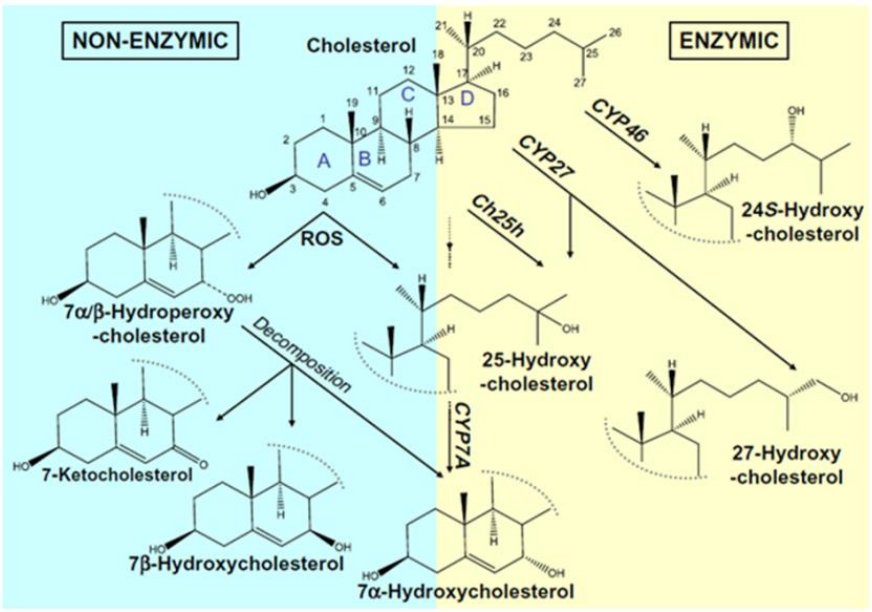

Figure 1.3. Oxidation of cholesterol induced by enzymes and reactive oxygen species [20].

Due to increased hydrophilicity and modified orientation, oxysterols are less able to tighten lipid packing than cholesterol, thus significantly changing membrane properties [14]. In addition, oxysterols are considerably more soluble in aqueous solution than cholesterol. Therefore, they easily cross plasma membranes and enter cells [30].

Oxysterols exhibit a multitude of roles in many biological processes, including both positive and negative [22]. Enzymatically oxidized sterols function in lipid metabolism as regulators of gene expression, substrates for bile acid synthesis, and transported forms of cholesterol during its delivery among tissues [21]. For example, 25-OH suppresses the generation of sterol regulatory element binding protein (SREBP) family, which is a transcription factor of genes in cholesterol synthesis. 24-OH is a ligand of liver X receptors (LRX) that regulate the expression of genes coding proteins associated with cholesterol

synthesis and export [20,21]. The activities of these oxysterols are important to maintain moderate cholesterol level in the body. However, it has been reported that some oxysterols induced by enzymatic oxidation of cholesterol (24-OH and 25-OH) have neurotoxic influences such as causing cell apoptosis at high concentrations [26,31]. Oxysterols derived from cholesterol autooxidation have been known as harmful factors. They have cellular cytotoxicity which decreases of cell growth and viability, changes membrane transport of small molecules, and induces apoptosis [32]. Furthermore, these oxidized sterols are implicated in the pathogenesis of some diseases such as atherosclerosis, lung disease, liver disease, cancer, and neurodegenerative illnesses [22]. For example, 7keto and 7βOH are found in oxidized LDL, the inducer of arterial injuries in atherosclerosis [33]. The involvement of 7βOH in lung cancer and other types of cancer has been demonstrated [34,35]. The deleterious effects of oxysterols are attributed to their ability to modify physicochemical properties of membranes [22]. Due to the dual role in many important biological events, oxysterols have received an increasing interest, especially in studies of pathophysiological mechanism of human diseases.

1.2. Cell membranes

1.2.1. Structure and function of cell membranes

1.2.1.1. Structure of cell membranes

Cell membranes tremendously vary in compositions and have a complicated structure. The most accepted model of membrane structure is the fluid mosaic model proposed by Singer and Nicolson (1972). According to this model, biological membranes are two-dimensional lipid bilayers containing proteins which freely float within the lipid bilayers (Figure 1.4) [3].

Lipid bilayer serves as the basic fluid structure of membranes. It is formed by the spontaneous aggregation of various amphiphilic lipids. Glycerophospholipids, cholesterol, and sphingolipids are major lipids in cell membranes, which contribute to 40-60%, 30-40%, and 10-20% of lipid composition, respectively [15]. Glycerophospholipids have a polar headgroup which contains a 3-carbon glycerol backbone, and two hydrocarbon tails that are saturated or unsaturated. Two abundant glycerophospholipids in cell membranes are 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) which are commonly used to prepare biomimetic membranes.

Sphingolipids are built from sphingosine molecules and fatty acid tails. The fatty acid chains of sphingolipids are often saturated, while their headgroups are larger than those of glycerophospholipids [3]. In the bilayer, lipid molecules expose polar headgroups to the hydrophilic lipid/water interface and bury hydrophobic side chains in the interior. The organization of lipid molecules is asymmetric between the two layers. In general, there are three main interactions existing among lipid molecules, including hydrophobic interaction of hydrocarbon tails, hydrophobic attraction at the hydrocarbon-water interface and repulsion of phospholipid headgroups (Figure 1.5) [38].

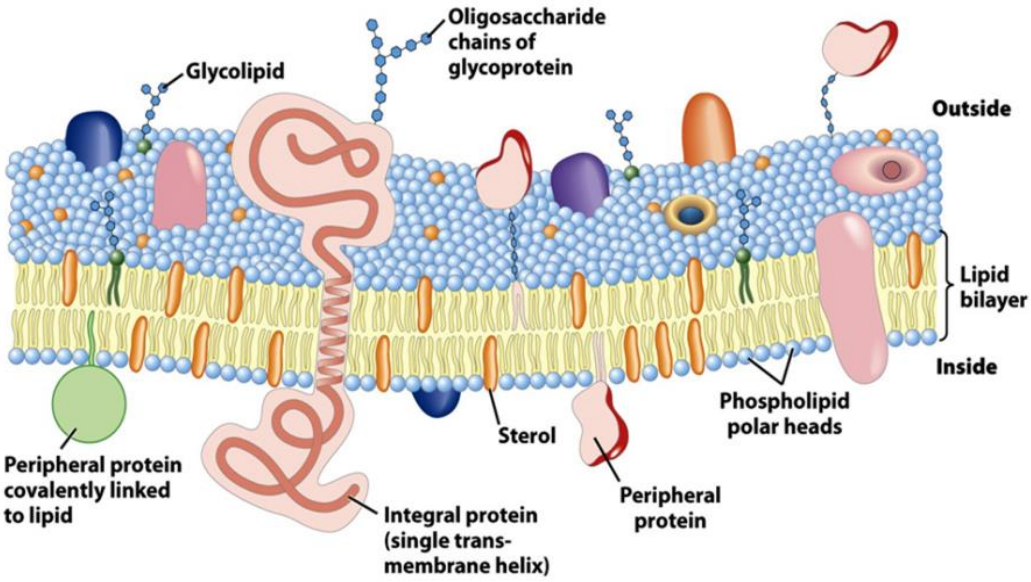

Figure 1.4. Structure of plasma membranes according to the fluid mosaic model [37].

Fluidity and phase behaviour are important physical properties of lipid bilayer. Fluidity, or the relative mobility an individual lipid molecule, refers to the viscosity which is measured by the resistance of lipid bilayer to gradual deformation by shear stress or tensile stress [39].This property depends on temperature and lipid composition of lipid bilayer. The bilayer can exist in a liquid (fluid) state in which hydrocarbon chains of lipid molecules are dis-ordered at lower temperature, or exist in a gel (rigid) state in which hydrocarbon chains are fully extended and closely packed at higher temperature. The change of lipid physical state resulting from a change in chain ordering is called a phase transition, and temperature required to induce the change is defined as chain-melting temperature (Tm) [3]. Phase behaviour of lipid bilayer is majorly governed by van der

Waals attraction between adjacent lipid molecules, which in turn depends on hydrocarbon-chain length and the presence of double bonds [40]. Each lipid species has a characterized Tm. Because cell membranes contain many kinds of lipid species, their phase behaviour is expected to be complicated. Lipid composition, especially cholesterol, controls fluidity, phase behaviour and other properties of cell membranes (discussed in section 1.1.1.3), thus changes in the composition of lipids and/or interactions among them significantly affect membrane properties and functions.

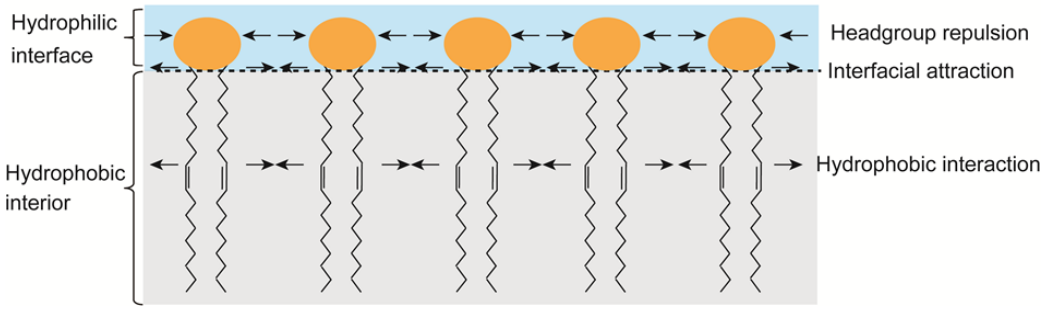

Figure 1.5. The interactions among phospholipids in lipid bilayer of cell membranes [38].

The fluid mosaic model indicates that most proteins mainly pass through the bilayer one or several times by the interaction between hydrophobic regions of proteins and nonpolar hydrocarbon tail of lipid molecules, called transmembrane proteins. Some others are able to attach to each surface of the lipid bilayer through nonconvalent interactions (peripheral membrane proteins). Membrane proteins account for approximately 30% of the proteome and perform most of membrane's functions [3].

Simons and Ikonen (1997) proposed the lipid raft model of membrane structure (Figure 1.6) which advances the understanding of membrane structure presented in the fluid mosaic model. The authors suggested that lipids have lateral organization in membranes due to preferential binding between cholesterols and sphingolipids, forming lipid microdomains which are called lipid rafts. These domains are enriched in cholesterol and sphingolipids, detergent-insoluble, and thicker than other parts of lipid bilayer. They serve as platforms for the binding of membranes proteins. Therefore, lipid rafts are important to many biological processes such as membrane trafficking and cellular signalling [41].

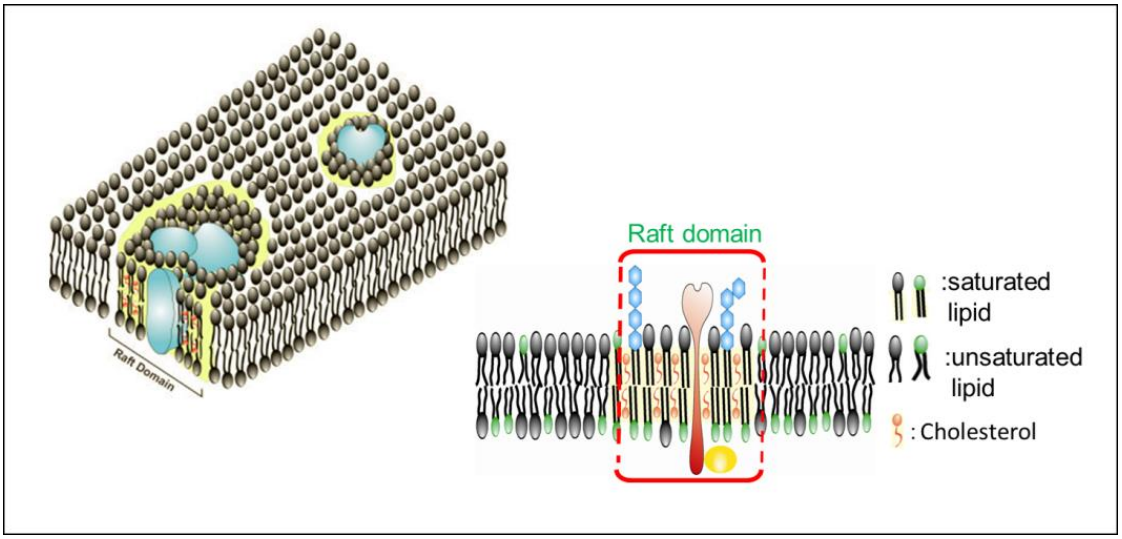

Figure 1.6. Lipid raft domains of cell membrane [36].

1.2.1.2. Function of cell membranes

Cell membranes play vital roles in the life of cells. First, they serve as boundaries of cells which separate and protect the cell from extracellular environment. Second, plasma membranes are essential for cellular metabolism. They selectively permeate and transport ion, organic molecules, and large particles needed for cell survival from the outside to the inside of cells and excrete metabolic products. Third, membranes are crucial in cell-to-cell communication and cell communication with the surrounding environment. Most of the proteins in membranes are receptors of cellular signaling pathways. They receive the information from external signals and subsequently trigger the responses of cells by changing its behavior. Last, cell membranes are required for other cellular processes such as cell adhesion, ion conductivity, and are the anchor of intracellular cytoskeleton [3].

Because of the above impacts, the integrity of membranes is fundamental to a healthy life of cells and the body. Many studies have shown that changes in membranes are involved in the pathogenesis of a variety of diseases. Membrane-related diseases are caused by interferences in receptor's activity, structural instability, modification in lipid state, and altered permeability [42]. Thus, unraveling the mechanism by which membranes are influenced by external stimuli, especially their interactions with external proteins are useful for finding the treatment of some human diseases.

1.2.2. Biomimetic membranes

Many biomimetic membrane systems have been developed and used in studies of biological membranes. In general, these model systems retain most indispensable features of biological membranes such as composition, structure, size, and permeability. In addition, they are simpler and are tailored with high precision [43]. Therefore, they enable researchers to exclude the complexity of cell membranes and investigate the role of specific components, especially lipid molecules, in behavior and function of membranes.

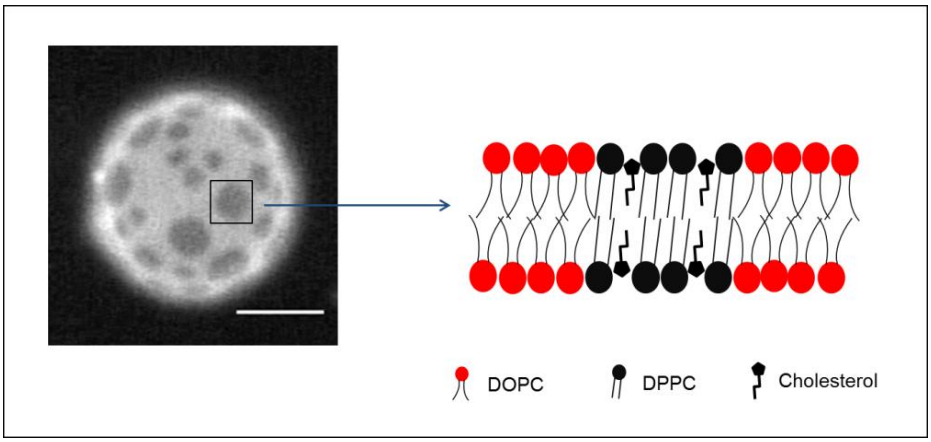

Figure 1.7. Fluorescent microscopic image of heterogeneous cell-sized lipid vesicle. The vesicle was prepared following the natural swelling method with the lipid composition of DOPC/DPPC/Cholesterol = 35/35/30 (molar ratio). Bright and dark regions indicate liquid-disordered and liquid-ordered phases, respectively. Scale bar is 10 μM.

Model membrane systems can be classified into four main kinds: Langmuir monolayers [44], supported bilayers [43], hybrid membrane systems [45,46], and lipid vesicles. Cell-sized lipid vesicles (cell-sized liposomes) are spherical model systems, which contain bilayers (unilamellar liposomes) and have size equal to or more than 10 μm (Figure 1.7) [36]. They contain one lipid phase (homogeneous) or more than one phase (heterogeneous). In heterogeneous lipid vesicles, there is phase separation by which lipid molecules organize in lateral compartments which are liquid-disordered (Ld) phase coexisting with liquid-ordered (Lo) or solid-ordered (So) phases [47]. In addition to their similarities to cell membranes in lipid composition, bilayer structure, and size like other model systems, cell-sized liposomes also have other similar properties to cell membranes such as curvature and transformation. They are able to provide a three-dimension space

essential for living cells, thus being real-world models of cells [48]. Moreover, these model membranes are large enough for researchers to observe, in real-time, any changes to their structure (membrane transformations). Therefore, cell-sized liposomes have been widely used to investigate transmembrane processes, encapsulation of macromolecules inside membranes, and membrane dynamics. They are commons platforms in studies on changes of membranes in the response to amyloid beta in pathology of Alzheimer's disease (AD) [36].

1.3. Amyloid beta (Aβ)-induced neurotoxicity in the pathology of Alzheimer's disease

1.3.1. Alzheimer's disease

Alzheimer's disease, first described by Alois Alzheimer in 1906 [49], is one of the commonest neurodegenerative disorders in humans. Common symptoms of the disease include short memory loss at the early stage (mild AD); long memory loss, confusion, and cognitive disabilities at later stages (moderate AD); inability to communicate and loss of body functions, finally leading to the death (severe AD) [50,51]. Regarding to pathological hallmarks, AD is characterized by extracellular plaques of Aβ fibrils, intracellular neurofibrillary tangles of hyperphosphorylated and misfolded tau protein, vascular damage resulting from extensive plaque deposition, and the loss of neuronal cells and synapses [52]. The disease can be associated with genetic abnormalities (familiar or early-onset AD) or with aging (sporadic or late-onset AD). Late-onset AD is highly prevalent, contributing to about 95-99% of people with the disease [53]

AD is the leading cause of dementia in older people, accounting for approximately 70% of all cases. According to World Health Organization, the number of people suffering dementia worldwide was about 35.6 million in 2006 and is predicted to rise to 65.7 million by 2030 and 115.4 million by 2050 [54]. In America, AD affects around 5.2 million people of all ages, 4% of Americans under age 65, and 11% of Americans over age 65. It is estimated that every 68 seconds, a person in America develops AD. Women contribute to two thirds of people with this disease [55]. Because of its widespread appearance and severity related to increasing life expectancy, AD has been one of the greatest threats to human health and healthcare systems worldwide [56].

1.3.2. Amyloid cascade hypothesis of Alzheimer's disease

Although there are many studies on AD, the mechanism by which the disease is triggered and developed in brain is not fully elucidated. Several hypotheses have been proposed to explain the pathogenesis of AD, including amyloid cascade [57], oxidative stress [58], cholinergic [59], and tau protein hypotheses [60], of which the amyloid cascade hypothesis (Hardy and Higgins, 1992) is the most influential [61]. This hypothesis posits that the deposition of amyloid beta (Aβ) peptide into plaques in brain tissue is the causative agent of AD, and the deposition directly induces formation of neurofibrillary tangles, vascular damages, and neuron loss. AD's pathology requires two crucial events which are (i) the generation of Aβ from amyloid precursor protein (APP), and (ii) Aβ-induced neurotoxicity to neuronal cells and Aβ-induced formation of neurofibrillary tangles. Amyloid cascade hypothesis was proposed based on histopathological and genetic information that clearly shows the association of Down syndrome having trisomy of APP gene and some APP gene mutations with a high risk of AD [57]. This original proposal was strongly supported by numerous studies for more than 20 years which have demonstrated that the accumulation and toxicity of Aβ, especially 42-residue isoform (Aβ-42) is tightly involved in AD pathogenesis. They also indicated that the production, deposition, and toxicity of Aβ are the result of a large number of distinct biochemical processes such as cholinergic, genomic, histopathologic, inflammatory, oxidative stress, and metabolic [62]. However, the process of Aβ-induced toxicity and the mechanism by which it triggers sand progresses the disease have not fully understood.

1.3.3. Production of Aβ

Aβ is generated from the cleavage of amyloid precursor protein (APP). APP (~ 100-130 kDa) is a single-pass transmembrane protein with large extracellular domains [36,62]. APP-coding gene locates in chromosome 21 [57] and undergoes alternative splicing, producing 8 different isoforms [63]. Three most common isoforms are the 695-residue isoform expressed predominantly in the CNS, the 751- and 770-residue expressed ubiquitously. It has been suggested that APP is important to cell signaling, cell and synaptic adhesion, synaptotrophic and neuroprotection, cognitive and synaptic functions [64]. APP is synthesized in large quantities in neurons. Newly produced protein is transported from the Golgi apparatus to cell membranes by endosomes. When it reaches cell membranes, it rapidly internalizes into the membranes. The protein can be

subsequently re-internalized into endosomes which transport it back to the cell surface or to lysosomes for degradation [65].

The processing of APP occurs in cell membranes, trans-Golgi network (TGN), and endosomes, following two main pathways, which are non-amyloidogenic and amyloidogenic. In the former pathway, the protein is cleaved in the extracellular domain by α-secretase and further cleaved in the transmembrane domain by γ-secretase, producing soluble N-terminal ectodomains (sAPPα), the identical intracellular C-terminal (AICD), and P3 (3 kDa) peptides. In the latter pathway, APP is first cleaved by β-site APP cleaving enzyme (BACE) and followed by γ-secretase, resulting in the formation of sAPPβ, AICD, and Aβ (Figure 1.8) [66]. The non-amyloidogenic processing is much more common in APP proteolysis (95%) and occurs in cell membranes, while the amyloidogenic is mainly performed in endosomes and TGN due to the predominant location of BACE in these intracellular compartments [36,63,65]. The formed Aβ is then dumped into the extracellular space by vesicle recycling [63].

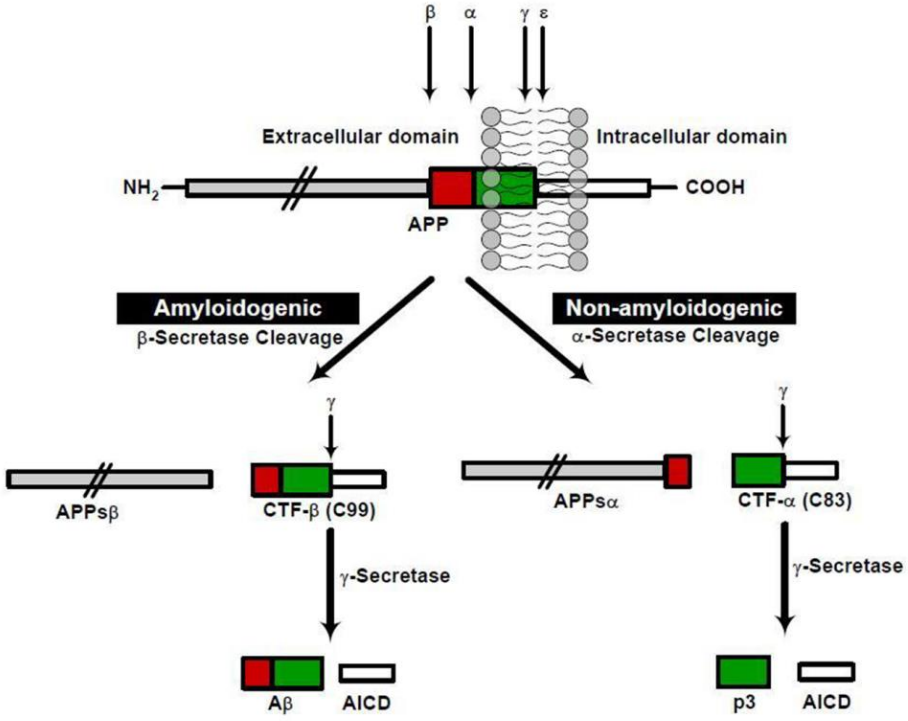

Figure 1.8. Proteolytic processing of amyloid precursor protein (APP) following non-amyloidogenic and amyloidogenic pathways. [66].

Changes in APP processing pathways due to genetic and metabolic abnormalities lead to the overproduction of Aβ or changes in ratio of Aβ isoforms. Some genetic mutations such as Down syndrome leading to the triplicated APP gene number and mutant APP genes causing a change in residues adjacent to the BACE cleavage site enhance Aβ formation [67]. Mutations in presenilin 1 (Psen1) and Psen2 which code two presenilins, components of γ-secretase, increase the generation of more toxic Aβ-42 isoform compared to 40-residue Aβ (Aβ-40) [68]. In addition, because three cleaving enzymes in APP processing are membrane-linked proteins, changes of membrane lipid composition can affect the activity of these enzymes, thus facilitating amylodogenic pathway [36,63]. Under normal physiological conditions, the cells are able to clear excess Aβ. There are three pathways of Aβ clearance in the brain, including (i) efflux of the peptide from CNS to circulation, (ii) intracellular proteolytic degradation in microglia and astrocytes, and (iii) extracellular degradation via insulin-degrading enzymes [68]. When these Aβ clearances are impaired (in late-onset AD) or Aβ production is significantly increased (in early-onset AD), an imbalance of the production and clearance of the peptide is formed, leading to its accumulation in the brain [69].

1.3.4. Structure and aggregation of Aβ

Amyloid beta (Aβ) denotes a peptide with an estimated weight of approximately 4.5 kDa and a hydrodynamic radius of 0.9 ± 0.1 nm [70]. The number of amino acids is 39-43, of which Aβ-40 and Aβ-42 are the most dominant isoforms, contributing to about 90% and 10% of all species, respectively [36,71]. Aβ is an amphiphilic molecule with a hydrophilic N-terminal region (including residues 1-27) and a hydrophobic C-terminus (including residues 28-43). The central region is from residues 16-22, which comprises a central hydrophobic core. There are 6 acidic amino acids and 3 basic amino acids in the hydrophilic part of Aβ. Therefore, the peptide is negatively charged at physiological pH and electrically neutral at weakly acidic pH [72]. According to dimensional structure, the native state of Aβ is a helix-turn-helix-like conformation, corresponding to its state in the precursor, APP [71]. After amyloidogenic processing of APP, Aβ is released in soluble form in aqueous environment. A large number of studies using a various structure-characterized techniques have demonstrated that the structure of Aβ monomers in aqueous environment is not stationary under physiological conditions. Some studies showed a random coil structure of the monomers [73]. Conversely, the others indicated that the monomers tend to partially fold,

producing a partly α-helical [74] or a β-sheets form [71]. The conformation of Aβ monomers depends on some factors such as temperature, pH, Aβ concentration, and it remarkably influences the amyloidogenecity [71,75].

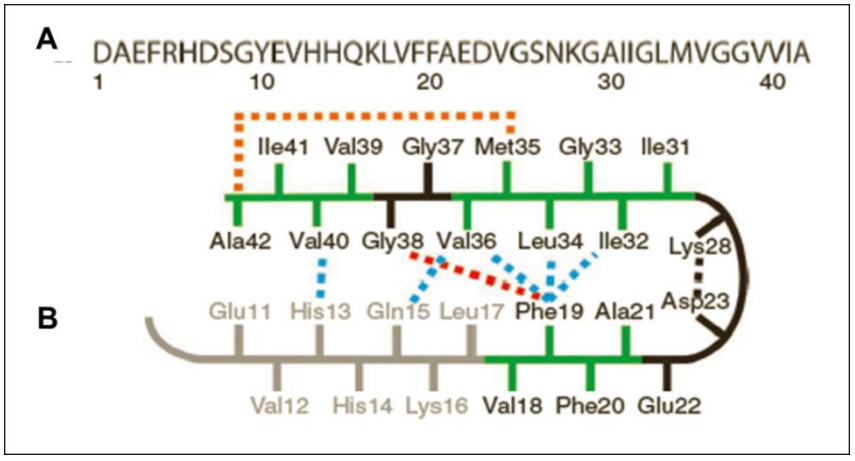

Figure 1.9. Sequence and structure of the amyloid beta (1-40) (Aβ-40) and amyloid beta (1-42) (Aβ-42) monomer units in fibrils. (A) Sequence of Aβ. (B) Structure of monomer units in Aβ-40 and Aβ-42 fibrils. In Aβ-40, side chain packing is observed between Phe19 and Ile32, Leu34 and Val36, and between Gln15 and Val36, resulting in β-turn-β structure (blue dashed line), while residues 1-10 is unstructured. In Aβ-42, the bindings between Phe19 and Gly38 (red dashed line) and between Met35 and Ala42 (orange dashed lines) produce β-turn-β structure in residue 18-42 [85]. The turn conformation is stabilized by hydrophobic interaction and a salt bridge among residues (green residues) in both isoforms [82].

Like other amyloid peptides, Aβ is prone to aggregate and form fibrils. The concentration required for spontaneous aggregation *in vitro* was reported at μM range [76]. The most prevalent model of Aβ aggregation considers this process as a nucleation-dependent polymerization. The process contains two major steps, which are (i) nucleation and (ii) fibril elongation [77,78]. In the first step, Aβ nuclei are formed from soluble Aβ monomers. The key event in this step is a conformational transition from unfolded/α-helix-enriched structure to β-sheet-enriched conformation occurring at a high peptide concentration or acidic pH, upon the ionic strength, and the presence of some ions or cell membranes [71-75]. The central region and hydrophobic C-terminus are thought to be

important for the transition [71]. β-sheet structure is tightly associated with self-aggregation [79], generating dimers, trimmers, and ultimately nuclei (*n*-mers) [78,80]. The structure of nuclei has not been characterized. The appearance of intermediate species including oligomers and protofibrils during Aβ fibrillation was reported. Some studies indicated that oligomers serve as nuclei of fibril growth [81,82]. Aβ oligomers vary in size [83] and structure. They may be globulomers (dodecamers) having globular shape, prefibrillar oligomers containing β-sheet structure [84,85], or disc-shaped pentamers composed of loosely aggregated strands [82]. Protofibrils are defined as small elongated oligomers [86] or precursor of fibrils which are generally curved and shorter than fibrils [81]. It has been demonstrated that oligomers and protofibrils are the primary toxic species of Aβ [85].

During the fibril elongation, Aβ nuclei polymerize in parallel and in-register orientation to form fibrils which feature β-sheet conformation [80,82]. Structure of monomer unit in Aβ was characterized. In Aβ-40, residues 11-40 form a β-strand-turn-β-strand (β-turn-β) conformation in which side chain packing is observed between Phe19 and Ile32, Leu34 and Val36, and between Gln15 and Val36, while residues 1-10 is unstructured. In Aβ-42, the bindings between Phe19 and Gly38 and between Met35 and Ala42 produce β-turn-β structure in residue 18-42 [87]. Ahmed and colleagues suggested that side chain packing in Aβ-42 can be formed between Phe19 and Leu34, Gln35 and Gly37, and between His13 and Gln15 [82]. The β-strand bends at Glu22, Asp23, and Lys28. The turn conformation is stabilized by hydrophobic interaction and a salt bridge among residues in both isoforms. (Figure 1.9) [82]. Driving forces bringing the nuclei together are nonnative side chain electrostatic and hydrophobic interactions. The assembly of β-sheets subsequently occurs by backbone-backbone interaction through intermolecular hydrogen bonds and finally by side chain-side chain interactions [80]. Aβ-42 aggregates faster than Aβ-40 due to two additional hydrophobic amino acids [81]. Furthermore, the reverse conversion of inert Aβ fibrils into neurotoxic protofibrils induced by membrane lipids was observed [88]. Since aggregated states are different in how they cause harm, the aggregation of Aβ plays important role in the peptide-induced neurotoxicity [78].

1.3.5. Cytotoxicity of Aβ

The accumulation of Aβ in the brain triggers a cascade of toxic processes, thus inducing an extensive synapse dysfunction and neuron loss, which are tightly associated

with cognitive impairment and dementia [57,89]. It has been reported that Aβ oligomers and protofibrils are majorly toxic species, and Aβ-42 is more harmful than Aβ-40 [81,85].

Aβ has directly multiple adverse effects on synapses and neurons. The peptide increases cellular oxidative stress by impairing the activity of ion-motive ATPases, glucose and glutamate transporters, GTP-binding proteins which cause lipid peroxidation [90]. The combination of the peptide with redox-active metals such as Fe^{2+} and Cu^{2+} are capable of oxygenating membrane lipids such as cholesterol [91]. Another effect is that Aβ induces dyshomeostasis of Ca^{2+} that serves fundamental roles in learning, memory, neuron survival and death. Cytosolic Ca^{2+} level is increased by Aβ-induced changes in calcium pumps and Aβ-formed ion channels in cell membranes [92]. In addition, the peptide is able to interact with different mitochondrial targets and induce dysfunction of mitochondria that in turn promotes ROS production [92]. Moreover, Aβ accumulation in endoplasmic reticulum (ER) activates ER stress, thereby enhancing Ca^{2+} efflux from this organelle to the cytosol [93]. Another effect of Aβ is to disrupt membrane receptors such as ionotropic glutamate receptors [90] that are important to synaptic activities [94]. These events alter synaptic functions and trigger neuron apoptosis.

Aβ accumulation has been demonstrated to induce the formation of intracellular neurofibrillary tangles, which are the characterized pathological hallmarks of AD and are also found in other neurodegenerative disease. They are composed of paired helical filaments of hyperphosphorylated tau proteins. Tau is microtubule-associated protein that plays important role in stabilization of microtubules [95]. This protein is natively unfolded [935 and is mainly found in the cytosol [94]. Aβ accumulation was demonstrated to alter the activity of several kinases and phosphatases that are able to phosphorylate tau and neurofilaments, leading to the formation of neurofibrillary tangles [89,96]. These tangles in turn induce cytoskeletal disruption, activate apoptosis proteins such as caspase-3 and ultimately cause apoptosis of neuronal cells [96,97].

Another primary consequence of Aβ accumulation is neuroinflammation. Accumulating evidences have shown that Aβ activates microglial and astrocytes [89], two most abundant glial cells in the CNS, by affecting some receptors such as specific toll-like receptor [98]. Microglia cells are important to initial responses of CNS to injuries, while astrocytes perform many functions such as brain organization, BBB formation, and injury response. They are able to phagocyte and degrade Aβ under normal physiological conditions. However, Aβ accumulation significantly changes the morphology and activity

of microglia and astrocytes, thus leading to production of inflammatory factors such as cytokines. These factors directly act on neurons and induce neurotoxic effects such as synaptic damages and neuron apoptosis [98].

1.3.6. Role of Aβ /membrane interaction in the peptide aggregation and cytotoxicity

Aβ is able to interact with cell membranes following three major pathways. In the first pathway, the peptide binds to some membrane receptors such as glutamate receptors and cellular, non-infectious form of prion protein (PrP^c) [99]. In the second pathway, the peptide specifically binds to gangliosides, a kind of sphingolipids of membranes [100]. They have a high affinity with Aβ, and the highest value was observed between monosialoganglioside G_{M1} (GM1) with Aβ-42 [101]. Because gangliosides mainly locate in lipid raft domain of cell membranes, lipid rafts serve as binding sites of the peptide [102]. The third pathway is the interaction of Aβ with the lipid bilayer of plasma membranes. The peptide can adsorb on membrane surface via electrostatic interaction [103]. On the other hand, it is capable of inserting into the lipid bilayer. The insertion is driven by hydrophobic interaction between hydrophobic C-terminal region of the peptide and non-polar hydrocarbon tail of membrane phospholipids [104]. The interaction between Aβ and membranes is strongly affected by membrane composition and properties [99]. Since cell membranes tremendously vary in composition, the mechanism of Aβ/membrane interaction is not fully understood.

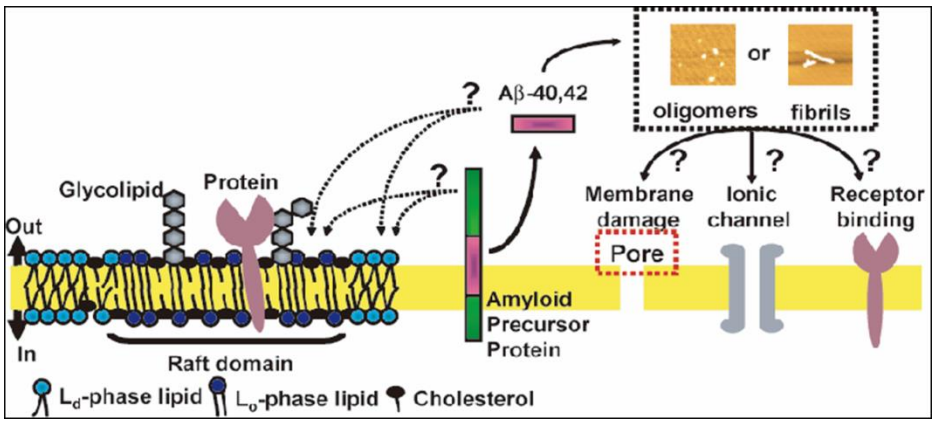

Figure 1.10. Proposed membrane-mediated cytotoxicity of amyloid beta [5]

Increasing evidences have demonstrated that Aβ/membrane interaction is the key event in Aβ aggregation and cytotoxicity (Figure 1.10) [2]. It has been reported that the concentration of Aβ in the extracellular fluid of the brain (at nM range) is much lower than the concentration required for spontaneous Aβ aggregation in vitro (at μM range) [76], and cell membrane is the platform of Aβ aggregation [102]. The peptide binds to GM1, adopts the conformational change from unfolded or α-helix-rich structure to β-sheet-rich, which subsequently undergoes the fibrillation [102]. Regarding Aβ cytotoxicity, the binding of Aβ with many membranes receptors alters their activities, thus enhancing oxidative stress, Ca^{2+} influx and perturbing cellular function (discussed in section 1.3.5). In addition, the peptide can insert into the lipid bilayer and form pores, which cause Ca^{2+} dyshomeostasis. The interaction of Aβ with membrane lipids causes lipid oxidation and changes membrane properties such as fluidity and domain structure, thereby disturbing the integrity and function of cell membranes [105,106]. Moreover, the endocytosis mediated by cell membranes is the process by which the cell absorbs Aβ which then causes toxicity to intracellular organelles [76,107]. Therefore, unravelling Aβ/membrane interaction is essential for understanding of AD's pathogenesis and therapeutics.

1.4. Effect of cholesterol and its oxidized derivatives on Aβ-induced neurotoxicity

1.4.1. Effect of cholesterol on Aβ-induced neurotoxicity

Cholesterol is considered as an important factor in AD. However, there are conflicting reports on whether cholesterol plays a risk or protective role in the disease. Early epidemiological studies suggested that high level of cholesterol is a susceptibility agent and using statin, which inhibits the activity of HMG-CoA enzyme in cholesterol biosynthesis, decreases the disease. Nevertheless, the direct relation between statin and the pathology of AD has not been well demonstrated, and some recent studies failed to show the beneficial effect of statins in AD treatment [108]. An increasing number of cellular and molecular studies have reported that cholesterol has remarkable effects in the production, aggregation, and cytotoxicity of Aβ, but its role as an accelerator or inhibitor of the disease is still disputable [5,108]. Many authors reported that some genes in cholesterol metabolism, especially ApoE, enhance Aβ accumulation in the brain [109]. In addition, membrane cholesterol disfavors the co-localization and interaction of APP and α-secretase [110], and promotes the binding of Aβ with lipid rafts [102], thereby increasing the amyloidogenecity. Others have argued that cholesterol loss in neuronal membranes enhances

Aβ generation [111], the interaction of Aβ with membranes, and the peptide's toxicity to neuronal cells [112].

In this study, we highlighted the effect of cholesterol on Aβ/cell-membrane interaction, which in turn significantly influences the aggregation and cytotoxicity of Aβ. Because cholesterol is an essential structural constituent and property modulator of cell membranes, the sterol is thought to modulate Aβ/cell-membrane interaction. However, whether cholesterol stimulates or hinders this interaction is uncertain, although there are many studies on this objective using both model membranes and living cells. Kakio and colleagues showed that cholesterol accelerates Aβ to bind to membrane GM1 by increasing the formation of GM1 cluster [113,114]. Similar conclusions were reported by Yahi et al. and Fantini et al. who demonstrated that cholesterol induces a change in GM1 conformation that is suitable for Aβ binding, thus enhancing Aβ/membrane interaction [115,116]. In addition, the direct interaction of oligomeric Aβ with membrane cholesterol with a higher affinity compared to Aβ/phospholipid interaction was indicated by Avdulov and colleagues [117]. Moreover, Wood and colleagues proposed that changes in the asymmetric distribution of cholesterol in neuronal plasma membranes by which the ratio between cholesterol in exofacial leaflet and cholesterol in cytofacial leaflet is higher can hinder the secretion or uptake of Aβ from or into cells [118]. Therefore, Aβ accumulation in neuronal cell membranes is increased by cholesterol. Yu and Zheng also reported that an increased cholesterol level modifies the structure and surface chemistry of membrane lipid bilayers, thus promoting the peptide to interact with the bilayers [119]. In contrast, many studies on neuronal cells and model membranes pointed out the inhibitory effect of cholesterol on Aβ association with cell membranes [104,112,120]. This is induced by the enhancing the influence of the sterol on membrane rigidity [104,120]. On the other hand, Ji et al. indicated that the cholesterol/phospholipid ratio determines Aβ's way of interacting with membranes. In low cholesterol DMPC vesicles, the peptide tends to position at the membrane surface and to adopt a β-sheet structure, while it partially inserts into the membranes and has an α-helix conformation at higher cholesterol concentrations [121]. Conversely, Devanathan et al. showed that cholesterol is not necessary for initial binding of Aβ to membranes, but the peptide will immediately finds and interacts with cholesterol when it inserts into membranes [122]. Regarding to membrane-mediated aggregation of Aβ, the role of cholesterol also remains controversial. Some reports indicated that cholesterol enhances Aβ aggregation by facilitating Aβ/GM1binding and changing the fluidity of cell

membranes [123,124]. However, Yip et al. demonstrated that the increase of cholesterol level in lipid bilayers induces the decreased number of mature fibrils [120]. These conflicting data indicates the need of further investigations of the link between cholesterol and Aβ/membrane interaction.

1.4.2. Effect of oxysterols on Aβ-induced neurotoxicity

The remarkable roles of cholesterol and its metabolism in the brain on Aβ production, aggregation, and cytotoxicity prompt researchers to investigate how oxysterols, which are derived from normally metabolic conversion and ROS-induced oxidation of cholesterol, influence the peptide. Since several oxysterols such as 24OH and 27OH function in regulating CNS cholesterol synthesis as well as in transport of cholesterol between CNS and periphery circulation, many studies aimed to unravel the role of these compounds in Aβ production and accumulation in the brain. Brown and colleagues showed that 24-OH and 27OH inhibit Aβ production by decreasing the amyloidogenic processing of APP in neuronal cells, but the former are approximately 1000-fold more potent than the latter [125]. The two oxysterols were also found to reduce the formation of Aβ in brain capillary endothelial cells, the anatomical basis of the BBB [126]. 22(R)OH is able to destabilize C-terminal fragments of APP and reduce Aβ generation as indicated by Koldamova et al [127]. On the other hand, the effect of oxysterols on Aβ clearance in the brain was investigated by many studies. They demonstrated that some oxysterols including 22(R)OH, 24OH, 25OH, and 27OH are ligands of liver X activated receptors (LXR), the key regulators of ApoE. Therefore, they stimulate the synthesis of ApoE [128], which tightly involves in the efflux of cerebral cholesterol and Aβ [69]. Additionally, 24OH and 27OH can upregulate the expression of ATP-binding cassette sub-family B member 1 (ABCB1) that hinders Aβ influx into the brain [129]. Moreover, a direct binding of 22(R)OH with Aβ-42 by docking of the oxysterol with a pocket formed by globular Aβ protects neuronal cells from the peptide toxicity [130]. This oxysterol and its derivative, caprospinol, have been considered as potential candidates for treatment of AD [131]. Besides the beneficial effects, some oxysterols have been indicated to have deleterious impact in Aβ-induced neurotoxicity. Björkhem et al. demonstrated an increased level of 27OH together with a decreased content of 24OH in the brain of AD patients [132], and Dasari et al. pointed out the ability of 27OH to increase the peptide production [133].

Other authors suggested a direct binding of 24OH with soluble Aβ-40, which contributes to the peptide accumulation [134] and 7βOH-induced enhancement of Aβ neurotoxicity [135]. In addition, small molecule products of cholesterol oxidation such as 4-hydroxynonenal and 3β-hydroxyl-5-oxo-5,6 secocholestan-6-al induce Aβ modifications, especially at Lys16, which render the peptide to form neurotoxic aggregates at physiological concentrations [136]. These studies have drawn a complicated link between oxysterols and AD's pathology.

Despite accumulating evidences revealing significant changes in membrane structure and properties caused by oxysterols, very little is known about the effect of these compounds on Aβ/cell-membrane interaction. Recently, Kim and colleagues indicated that the substitution of membrane cholesterol with 7βOH and 7keto induce a drastic reduction of the lysis tension of model membranes, thus promoting Aβ insertion into the membranes [137]. As far as we are aware, there is no report on the effect of oxysterols on membrane-mediated aggregation of Aβ. The current understanding is poor relative to a variety of oxysterols and their influences on membranes. This highlights the importance of more extensive studies on Aβ/membrane interaction upon the effect of oxysterols.

1.5. Research objective and dissertation outline

1.5.1. Research objectives

The understanding obtained from plethora of previous studies clearly shows that the interaction of Aβ with cell membranes plays crucial roles in Aβ aggregation and cytotoxicity, key processes in the pathogenesis of AD. Cholesterol, with the function as an essential structural component and property modulator of cell membranes, remarkably alters Aβ/membrane interaction. However, the role of cholesterol as a protective factor or a deleterious agent remains controversial. Oxysterols, which are derived from cholesterol oxidation upon the effect of enzymes, oxidative stress, or Aβ combining with redox-active metals, have a high ability to change the structure and properties of cell membranes. Nevertheless, their effects on Aβ/membrane interaction have not been well understood.

This dissertation aimed to investigate the role of cholesterol and oxysterols on the interaction of Aβ with the lipid bilayer of membranes and membrane-mediated aggregation of the peptide. Therefore, studies of the dissertation focused on:

1. Investigation of how cholesterol and two commonly occurring oxysterols, 25OH and 7keto, influence the interaction of Aβ-40 and Aβ-42 aggregated species with the lipid bilayer of a homogenous membrane system and associated membrane dynamics.
2. Elucidation of the effect of cholesterol and 7keto on Aβ-42 protofibrils localization in lateral lipid compartments of a heterogeneous membrane system.
3. Estimation of how cholesterol and 7keto affect the interaction of Aβ-42 protofibrils with Jurkat T cell membranes.
4. Investigation of the influence of cholesterol- and 7keto-containing homogeneous model membranes on Aβ-42 self-assembly implicated in neurotoxicity.

The result of this study will contribute to further understandings of the mechanism by which cholesterol and cholesterol oxidation modulate the interaction between Aβ and cell membranes. These understandings are important to unravel the pathogenesis of Alzheimer's disease and propose potential therapeutics for the disease.

1.5.2. Outline of dissertation

The dissertation is divided into 6 chapters with different objectives.

Chapter 1 provides a comprehensive background of the study. By reviewing related studies, I demonstrated a general overview of structure, metabolism, and function of cholesterol and oxysterols. Current understandings of the amyloid cascade hypothesis of AD and the impact of Aβ/cell-membrane interaction in Aβ aggregation and cytotoxicity were presented. Moreover, the controversial role of cholesterol and potential impact of oxysterols in Aβ/cell-membrane interaction were highlighted. This background indicates the need of more investigation on the link between cholesterol, oxysterols and Aβ/cell-membrane interaction which is the main purpose of this study.

In chapter 2, I used homogenous cell-sized liposomes to study how Aβ interacts with the lipid bilayer of homogenous membranes containing cholesterol and oxysterols. 25OH and 7keto was studied because they are major products of enzymatic oxidation and autooxidation of cholesterol, respectively. In addition, their harmful effects on cells have been reported. All aggregated species (small oligomers, protofibrils, and fibrils) of two abundant Aβ isoforms (Aβ-40 and Aβ-42) were used. The Aβ/membrane interaction was

analysed through localization of the peptide in membranes and associated membrane dynamics.

Chapter 3 is an investigation on the influence of cholesterol and 7keto on Aβ-42 protofibrils localization in lateral lipid compartments of heterogeneous model membranes which is corresponding to the heterogeneity structure of cell membranes. I used 7keto and Aβ-42 protofibrils because results of chapter 2 reveal a high ability of 7keto to promote the peptide localization in membranes and the highest tendency of these Aβ species to interact with membranes. Changes in fluidity of membrane lipid phases induced by cholesterol and 7keto were estimated to explain their influences on Aβ/membrane association.

Chapter 4 describes how cholesterol and 7keto affect the association of Aβ-42 protofibrils with Jurkat T cell membranes. Aβ-induced changes in viability and intracellular Ca^{2+} content of the cells under effect of cholesterol and 7keto were also estimated. The highlight of this chapter is the usage of biological membranes and living cells instead of model membranes systems. Results of this chapter were discussed by the understanding obtained from studies on model membranes.

Chapter 5 shows the effect of cholesterol- and 7keto-containing homogeneous model membranes on the aggregation of Aβ-42. The kinetics of Aβ aggregation, the morphology and ability of some Aβ aggregates to interact with Jurkat T cells were measured. Studies in this chapter indicated the link between membrane lipid composition and Aβ aggregation, which plays an important impact in the peptide-induced cytotoxicity.

Finally, chapter 6 summarizes conclusions obtained from the studies. Moreover, the prospects of this work were discussed.

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Chapter 2

Effect of cholesterol and oxysterols on the interaction of Alzheimer's amyloid beta with model membranes and associated membrane dynamics

Abstract

The interaction of amyloid beta (Aβ) with cell membranes has been shown to be influenced by Aβ conformation, membrane physicochemical properties and lipid composition. However, the effect of cholesterol and its oxidized derivatives, oxysterols, on Aβ-induced neurotoxicity to membranes is not fully understood. In this chapter, I employed homogeneous model membranes to investigate the localization of Aβ in membranes and the peptide-induced membrane dynamics in the presence of cholesterol and 7-ketocholesterol (7keto) or 25-hydroxycholesterol (25OH). The results have indicated that oxysterols rendered membranes more sensitive to Aβ, in contrast to role of cholesterol in inhibiting Aβ/membranes interaction. Two oxysterols had different impacts owing to distinct positions of the additional oxygen group in their structures. 7keto-containing cell-sized liposomes exhibited a high propensity toward association with Aβ, while 25OH systems were more capable of morphological changes in response to the peptide. Furthermore, I have shown that 42-amino acid Aβ (Aβ-42) protofibrils had higher association with membranes, and caused membrane fluctuation faster than the same aggregated species of 40-amino acid isoform (Aβ-40). These findings suggest the inhibiting role of cholesterol and enhancing effect of oxysterols on the interaction of Aβ with membranes. They contribute to clarify the harmful impact of cholesterol on Aβ-induced neurotoxicity by means of its oxidation.

Keywords: Oxysterols, Amyloid beta, Model membranes, Aβ localization, Membrane dynamics

2.1. Introduction

The interaction of Alzheimer's amyloid beta (Aβ) with cell membranes is a reportedly crucial event in the pathogenesis of Alzheimer's disease (AD) [1,2]. Unfolded Aβ oligomers arising from amyloidogenic processing pathway of a transmembrane protein, amyloid precursor protein (APP), specifically binds to neuronal membranes in lipid raft domains because the peptide has a high affinity for membrane sphingolipids. The binding induces a conformation change of Aβ from unfolded/α-helix-rich structure to β-sheet, leading to the generation of fibril nuclei. The nuclei subsequently aggregate to form toxic amyloid oligomers, protofibrils and less toxic mature fibrils [3-5]. The critical concentration of Aβ aggregation in membranes has not been reported. However, it may be lower than Aβ critical concentration in solution (1-30 μM) [6]. The presence of Aβ species, especially oligomers and protofibrils, trigger a cascade of harmful interactions with cell membranes including their insertion into membranes, which has been reported to (i) cause channel/pore formation [7,8], (ii) oxidize membrane lipids resulting in loss of membrane integrity [9,10], and (iii) internalize into the cell, thus influencing mitochondria, lysosomes and inducing their dysfunction followed by oxidative stress and apoptosis [11-13]. It has been shown that Aβ/membrane interaction is controlled by conformation of the peptide, membrane physicochemical properties and membrane lipid composition. One of the most important membrane components is cholesterol. The sterol is able to tightly pack with sphingolipids and glycerophospholipids, thus forming lipid raft domains and modulating membrane properties [14,15]. The mechanism by which cholesterol influences the peptide's interaction with the membranes, and/or the peptide's aggregation is not well understood. Some studies have reported that cholesterol decreases the Aβ-induced changes in structure and morphology of lipid rafts, hinder the β-sheet formation in membranes, thereby reducing the peptide insertion, aggregation and cytotoxicity [16-18]. Conversely, other researches have demonstrated that cholesterol directly binds to the peptide as soon as it inserts into the lipid bilayer and accelerates its recruitment and oligomerization [3], in agreement with epidemiological and clinical studies about toxic effect of high cholesterol concentration in AD's pathogenesis (reviewed in [19]).

Oxysterols are oxidized derivatives of cholesterol [20]. There are two main categories of oxysterols, those oxygenated on the sterol ring such as 7-ketochoelsterol (7keto), 7α/β-hydroxycholesterol (7α/βOH) and those oxygenated on the side-chain including 24(S)-hydroxycholesterol (24(S)OH) and 25-hydroxycholesterol (25OH) [20,21].

In general, the former class is produced by reactive oxygen species (ROS), while the formation of the latter is attributed to enzymes [20,22]. Aβ has also been reported to have cholesterol-oxidizing activity, producing mainly 4-cholesten-3-one [23] and 7βOH [24]. In comparison with cholesterol, oxysterols possessing one or more supplementary oxygen groups (hydroxyl, carbonyl, or epoxide) are more hydrophilic and different in three-dimensional shape and orientation in membranes [20]. Oxysterols exhibit both positive and negative biological roles. Some of them (24(S)OH and 27OH), at physiological concentrations, play an important impact in cholesterol homeostasis, sterol biosynthesis, and cell signalling via their interaction with receptor proteins [25], while others (7keto, 7α/β-OH, 25OH, even 24(S)OH at μM concentrations) have harmful effects and contribute to some human diseases such as atherosclerosis [26-28].

In AD's pathogenetic studies, oxysterols have received a lot of attention mainly as markers and protectors of the brain from Aβ's neurotoxicity. Data obtained from *in vitro* studies has suggested that 24(S)OH and 27OH inhibited Aβ production by modulating expression of genes coding APP and β-secretase, an important enzyme in amyloidogenic APP processing pathway [29,30]. Moreover, a 22R-hydroxycholesterol's derivative, caprospinol, has been considered as a potential drug candidate in AD treatment because it is able to bind to Aβ(1-42) isoform (Aβ-42), reduce its cytotoxicity, and clean the peptide small oligomers presenting in mitochondria [31]. As far as we are aware, there is less evidence on the negative effects of oxysterols on Aβ/membrane interaction although their capability of changing membrane structure and properties has been reported [20,32-34]. Because oxysterols have a different orientation in membrane compared to cholesterol, they are less able to condense lipids, thus modifying some physical properties of membrane such as collapse pressure and influencing raft domains [35,36]. Since membrane properties and domains structure remarkably affect the interaction between Aβ and membranes as discussed previously, it is reasonable to suppose that oxysterols can facilitate this interaction. The effect of 7keto and 7βOH on enhancing Aβ insertion into the lipid bilayer by decreasing intermolecular cohesive interaction was discovered by Kim et al. [37]. However, the impact of oxysterols on Aβ/membrane interaction remains an important subject that needs to be elucidated.

In this chapter, I was interested in the Aβ-induced dynamics of model membranes containing cholesterol, 7keto or 25OH. I chose these oxysterols in order to investigate the role of not only the presence of an additional oxygen group but also its relative location in

the molecule. Briefly, 7keto is a major auto-oxidative species which has an extra ketone group in ring structure and serves as an inhibitor of raft domains formation [20]. This oxysterol has been showed to be a risk factor in atherosclerosis [26]. 25OH, generated from enzymatic oxidation of cholesterol, possesses an additional hydroxyl group in the side-chain and promotes the formation of rafts [20]. This oxysterol was found to be a regulator of cholesterol biosynthesis [38] and an important factor of immunity [39]. I am aware that 24(S)OH and 22(R)OH play important roles in AD pathogenesis [30,31]. However, 25OH was chosen because it is more affordable. Moreover, both 25OH and 24(S)OH have been reported to induce apoptosis of neuronal cells at high concentrations [28,29]. Cell membrane dynamics such as membrane fluctuation and vesicle formation is driven by dynamic movement of molecules in membranes and structured reorganization of the membrane constituents upon the introduction of external stimuli [40,41]. Thus, the study about biomimetic membrane dynamics without protein is an ideal platform for investigating the mechanism by which lipid components control the interaction of the peptide with membranes [42]. Previously, Aβ-40 was reported to induce formation of sphero-stomatocyte, and this pathway of membrane transformation reveals a deeper insertion of the most toxic Aβ-40 oligomers into DOPC cell-sized liposomes (model membranes) compared to other species [43]. Here, I have shown that the presence of cholesterol (at 50% of membrane lipid composition) annulled the 'toxic' effect of Aβ on model membranes. In contrast, 7keto (at 10% of membrane lipid composition) significantly facilitated Aβ localization in membranes, while 25OH stimulated peptide insertion and subsequent membrane transformation. In addition, I have demonstrated that Aβ-42 protofibrils had a higher potential to interact with membranes compared to Aβ-40 protofibrillar species.

2.2. Materials and Methods

2.2.1. Materials

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol were purchased from Avanti Polar Lipids (USA). Two cholesterol oxidized derivatives, 7keto and 25OH, were obtained from Sigma-Aldrich (USA). Amyloid β proteins (Human, 1-40 and 1-42), were from Peptide Institute Inc. (Japan). Hilyte Fluor^{TM} 488-labelled Aβ-40 and Aβ-42 (λex=503 nm, λex=528 nm) were obtained from Anaspec, Inc. (USA). Olive oil, chloroform, and methanol were purchased from Wako Pure Chemicals, Kanto-Chemical,

and Nacalai Tesque (Japan), respectively. Deionized water was obtained using an ultraviolet water purification system (Millipore S.A.S, France).

2.2.2. Preparation of water-in-oil (W/O) homogeneous microdroplets

Four W/O microdroplet systems, including DOPC (only DOPC), Chol (DOPC/Chol = 50/50, molar ratio), 7keto (DOPC/Chol/7keto = 50/40/10, molar ratio), and 25OH microdroplets (DOPC/Chol/25OH = 50/40/10, molar ratio), were prepared using the simple mixing procedure [44,45]. Briefly, lipid mixtures dissolved in chloroform/methanol (2/1, v/v) were contained in glass test tubes. After the evaporation of organic solvent under a gentle nitrogen stream, a thin lipid film was formed at the bottom of tubes. The film was dried in a desiccator for about 3 h, and was subsequently sonicated with olive oil for 60 min at 37 ºC, followed by a vortex mixing to form microdroplets. The final lipid concentration was 0.2 mM.

2.2.3. Preparation of cell-sized homogeneous liposomes

Three kinds of cell-sized liposomes, including Chol (DOPC/Chol = 50/50 molar ratio), 7keto (DOPC/Chol/7keto = 50/40/10 molar ratio), and 25OH liposomes (DOPC/Chol/25OH = 50/40/10 molar ratio), were prepared by the natural swelling method as described previously [44,45]. In particular, lipid mixtures were dissolved in chloroform/methanol (2:1, v/v) in glass test tubes. The organic solvent was evaporated using a nitrogen gas flow to produce a thin film. The film was then dried under vacuum condition for 3 h and was hydrated with deionized water overnight at 37 ºC. The final concentration of lipid was 0.2 mM.

2.2.4. Preparation of Aβ-40 and Aβ-42 aggregation species

Aβ-40 and Aβ-42 aggregation species were prepared using the same method as in our previous studies [46]. Briefly, Aβ peptides dissolved in Tris buffer (20 mM, pH 7.4) at 80 μM concentration were incubated at 37 ºC for various periods to form various stages of aggregation. Small oligomeric, protofibrillar, and fibrillar species of Aβ-40 were obtained from incubations for 0 h, 24 h, and 120 h, respectively. In the case of Aβ-42, the periods of

incubation were 0 h, 12 h, and 48 h, producing small oligomers, protofibrils, and fibrils, respectively.

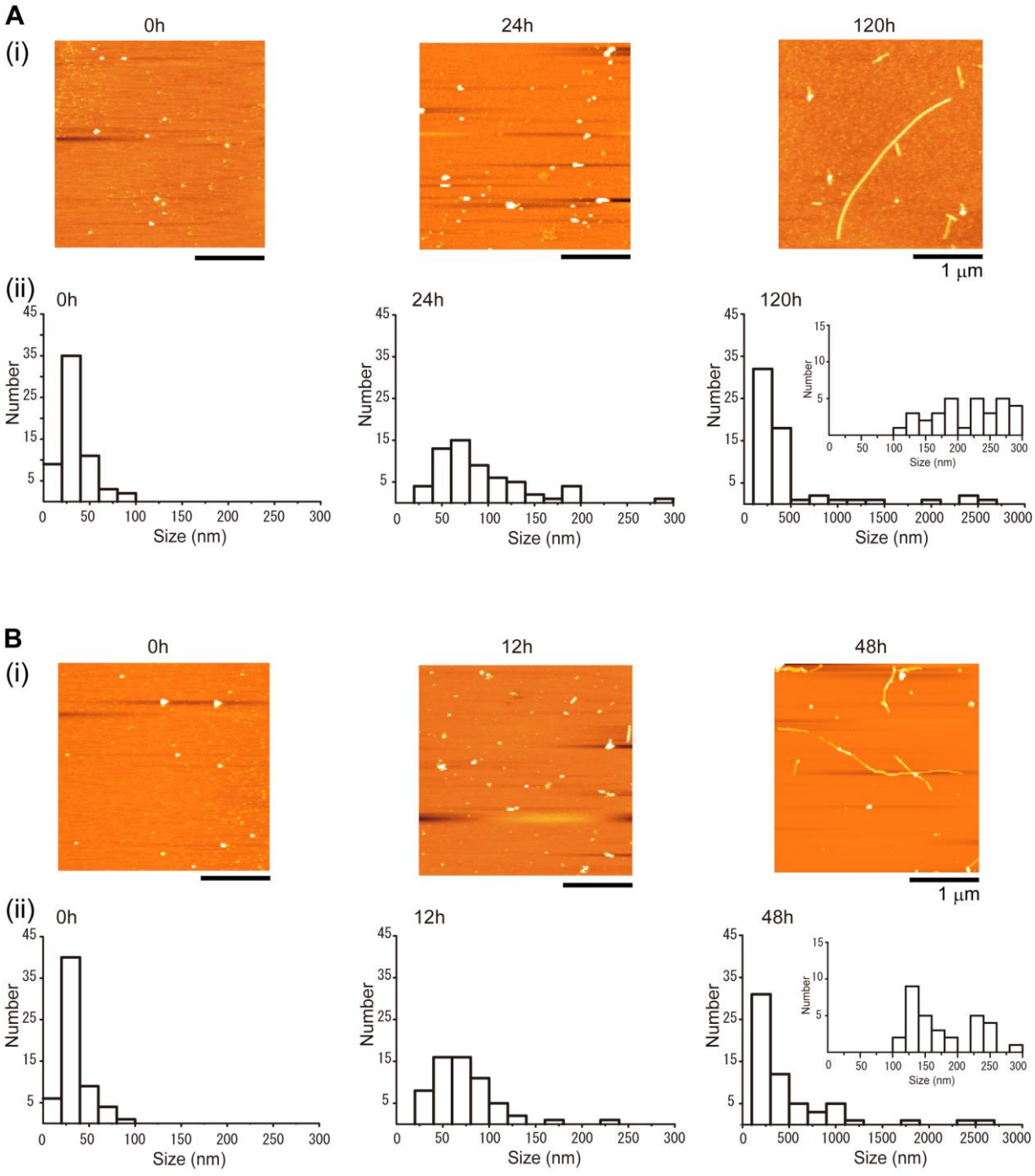

Figure 2.1. The conformation of Aβ aggregation species after incubation in different times at 37 ºC in 20 mM Tris buffer (pH = 7.4). (A) Typical AFM images (i) and size distributions (ii) of Aβ-40 species (n = 60), (B) Typical AFM images (i) and size distributions (ii) of Aβ-42 species (n = 60).

2.2.5. Morphological observation of Aβ

The morphology of Aβ-40 and Aβ-42 species was observed using AFM (SPA400-SPI 3800, Seiko Instruments Inc., Japan). 5 μM of Aβ-42 solution was uniformly spread and immobilized in a mica plate (Furuuchi Chemical Co., Japan). The mica was subsequently washed three times with 50 μl of deionized water to exclude Tris buffer molecules and was dried under the vacuum condition. The sample was measured by AFM equipped with a calibrated 20 μm xy-scan, 10 μm z-scan range PZT-scanner and a scanning silicon nitride tip (SI-DF3, spring constant = 1.6 N/m, frequency resonance = 28 kHz, Seiko Instruments Inc.) in a dynamic force mode (DFM) [40]. All AFM operations were performed in an automated moisture control box with 30–40% humidity at room temperature. The length of Aβ-42 aggregates was analysed using Image J software and is presented in Figure 2.1.

2.2.6. Observation of Aβ localization in membranes

Suspension of microdroplet (5 μL) was mixed with 5 μM fluorescent Aβ solution (5 μL) followed by a gentle tapping. The resultant mixture (5 μL) was placed on a glass slide and immediately used for microscopy observation. Observation of Aβ localization was performed using laser scanning confocal microscopy (Olympus FV-1000, Japan) at room temperature (~ 21.5 ºC) within 2 min of sample preparation. The amount of Aβ locating in membranes was estimated by fluorescent intensity values analysed using Image J software. The data are expressed as means ± standard error (SE). Comparisons between the different membranes were performed using ANOVA followed by Bonferroni's post comparison test. Fluorescent-labelled Aβ has been used by many researches on Aβ cellular binding, uptake and toxicity. It was also reported that the uptake of labelled and unlabelled protofibrillar Aβ by neuronal cells are similar [47]. In addition, the duration of liposome observation in our work was short (for 2 min). Thus, the effect of quenching is not a significant problem.

2.2.7. Detection of Aβ-induced membrane dynamics

Aβ was introduced into liposome suspension at the final concentration of 1 μM dissolved in 0.5 mM Tris buffer (pH = 7.4). This concentration of Tris has been shown not to influence membrane stability [43]. Liposome suspension (5 μL) and Aβ solution (5 μL)

were mixed, transferred immediately in a circular silicone well (0.1 mm) placed on a glass slide, and covered with a glass cover. Observation of membrane dynamics was carried out within 2-min introduction of the peptide to liposome suspension using a phase-contrast microscope (Olympus BX50, Japan) at room temperature. The total period of real-time observation was 20 min. The images were recorded on a hard-disk drive at 30 frames s^{-1} and processed using Image J software [46]. Membrane fluctuation was analyzed as a function of radius and its distribution $r(\theta,t)$ ($\theta = 2\pi/n$, n = 0, 1, 2,…, 100) [48]. A liposome is considered to be fluctuating when the value $\sigma = \langle sqr(r(\theta)-\langle r\rangle)^2\rangle/\langle r\rangle$ is equal to and/or more than 1.3% [49].

2.3. Results and discussion

2.3.1. Effect of cholesterol on the interaction of Aβ with model membranes

I first clarified the role of cholesterol in Aβ/model membranes interaction by comparing cholesterol-containing (DOPC:Cholesterol) membranes with only DOPC-containing systems. W/O microdroplet systems, which are cell-sized monolayer vesicles, were employed in the peptide localization study because it has high resistance to physical stress and can easily encapsulate biological macromolecules [50]. As can be seen in Figure 2.2, the amount of Aβ aggregated species localizing in Chol microdroplets was significantly lower than those associating closely with DOPC systems. This result suggested that the presence of cholesterol hinders Aβ localization in membranes.

Cholesterol membrane transformation induced by Aβ was studied using cell-sized bilayer vesicles, also called liposomes on the grounds that they are not only comparable to actual cell membrane in size, lipid composition and bilayer structure, but also controllable so that researchers can capture in real time a direct observation of membrane changes under desirable conditions [43]. In comparison with DOPC liposomes, which were reported previously [43,51], Chol systems exhibited a lower frequency of transformation upon addition of Aβ under most conditions (Aβ-40 protofibrils and all Aβ-42 aggregation species) (Figure 2.3A).

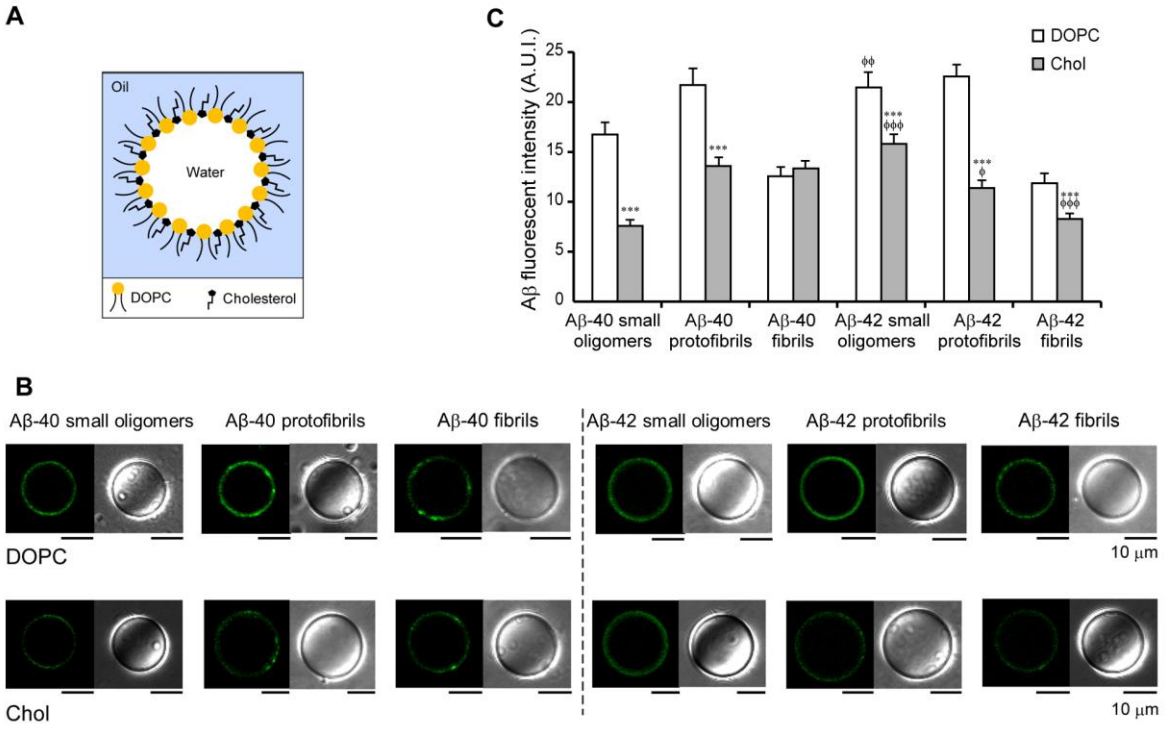

Figure 2.2. The localization of amyloid beta (Aβ) in DOPC- and cholesterol- containing membranes. (**A**) Schematic illustration of W/O microdroplet systems. (**B**) Representative confocal scanning microscopy images of DOPC microdroplets and Chol systems showing Aβ absorption. (**C**) Fluorescent intensity values representing the amount of Aβ absorbed in DOPC microdroplets (white, as the control), and Chol systems (DOPC/Chol = 50/50, bright gray). The values are mean ± SE of three replicates. The symbols *** indicate significant differences of Chol microdroplets relative to the control with $P \leq 0.01$. The symbols ϕ, ϕϕ, and ϕϕϕ that indicate significant differences of Aβ-42 compared to Aβ-40 with $P \leq 0.1$, $P \leq 0.05$, and $P \leq 0.01$, respectively. Scale bars are 10 μm.

Moreover, the remarkable distinction between the two systems was in pathways of membrane transformation. DOPC liposomes displayed three main pathways, including fluctuation, exo-tube/bud, and stomatocyte (Figure 2.3B). I supposed that exo-tube/bud and stomatocyte are big changes in membrane morphology which could be related to exocytosis and endocytosis respectively, and affect spatial localization of membrane receptors [43]. Since endocytosis has been shown to be involved in Aβ uptake of cells [52], the peptide may internalize into the liposomes by endovesicles during stomatocyte formation. Therefore, I placed more attention in stomatocyte formation. This transformation pathway was observed in liposomes exposed to Aβ protofibrils, one of

primary neurotoxic species, and the peptide fibrils which can be unfolded into pre-fibril structure upon their interaction with membrane molecules [42].

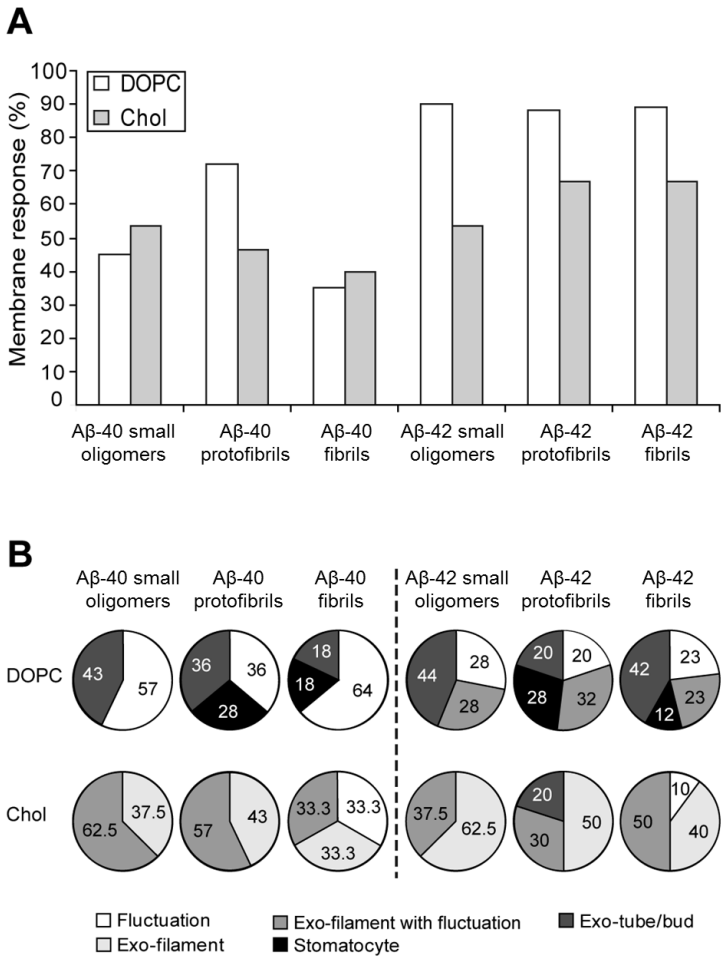

Figure 2.3. The Aβ-induced membrane transformation of DOPC and Chol liposomes. (**A**) The percentage of liposomes which showed membrane transformation in the response to Aβ. DOPC liposomes (white) [43,51], and Chol systems (bright gray) (n = 20). The presence of Aβ species is shown below each corresponding column. (**B**) Distribution profile of transformation pathways of DOPC and Chol liposomes (n = 20). The presence of Aβ species is shown below above each corresponding pie chart.

On the other hand, Chol liposomes induced exo-filament without fluctuation as a major transformation pathway caused by the peptide (Figure 2.3B). Under our experimental conditions, membrane fluctuation was attributed to Aβ insertion into membrane. Therefore, exo-filament without fluctuation was not involved the

peptide/membrane interaction. It could be caused by an osmotic disparity due to different Aβ concentrations between the outside and the inside of liposomes. Other major transformation pathways of cholesterol systems were fluctuation and exo-filament with fluctuation. Especially, exo-tube/bud was only displayed after the introduction of Aβ-42 protofibrils, and stomatocyte formation was not observed in any condition (Figure 2.3B). This finding indicates the role of cholesterol in suppressing 'significant' transformation of membranes induced by Aβ. This indicated that the sterol may preserve the integrity of cell membranes upon the effect of Aβ and inhibit Aβ internalization into the cytosol.

2.3.2. Localization of Aβ in oxysterol-containing membranes

The effect of oxysterols (7keto or 25OH) on localization of Aβ in model membranes was studied using Chol microdroplets as the control. Figure 2.4 shows that the presence of either 7keto or 25OH in microdroplets facilitated all the peptide aggregated species to locate in membranes compared to the control. Comparing the two oxysterols, 7keto had a much higher efficiency in modulating Aβ association with membranes than 25OH, especially in the case of Aβ small oligomers and protofibrils. These oxysterols differ in the location of the additional oxygen group. Thus, my data showed that the association of Aβ with membranes was affected by not only the presence of the additional polar group but also its relative position in oxysterols molecules.

The association of Aβ with membranes is first attributed to Aβ adsorption in membrane surface which can be followed by the insertion of the peptide into membranes. Because Aβ is an amphiphilic molecule, it has a pronounced surface activity and is able to adsorb at the interface. Aβ adsorption in membranes has been reported to be lower at high surface pressure [54]. The different effects of cholesterol, 7keto and 25OH on this association may be discussed in terms of their influences on membrane physical properties.

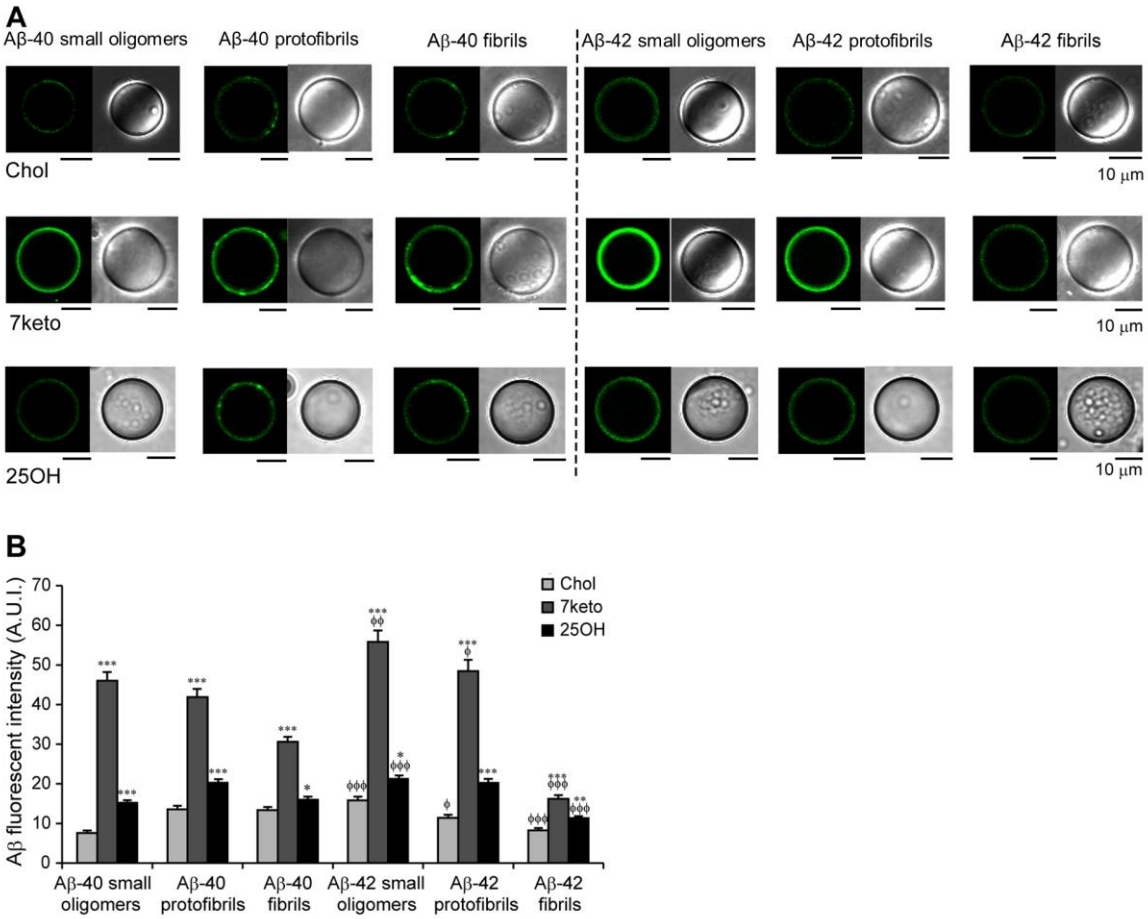

Figure 2.4. Localization of amyloid beta (Aβ) in oxysterols-containing microdroplet membranes. (**A**) Representative confocal scanning microscopy images of Chol and oxysterols microdroplet systems showing Aβ absorption. The presence of Aβ species is shown below each corresponding column or above each image. (**B**) Fluorescent intensity values reflecting the amount of Aβ absorbed in Chol microdroplets (DOPC/Chol = 50/50, bright gray, as the control), 7keto systems (DOPC/Chol/7keto = 50/40/10, dark gray), and 25OH systems (DOPC/Chol/25OH = 50/40/10, black). The values are mean ± SE of three replicates. The values are mean ± SE of three replicates. The symbols *, **, and *** indicate significant differences of Chol microdroplets relative to the control with $P \leq 0.1$, $P \leq 0.05$, and $P \leq 0.01$, respectively. The symbols ϕ, ϕϕ, and ϕϕϕ that indicate significant differences of Aβ-42 compared to Aβ-40, with $P \leq 0.1$, $P \leq 0.05$, and $P \leq 0.01$, respectively. Scale bars are 10 μm.

Due to the orientation in which the plane of rigid tetracyclic ring is perpendicular to lipid bilayer surface and hydroxyl group pointed at the membrane-water interface,

cholesterol is able to order the upper part of acyl chain of the neighbor phospholipids, and enhance cohesive interactions among molecules inside the bilayer. On the other hand, because of the introduction of an additional oxygen group near OH group at C3, ring-oxygenated oxysterols often produce a marked tilt with respect to the membrane-water interface. Side-chain-oxygenated oxysterols tend to orient horizontally so that both of polar groups are exposed to the hydrophilic interface, or they may adopt an inverted orientation where the side chain OH group is at the interface of membranes. These conformations render oxysterols less effective in ordering phospholipids compared to cholesterols and change membrane properties [53]. Therefore, 7keto and 25OH membranes have noticeably lower surface than Chol membranes, lead to the higher amount of the peptide in membranes containing oxysterols. Although the surface pressure of pure 25OH is lower than that of 7keto, it has reported that monolayers containing a small amount of 25OH exhibit higher surface pressure relative to monolayers with a similar levels of 7keto [35]. Yoda et al. indicated that the presence of 7keto at 6.5% concentration in DOPC/Chol monolayer more significantly decreases the surface pressure of the monolayer than 25OH [34]. In addition, a surface compression modulus (C_s^{-1}) measurement found the higher C_s^{-1} of 25OH membranes compared to 7keto systems. Maltseva and Brezesinski pointed out that compressed monolayers are capable of decreasing Aβ absorption [54]. These properties may accounts for higher absorption of Aβ in 7keto membranes than that in 25OH systems.

Some studies have indicated the direct binding of some oxysterols including 24(S)-hydroxycholesterol, 22(R)-hydroxycholesterol and its derivatives to Aβ. This binding is induced by docking of oxysterols with a pocket formed by globular Aβ and strongly depends on oxysterols and Aβ conformation [55]. Since this interaction seems to be difficult when oxysterols locate in membranes and the Aβ which I studied was not globular, we did not discuss this mechanism in my study. However, I plan to study whether there is a direct biding between 7keto, 25OH and Aβ in further research.

2.3.3. Effect of oxysterols on Aβ-induced membrane fluctuation

In order to investigate further the interaction of Aβ with oxysterols-containing membranes, membrane fluctuation was characterized.

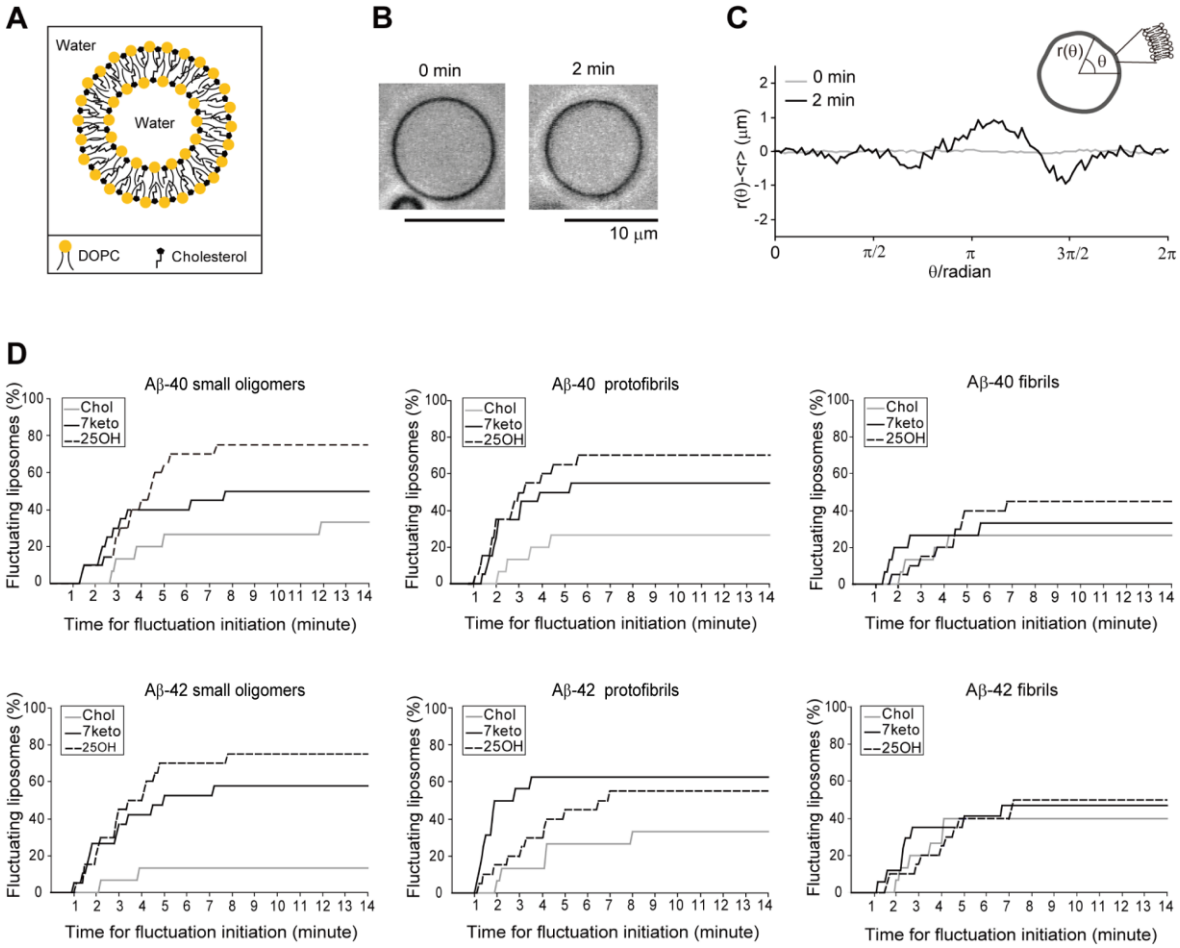

Figure 2.5. The effect of oxysterols on membrane fluctuation in response to Aβ species. (**A**) Scheme of cell-sized liposomes. (**B**) Typical images of a fluctuating lipid vesicles captured using phase-contrast microscope. (**C**) Degree of membrane fluctuation 0 and 2 minutes after its exposure to Aβ. Plotted the value of (r(θ)-<r>) in each θ (θ = 2π/n, n = 0, 1, 2,…. 100). (**D**) Percentage of lipsomes which started fluctuating in a given time of observation. Chol-containing cholesterol liposomes (gray, as the control), 7keto-containing liposomes (black), and 25OH-containing those (dash) (n = 20). The presence of Aβ species is shown above each corresponding line chart.

Fluctuation was the first step of most membrane transformation pathways observed in liposomes [43]. It has been reported that this type of membrane dynamics is induced by a decrease in volume (V) to area (A) ratio upon external physical stresses or surfactants [34]. These external stresses cause a reduced volume or membrane excess area, thus decreasing V/A ratio. My group reported previously that the movement of oxysterols toward membrane surface prompts membranes to expand their area and then fluctuate [49].

In the case of membranes exposed to Aβ, I proposed that the insertion of the peptide and/or recruitment of free lipid molecules and small vesicles to the considered liposomes mediated by the peptide lead to an increase of membrane area and the resultant fluctuation [43,51].

We calculated the percentage of fluctuating liposomes in a time-dependent manner in order to elucidate the effect of oxysterols on Aβ penetration into membranes. Figure 2.5 reveals that oxysterols-containing liposomes were more responsive to fluctuation induced by Aβ small oligomers and protofibrils compared to those containing cholesterol. In these conditions, the percentage of fluctuating cholesterol liposomes was considerably less than that of oxysterol systems. However, in the presence of fibrillar species, the membrane fluctuation was not significantly different among three concerned membrane systems. The data also showed a higher effect of 25OH on Aβ-induced membrane fluctuation compared to 7keto although the latter was more able to mediate the peptide localization in membranes. The time needed for 50% of 25OH liposomes to start fluctuation was shorter than that needed for 7keto liposomes in most conditions, except for in the introduction of Aβ-42 protofibrils (Table 2.1).

Table 2.1. The effect of oxysterols on membrane fluctuation induced by Aβ. The values represent the time required for 50% of liposomes to start fluctuating in the exposure to Aβ species.

Membrane systems	Time (minute)					
	Aβ-40 monomers	Aβ-40 protofibrils	Aβ-40 fibrils	Aβ-42 monomers	Aβ-42 protofibrils	Aβ-42 fibrils
Cholesterol	*	*	*	*	*	*
7-ketocholesterol	7.7	3.9	*	5.0	1.9	*
25-hydroxycholesterol	4.4	3.0	*	3.4	6.5	7.2

(* Could not be observed)

These results clearly show the role of oxysterols, especially 25OH, in enhancing Aβ insertion into membranes and are consistent with a previous study that has reported on the capability of 7keto and 7βOH to promote the peptide penetration into membranes [37].

This distinction could be attributed to how the extra-oxygen group of oxysterols influences membrane property. The insertion of Aβ into membranes requires a vacant space in the lipid bilayer interior and the energy for the space formation is proportional to the membrane area expansion modulus [56]. The presence of the additional polar group decreases the condensation of oxysterol membranes, thus rendering them easier to expand in comparison with cholesterol (Figure 2.6). Mintzer et al. reported that 25OH has the lowest condensation effect on the monolayer, followed by 7keto, and cholesterol exhibits the highest effect [35]. As a consequence, oxysterols, especially 25OH, facilitates Aβ penetration into membranes.

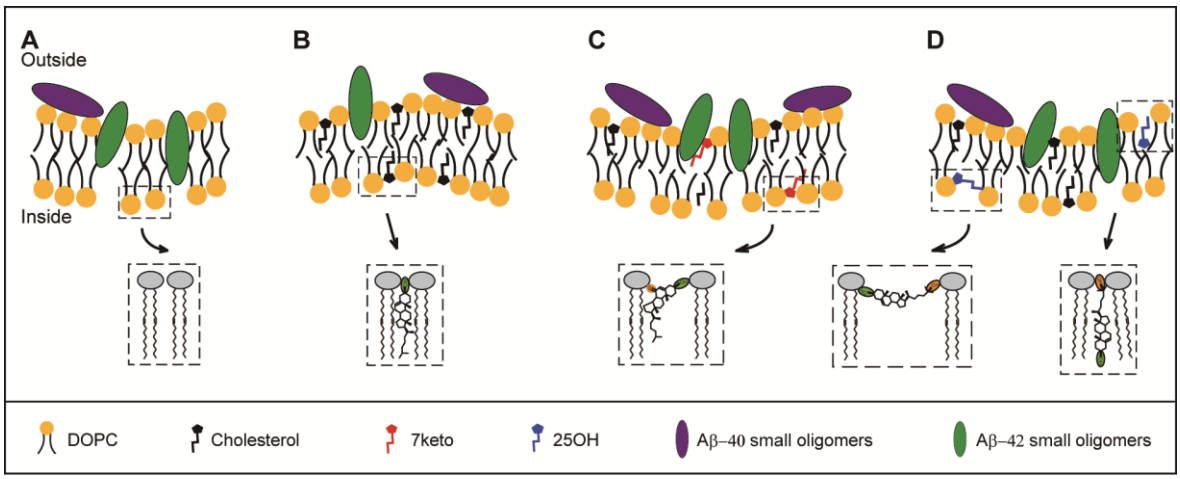

Figure 2.6. Schematic illustration of the effect of cholesterol and oxysterols on the interaction between Aβ and membranes. (A) DOPC membrane, (B) Chol-containing membrane, (C) 7keto-containing membrane, and (D) 25OH-containing membrane.

2.3.4. Effect of oxysterols on Aβ-induced membrane transformation

Aβ-induced transformation of liposomes containing oxysterols was studied to clarify the effect of oxysterols on membrane stability in the presence of the peptide. Similar to the membrane fluctuation experiments, I found that 7keto or 25OH liposomes have a higher propensity to change in morphology in the presence of most Aβ aggregated forms compared to Chol systems, and 25OH liposomes were slightly more sensitive to the peptide than 7keto systems (Figure 2.7). Five transformation pathways were observed in oxysterol liposomes (Figure 2.8). Distribution profile of these pathways significantly

showed the difference in response to Aβ between oxysterols and cholesterol membranes (Figure 2.9).

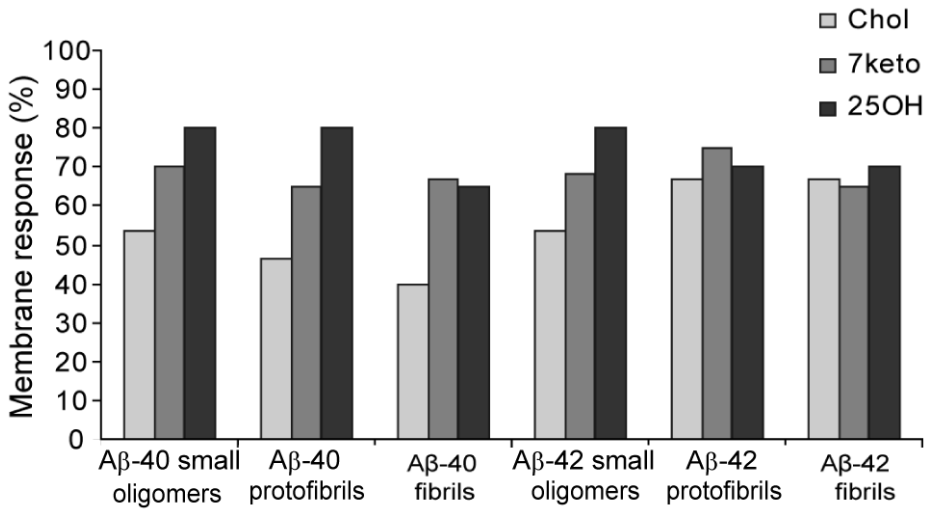

Figure 2.7. The percentage of oxysterols-containing liposomes which showed membrane transformation in response to Aβ. Chol liposomes (bright gray, as the control), 7keto systems (dark gray), and 25OH systems (black) (n = 20). The presence of Aβ species is shown below each corresponding column.

Although the percentage of transformed cholesterol liposomes was high (from 40% to 67%), they induced mainly exo-filament without fluctuation. In contrast, oxysterols liposomes only exhibited exo-filament without fluctuation at a high percentage in the presence of Aβ fibrils. This indicated that oxysterols render the lipid bilayer less stable in the presence of the peptide compared to cholesterol. In addition to four types of transformation pathways that were observed in cholesterol systems, both 7keto liposomes and 25OH systems showed stomatocyte formation in response to the peptide. Since stomatocyte formation may involve in Aβ internalization into liposomes, this data suggested that oxysterols not only accelerate Aβ penetration into membranes but also induce the peptide uptake of liposomes (Figure 2.8 and 2.9).

7keto and 25OH liposomes exhibited some differences in Aβ-induced transformation. The latter has a noticeably higher stomatocyte formation after introduction of protofibrillar species (19% and 21.5% responded to Aβ-40 and Aβ-42 protofibrils, respectively) compared to the former (0% and 8% responded to Aβ-40 and Aβ-42 protofibrils, respectively) (Figure 2.9). In the presence of small oligomeric species, the

percentage of 25OH liposomes showing stomatocyte was similar to that of 7keto systems. However, 25OH liposomes also underwent exo-tube/bud formation, another big change of membrane morphology, in addition to stomatocyte pathway (Figure 2.8 and 9). These results indicate that 25OH has slightly higher potential to promote peptide insertion and decrease membrane stability in comparison with 7keto.

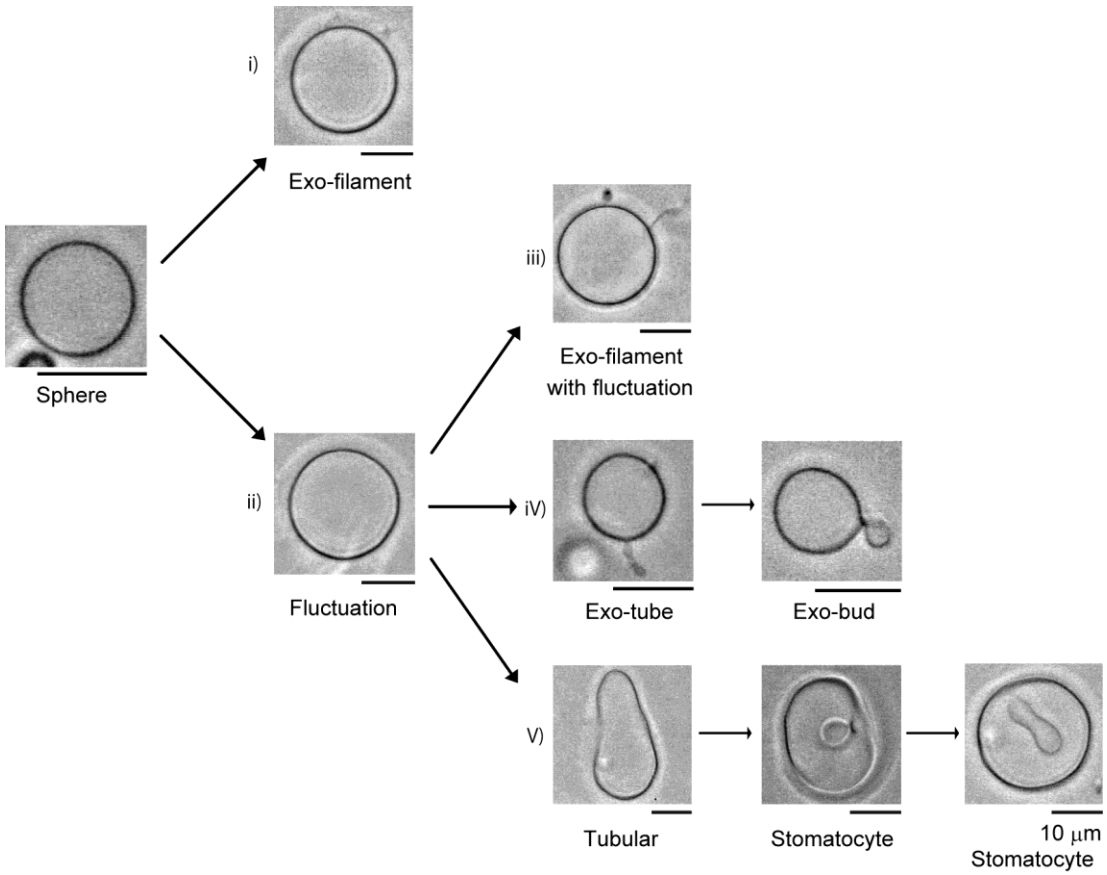

Figure 2.8. Membrane transformation pathways of oxysterol liposomes in response to the presence of Aβ aggregation species. Five pathways were observed: (i) fluctuation, (ii) exo-filament, (iii) exo-filament with fluctuation, (iv) exo-tube/bud, and (v) stomatocyte. Scale bars are 10 μm.

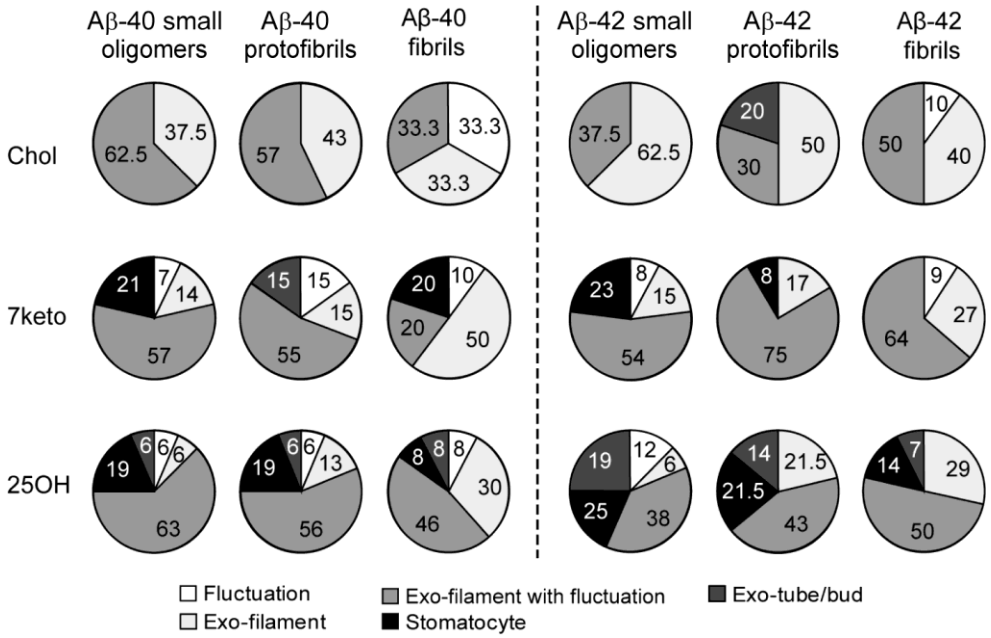

Figure 2.9. The effect of oxysterols on Aβ-induced membrane transformation. Distribution profile of transformation pathways of Chol, 7keto, and 25OH liposomes ($n \geq 20$), showing the percentage of liposomes which underwent a given pathway. The presence of Aβ species is shown below above each corresponding pie chart.

As discussed previously, oxysterols facilitated the insertion of Aβ into membranes, thereby increasing the morphological change of membranes. The appearance of stomatocyte transformation pathway in only oxysterols liposomes (and not in cholesterol only liposomes) could be explained by differences in flip-flop between two membranes leaflets of these sterols. Flip-flop rate of membrane lipids is affected by the molecular hydrophilicity [31]. Cholesterol, with a single hydroxyl group, possesses a fast flip-flop rate, while oxysterols flip-flops more slowly because of two polar groups [32,57]. When Aβ inserts into the outer layer of membranes, it causes an increase in the area of this layer. Various experiments have shown that an area difference between two leaflets induces the bending of membranes [58]. In this case, membranes first bend outwardly, and a fast movement of cholesterol from the inner layer to the outer can induce the formation of positive curved regions (exo-filament with fluctuation and exo-tube/bud) which is able to alleviate the area difference between two layers (Figure 2.6). In oxysterols-containing membrane, the change in the outer leaflet area could not be quickly relieved due to a slow flip-flop rate of these sterols. Consequently, Aβ can penetrate more deeply, resulting in

bigger change in both membrane leaflets. Thus, both positive and negative curvatures (stomatocyte pathway) were achieved (Figure 2.6).

This explanation similarly applies to the case of DOPC liposomes which were previously demonstrated to form stomatocyte pathway with high percentages in the presence of Aβ protofibrils (28% in the presence of Aβ-42 and Aβ-40) and fibrils (18% and 12% in the presence of Aβ-42 and Aβ-40, respectively) [43,51]. These values are slightly higher than those observed in oxysterols systems (Figure 2.7). It has been reported that DOPC has a slower flip-flop rate compared to sterols because of its large polar headgroups [32]. Therefore, DOPC membranes are more capable of stomatocyte formation than sterol-containing systems (Figure 2.6). Our discussion is in a good agreement with a previous result which indicated that stomatocyte pathway accompanied with Aβ-40 deep insertion into membranes [43].

2.3.5. Different propensities of Aβ-40 and Aβ-42 to homogeneous model membranes

I was also interested in understanding how the two most abundant Aβ isoforms associate with membranes. Figure 2.2 and Figure 2.4 show that the amount of Aβ-42 small oligomers localizing in all considered microdroplet systems was significantly higher than that of Aβ-40 ($P \leq 0.05$). This result was in line with previous studies which reported that Aβ-42 is more 'amyloidogenic' than Aβ-40 [42], suggesting strongly that amyloid beta association with membrane lipids maybe lead to 'amyloidogenicity. The localization of Aβ-40 and Aβ-42 protofibrils in Chol and 7keto microdroplets is less significantly different ($P \leq 0.1$), while that in DOPC and 25OH systems is not significantly different ($P > 0.1$). This suggested that in protofibrillar form, the less amyloidogenic isoform is likely to have the similar capability for association with membranes as the more amyloidogenic one. In addition, the localization of both isoforms dramatically decreased in the case of fibrillar species. The Aβ-40 fibrils were more prone to locate in microdroplets compared to Aβ-42.

Regarding to membrane fluctuation, Aβ-42 has a higher ability to induce fluctuation of oxysterol membranes compared to Aβ-40 (Figure 2.5 and Table 2.1) as demonstrated by shorter times needed for 50% of liposomes to start fluctuation. This indicated that the former is more able to insert into membranes than the latter. Moreover, the significant difference in the effect of two amyloid peptide isoforms on membrane

transformation was found in cholesterol liposomes and 7keto systems. Aβ-42 protofibrils induced exo-tube/bud formation in cholesterol membranes and stomatocyte in 7keto vesicles, both of which were not observed in the presence of Aβ-40 protofibrils, suggesting a deeper insertion of Aβ-42 species into these membranes [Figure 2.8]. Nevertheless, there was a contrast in the influence of protofibrillar species on 25OH liposomes. The time for fluctuation initiation of 50% of 25OH liposomes induced by Aβ-40 was more than two times shorter than that caused by Aβ-42 (Table 2.1).

My studies about Aβ localization in microdroplets and the peptide-induced membrane dynamics indicated the higher interaction of the more 'amyloidogenic' compared to the less 'toxic' isoform. Due to two additional water-repelled amino acids at C-terminal region, the ratio of hydrophobicity to hydrophilicity in Aβ-42 is increased in comparison with Aβ-40 [59]. Thus, I supposed that the former isoform may have more pronounced surface activity than the latter, accounting for a higher amount of small oligomeric and protofibrillar species of the former adsorbing in model membranes. In addition, this property may enable Aβ-42 small oligomers and protofibrils to insert faster than similar aggregates of Aβ-40 on the ground that hydrophobic interaction between hydrophobic C-terminus of the peptide and nonpolar hydrocarbon tail of phospholipids is the driving force Aβ penetration into the lipid bilayer of membranes [60]. However, further studies are necessary in order to elucidate the mechanism of the different impact of the two most abundant Aβ isoforms on cellular membranes.

2.4. Conclusions

In conclusion, I have investigated the impact of oxysterols on localization of Aβ-40 and Aβ-42 in membranes and the peptides-induced membrane dynamics. I have demonstrated that the presence of cholesterol inhibited Aβ association with membranes and stabilized membranes. In contrast, oxysterols mediated Aβ localization and Aβ-induced membrane transformation. Moreover, I have shown that the influence of oxysterols on the interaction of Aβ with membranes depended not only on the presence of an extra oxygen group but also its position in molecules. 7keto, a product of sterol ring oxidation, increased Aβ localization due to its high potential for absorption of peptide in membranes, while 25OH, a side-chain-oxygenated oxysterols, enhanced membrane transformation by facilitating the peptide insertion into the bilayers. The results have also revealed that Aβ-42 pre-fibrillar species (small oligomers and protofibrils) exhibited a

higher ability to interact with membranes compared to Aβ-40 species. These findings are important and aid in understanding the effect of membrane lipid composition, especially cholesterol and its oxidation, on the Aβ-induced Alzheimer's neurotoxicity

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Chapter 3

Interaction of amyloid beta (1-42) protofibrils with membrane lateral compartments under the influence of cholesterol and 7-ketocholesterol

Abstract

Lateral organization of the lipids is one of characterized structural features of cell membranes and contributes to some biological processes. Although cholesterol is important to lipid organization, it is not fully understood how the sterol influences the interaction of Aβ with membrane lateral compartments. In this chapter, I have shown that cholesterol decreased the localization of Aβ-42 protofibrils in solid-ordered domains and increased that in liquid-ordered domains. The sterol changed the amount of Aβ associating with liquid-disordered (Ld) phase in different tendencies depending on the composition of heterogeneous membrane systems. These effects were associated with cholesterol's ability to alter the fluidity of lipid phases. In addition, 7-keto majorly enhanced the fluidity and interaction of Ld phase with protofibrillar Aβ-42. These results demonstrate the direct influence of cholesterol and its oxidized derivatives on Aβ interaction with lipid domains of model membranes.

Keywords: Cholesterol, 7-ketocholesterol, Amyloid beta localization, Homogeneous membranes, Liquid-disordered phase, Liquid-ordered phase, Solid-ordered phase, Membrane fluidity

3.1. Introduction

Lateral heterogeneity is one of intrinsic structural properties of cell membranes. Due to the phase behavior of different lipid species, it has been indicated that cell membranes consist of some lipid domains which are different in composition, physical properties and functions [1]. Lipid raft hypothesis (Simons and Ikonen, 1997) reported that the preferential binding of cholesterol to sphingolipids results in the formation of closely packed sphingolipid-cholesterol clusters coexisting with unsaturated-phosphatidylcholines-containing regions. Sphingolipid-cholesterol clusters are known as lipid raft domains which are detergent-insoluble, thicker and more ordered than other parts of membranes [2]. On other hand, unsaturated-phosphatidylcholines-containing regions are fluid and corresponding to liquid phase of membrane lipid bilayers [1,2]. Lipid rafts serve as platforms for the attachment of membrane proteins during some biological processes including signal transduction and membrane trafficking [2]. Therefore, these domains are important to functions of cell membranes and their interaction with external proteins.

Cholesterol, a vital component of lipid rafts, has been widely implicated in the pathogenesis of Alzheimer's disease (AD) [3-5]. The role of cholesterol in this neuro-degenerative illness is attributed to (i) the association of some genes in cholesterol metabolism such as ApoE with AD [6,7], especially (ii) the ability of cholesterol to modulate membrane physiochemical properties which mediate the production [8], aggregation, and cytotoxicity of amyloid beta (Aβ-42) peptide, the neurotoxic inducer in AD [9-11]. Cholesterol contributes to 30-40% of membrane lipid composition [12]. It features a planar four-ring steroid structure with a hydroxyl group at the C3 and a hydrophobic isooctyl side-chain attaching to another end. This unique structure allows cholesterol to orient perpendicularly to the lipid bilayer plane in which the rigid, hydrophobic steroid ring interacts with and reduces the mobility of adjacent phospholipid's hydrocarbon tails. Thus, cholesterol enhances the packing of membrane lipids and affects the fluidity, permeability, and phase separation of membranes [13,14]. Some researchers, including my group, have recently proposed that the oxidation of cholesterol caused by reactive oxygen species (ROS), enzymes, and Aβ/Cu^{2+} complex significantly influences Aβ-induced cytotoxicity. Having an additional oxygen group, oxidized derivatives of cholesterol (oxysterols) are more hydrophilic and less capable of packing lipids closely than cholesterol [15,16]. Therefore, some of them can increase the fluidity of membranes and promote Aβ insertion into model membranes [16-18].

Although there are many studies on the impact of cholesterol in Aβ-induced neurotoxicity, the mechanism by which the compound affects the interaction of Aβ with membrane domains is not fully understood. Recent studies have emphasized the importance of lipid rafts in AD as the sites for Aβ binding and aggregation [19]. They indicated that Aβ specifically binds to glycosphingolipid GM1 of lipid rafts [20], and cholesterol accelerates this interaction by turning a change in GM1 conformation that is suitable for Aβ recognition [21] and facilitating GM1 cluster formation [22]. In addition to glysosphingolipid/Aβ binding, the peptide has been suggested to directly associate with the lipid bilayer of membrane domains. Some authors reported that lateral compartments of model membranes interact differently with Aβ depending on their structure and properties [23,24]. Since cholesterol remarkably mediates the structure and features of membrane domains, it may modulate the association of Aβ with the lipid bilayer of these domains.

This chapter aims to investigate the effect of cholesterol and its oxidized derivative, 7-ketocholesterol (7keto), on the association of protofibrillar Aβ-42 with different lipid phases of heterogeneous membranes. In a previous study (and discussed in chapter 2 of this thesis), I demonstrated that cholesterol and two oxysterols, including 7keto and 25-hydroxycholesterol (25OH), significantly influenced the interaction of Aβ with homogeneous model membranes. The effect of 7keto on Aβ localization in membranes was higher than 25OH [18]. In addition, this oxysterol is a major product of cholesterol auto-oxidation induced by ROS, and it is a harmful factor in some human diseases such as atherosclerosis [25]. The study also showed a higher ability of Aβ-42 protofibrils to interact with membranes compared to Aβ-40 protofibrils, consistent with other publications [26]. With these understandings, I used 7keto and Aβ-42 protofibrils for my further studies. I employed a GM1-free heterogeneous cell-sized liposome system, composed of cholesterol, equal concentrations of unsaturated phospholipid and saturated phospholipid. This ternary model membrane tends to form a liquid-disordered (Ld) phase coexisting with solid-ordered (So) domains at low cholesterol fraction (0-10%) or liquid-ordered (Lo) domains at higher cholesterol levels (10-45%) [27]. It has been reported that Ld and So are considered as liquid and gel states of biological membranes, whist Lo domains are lipid raft-like domains [28]. My results have revealed that cholesterol significantly influences the interaction of Aβ-42 with all phases of heterogeneous membranes, while 7-ketocholesterol mainly enhanced Aβ-42 localization in Ld phase. A Laurdan generalized polarization (GP) measurement has shown the cholesterol- and 7keto-

induced changes in fluidity of membrane phases which may influence their effects on Aβ-42/membrane-phase interaction.

3.2. Materials and Methods

3.2.1. Materials

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 (USA). 7-ketocholesterol (7keto) was obtained from Sigma-Aldrich (USA). Amyloid beta protein (Human, 1-42) (Aβ-42) and Hilyte Fluor^{TM} 488-labelled (λex=503 nm, λex=528 nm) Aβ-42 (fluorescent Aβ-42) were from Peptide Institute Inc. (Japan) and Anaspec, Inc. (USA), respectively. Rhodamine b 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (Rho-DHPE) (λ_{ex}=560 nm, λ_{em}=580 nm) and 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) (λ_{ex}=363 nm, λ_{em}=497 nm) were from Invitrogen (USA). Chloroform, methanol, and Tris(hydroxymethyl)aminomethane (Tris) were purchased from Takara Bio Inc., Kanto-Chemical, and Nacalai Tesque (Japan).

3.2.2. Preparation of cell-sized heterogeneous liposomes

Cell-sized heterogeneous liposomes were prepared following the natural swelling method [29]. Lipid mixture was dissolved in chloroform/methanol (2:1, v/v) at the final concentration of 0.1 mM in a glass tube. A gentle nitrogen stream was used to evaporate the solvent. The tube was subsequently dried in a desiccator for 3 h, resulting in a thin lipid film at the bottom of tube. The film was swollen with Tris buffer (1.25 mM, pH = 7.4) overnight at 37 ^{o}C to form lipid vesicles.

So/Ld liposomes were prepared with two different cholesterol concentrations, Chol 0% (DOPC/DPPC/Cholesterol = 50/50/0, molar ratio) and Chol 10% (DOPC/DPPC/Cholesterol = 45/45/10, molar ratio). Similarly, Lo/Ld liposomes with different cholesterol levels were prepared, including Chol 20% (DOPC/DPPC/Cholesterol = 40/40/20, molar ratio) and Chol 30% (DOPC/DPPC/Cholesterol = 35/35/30, molar ratio). In order to study the effect of 7keto, we used Chol 30% Lo/Ld liposomes because this cholesterol concentration is nearly similar to that in biological membranes [12]. Cholesterol was partially replaced with 7keto at 75/25 (Chol/7keto) molar ratio. To

visualize membrane lateral compartments, Rho-DHPE, a specific probe of DOPC, was added into lipid mixture at 0.05% (mol/mol) concentration. The final concentration of lipid was 0.1 mM.

3.2.3. Preparation of protofibrillar Aβ-42

We first prepared 200 µM Aβ-42 solutions by dissolving Aβ-42 powder in 0.02% (v/v) ammonia solution and stored the solutions at -80 ^{o}C. Before doing experiments, fluorescence-labelled Aβ-42 and Aβ-42 were mixed at 1/2 (fluorescence-labelled Aβ-42/Aβ-42) molar ratio. The mixture was diluted in Tris buffer (20 mM, pH 7.4) at 80 µM concentration, subsequently incubated at 37 ^{o}C for 12 h. The morphology of Aβ-42-42 was measured by atomic force microscopic observation (SPA400-SPI 3800, Seiko Instruments Inc., Japan) and analysed by Image J software [18,24] as presented in Figure 2.1 in chapter 2.

3.2.4. Observation of protofibrillar Aβ-42 localization in model heterogeneous membranes

Fluorescence-labelled Aβ-42 protofibrils were added to liposome suspension at final concentration of 5 µM, followed by a gentle tapping. The resultant mixture was immediately poured into a silicon well (0.01 mm) placed on a glass slide and was used for confocal microscopy observation (Olympus FV-1000, Japan) at room temperature (~ 21.5 ^{o}C) within 2 min. This observation period is short enough to avoid the effect of fluorescence quenching [18]. Membrane lateral compartments and protofibrillar Aβ-42 were visualized after the excitation of Rho-DHPE and fluorescence-labelled Aβ-42. The intensity of fluorescent Aβ-42 was subsequently analyzed and used to estimate Aβ-42 localization in membranes. The data were expressed as means ± SE of three independent experiments. Comparisons between the different membranes were performed using ANOVA followed by Bonferroni's post comparison test.

3.2.5. Measurement of membrane fluidity

The fluidity of membrane lateral compartments was measured using excitation generalized polarization (GP) distribution of Laurdan [30]. Liposomes were labeled with 0.2% (mol/mol) Laurdan. The fluorescent emission of the label was detected at 421 and 519 nm using confocal scanning microscopy. Laurdan GP value was calculated following

the equation $GP = (I_{421} - I_{519})/(I_{421} + I_{519})$, in which I_{421} and I_{519} are average fluorescence intensity of Laurdan detected at 421 nm and 519 nm, respectively [30].

3.3. Results and discussion

3.3.1. Cholesterol-induced modulation of protofibrillar Aβ-42 association with membrane lateral compartments.

To elucidate the effect of cholesterol on protofibrillar Aβ-42's association with membrane lateral compartments, I first introduced the peptide to So/Ld lipid vesicles containing different levels (0% and 10%, molar ratio) of cholesterol. Microscopic observation revealed that cholesterol affected not only the location but also the intensity of Aβ localization in heterogeneous membranes. In the absence of the sterol (Chol 0% vesicles), Aβ protofibrils localized in both So and Ld phase (Figure 3.1A(i)). The selective localization of Aβ in So domain as well as its random distribution in both So and Ld phases has been reported [23,24]. The new of this studies is to show that the presence of cholesterol in the vesicles (Chol 10% vesicles) strongly decreased the amount of Aβ protofibrils partitioning in So domains, while that in Ld phase was significantly increased (Figure 3.1A(ii)). This implies that the sterol is able to inhibit the interaction of the peptide with So domains and facilitate its association with Ld phase.

The influence of cholesterol on protofibrillar Aβ-42 localization in Lo/Ld membrane systems was then investigated. I used a cholesterol concentration similar to that in biological membranes (30% molar ratio) [12] and a slightly lower concentration (20%). A higher cholesterol level was not used because of the difficulty in preparation of stable Lo/Ld cell-sized vesicles. Figure 3.1B shows that at low concentration of cholesterol (20%), Aβ-42 localized mainly in Ld phase, in agreement with Morita et al. [23]. When cholesterol level was increased to 30%, Aβ-42 association in Ld phase was reduced. In addition, the peptide was able to associate with Lo domains although its amount was lower than that in Ld phase. (Figure 3.1B).

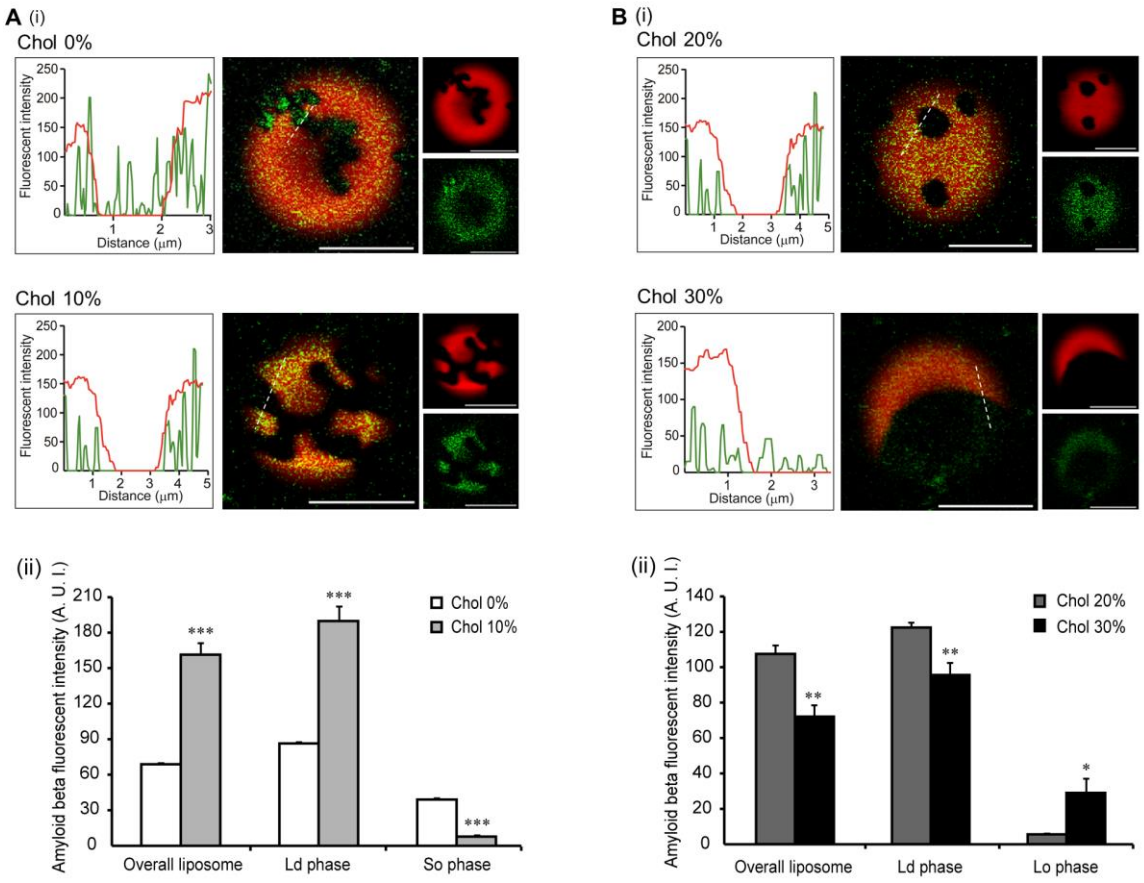

Figure 3.1. Effect of cholesterol on the localization of protofibrillar amyloid beta (1-42) in heterogeneous model membranes. (**A**) Representative confocal microscopy images (i) and fluorescence intensity values (ii) reflecting the amount of Aβ localizing in So/Ld phase-seperated membranes. Red and black regions indicate Ld and So domains, respectively. (**B**) Representative confocal microscopy images (i) and fluorescence intensity values (ii) reflecting the amount of Aβ associating with Lo/Ld phase-seperated membranes. Red and black regions indicate Ld and Lo domains, respectively. The values are mean ± SE of three replicates. The symbols *, **, and *** show significant differences with $P \leq 0.1$, $P \leq 0.05$, and $P \leq 0.01$. Scale bars are 5 μm.

This result in combination with the study of So/Ld membranes clearly demonstrated cholesterol as a modulator of the direct interaction of protofibrillar Aβ-42 with lipid bilayer of membrane lateral compartments (summarized in Table 3.1). Briefly, membrane cholesterol enabled the protofibrils to interact with Lo (raft-like) domains at a

moderate level (30%). On the other hand, loss of cholesterol (0%) strongly enhanced Aβ-42 localization in So phase which can exist in lipid domains of cell membranes deficient in the sterol. Cholesterol could increase or decrease Aβ-42 association with Ld phase depending on the composition of the heterogeneous membrane system (Figure 3.1). These findings contributed to understanding of the role of cholesterol in membrane's lipid raft/Aβ-42 interaction. They suggested that in addition to the effect on lipid-raft-GM1/Aβ-42 binding as reported previously, cholesterol is able to modulate the direct interaction of protofibrillar Aβ-42 with lipid bilayer of this domain.

Table 3.1. Summary of protofibrillar amyloid beta (1-42) localization in heterogeneous model membranes upon the effect of cholesterol.

Membrane systems	Low cholesterol	High cholesterol
So/Ld	So and Ld	Ld
Lo/Ld	Ld	Ld (mainly) and Lo

3.3.2. Effect of 7keto on the localization of protofibrillar Aβ-42 in membrane lateral compartments

It is widely accepted that oxidative stress is a harmful factor contributing to the pathology of AD [31]. In chapter 2, I discussed the enhancing impact of 7keto on Aβ association with homogeneous model membranes [18]. Here, I sought to assess whether the presence of 7keto in membranes affects the ability of Aβ-42 protofibrils to localize in membrane lateral compartments. Lo/Ld heterogeneous lipid vesicles containing 30% of cholesterol (Chol 30% vesicles) were used because this cholesterol concentration is closer to the concentration in biological membranes [12]. 7keto was introduced to Chol 30% lipid vesicles at 7.5 mM concentrations which closely corresponds to the level detected in rat hippocampus after kainite excitotoxicity (2500-3100 ng/g tissue, or 6.24 – 7.5 μM) [32].

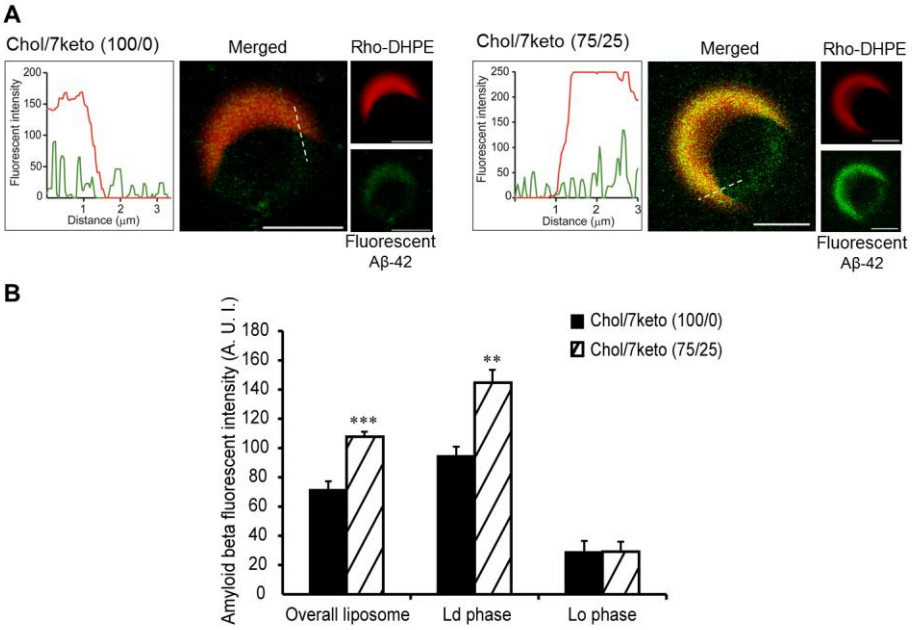

Figure 3.2. Effect of 7-ketocholesterol on localization of amyloid beta (1-42) protofibrils in membranes. (**A**) Representative confocal microscopy images (**B**) and fluorescence intensity values reflecting the amount of Aβ-42 in Lo/Ld phase-separated membranes. Red and black regions indicate Ld and Lo domains, respectively. The values are mean ± SE of three replicates. The symbols ** and *** show significant differences with $P \leq 0.05$ and $P \leq 0.01$. Scale bars are 5 μm.

As can be seen in Figure 3.2A, the position of Aβ-42 protofibrils localizing in heterogeneous membranes containing 7keto was not changed compared to membranes without the oxysterols. Meanwhile, the amount of protofibrillar Aβ-42 localizing in overall Lo/Ld membrane systems was significantly increased by the substitution of membrane cholesterol with 7keto (Figure 3.2B). The increase was influenced by a higher recruitment of the protofibrils to Ld phase of 7keto-containing lipid vesicles relative to cholesterol-containing systems. The interaction of Aβ-42 protofibrils with Lo domains was not significantly changed in the presence of 7keto in comparison with that in membranes without the oxysterol (Figure 3.2B). This indicated that 7keto tended to mainly affect the association of the peptide with Ld phase of heterogeneous membranes. Consistent with the study on homogeneous membranes (presented in chapter 2), the finding suggested that the

oxidation of membrane cholesterol induced by oxidative stress may enhance Aβ/membranes interaction, thus being a risk factor of Aβ-induced toxicity in AD's pathology.

3.3.3. Effect of cholesterol and 7keto on the fluidity of heterogeneous membranes

Many studies have reported that Aβ-42/cell-surface interaction strongly depends on the physicochemical properties of membranes including membrane fluidity [33,34]. Therefore, to understand how cholesterol and 7keto influence this interaction, I studied their effects on the fluidity of heterogeneous membranes using generalized polarization (GP) of Laurdan, one of common fluorescent markers of membrane fluidity [30]. Featuring a large excited state dipole moment and spectral sensitivity to the polarity of its environment, the probe shows emission maximum at 490 nm in a polar environment and at 440 nm in nonpolar one. Environment polarity was determined by the number of water molecules existing in the lipid bilayers. It has been reported that the extent of water molecules to penetrate into the lipid bilayer is affected by lipid packing and membrane fluidity. Therefore, measurement of Laurdan GP by comparing fluorescent intensities at the two wavelengths is able to give information about membrane properties [30,35]. A more fluid environment has a lower GP value compared to a less fluid environment.

Figures 3.3 and 3.4 show that cholesterol induced significant changes in the fluidity of all heterogeneous membrane's lipid phases. The presence of cholesterol in So/Ld lipid vesicles decreased GP value of overall vesicles, representing an increase of fluidity. GP value of Ld phase was also reduced, suggesting that the phase was more fluid. Conversely, the So phase became more rigid as indicated by a higher GP value compared to membranes without cholesterol (Figure 3.3). This result seems to be contrary to previous findings of the cholesterol's rigidifying effect on liquid phase and fluidifying effect on gel phase [36]. However, my results agreed with Parassi and colleagues who demonstrated that adding cholesterol renders gel phase more ordered. By comparing the histogram of GP values measured in DOPC only and DPPC only vesicles with that of DOPC/DPPC systems, the authors also pointed out that the So and Ld domains of the binary vesicles are not simply pure gel and liquid phases, respectively [29]. In agreement, I propose that there were some DOPC molecules in gel phase as well was DPPC molecules in liquid phase of the mixture vesicles. When cholesterol was present in the vesicles, it may compete with

DOPC for interaction with DPPC due to the preferential biding of the sterol with saturated lipids. Thereby, DOPC molecules are excluded from So domains and a similar exclusion of DPPC from Ld domains occurs. As a result, So domains mainly consisted of saturated phospholipids and cholesterol, thus becoming more rigid compare to So domains of DOPC/DPPC vesicles. On the other hand, Ld phase of membrane systems containing cholesterol was more fluid without DPPC.

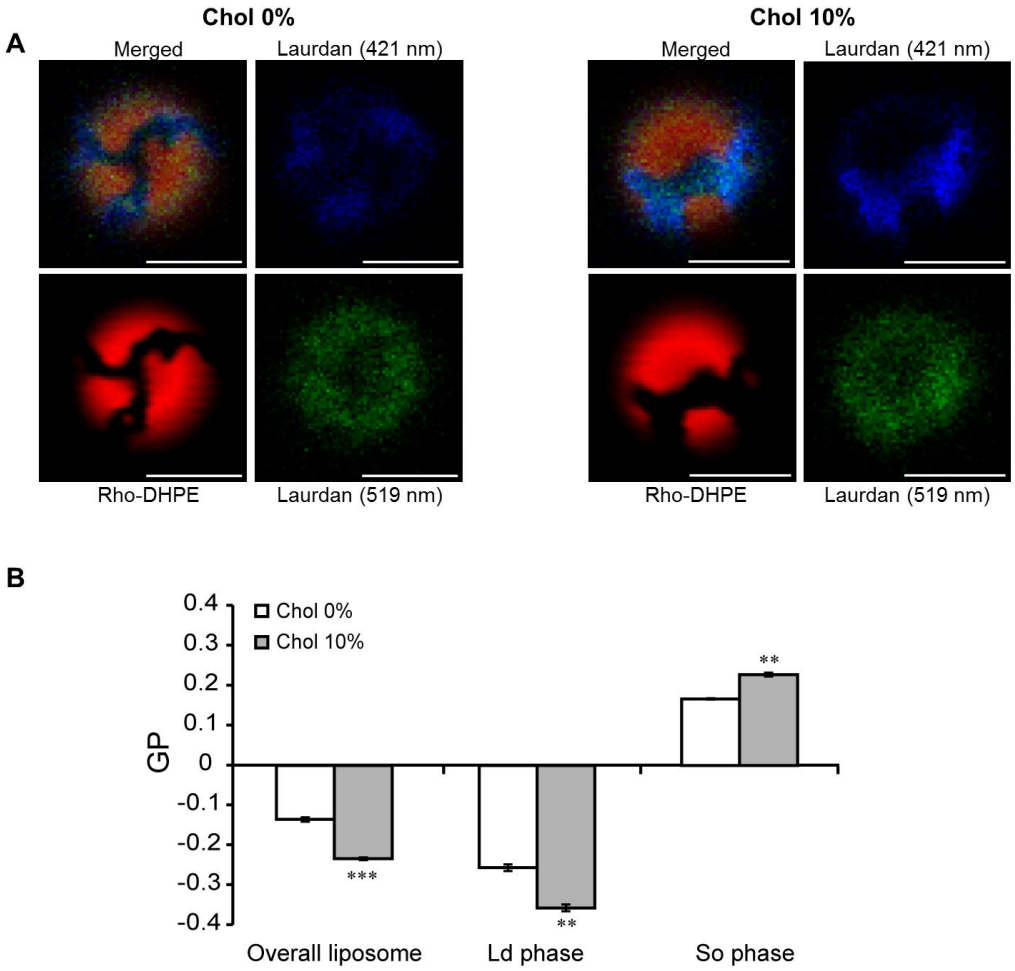

Figure 3.3. Effect of cholesterol on the fluidity of So/Ld heterogeneous model membranes. (**A**) Representative confocal microscopy images of Laurdan emission, (**B**) GP values of So/Ld phase-separated membranes. Red and black regions indicate Ld and So domains, respectively. The values are mean ± SE of three replicates. The symbols ** and *** show significant differences with $P \leq 0.05$ and $P \leq 0.01$, respectively. Scale bars are 5 μm.

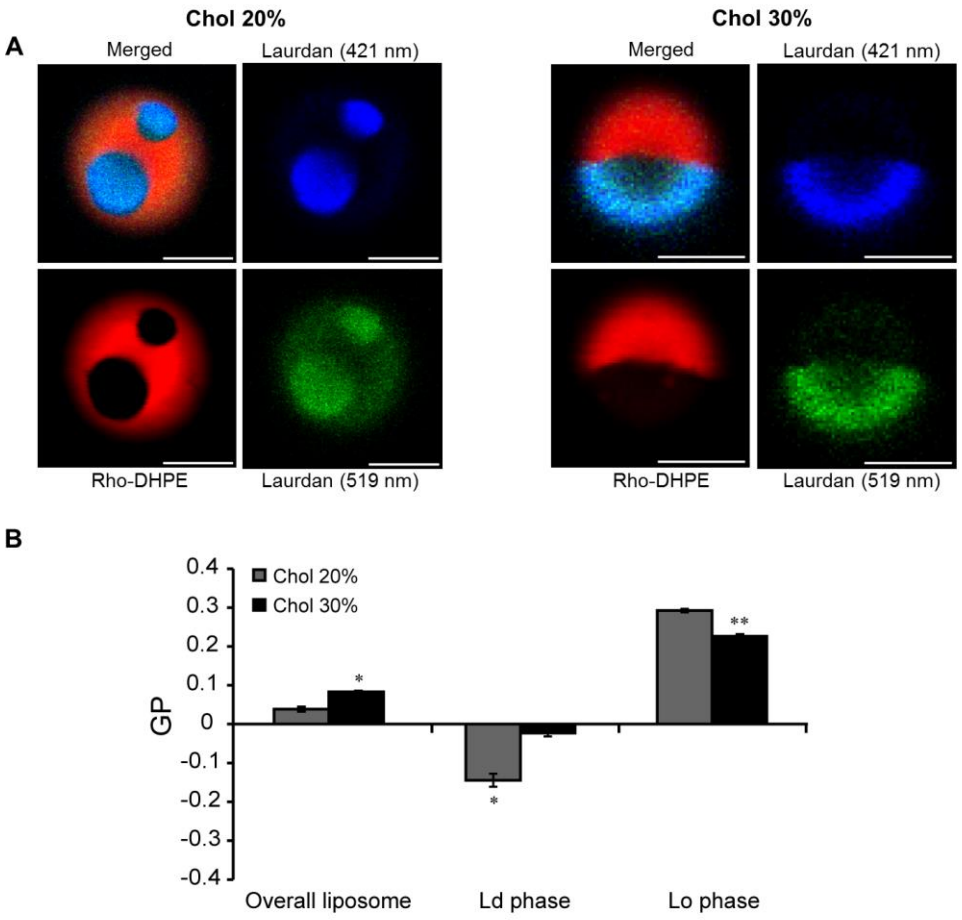

Figure 3.4. Effect of cholesterol on the fluidity of Lo/Ld heterogeneous model membranes. (**A**) Representative confocal microscopy images of Laurdan emission, (**B**) GP values of Lo/Ld phase-separated membranes. Red and black regions indicate Ld and Lo domains, respectively. The values are mean ± SE of three replicates. The symbol * and ** show significant differences with $P \leq 0.1$ and $P \leq 0.05$. Scale bars are 5 μm.

In case of Lo/Ld systems, overall liposomes and Ld phase had a higher GP value when cholesterol concentration increased, implying that they were more ordered. By contrast, Lo phase became more dis-ordered upon increase in cholesterol level, as demonstrated by a reduction of GP value (Figure 3.4). This is consistent with previous report of cholesterol effect on membrane fluidity [36].

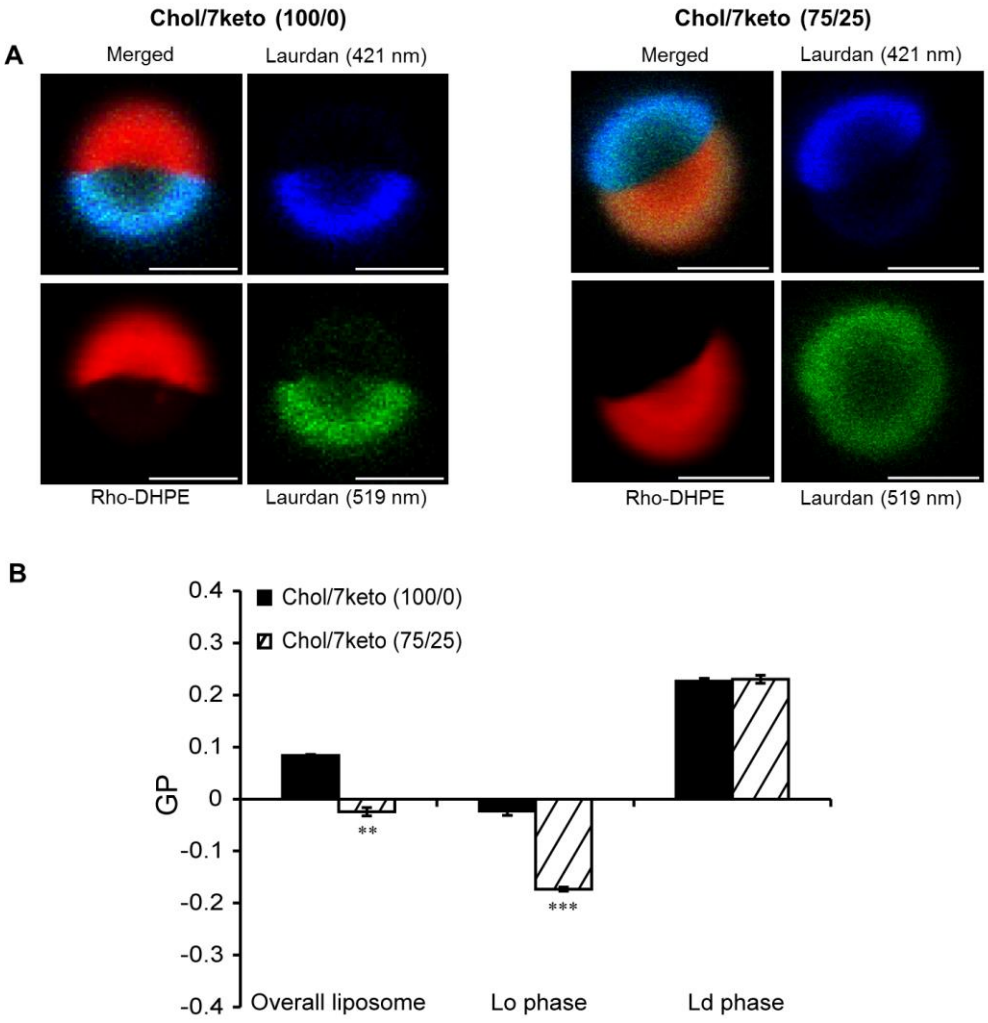

Figure 3.5. Effect of 7-ketocholesterol on the fluidity of Lo/Ld heterogeneous model membranes. (**A**) Representative confocal microscopy images of Laurdan emission, (**B**) GP values of Lo/Ld phase-separated membranes. Red and black regions indicate Ld and So domains, respectively. The values are mean ± SE of three replicates. The symbols ** and *** show significant differences with $P \leq 0.05$ and $P \leq 0.01$, respectively. Scale bars are 5 μm.

In 7keto-containing lipid vesicles, GP values of overall liposomes and Ld phase were remarkably lower than the system without the oxysterol, while that of Lo phases was not noticeably changed (Figure 3.5). This indicated that 7keto mainly affected Ld phase, rendering it more fluid and the oxysterol did not influence the fluidity of Lo phase. The ability of 7keto to increase membrane fluidity may involve an orientation in lipid bilayer.

Due to an additional carbonyl group, 7keto tends to adopt a tilt with respect to membrane surface so that both oxygen groups are exposed to the hydrophilic interface. This orientation weakens hydrophobic interaction between the rigid, hydrophobic steroid rings of the oxysterol with adjacent phospholipid's hydrocarbon tails, thus decreasing 7keto's capability of reducing the mobility of hydrocarbon tails and packing membrane lipids [37]. Therefore, membranes become more fluid.

Laurdan GP measurement clearly demonstrated that cholesterol was able to change the fluidity of both Ld phase and Lo phase of Lo/Ld heterogeneous membranes, whilst 7keto most likely influenced the property of Ld phase. We explained this result based on their different orientations and effects on phospholipid/phospholipid interaction in membrane phases. The major phospholipid species of liquid-ordered phase is DPPC [27] which are densely packed by strongly hydrophobic interaction along straight, long hydrocarbon chains. In contrast, DOPC molecules which mostly exist in liquid disordered phase [27] are difficult to pack together due to kinks generated from cis-double bonds in acyl chains (Figure 3.6) [13]. As discussed previously, cholesterol orients perpendicularly to the plane of lipid bilayers. When cholesterol partitions in Lo phase, its plate-like rings and hydrophobic isooctyl side-chain interact with the upper part of hydrocarbon chains of adjacent DPPC. The presence of a bulky tetra-ring structure and short acyl chain can induce a larger space between lower parts of two neighboring phospholipid's side chains, thus weakening their hydrophobic interaction. Therefore, Lo phase becomes more fluid. Although cholesterol preferentially binds to DPPC, this sterol is able to interact with DOPC in Ld phase. The orientation of cholesterol in this phase is similar to that in Lo phase (Figure 3.6). As a consequence, the sterol can strongly interact with hydrocarbon chains of adjacent DOPC molecules. The hydrophobic interaction between DOPC and cholesterol is stronger than DOPC/DOPC interaction, resulting in a more densely packed Ld phase [38].

On the other hand, the orientation of 7keto in Lo phase and Ld phase may be different. Massey and Pownall reported that the driving force determining the orientation of 7keto in tightly packed Lo phase is the increased van der Waals attractive interaction between the oxysterols and hydrocarbon chain of phospholipids. Therefore, 7keto orients quasi-perpendicular like cholesterol. As a result, the substitution of cholesterol with 7keto does not significantly change the fluidity of Lo phase. Nevertheless, the driving force of

7keto orientation in Ld phase is hydrogen binding of the carbonyl group with surface polar groups of DOPC. This enables the oxysterol to tilt with respect to the surface, leading to a decreased packing of lipids and an increased fluidity of this phase [38]. The authors also indicated that 7keto has stronger tendency to partition into Ld phase compared to cholesterol [38], which is attributed to a significant effect of 7keto on this phase.

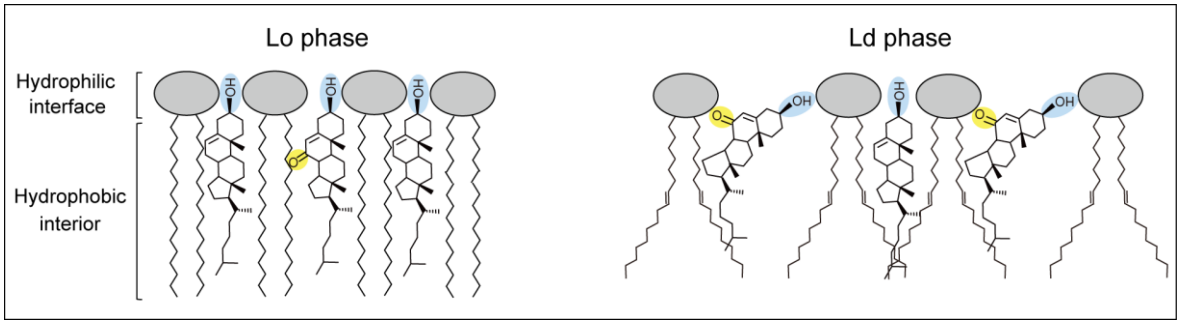

Figure 3.6. Orientation of cholesterol and 7-ketocholesterol in Lo and Ld phases of heterogeneous model membranes [38].

3.3.4. Influence of cholesterol and 7keto in protofibrillar Aβ-42 localization in membrane lateral compartments by changing membrane fluidity

Microscopic observation and Laurdan GP study suggested that cholesterol and 7keto may influence the localization of Aβ-42 protofibrils in heterogeneous membranes through their effect on membrane fluidity (Table 3.2). In So phase, the presence of cholesterol decreased the fluidity and the localization of Aβ-42 protofibrils. Previous studies showed that the peptide adsorbs to the surface of rigid gel phase [23,24]. However, the adsorption was not detected at a high membrane surface pressure (above 30 mNm^{-1}) [34]. The addition of cholesterol induced a higher rigidification of So phase (Figure 3.4), correlating with an increase of compressed state and surface pressure of the phase. Therefore, the Aβ-42 adsorption on this phase was inhibited (Figure 3.1A).

The peptides interact with Lo and Ld phases via a direct insertion into the liquid phase [23,39]. The insertion is mainly driven by hydrophobic interaction between the linear and highly lipophilic region containing residues 25-35 of Aβ with hydrophobic part of membrane lipids [40]. Scala and colleagues reported that this interaction is specific for

cholesterol and the peptide [41]. D'Errico et al. also reported that the peptide is able to interact with hydrocarbon chain of phospholipids [40]. In our study, we have indicated that there is interaction between Aβ and cholesterol-containing membranes as well as oxidized cholesterol-containing membranes. Varying the concentration of cholesterol and the presence of cholesterol oxidized derivative (7keto) do have an effect on how and where (which phase) Aβ interacts with the membrane. Our work has also shown that cholesterol and 7keto do alter membrane fluidity, and we have attributed the degree of Aβ interaction with membrane systems, largely to the changes in fluidity. The degree of Aβ insertion into the membrane is dependent on the fluidity of the bilayer. A similar conclusion on the impact of fluidity was reported previously [40,42]. Membrane fluidity influences area expansion modulus which is proportional to the energy needed for the formation of a vacant space in the lipid bilayer interior for Aβ insertion [23,43].

Table 3.2. Correlation of amyloid beta (1-42) protofibrils localization in heterogeneous membrane phases and membrane fluidity upon the effect of cholesterol and 7keto.

Membrane systems	Phases	Low cholesterol		High cholesterol		Presence of 7keto	
		Fluidity	Aβ-42 Localization	Fluidity	Aβ-42 Localization	Fluidity	Aβ-42 Localization
So/Ld	So	Low	High	Decreasing	Decreasing	—(*)	—(*)
	Ld	High	Low	Increasing	Increasing	—(*)	—(*)
Lo/Ld	Lo	Low	Low	Increasing	Increasing	No change	No change
	Ld	High	High	Decreasing	Decreasing	Increasing	Increasing

(*) So/Ld membranes were not formed with 7keto

According to our experimental data, higher cholesterol level made Lo phase more fluid (Figure 3.4B), thus rendering them easier to expand in comparison with cholesterol. Thereby, the protofibrils penetrationed into this phase was promoted (Figure 3.1B). An opposite effect of the sterol was observed in Ld phase. The phase became more rigid at higher cholesterol level, thus the insertion of Aβ-42 into the lipid bilayer was decreased (Figure 3.4B). The substitution of 7keto for cholesterol significantly increased the fluidity

of Ld phase (Figure 3.5), rendering the lipid bilayer of this phase easier to expand. This resulted in a higher amount of Aβ-42 associating with Ld phase (Figure 3.3). Our results of the correlation between membrane fluidity and Aβ-42/membranes interaction was in an agreement with previous study on neuronal cells which demonstrated that the interaction of Aβ with cell surface are mediated by membrane cholesterol and its effect on membrane fluidity [42].

3.4. Conclusions

In conclusion, I have demonstrated that cholesterol affect the localization of protofibrillar Aβ-42 in all membrane lateral compartments. Briefly, the loss of cholesterol strongly enhanced Aβ-42 localization in So phase which can exist in lipid rafts of cell membranes deficient in the sterol. On the other hand, the increase of cholesterol level enabled the protofibrils to interact with Lo (raft-like) domains. Cholesterol could increase or decrease Aβ-42 association with Ld phase depending on the composition of the heterogeneous membrane system. A decrease in basal cholesterol level increases Aβ-42 association with T cell membranes. The effect of cholesterol on Aβ-42 association with membrane lateral compartments was mediated by its ability to change the fluidity of membrane compartments. Moreover, I have shown that the presence of 7keto in membranes majorly promoted the protofibrils to partition into Ld phase because this oxysterol induced a significant increase in fluidity of this phase. The enhancing effect of 7keto on Aβ-42/T-cell interaction was also observed. Our findings suggest that a moderate level of cholesterol is important in maintaining the membrane fluidity which prevents the recruitment of high levels of Aβ in membranes, while cholesterol oxidation appears to be a risk factor in Aβ-induced cytotoxicity.

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Chapter 4

Effect of cholesterol and 7-ketocholesterol on interaction of amyloid beta (1-42) protofibrils with biological membranes

Abstract

With the understanding obtained using biomimetic membrane systems, I further advanced my study on the effect of cholesterol, 7keto on protofibrillar Aβ-42/membrane interaction and used Jurkat T-cells. I have shown that the loss of membrane cholesterol strongly enhanced the interaction of Aβ-42 protofibrils with Jurkat T cells and decreased the viability the cells exposed to the protofibrils relative to cells with basal cholesterol content. On the other hand, these processes were not significantly influenced by the increase of membrane cholesterol level. In comparison with cholesterol, 7keto had a high ability to enhance the localization of Aβ-42 protofibrils in Jurkat T cell membranes and increase the effects of the peptide which reduce cell viability and increase cytosolic Ca^{2+} content of the cells. These results suggest that cholesterol has the beneficial role in Aβ-induced toxicity to Jurkat T cells, while 7keto may be a harmful factor in this process.

Key words: Cholesterol, 7-ketocholesterol, Jurkat T-cells, Aβ-42 protofibrils, Aβ-42 localization, Cell viability, Intracellular Ca^{2+} level

4.1. Introduction

Amyloid beta (Aβ)-induced cytotoxicity is the central event in the pathogenesis of Alzheimer's disease (AD), which causes amyloid plaques, neurofibrillary tangle formation, vascular damage, and neuronal cell loss [1]. Accumulating evidences have demonstrated that Aβ-induced cytotoxicity is mainly mediated by the interaction of the peptide with cell membranes [2]. Monomeric Aβ binds to ganglioside membrane receptors, and subsequently adopts a change from α-helix-rich to β-sheet-rich conformation, which in turn forms oligomers, protofibrils, and fibrils [3,4]. Another receptor of the peptide is a cellular, non-infectious form of prion protein (PrP^c) which was shown to specifically interact with Aβ oligomers at nanomolar amyloid concentration [5]. In addition, Aβ species are able to insert into lipid bilayer of membranes [3,6]. After binding to cell membranes, the peptide triggers a cascade of harmful processes, including (i) disruption of intracellular Ca^{2+} homeostasis which plays crucial role in neuron survival and death [7,8], (ii) oxidative stress of membrane lipids [9], (iii) interference in membrane receptors [10], and (iv) dysfunction of mitochondria [11]. These processes finally contribute to changes in function and structure of synapses, neuron death as well as microgliosis and cytokine elevation which are associated with brain inflammation [12].

The interaction of Aβ with cell membranes is influenced by lipid composition, especially cholesterol, which is the vital structural constituent of cell membranes [13,14]. The role of the sterol in this interaction has been investigated by numerous studies using neuronal cells [14-16]. Most of them reported that loss of membrane cholesterol significantly enhanced Aβ association with cell membranes, while the increase in cholesterol level precludes this interaction.

7-ketocholesterol (7keto) is a major product of cholesterol oxidation induced by reactive oxygen species (ROS) [17]. This compound has been considered as one of the most harmful oxysterols [18]. It causes apoptosis in non-neuronal cells at micromolar concentrations [19] and plays an important role in atherosclerosis [20]. 7keto is able to increase exocytosis and neurotrasmitter release which are involved in neuroinflammation [21]. Recently, the effect of 7keto on Aβ interaction with model membranes has been investigated. The oxysterol facilitates insertion of the peptide into membrane lipid bilayer by changing its lysis tension [22]. In agreement, as discussed in chapter 2 and 3, my previous studies have demonstrated that 7keto facilitates Aβ to associate with homogeneous membranes [23] and Ld phase of heterogeneous membranes. However, very

little is known about the interaction of the peptide with biological membranes in the presence of 7keto.

It has been reported that T cell, a type of white blood cells playing a central role in immunity system [24], is a target of Aβ [25]. A systemic T-cell activation by Aβ was detected in the brain of AD mouse model and patients [26,27]. The association of the peptide with T cells may play both deleterious and beneficial roles in inflammatory process during AD pathogenesis [25,28]. Some studies suggested that peripheral T cells secrete cytokines in response to Aβ. The cytokines subsequently enter the central nervous system and activate microglia, which are important cells in responsiveness of the brain to injury and infection [12]. These cells secrete some kinds of pro-inflammatory cytokines such as tumor necrosis factor-α, thus promoting brain inflammation [29]. On the other hand, Aβ-reactive T cells are able to produce anti-inflammatory factors in some conditions, resulting in immune responses to the peptide, for example an increased clearance of Aβ plaque [28,30]. Therefore, T cells are used for immunotherapy of AD [28]. Besides the reported ability to respond to Aβ, as far as I am aware, T cells possess some advantages in studies on the interaction of cell membranes with proteins. The organization and function of membrane components, especially lipid rafts, during cellular signalling upon various external stimuli are well understood [31,32]. In addition, they have spherical shape and float separately in cultured medium, so other factors that influence protein/membrane interaction such as membrane curvature and the cluster of cells can be excluded. Therefore, I proposed to use T cell as a reasonable model in study of the interaction between biological membranes with Aβ.

With these understandings, I here sought to investigate the effect of cholesterol and 7keto on the interaction between Aβ-42 protofibrils with cell membranes using Jurkat T cells. The localization of protofibrillar Aβ-42 in membranes, Aβ-42-induced changes in membrane permeability to Ca^{2+} and cell death upon the presence of 7keto has been studied in comparison with cholesterol. I have demonstrated that 7keto significantly increased the protofibril association with cell membranes compared to cholesterol. Moreover, the oxysterols enhanced the effect of Aβ-42 protofibrils on membrane permeability and cell death. These results contribute to clarify how the oxidation of cholesterol influences Aβ-induced toxicity.

4.2. Materials and methods

4.2.1. Materials

Jurkat human leukemic T cell line (Jurkat T cells) was from Riken cell bank (Japan). Water-soluble cholesterol, 7-ketocholesterol (7keto), methyl-β-cyclodexin (MβCD), bovine serum albumin (BSA), and trypan blue were obtained from Sigma-Aldrich (USA). Amyloid beta protein (Human, 1-42) (Aβ-42) and Hilyte Fluor^{TM} 488-labelled (λex=503 nm, λex=528 nm) Aβ-42 were from Peptide Institute Inc. (Japan) and Anaspec, Inc. (USA), respectively. Roswell Park Memorial Institute 1640 (RPMI1640) medium, fetal bovine serum (FBS), Pluronic F-127, Fluo-3 AM, and Alexa Fluor 488 conjugated cholera toxin subunit B (CT-B) (λ_{ex}=560 nm, λ_{em}=580 nm) were from Invitrogen (USA). Phosphate buffer salts (PBS), and Tris(hydroxymethyl)aminomethane (Tris) were purchased from Takara Bio Inc. and Kanto-Chemical (Japan), respectively.

4.2.2. Preparation of protofibrillar Aβ-42

200 μM Aβ-42 solutions were prepared by dissolving Aβ-42 powder in 0.02% (v/v) ammonia solution and stored the solution at -80 ^{o}C. Before experiments, the Aβ-42 solution was diluted to 80 μM concentration with Tris buffer (20 mM, pH 7.4), and subsequently incubated at 37 ^{o}C for 12 h [23]. To prepare fluorescence protofibrillar species, fluorescence-labelled Aβ-42 and normal Aβ-42 solutions was mixed at 1:2 molar ratio before the incubation [33]. The morphology of Aβ-42 was measured by atomic force microscopic observation (SPA400-SPI 3800, Seiko Instruments Inc., Japan), analysed by Image J software, and presented in Figure 2.1. (chapter 2).

4.*2.3. Cell culture and visualization of lipid rafts*

Jurkat T cells were cultured in RPMI1640 medium supplemented with 10% (v/v) FBS. They were maintained in a humidified atmosphere with the presence of 5% (v/v) CO_2 at 37 ^{o}C. Lipid rafts were labelled by treating cells with 15 μg/ml CT-B, which specifically binds to GM1 in lipid rafts [34], and 0.02% (v/v) BSA in PBS at 0 ^{o}C for 30 min, followed by an incubation at 37 ^{o}C for 10 min.

4.2.4. Modulation of membrane cholesterol and 7keto levels

Membrane cholesterol of Jurkat T cells was depleted by treating cells with 2 mM MβCD in non-serum RPMI1640 medium for 10 min at 37 °C. It has reported that MβCD is able to remove cholesterol from plasma membranes [31]. In order to increase cholesterol content, Jurkat T cells were treated with 1mM soluble cholesterol in PBS for 10 min at 37 °C. Membrane cholesterol level was measured using cholesterol cell-based detection assay kit (Cayman, USA) (Figure 4.1). 7keto was added to cell membranes by treating Jurkat T cells with 7keto solution at two different concentrations (5 μM and 10 μM) for 10 min at 37 °C.

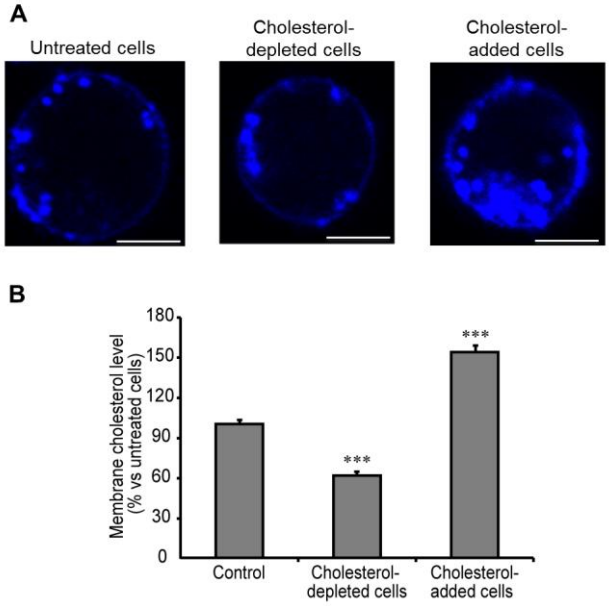

Figure 4.1. Modulation of membrane cholesterol levels in Jurkat T cells. (**A**) Representative confocal microscopy images showing the levels of membrane cholesterol in Jurkat T cells. (**B**) The quantity of membrane cholesterol of untreated, cholesterol-depleted, and cholesterol-added cells. Scale bars are 10 μm.

4.2.5. Observation of protofibrillar Aβ-42 localization in Jurkat T cells

Fluorescence-labelled Aβ-42 was added to T cell suspension at the final concentration of 5 μM concentration. The resultant mixture was poured into a silicon well (0.01 mm) placed on a glass slide and used for confocal microscopy observation (Olympus FV-1000, Japan) at room temperature (~ 21.5 ºC) within 2 min. This observation period is short enough to avoid the effect of fluorescent quenching [23]. Membrane lateral

compartments and Aβ-42 were visualized after the excitation of CT-B and fluorescence-labelled Aβ-42. The intensity of fluorescent Aβ-42 was subsequently analyzed and used to estimate the peptide localization in membranes.

4.2.6. Measurement of intracellular Ca^{2+} level

The intracellular Ca^{2+} level of cells was measured using Fluo3-AM, a visible light excitable Ca^{2+} chelating fluorescence label [35]. Untreated, cholesterol-added, and 7keto-added cells cultured in 48-well plates were exposed to 10 μM Aβ-42 protofibrils for 1 h [36]. After washed three times with serum-free RPMI1640 medium, the cells were loaded with 10 μg/ml Fluo3-AM in the presence of 0.14 mg/ml plorunic F127 and PBS at 37 °C for 20 min. The cells were diluted five times with 0.01% (w/v) BSA in PBS and were incubated at 37 °C for 40 min. Fluo3-AM-loaded cells were then washed three times, resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, and used for confocal scanning microscopic observation. Intercellular Ca^{2+} level was measured by analyzing emitted Fluo3-AM fluorescence intensity obtained from its excitation at 488 nm [37]. The data are expressed as means ± standard error (SE) of three independent experiments. Comparisons between the different membranes were performed using ANOVA followed by Bonferroni's post comparison test.

4.2.7. Measurement of cell viability

Trypan blue exclusion assay was used to estimate Jurkat T cell viability upon the effect of Aβ-42 protofibrils [38,39]. Untreated, cholesterol-depleted, cholesterol-added, and 7keto-added cells cultured in 48-well plate were exposed to 10 μM protofibrillar Aβ-42 for 24 h. ThT assay showed that the conformation of protofibrillar Aβ-42 did not significantly change in this condition (data was not shown). The cells were then washed two times with PBS and treated with trypan blue at the final concentration of 0.01% (w/v) in PBS for 10 min at room temperature (~ 21.5 ºC). The number of dead cells and viable cells were counted using a hemocytometer. The cell having a clear cytoplasm was defined as a viable cell, while a dead cell is the one with a blue cytoplasm [8]. Cell viability was calculated following the formation: cell viability = total viable cells /total cells. The data are expressed as means ± standard deviation (SD) of three independent experiments.

Comparisons between the different membranes were performed using ANOVA followed by Bonferroni's post comparison test.

4.3. Results and discussion

4.3.1. Effect of cholesterol on protofibrillar Aβ-42 localization in biological membranes and Aβ-42-induced cytotoxicity

Although many studies have demonstrated a modulating role of cholesterol on Aβ-42 interaction with neuronal cell membranes, the effect of this sterol on Aβ-42/T-cells binding has not been investigated. Therefore, I first clarify the association of the peptide and its cytotoxicity to Jurkat T cells upon changes in membrane cholesterol level.

I found that Jurkat T cells with basal cholesterol content (the control) are able to associate with Aβ-42 protofibrils (Figure 4.2A). Green and red colors in microscopic images represent protofibrillar Aβ-42 and lipid rafts of cell membranes, respectively. Microscopic observation revealed that the peptide mainly associated with membranes, and did not internalize into the cells. This was different from the interaction of Aβ-42 with neuronal cells in which the internalization of the peptide was observed in addition to the Aβ localization in membranes [15]. Moreover, there were tangle-like yellow regions attaching to the outside of membranes that are the merging of green fluorescence emission of Aβ-42 with red fluorescent emission of CT-B, a specific label of lipid raft domains of membranes. They indicated that lipid raft's constituents might be partially expelled from the membranes in response to Aβ-42 protofibrils, and the protofibrils co-localized with these constituents (Figure 4.2A).

Significant changes in membrane cholesterol did not alter the position but remarkably influenced the intensity of Aβ-42 localizing in Jukart T cells. When cholesterol was depleted by approximately 40% of initial content, the amount of protofibrils associating with cells was 2.15 times higher than that in control cells. This implied that the loss of membrane cholesterol strongly increased the peptide localization in cells. On the other hand, the amount of protofibrillar Aβ-42 partitioning in cells enriched in cholesterol (40% higher than the basal content) was not significantly different compared to control cells, suggesting that the increase of membrane cholesterol did not influence the protofibrils/T-cell membranes association (Figure 4.2B). The results indicated that Aβ

interacts with lipid rafts of cell membranes and the interaction was influenced by cholesterol.

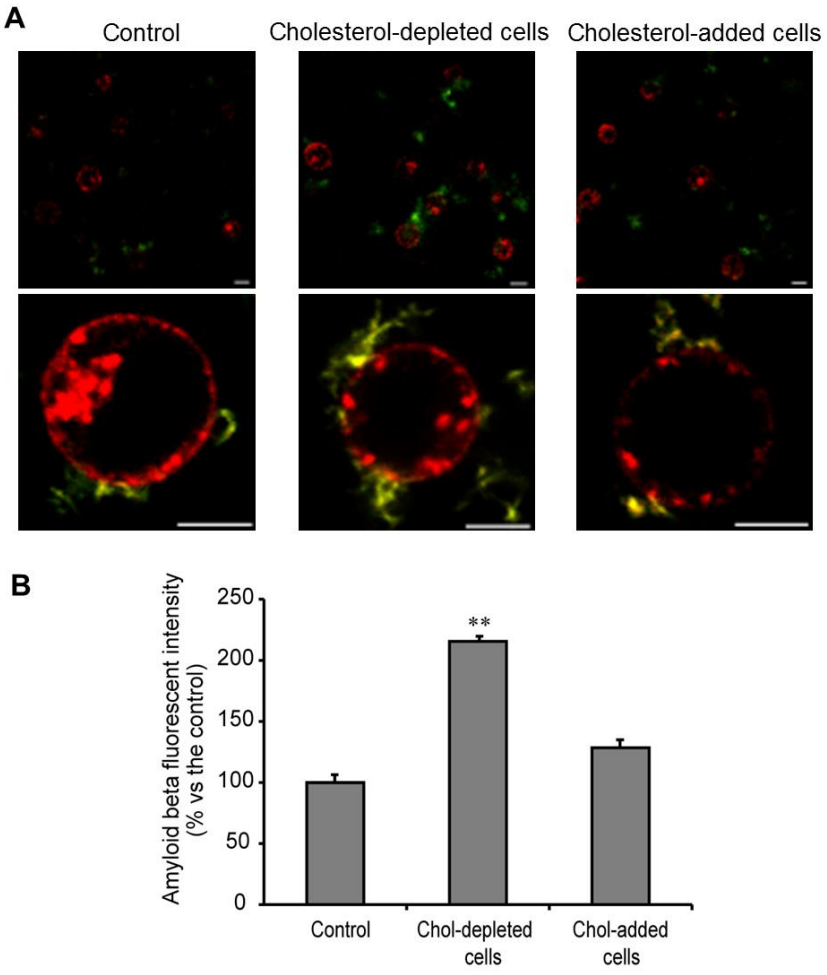

Figure 4.2. Effect of cholesterol on protofibrillar Aβ-42 association with Jurkat-T-cell membranes. (**A**) Representative confocal microscopy images showing the localization of Aβ-42 protofibrils in cell membranes. (**B**) Fluorescence intensity values reflecting the amount of Aβ-42 protofibrils localizing in cell membranes. The values are mean ± SE of three replicates. The symbol ** indicates significant differences with $P \leq 0.05$. Scale bars are 10 μm (above images) and 5 μm (below images).

The result was consistent with my results obtained from model membrane study which demonstrated that solid-ordered (So) phase, which corresponds to gel phase of biological membrane, had a significantly high Aβ-42 localization in the absence of cholesterol (discussed in chapter 3). It is in an agreement with previous studies on neuronal

cells, which showed an enhancement of toxic amyloid oligomer/membrane binding upon membrane cholesterol depletion [15,40]. The effect of membrane cholesterol on Aβ-42/membrane interaction was explained by cholesterol-induced changes in membrane fluidity. Yip and colleagues indicated that membrane fluidity was remarkably increased when cholesterol was decreased by more than 10% of the basal content, thus promoting the peptide to insert into membranes [16]. Our study on model membranes also showed that So phase without cholesterol is more fluid than in the presence of the sterol. Moreover, these findings confirm that Jurkat T cells can be used model cells to study the interaction of Aβ with cell membranes.

To assess the effect of membrane cholesterol on protofibrillar Aβ-42-induced cytotoxicity to Jurkat T cells, the viability of cells with different cholesterol levels in the absence and presence of the peptide was estimated using trypan blue exclusion assay. This assay is a fast and sufficient method to assess cell viability. It is based on the principle that the membrane of viable cells has a selective permeability, so it does not absorb certain dyes such as trypan blue [38].

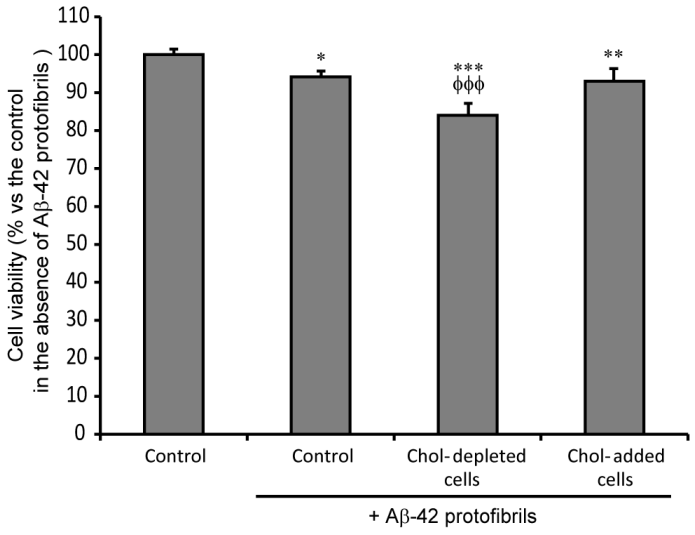

Figure 4.3. Effect of membrane cholesterol on the viability of Jurkat T cells. The values are mean ± SD of three independent replicates. The symbols *, **, and *** indicate significant differences between the cells exposed to Aβ-42 protofibrils and the control cells in the absence of the peptide ($P \leq 0.1$, $P \leq 0.05$, and $P \leq 0.01$, respectively). The symbol ϕϕϕ indicates a significant difference between cholesterol-depleted cells and the control cells in response to Aβ-42 protofibrils ($P \leq 0.01$). Scale bars are 10 μm.

In the absence of Aβ-42 protofibrils, changes in membrane cholesterols did not significantly affect cell viability (data was not shown). The presence of Aβ-42 protofibrils induced a small loss of cell viability (Figure 4.3). This suggested that the peptide is slightly harmful to Jurkat T cells at basal cholesterol content. In comparison with neuronal cells [15], Aβ-42-induced decrease in viability of T cells was much smaller, correlating with previous studies on different effects of Aβ on T cells and neuronal cells. It has been shown that the major influence of Aβ on the former cells is activating their secretion of inflammatory factors, while the peptide is able to induce apoptosis of the latter. When cholesterol was added to membranes, cell viability upon the presence of Aβ was nearly similar to cells with basal cholesterol level, implying that high membrane cholesterol contents did not affect Aβ cytotoxicity to Jurkat T cells. In contrast, the partial depletion of cholesterol from membranes significantly increased the peptide toxicity to Jurkat T cells as indicated by a higher reduction in cell viability. This influence of cholesterol was also observed in neuronal cells [15,16].

4.3.2. Effect of 7keto on protofibrillar Aβ-42 localization in biological membranes

The effect of 7keto on protofibrillar Aβ-42/Jurkat-T-cell membrane interaction was subsequently investigated. 7keto was introduced to cell membranes at 5 and 10 μM concentrations which are similar to the level detected in rat hippocampus after kainite excitotoxicity (2500 – 3100 ng/g tissue or 6.25 – 7.5 μM) [38]. These 7keto concentrations were lower than the concentration that was used to induce cell apoptosis (50 μM) [41]. The trypan blue exclusion assay demonstrated that cell viability was not affected by the presence of cholesterol or 7keto at these levels (data was not shown).

As can be seen in Figure 4.4A, the position of protofibrillar Aβ-42 localizing in Jurkat T cells was not changed in the presence of 7keto. However, the introduction of the oxysterol to Jurkat T cells strongly facilitated the protofibrils to associate with membranes, indicated by a remarkably higher Aβ-42 localization in 7keto-added cells compared to the cells with basal sterol content. The enhancing effect of 7keto on Aβ-42/T-cell-membrane interaction was concentration-dependent. In contrast, the cells which were treated with cholesterol at the same levels did not show changes in Aβ localization compared to the

control (Figure 4.4B). The result clearly demonstrated that the oxysterol had a higher ability to increase Aβ/membrane interaction than cholesterol, consistent with my studies on model membranes (chapter 2 and 3). Due to differences in hydrophobicity and orientation in lipid bilayer from cholesterol, 7keto has been reported to increase the fluidity of model membranes. In this chapter, I proposed that the presence of 7keto in cell membranes induced a significant enhancement of membrane fluidity, thus promoting the protofibrils association with cell membranes.

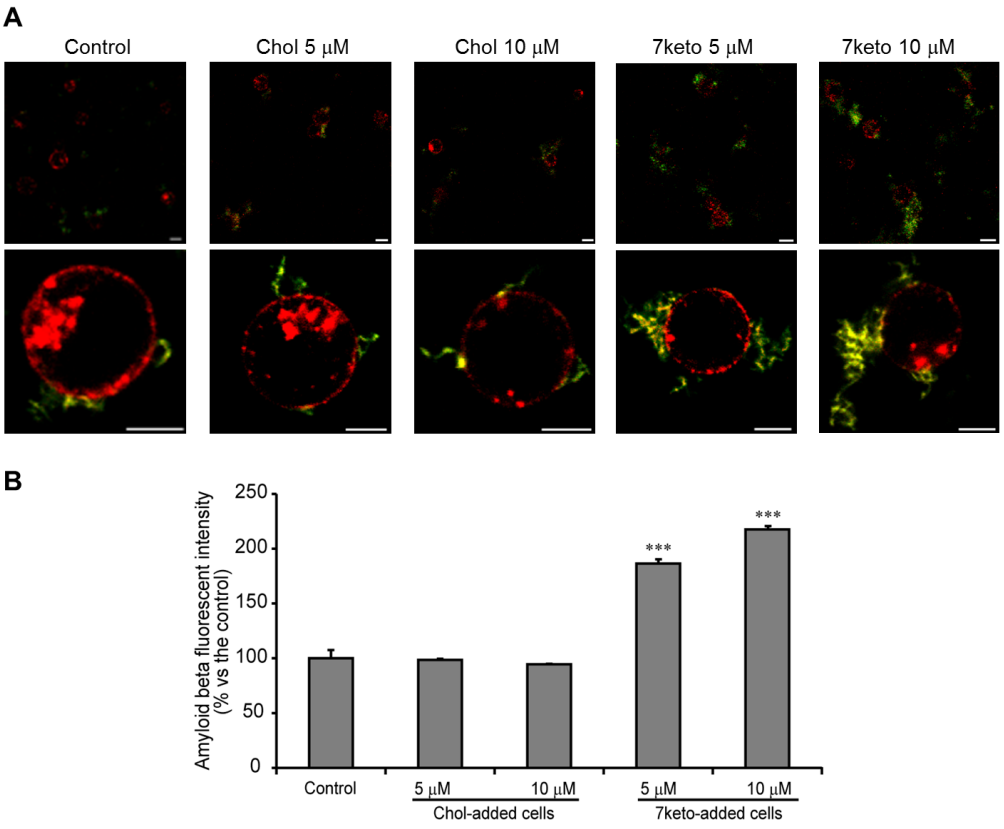

Figure 4.4. Effect of 7-ketocholesterol on protofibrillar Aβ-42 localization in Jurkat-T-cell membranes. (**A**) Representative confocal microscopy images showing the localization of Aβ-42 protofibrils in cell membranes. (**B**) Fluorescence intensity values reflecting the amount of Aβ-42 protofibrils localizing in cell membranes. The values are mean ± SE of three replicates. The symbol *** indicates significant differences with $P \leq 0.01$. Scale bars are 10 μm (above images) and 5 μm (bellow images).

Yellow regions existing in membranes revealed the co-localization of Aβ with lipid rafts (Figure 4.4.A). This suggested that the peptide mainly interact with lipid rafts of cell

membranes and 7keto may affect this interaction. The result seem to be different from the study using biomimetic membranes that suggested that 7keto mainly facilitated Aβ-42 localization in liquid-disordered (Ld) phase and did not influence that in liquid-ordered (Lo) phase which has been considered as the raft-like domains (discussed in chapter 3). I was aware that the interaction of Aβ with biological membrane may be different from its interaction with a simple, protein-free model membrane system due to the complicated structure of cells. For example, cell membranes consist of not only DOPC, DPPC, cholesterol but also many other lipid species [24]. Cholesterol and sphingolipid are major constituents of lipid raft domains [2], among which GM1, a kind of sphingolipids, has reported to specifically binds to Aβ [42]. GM1/Aβ interaction may be more prominent than the absorption of the peptide in lipid bilayer of membranes. The presence of 7keto can facilitate Aβ insertion into the lipid bilayer as well as GM1/Aβ interaction. Despite of the current disadvantages, biomimetic membranes are still useful models because they are similar to cell membranes in size, structure, lipid composition and are easily used under a controlled environment [43].

4.3.3. Effect of 7keto on protofibrillar Aβ-42-induced changes in intracellular Ca^{2+} level

Plasma membrane of eukaryotic cells has a selective permeability to Ca^{2+} which is important signaling molecule. In general, the intracellular Ca^{2+} is stored in some organelles such as endoplasmic reticulum. The concentration of free Ca^{2+} in cytosol (~ 10^{-4} mM) is much lower than extracellular Ca^{2+} concentration (~ 1-2 mM), producing a Ca^{2+} gradient across cell membranes [24]. Ca^{2+} gradient plays essential roles in signaling pathways which trigger some biological process, including inflammatory responses, fertilization [24], neuron survival and death [12]. Therefore, it is very important for cells to maintain this gradient. Extracellular Ca^{2+} enter into cytosol when Ca^{2+} channels in plasma membranes are opened in response to ligand binding, and intracellular Ca^{2+} are subsequently excluded from the inside to the outside of cells by Ca^{2+} pumps [24]. It has been reported that Aβ is able to enhance Ca^{2+} influx [7,44], thus perturbing Ca^{2+} homeostasis of cells as one of the earliest biochemical consequences of the peptide/membranes interaction [36].

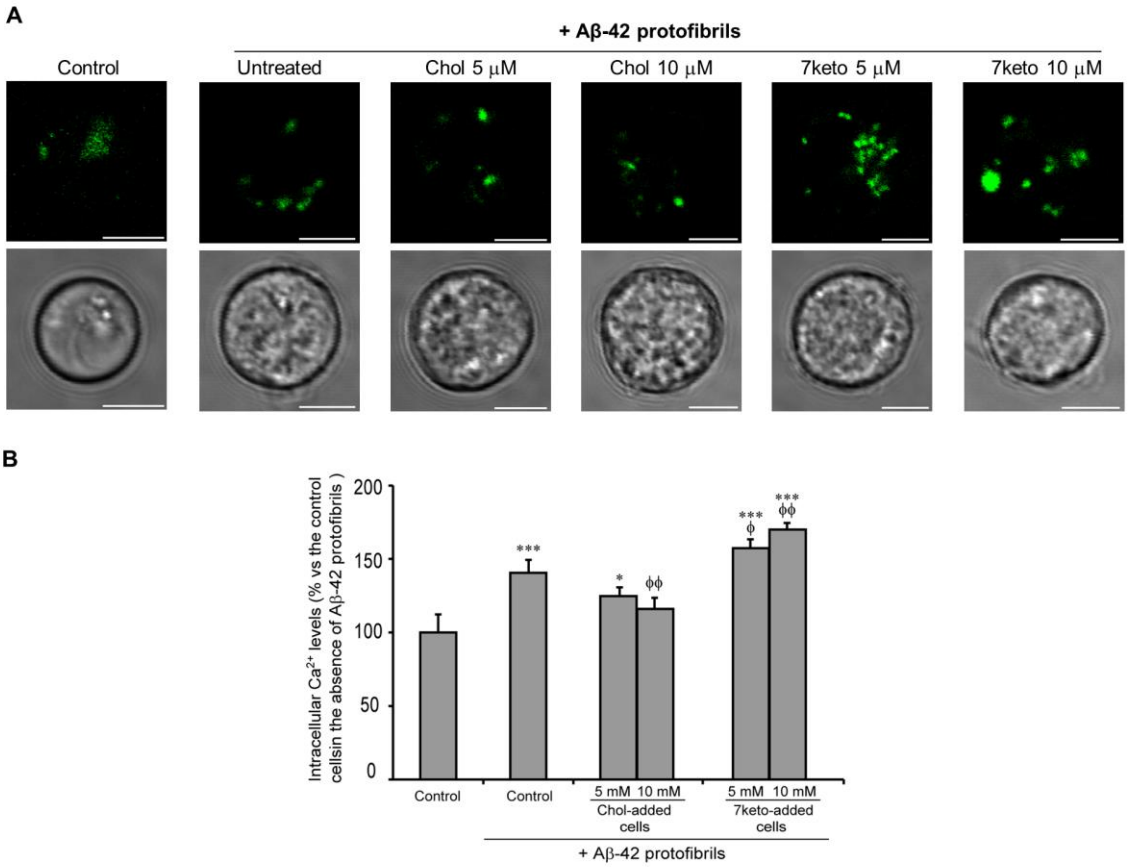

Figure 4.5. Intracellular Ca^{2+} levels of Jurkat T cells with different cholesterol and 7-ketocholesterol contents in the exposure to Aβ-42 protofibrils. (A) Representative confocal microscopy (above) and differential interference microscopic (below) images of control, cholesterol-added, and 7-ketocholesterol-added cells exposed to Aβ-42 protofibrils. (B) The quantity of intracellular Ca^{2+} of Jukat T cells reflected by fluorescence intensity of Fluo3-AM. The values are mean ± SE of three replicates. The symbols * and *** indicate significant differences between the cells exposed to Aβ-42 protofibrils and the control cells in the absence of the peptide ($P \leq 0.1$ and $P \leq 0.01$, respectively). The symbols ϕ and ϕϕ indicate significant differences between cholesterol- or 7keto-added cells and the control cells in response to Aβ-42 protofibrils ($P \leq 0.1$ and $P \leq 0.05$, respectively). Scale bars are 10 μm.

In order to further understand whether 7keto changes the effect of protofibrillar Aβ-42 on Jurkat T cells, I assessed Aβ-42-induced changes in cytosolic Ca^{2+} level in the absence and presence of the oxysterol. Jurkat T cells with different cholesterol and 7keto

levels were cultured in RPMI1640 medium which contains approximately 0.424 mM Ca^{2+} concentration [45] and exposed to Aβ-42 protofibrils. Fluo3-AM, a labeled calcium indicator, was used to measure intracellular Ca^{2+} content. Since the indicator shows an increased fluorescence emission upon Ca^{2+} binding [35], the intensity of Fluo3-AM correlates with the amount of Ca^{2+} in cytosol.

First, I estimated the effect of Aβ-42 protofibrils on cytosolic Ca^{2+} levels of Jurkat T cells with basal cholesterol (the control cells). Figure 4.5 reveals a 40% higher cytosolic Ca^{2+} level of cells exposed to the protofibrils relative to the cells in absence of the protofibrils. This indicated that the protofibrillar Aβ-42 increased influx of Ca^{2+} from the extracellular medium to the cytosol of cells, thus altering intracellular Ca^{2+} homeostasis. This effect is similar to the reported effect of Aβ-42 on neuronal and fibroblast cells [7,14].

When 7keto was added into Jurkat T cells, Ca^{2+} level changes induced by protofibrillar Aβ-42 was increased, corresponding to higher intracellular Ca^{2+} contents (17% and 30% higher Ca^{2+} content at 5 μM and 10 μM 7keto concentration, respectively) compared to cells with basal sterol levels that were treated similarly. Conversely, the presence of cholesterol at the same concentrations (5 μM and 10 μM) tended to decrease the Ca^{2+} changes, as represented by lower cytosolic ion concentration (Figure 4.5). The results suggested that 7keto is highly able to enhance the effect of membranes/Aβ-42 protofibrils interaction on Ca^{2+} content compared to cholesterol. Evangelisti and colleagues have recently shown that the recruitment of Aβ-42 oligomers in cell membranes leads to Ca^{2+} dyshomeostasis of fibroblasts from AD patients. In agreement, I supposed the increasing influence of 7keto on protofibrillar Aβ-42-induced changes in Ca^{2+} content of Jurkat T cells (Figure 4.5) was due to the ability of 7keto to promote the peptide to associate with cell membrane (Figure 4.4).

Accumulating evidences show that Aβ increases cytosolic Ca^{2+} level of cells by interfering with ion channels located in plasma membranes (L-type calcium channels, N-methyl D-aspartyl receptor) and those existed in membrane of endoplasmic reticulum (ER) which is one of intracellular Ca^{2+} stores (ryanodine receptor, Inositol(1,4,5)P3 receptor, sarco endoplasmic reticulum calcium ATPase). In addition, Aβ is capable of forming Ca^{2+}-selective pores in cell membranes [46]. Kawahara and Kuroda reported that cholesterol can block Aβ-induced changes in intracellular Ca^{2+} content in neuronal cells because it affects membrane fluidity which in turn influences the formation of Aβ pores [47]. In this study, I

proposed that the presence of 7keto resulted in the increase of membrane fluidity, thus facilitating the formation of Aβ pores in membranes. Moreover, 7keto induced a higher recruitment of Aβ-42 protofibrils in membrane of Jurkat T cells. Consequenly, the effect of the peptide on the activity of Ca^{2+} channels was enhanced, resulting in increased cytosolic Ca^{2+} content. Furthermore, it has been reported that changes in cytosolic Ca^{2+} level also results from an increased efflux of the ion from ER to the cytosol upon the effect of Aβ [48]. The peptide internalizes into cells via endocytosis [49]. The high accumulation of Aβ inside cells induces ER stress during which Ca^{2+} channels in ER membrane are disturbed, causing an enhanced efflux of Ca^{2+} [50]. My studies on model demonstrated that 7keto increases membrane fluidity and promotes model membranes to form stomatocyte transformation pathway which might be corresponding to endocytosis of biological membranes in the presence of Aβ. This is consistent with Ma et al. who indicated that impact of 7keto in causing exocytosis of neuronal cells. From these understanding, I supposed that the 7keto may facilitate the internalization of Aβ protofibrils into cells, thus increasing ER stress and Aβ-induced changes of intracellular Ca^{2+} level.

4.3.4. Effect of 7keto on protofibrillar Aβ-42-induced toxicity to cells

The above studies demonstrated that 7keto significantly increased protofibrillar Aβ-42/cell membranes association and its effect on intracellular Ca^{2+} homeostasis of Jurkat T cells. I also found that Aβ-42 protofibrils slightly decreased the viability of T cells. Herein, I sought to investigate how the oxysterol changes the influence of Aβ-42 protofibrils on cell viability. Jurkat T cells having basal cholesterol or cholesterol-enriched and 7keto-enriched content was exposed to the protofibrils for 24 h and estimated cell viability using Trypan blue exclusion assay.

The introduction of 7keto into Jurkat T cells did not significantly changes cell viability in the absence of Aβ-42 protofibrils (data was not shown). However, the viability of 7keto-added Jurkat T cells exposed to Aβ-42 protofibrils was moderately lower than that of T cells with basal cholesterol content (Figure 4.6). This implied that the cytotoxicity of the peptide to T cells was increased by 7keto. This change was not observed in the introduction of cholesterol at similar concentrations. In particular, the viability of Jurkat T cells was slightly enhanced (Figure 4.6). As discussed in part 4.1 of this chapter, a

significant depletion of membrane cholesterol (approximately 40% of basal content in this study) is capable of increasing Aβ-42-induced death of cells, while the enrichment of cholesterol might play an inhibitory effect on the toxicity to cells [16]. My results showed that the presence of a cholesterol-oxidized derivative, 7keto, at low concentration (5 μM and 10 μM) could promote the harmful effect of Aβ-42 protofibrils on cells.

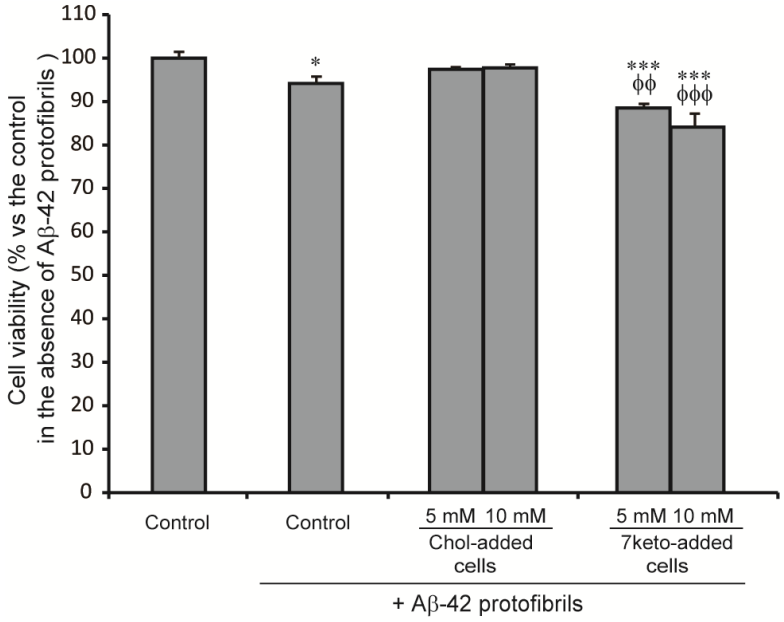

Figure 4.6. Effect of 7-ketocholesterol on the viability of Jurkat T cells. The values are mean ± SD of three independent replicates. The symbols * and *** indicate significant differences between the cells exposed to Aβ-42 protofibrils and the control cells in the absence of the peptide ($P \leq 0.1$ and $P \leq 0.01$, respectively). The symbols ϕϕ and ϕϕϕ indicate significant differences between 7keto-added cells and the control cells in response to Aβ-42 protofibrils ($P \leq 0.1$ and $P \leq 0.05$, respectively). Scale bars are 10 μm.

4.4. Conclusions

Jurkat T cells, a kind of white blood cell, were successfully used to investigate the effect of cholesterol and 7keto on protofibrillar Aβ-42/cell membranes interaction. We have demonstrated that (i) a significant decrease in membrane cholesterol content increased Aβ-42 protofibril association with T cells and effect of the protofibrils on cell

viability, while the increase in cholesterol level did not significant change these processes; (ii) 7keto had a high ability to enhance the localization of Aβ-42 protofibrils in Jurkat T cell membranes; (iii) the effect of protofibrillar Aβ-42/cell membranes interaction on the permeability of Jurkat T cells to Ca^{2+} was promoted by 7keto; (iv) the oxysterol promoted the peptide's negative effect on T cell viability. The findings indicated that 7keto may be a harmful factor in Aβ-induced toxicity. As far as I am aware, this study is the first report of 7keto effect on the interaction between Aβ-42 with cell membranes. It contributes an important part to understanding the impact of cholesterol oxidation as well as oxidative stress in Aβ-induced brain injury and Aβ cytotoxicity during AD's pathology.

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Chapter 5

Effect of cholesterol and 7-ketocholesterol on model membrane-mediated aggregation of amyloid beta (1-42)

Abstract

It has been reported that cell membranes mediate the aggregation of amyloid beta (Aβ), one of critical pathogenic events in Alzheimer's disease (AD). However, the effect of cholesterol, a vital structural component of cell membranes, and its oxidized derivatives on membrane-mediated aggregation of Aβ is not well understood. In this chapter, I aimed to investigate how cholesterol and 7keto influence Aβ-42 aggregation in the presence of homogeneous model membranes. I have shown that the presence of DOPC vesicles noticeably increased Aβ nucleation and inhibited the fibril elongation. On the other hand, when cholesterol was present in the vesicles, the kinetics of nuclei formation was moderately hindered and that of fibrillar Aβ-42 growth was considerably accelerated. The partial substitution of membrane cholesterol with 7keto slightly enhanced the formation of nuclei from monomers and remarkably inhibited fibril elongation. Moreover, cholesterol-containing vesicles induced a faster formation of fibrils which has a considerable propensity to cells, while 7keto-containing vesicles inhibited the formation of fibrils, maintained the peptide in protofibrillar aggregates which were highly able to localize in cells. Since the cytotoxicity of Aβ remarkably depends on the aggregated state, these results suggested that cholesterol hinders Aβ cytotoxicity to cells by accelerating the formation of fibrils, while 7keto mediates Aβ cytotoxicity by inhibiting the conversion of protofibrils to mature fibrils.

Keywords: Cholesterol, 7-ketocholesterol, Amyloid beta aggregation, Amyloid beta morphology, Amyloid beta localization, Homogeneous model membranes

5.1. Introduction

The aggregation of soluble monomeric amyloid beta (Aβ) peptide into fibrils is one of principal pathogenic events in Alzheimer's disease (AD), the most common neurodegenerative illness of late life [1,2]. It has been shown that Aβ aggregation is a nucleation-dependent polymerization process, including two main steps: (i) nucleus formation and (ii) fibril elongation [3]. In the first step, soluble monomeric Aβ, which is generated from the amyloidogenic cleavage of a transmembrane amyloid precursor protein (APP), undergoes a misfolding from random coil to β-sheet structure [4,5] and/or adopt a conformational switching from α-helical-enriched conformation to β-sheet-enriched one under various conditions [6]. The β-sheet conformation is prone to self-aggregation [7], producing dimers, trimmers, and eventually nuclei (n-mers) [3]. In the next step, Aβ nuclei trigger the formation of intermediate oligomers or protofibrils [8]. The intermediates subsequently bind end-to-end or laterally, forming insoluble fibrils [3,9]. These fibrils deposit into extracellular neuritic plaques that are recognized as characterized hallmarks of AD [1]. Accumulating evidences show that Aβ toxicity significantly depends on the aggregated state. Intermediate species including oligomers and protofibrils are more toxic than soluble monomers and mature fibrils [9-11] due to its ability to (i) interact with cell membranes, thus compromising membrane integrity [13] (ii) internalize into cells, leading to the dysfunction of mitochondria [14], and (iii) directly disturb synaptic function [2]. Therefore, controlling Aβ aggregation and the formation of neurotoxic species has become one of the emerging therapeutic strategies in AD treatment [15,16].

Cell membranes are remarkably associated with Aβ aggregation. They serve as an aggregation matrix which seeds and facilitates fibrillar Aβ-42 formation [17,18]. Many studies on model membranes have demonstrated that the peptide specifically binds to a cluster of GM1 ganglioside, a major constituent of membrane lipid raft domains. The peptide subsequently changes its conformation to adopt a β-sheet-enriched structure, which in turn becomes a nucleus for amyloid recruitment and fibrillation [19-21]. Membrane-mediated aggregation of Aβ-42 is dependent on various factors such as peptide density [19] and membrane lipid composition [19,22]. In addition to the central role of GM1 content as discussed above, other membrane lipids have been reported to influence the rate of Aβ assembly. Aβ preferably accumulated in positively charged (DOTAP) lipid bilayers than negatively charged (DOPG) and neutral (DOPC, DPPC) systems [23]. Moreover, a faster

accumulation of the peptide was detected in oxidized phospholipid-containing monolayers compared to membranes composed of unoxidized phospholipids [24].

Cholesterol is a prominent component of membranes [25] and an important factor in the pathogenesis of AD [26,27]. However, the effect of cholesterol on membrane-mediated aggregation of Aβ is not well understood. Some studies have demonstrated that this sterol indirectly influences Aβ/membrane interaction and following membrane-mediated aggregation of the peptide by accelerating Aβ/GM1 binding [28,29]. My focus is different in that I sought to study how the direct Aβ/lipid-bilayer interaction modulated by cholesterol affects the assembly of amyloid peptide on membrane. The ability of cholesterol to change physicochemical property of membrane lipid bilayers, thus inhibiting [30-32] or promoting the direct Aβ interaction with the bilayers [33] has been proposed by many researches including my group. The effect of cholesterol-induced changes of lipid bilayers on Aβ fibrillation was reported by several studies, but the data was contradicting [27,34]. In addition, cholesterol is a target of cellular oxidation induced by reactive oxygen species (ROS), enzymes, and Aβ in combination with Cu^{2+}, generating various oxidized derivatives (oxysterols) [35,36]. 7-ketocholesterol (7keto) is one of major products of ROS-caused cholesterol oxidation [35]. It has recently been reported to facilitate Aβ insertion into lipid bilayers [37,32]. Unravelling how 7keto influences Aβ aggregation on lipid bilayer is important to understand the impact of cholesterol oxidation in Aβ-induced toxicity.

In this chapter, I sought to investigate the kinetics of Aβ-42 aggregation in the presence of cholesterol-containing and 7keto-containing lipid vesicles. Moreover, the morphology and cytotoxicity of resultant amyloid aggregates were studied. Lipid vesicles have been developed as model cell membrane systems, and widely used to study Aβ-42/membrane interaction [22,38]. In previous studies (as discussed in chapter 2, 3, and 4), I successfully used lipid vesicles containing DOPC, a kind of glycerophospholipids which contribute to 40-60% of membrane lipid composition [25], cholesterol, and 7keto to clarify the inhibiting effect of cholesterol and enhancing effect of 7keto on Aβ association with membranes [32]. Here, I have shown that the presence of lipid vesicles significantly influenced Aβ-42 fibrillation. Briefly, DOPC systems facilitated amyloid nucleus formation and inhibited fibril elongation. In contrast, cholesterol-containing vesicles strongly inhibited the kinetics of nucleus formation and accelerated fibrillar Aβ-42 growth. The partial substitution of membrane cholesterol with 7keto influenced the kinetics of Aβ-42 fibrillation in a similar tendency to DOPC systems. However, 7keto-containing lipid bilayers remarkably

inhibited fibril elongation compared to DOPC bilayers. Studies on the peptide morphology and association with cell membranes have revealed that cholesterol-containing lipid bilayers promoted the formation of fibrillar Aβ-42, while 7keto systems enhanced the appearance of more toxic protofibrils.

5.2. Materials and methods

5.2.1. Materials

Two kinds of membrane lipids, including 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol were purchased from Avanti Polar Lipids (USA). 7-ketocholesterol (7keto) and bovine serum albumin (BSA) were from Sigma-Aldrich (USA). Amyloid beta protein (Human, 1-42) (Aβ-42) and Hilyte Fluor^{TM} 488-labelled (λex=503 nm, λex=528 nm) Aβ-42 were obtained from Peptide Institute Inc. (Japan) and Anaspec, Inc. (USA), respectively. Roswell Park Memorial Institute 1640 (RPMI1640) medium and fetal bovine serum (FBS) were from Invitrogen (USA). Jurkat human leukemic T cell line (Jurkat T cells) was from Riken cell bank (Japan). Thioflavin T (ThT), phosphate buffer salts (PBS), chloroform, Tris(hydroxymethyl)aminomethane (Tris), and methanol were purchased from Tokyo chemical industry co., Takara Bio Inc., Kanto-Chemical, and Nacalai Tesque (Japan), respectively. Deionized water was obtained using an ultraviolet water purification system (Millipore S.A.S, France).

5.2.2. Lipid vesicle preparation

DOPC (DOPC only), Chol (DOPC/cholesterol = 50/50 molar ratio), and 7keto (DOPC/cholesterol/7keto = 50/40/10 molar ratio) lipid vesicles were prepared following natural swelling method [32,39]. Lipid mixture was dissolved in chloroform/methanol (2/1, v/v) in a glass tube at the final concentration of 0.2 mM. The solvent was subsequently removed by evaporating the tube under a gentle nitrogen stream and drying in a desiccator for 3 h, resulting in a thin lipid film at the bottom of tube. The film was swollen with Tris buffer (20 mM, pH = 7.4) overnight at 37 ^{o}C to form lipid vesicles. A phase-contrast microscopy (Olympus BX50, Japan) was employed to estimate the vesicle formation.

5.2.3. Aβ-42 incubation

Aβ-42 was incubated following the method as reported previously [40,41]. First, 200 μM Aβ-42 solutions were prepared by dissolving Aβ-42 powder in 0.02% ammonia and stored at -80 °C. The peptide solution was then diluted and incubated in the absence or presence of lipid vesicles (lipid vesicles/peptide = 5/4, v/v) in Tris buffer (20 mM, pH 7.4) at 80 μM concentration in various incubation periods (0h, 6h, 12h, 24h, 36h, and 48h). In Aβ-42 localization experiments, fluorescence-labelled Aβ-42 and Aβ-42 were mixed at 1/1 ratio (v/v) before the incubation [41].

5.2.4. Cell culture and visualization of lipid rafts

T cells were cultured in RPMI1640 medium supplemented with 10% FBS (v/v) and maintained in a humidified atmosphere with 5% CO_2 (v/v) at 37 °C. To visualize lipid rafts, cells were treated with 15 μg/ml CT-B which specifically binds to GM1 in lipid rafts [42] and 0.02% BSA in PBS at 0 ºC for 30 min, followed by an incubation at 37 ºC for 10 min.

5.2.5. Measurement of Aβ-42 aggregation

The aggregation of Aβ-42 was assessed by ThT fluorescence assay [43,44]. The peptide incubated in different conditions was diluted in 20 mM Tris buffer at 20 μM concentration and subsequently added into 5 μM ThT solution contained in a transparent cell. The cell was immediately placed in FP-6500 spectrofluophotometer (Jasco, Japan) to detect ThT fluorescence after an excitation at 450 nm and an emission at 483 nm.

5.2.6. Kinetic analysis of Aβ-42 aggregation

The kinetics of Aβ-42-42 aggregation was analysed using the autocatalytic reaction model reported by Sabaté et al. [3]. This model considers Aβ-42 aggregation as a two-step autocatalytic reaction from monomers to aggregated species and its kinetics is controlled by nucleation rate and elongation rate. ThT fluorescence intensity data was fitted to this model using equation $f = \rho\{\exp[(1+\rho)kt]-1\}/\{1+\rho\exp[(1+\rho)kt]\}$ where f is the fraction of fibrillar form; $k = k_e a, k_e$ is elongation rate constant, a is the initial concentration of Aβ-42 in the solution; $\rho = k_n/k$, k_n is nucleation rate constant.

5.2.7. Characterization of Aβ-42 aggregate morphology

Atomic force microscopy (AFM) was used to image and characterize the morphology of Aβ-42 aggregates derived from the incubation of Aβ-42 alone or with lipid vesicles. In order to prepare AFM samples, 5 μM of Aβ-42 solution was uniformly spread and immobilized in a mica plate (Furuuchi Chemical Co., Shinagawa, Tokyo, Japan). Then, the mica was washed three times with 50 μl of deionized water to exclude Tris buffer molecules and was dried under the vacuum condition. The sample was measured by AFM (SPA400-SPI 3800, Seiko Instruments Inc., Japan) equipped with a calibrated 20 μm xy-scan, 10 μm z-scan range PZT-scanner and a scanning silicon nitride tip (SI-DF3, spring constant = 1.6 N/m, frequency resonance = 28 kHz, Seiko Instruments Inc.) in a dynamic force mode (DFM) [40]. All AFM operations were performed in an automated moisture control box with 30–40% humidity at room temperature. The length and height of Aβ-42 aggregates were analysed using Image J and SPI software, respectively [32].

5.2.8. Observation of Aβ-42 localization in Jurkat T cells

The mixture of 10 μM fluorescence-labelled Aβ-42 aggregates and Jurkat T cell suspension was poured into a silicon well (0.1 mm) placed on a glass slide and immediately used for confocal microscopy observation (Olympus FV-1000, Japan) at room temperature (~ 21.5 ºC) [31]. Aβ-42 localization was detected after the excitation of fluorescence-labelled Aβ-42 at 495 nm. The intensity of fluorescent Aβ-42 representing the amount of the peptide localizing in cells was subsequently analyzed using FV10-ASW 1.7 software (Olympus, Japan). The data is expressed as means ± SE of three independent experiments. Comparisons between the different membranes were performed using ANOVA followed by Bonferroni's post comparison test.

5.3. Results

5.3.1. Effect of cholesterol- and 7keto-containing model membranes on the kinetics of Aβ-42 aggregation

ThT assay was employed to investigate the effect of cholesterol-containing and 7keto-containing lipid vesicles on Aβ-42 aggregation kinetics. This assay is a common analytical technique for detecting the degree of amyloid fibrillation. Its mechanism is based on the

ability of ThT to show enhanced fluorescence emission at 483 nm wavelength upon the binding to the β-sheet of Aβ-42 peptide, while that of free ThT is observed at 445 nm [43,44]. ThT assay provided us the fraction of ThT fluorescence intensity, correlating to the extent of fibrils in solution, as a function of time. This data was subsequently analyzed using the autocatalytic reaction model to assess the time course curves of the kinetics of fibrillar Aβ-42 formation from monomers. This model assumes that Aβ-42 fibrillation follows two reactions: (1) $nM \xrightarrow{k_n} P_n$ (nucleus formation step) and (2) $M + P_n \xrightarrow{k_e} P_{n+1}$ (elongation step), where M is monomeric peptide, P_n is nucleus of fibrils, P_{n+1} is elongated fibril with $n+1$ molecules of Aβ-42, k_n is nucleation rate constant, and k_e is elongation rate constant. k_n and k_e are two key parameters that control the kinetics of amyloid fibrillation process [3].

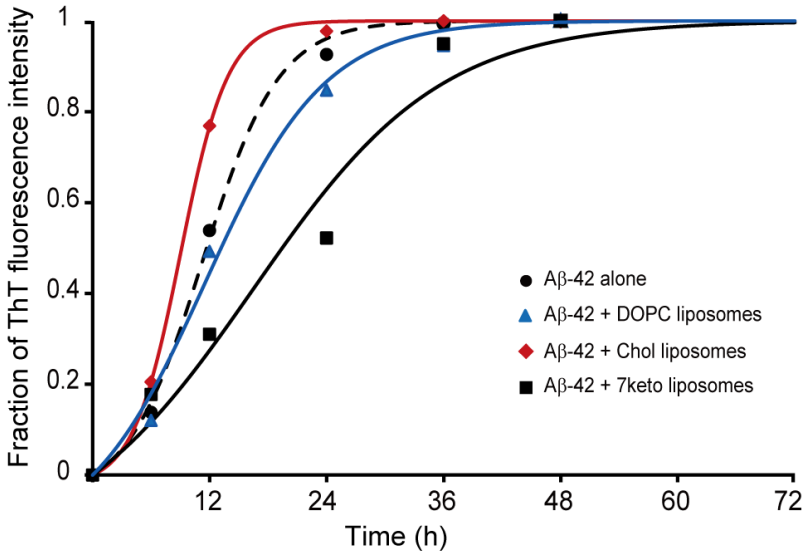

Figure 5.1. Time course curves of Aβ-42 fibrillation in the absence (dash, black) and presence of DOPC vesicles (blue), DOPC/Chol (50/50) vesicles (red), and DOPC/Chol/7keto (50/40/10) vesicles (black). Each point is the average value of three independent experimental measurements. Lines are obtained from fitting the experimental data to the equation of the autocatalytic reaction model (presented in section 5.2.6).

Figure 5.1 shows time course curves of fibrillar Aβ-42 formation from monomers in the absence and presence of three lipid vesicle systems. The aggregation without vesicles was used as the control. The peptide in the absence of lipid vesicles exhibited a typical sigmoidal

curve as reported previously (Figure 5.1) [3,45]. The sigmoidal curve starts with lag phase in which nucleus formation is detectable, subsequently proceeds on an explosive elongation phase corresponding to a rapid fibril growth, and reaches the equilibrium when most peptide in solution has aggregated into fibrils [46]. The presence of lipid vesicles changed the time course curve of amyloid fibrillation (Figure 5.1). After addition of DOPC vesicles, the lag phase of the curve was slightly shortened, while the elongation phase was significantly delayed compared to those detected without additives. Conversely, the curve obtained with vesicles composed of both DOPC and cholesterol (Chol vesicles) was not changed in lag phase, and it had a faster elongation phase. 7keto-containing vesicles induced remarkable changes in aggregation-kinetic curves vs. time course of Aβ-42. Lag phase was not clearly observed in contrast to a visible lag phase of time course curves assessed in three other conditions. In addition, the speed of elongation phases was slowed down (Figure 5.1). The data suggested that all three studied model membranes influenced the aggregating process of Aβ-42. DOPC and 7keto lipid vesicles inhibited the process, while cholesterol-containing membranes facilitated Aβ-42 fibrillation. The inhibitory effect of 7keto vesicles was higher than that of DOPC systems.

Table 5.1. Nucleation and elongation rate constants of Aβ-42 aggregation in the absence and presence of DOPC vesicles (DOPC = 100), Chol vesicles (DOPC/Chol = 50/50), and 7keto vesicles (DOPC/Chol/7keto = 50/40/10).

Samples	k_n (s^{-1})	k_e (L mol^{-1} s^{-1})
Aβ-42 alone	3.73×10^{-6}	3.378
Aβ-42 + DOPC liposomes	5.97×10^{-6}	1.968
Aβ-42 + Chol liposomes	2.74×10^{-6}	5.573
Aβ-42 + 7keto liposomes	4.4×10^{-6}	1.227

In order to clarify how lipid vesicles influence the kinetics of two major steps in Aβ-42 fibrillation process, which are nucleus formation and fibril elongation, I estimated

nucleation rate constant, k_n, and elongation rate constant, k_e by fitting the experimental data of ThT intensity to the equation of autocatalytic reaction model. Sabaté et al. proposed that $k_n \ll 1$ because nucleus formation associates with a series of thermodynamically unfavourable steps, and $k_e \gg 1$ since further addition of soluble peptide to nuclei is thermodynamically favourable [3]. As can be seen in Table 5.1, the assembly of Aβ-42 alone had a nucleation constant of 3.73×10^{-6} s^{-1} and an elongation constant of 3.38 L mol^{-1} s^{-1}. The kinetic constants were considerably influenced by the composition of lipid vesicles (Table 5.1). When DOPC vesicles were present, the aggregation of Aβ-42 peptide afforded a higher k_n and a smaller k_e compared the process without vesicles. The kinetic constants demonstrated that DOPC vesicles facilitated nucleus formation and hindered the growth of fibrils. The effect of DOPC membranes in two major steps of Aβ-42 fibrillation was nearly the same. In case of Aβ-42 aggregation with Chol vesicles, nucleation rate constant was decreased by 1.35 fold, while elongation rate constant was increased by 1.65 fold relative to the control. In comparison with DOPC vesicles, this Aβ-42 aggregation had a 2.18-fold lower nucleation rate constant and a 2.83-fold higher elongation rate constant (Table 5.1). This indicated an inhibited nucleus formation and a significantly accelerated fibril elongation mediated by cholesterol-containing membranes, and also the predominant effect of cholesterol-containing membranes on the growth of fibrils. Some previous studies suggested that cholesterol promoted the already-formed aggregation of Aβ-42 on 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)/cholesterol [47] and sphingomyelin (SM)/cholesterol mixed bilayers [28]. The effect of 7keto vesicles on the two kinetic constants was opposite to that of Chol vesicles. In the presence of 7keto vesicles, nucleation rate constant was increased by 1.2 fold, while elongation rate constant was decreased by 2.75 fold compared to that without vesicles. With respect to Chol vesicles, 7keto systems mediated Aβ-42 fibrillation with a 1.61-fold higher nucleation rate and a 4.54-fold smaller elongation rate (Table 5.1). These results suggest that 7keto vesicles could maintain the existence of Aβ-42 in intermediate states (oligomers and protofibrils) by accelerating nucleus formation and hindering fibril growth. Despite numerous studies on membrane-mediated Aβ-42 aggregation, this is the first report on how cholesterol oxidized derivatives influence the process.

5.3.2. Morphology of Aβ-42 aggregates under the influence of cholesterol- and 7keto-containing model membranes

In order to further understand Aβ-42 aggregation under the effect of cholesterol- and 7keto-containing model membranes, I characterized the morphology of some amyloid aggregates using AFM. This imaging technique has been extensively employed in studies of amyloid fibrillation and toxicity due to its ability to capture nanoscale morphological structure of the peptide [48].

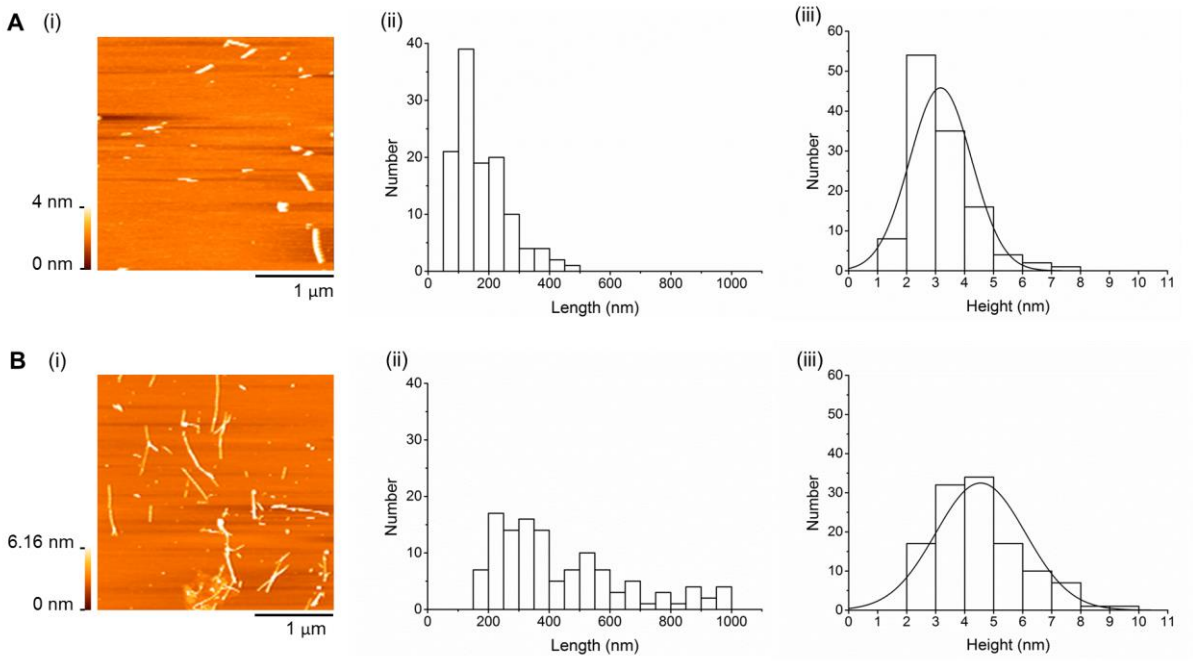

Figure 5.2. Morphology of Aβ-42 aggregates obtained from aggregation in the absence of lipid vesicles. (**A**) Representative AFM images (i), length distribution (ii), and height distribution (iii) of Aβ-42 aggregates obtained from 12-h incubation without lipid vesicles. (**B**) Representative AFM images (i), length distribution (ii), and height distribution (iii) of Aβ-42 aggregates obtained from 24-h incubation without lipid vesicles.

First, I measured Aβ-42 morphology after its incubation in the absence of lipid vesicles (also called in buffer) for 12 h and 24 h as the controls. AFM images revealed that after 12 h of incubation alone, peptide aggregates had a rod-like shape with height and length largely in the range from 1 to 4 nm (81.1%) and from 50 to 250 nm (82.5%), respectively (Figure 5.2A). This clearly showed that Aβ-42 was mostly protofibrillar [49,50]. Aβ-42 protofibrils was defined as small elongated oligomers, which are intermediate species in the

pathway of fibril formation from soluble monomers [8]. At 24-h incubation, I observed longer, branched or linear particles. The aggregates having length of 200-1000 nm contributed to about 95%. The average height of Aβ aggregates is 4.54 nm (Figure 5.2B). These results indicated that Aβ-42 fibrils were formed [51,52]. Therefore, Aβ aggregates obtained from 12-h incubation and 24-h incubation in buffer were called Aβ protofibrils and fibrils, respectively. The morphology of Aβ-42 aggregates was consistent with its fibrillation process demonstrated by time course curves in Figure 5.1.

In case of the peptide incubated with lipid vesicles, I placed an attention to the aggregates obtained from 24-h incubation because time course curves revealed that there were significantly differences among aggregations of Aβ-42 with different lipid vesicles at this incubation time (Figure 5.1). For the aggregation with cholesterol-containing membranes, Aβ-42 aggregates at 12-h incubation were additionally measured to confirm whether Chol vesicles induce a faster formation of fibrils compared to the aggregation without vesicles.

I found that when Aβ-42 was incubated with DOPC vesicles for 24h, it had branched, fibrillar shape and length distribution similar to the aggregates obtained from 24-h incubation in buffer (Aβ-42 fibrils) (Figures 5.2B and 5.3A). However, there was a significant decrease in the height of the aggregates that largely distributed in 1-4 nm range (77%) like protofibrils obtained from 12-h incubation of Aβ-42 alone (Figures 5.2A(iii) and 5.3A(iii)). A similar finding was reported by Hane and colleagues [23]. This data correlates well with the kinetic study which presented a decreased elongation of Aβ-42 incubated with DOPC vesicles (Figure 5.1).

In the presence of Chol vesicles, amyloid fibrils with typical height and length distributions was observed after 12 h of incubation, implying a faster formation of fibrils than other conditions. However, these fibrils tended to form clusters, in contrast to fibrils from 24-h incubation alone which appeared separately (Figure 5.2B and Figure 5.3B). As incubation time increased, the morphology of fibrils likely not to significantly change (Figure 5.3C).

Conversely, Aβ-42 aggregates detected from 24-h incubation with 7keto vesicles mainly had protofibrillar morphology (Figure 5.3D). 85% of aggregates were 1-4 nm high and 50-250 nm long. This implicated that fibril formation of Aβ-42 from monomers was hindered in presence of 7keto vesicles. These results of morphological analysis of Aβ-42 was in a good agreement with kinetic study using ThT assay, showing that Aβ-42 fibrillation was remarkably accelerated by cholesterol-containing membranes and inhibited by 7keto-containing membranes.

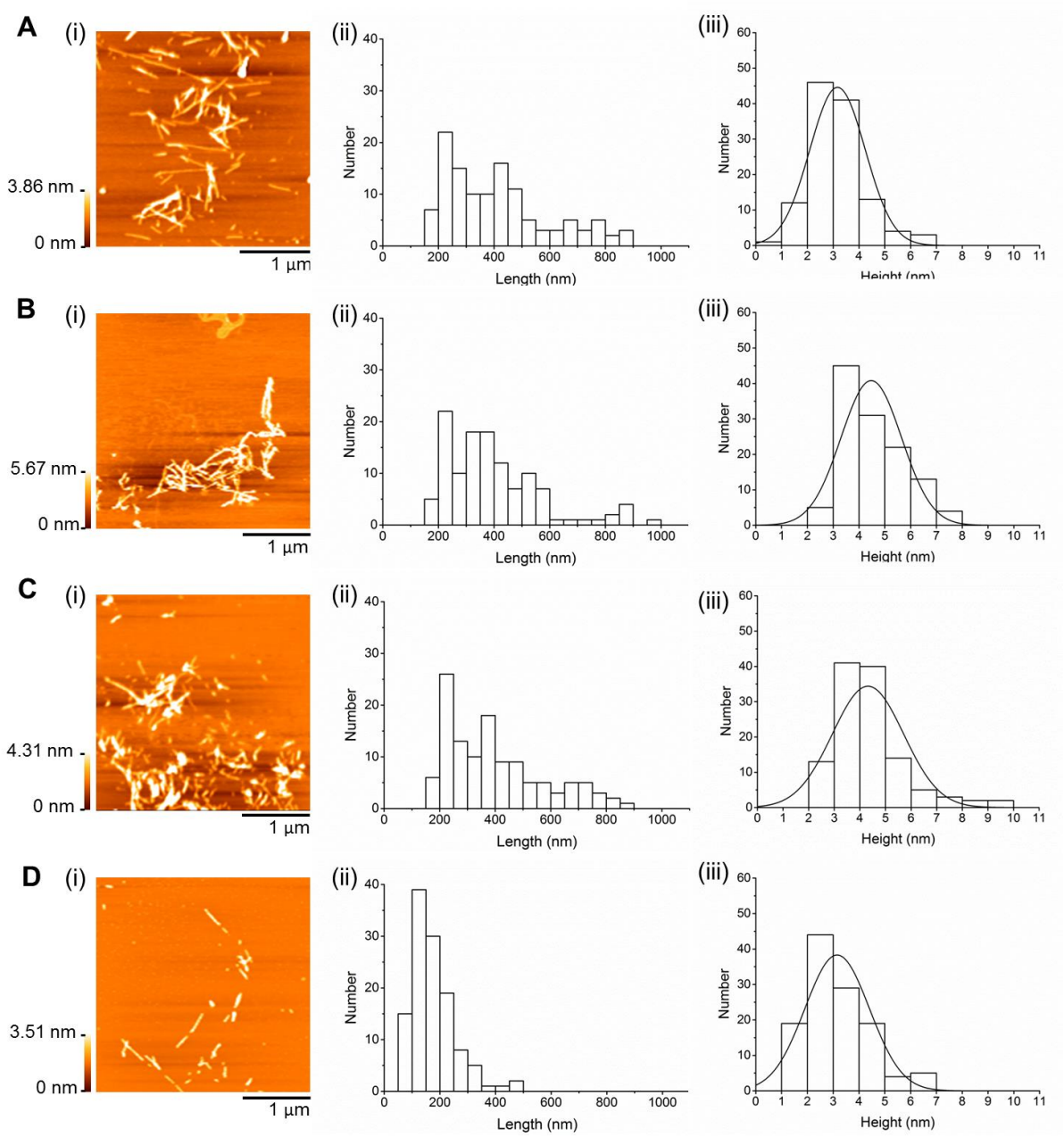

Figure 5.3. Morphology of Aβ-42 aggregates obtained from aggregation in the presence of lipid vesicles. (**A**) Representative AFM image (i), length distribution (ii), and height distribution (iii) of Aβ-42 aggregates obtained from 24-h incubation with DOPC (DOPC = 100) vesicles. (**B**) Representative AFM image (i), length distribution (ii), and height distribution (iii) of Aβ-42 aggregates obtained from 12-h incubation with Chol (DOPC/Chol = 50/50) vesicles. (**C**) Representative AFM image (i), length distribution (ii), and height distribution (iii) of Aβ-42 aggregates obtained from 24-h incubation with Chol vesicles. (**D**) Representative AFM image (i), length distribution (ii), and height distribution (iii) of Aβ-42 aggregates obtained from 24-h incubation with 7keto (DOPC/Chol/7keto = 50/40/10) vesicles.

5.3.3. Localization of Aβ-42 aggregates incubated with cholesterol- and 7keto-containing lipid vesicles in cell membranes

With the understanding of the morphology of Aβ-42-42 aggregates upon the effect of cholesterol- and 7keto-containing lipid vesicles, I assessed the localization of these aggregates in cell membranes using Jurkat T cells. The ability of Aβ-42 to associate with membrane is one important criterion of its toxicity [10]. My previous studies (presented in chapter 4) successfully used Jurkat T cells to investigate protofibrillar Aβ-42/cell-membrane interaction. I also labeled lipid rafts with CT-B to study where the aggregates locate in different positions in membranes.

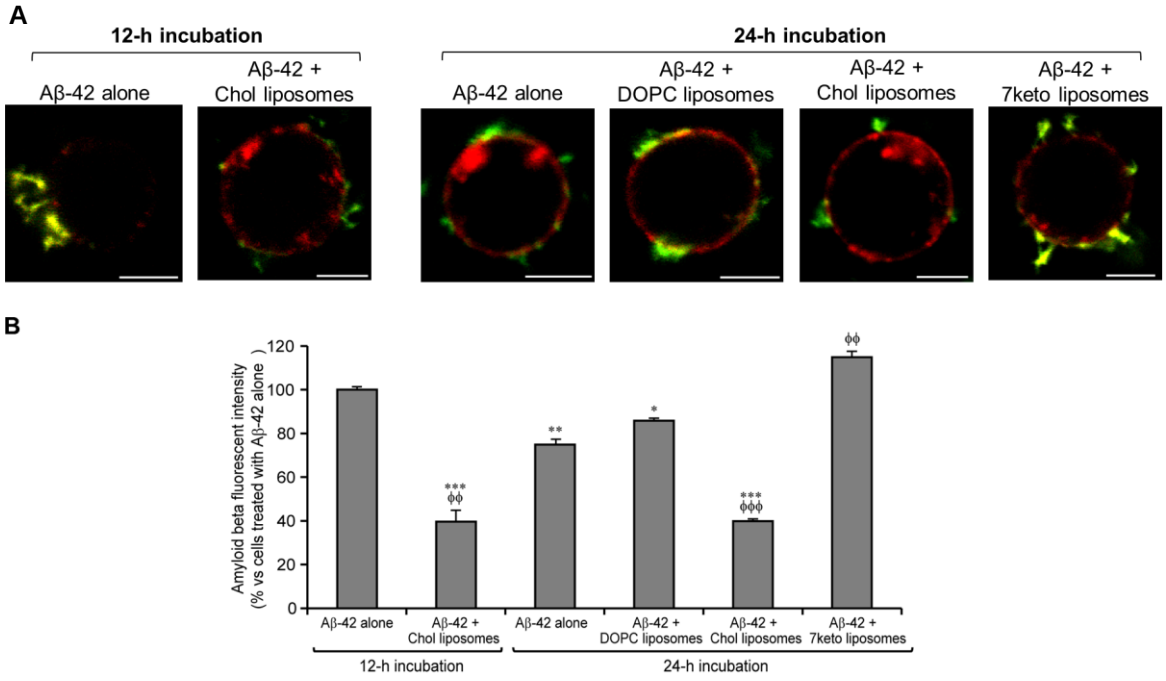

Figure 5.4. Localization of protofibrillar Aβ-42 aggregates obtained from the incubation with and without lipid vesicles in Jurkat T cell membranes. (**A**) Representative confocal microscopy images showing the association of Aβ-42 protofibrils with cell membranes. (B) Fluorescence intensity values reflecting the amount of Aβ-42 protofibrils localizing in cell membranes. The symbols *, **, and *** indicate significant differences of Aβ-42 aggregates obtained from different incubation conditions relative to the aggregate from 12-h incubation in buffer ($P \leq 0.1$, $P \leq 0.05$, and $P \leq 0.01$, respectively). The symbols ϕϕ and ϕϕϕ indicate significant differences of Aβ-42 aggregates obtained from different incubation conditions compared to the aggregate from 24-h incubation in buffer ($P \leq 0.05$ and $P \leq 0.01$, respectively). Scale bars are 10 μm.

Figure 5.4 shows different abilities of Aβ-42 aggregates, which were obtained from incubation of Aβ-42 monomers without or with three kinds of lipid vesicles, to associate with biological membranes. Regarding to the aggregates from incubation without lipid vesicles, the amount of Aβ-42 protofibrils localizing in T cells was significantly higher than that of Aβ-42 fibrils. This indicating the higher propensity of the protofibrils to membranes compared with fibrils, consistent with my previous study on model membranes (discussed in chapter 2) and other studies on neuronal cells [10,11]. In addition, both Aβ-42 aggregates from 12-h incubation and that from 24-h incubation majorly partitioned in cell membranes, and they co-localized within the lipid rafts as represented by yellow regions (Figure 5.4A).

The Aβ-42 aggregates from 24-h incubation with DOPC vesicles were more able to localize in Jurkat T cells than those incubated in buffer, corresponding to a moderately higher amount of the aggregates associating with cells (Figure 5.4). This result together with the decreased height of the aggregates demonstrated by AFM measurement suggested that the fibrillar particles observed in 24-h incubation with DOPC were different from typical short fibrils.

When Aβ-42 was incubated with Chol vesicles, the aggregates obtained in 12-h incubation and 24-h incubation had a similar capability of localizing in Jurkat T cells. The amount of both Aβ-42 incubated for 12 h and 24 h localizing in cells were 2.5-fold and 1.9-fold lower than those incubated without lipid vesicles, respectively, implying that the ability of these fibrils to interact with cell membranes was the lowest (Figure 5.4).

In contrast, the incubation of the peptide with 7keto vesicles for 24 h produced the aggregates with a high propensity to Jurkat T cells. The amount of these aggregates associating with cell membranes was considerably higher (40%) than that of fibrils from 24-h incubation alone and slightly higher (15%) than that of typical protofibrils (Figure 5.4B). Confocal microscopy images clearly showed as distinct yellow regions (green Aβ-42 merged with red CT-B, the specific label of lipid raft domains) in the association of Aβ-42 incubated with 7keto vesicles for 24 h with Jurkat T cells. These regions were not observed in aggregates incubated with other lipid vesicles (Figure 5.4A). I previously demonstrated that protofibrillar Aβ-42 associate with raft domains of cell membranes and it is able to induce the partial exclusion of raft's components from membranes (discussed in chapter 4). The microscopic observation in this chapter revealed that Aβ-42 aggregates from 24-h incubation with 7keto vesicles behaved similarly to typical protofibrils not only in the degree of

association but also position in Jurkat cell membranes. These results indicated that the presence of 7keto-containing membranes extended the appearance of Aβ-42 in protofibrillar state which is more interactive with cell membranes.

5.4. Discussion

My results clearly showed that model membranes mediated the fibrillation of Aβ-42 and its effect was changed by cholesterol and its oxidized derivative, 7keto. Many studies concluded that Aβ-42 can aggregate on membrane surface and this aggregation is more favored than that in buffer [20,21]. In agreement, I demonstrated that the formation of Aβ-42 nuclei was promoted by the addition of DOPC vesicles, indicated by a higher nucleation rate constant compared to that without lipid vesicles [Table 5.1]. Moreover, DOPC vesicles inhibited the elongation of nuclei to form fibrils, consistent with a previous study of Aβ-42 assembly on planar lipid bilayers which reported that a 24-h incubation of the peptide on DOPC bilayers produces a lower fraction of fibrils relative to the incubation in buffer [23]. The mechanism of Aβ-42 aggregation on GM1-free lipid bilayers has been clarified in some previously studies [23,28]. They demonstrated that Aβ-42 monomers adsorb to lipid bilayers upon binding of the hydrophilic N (1-27 residues) terminus of the peptide to polar head group of phospholipid. Hydrogen bonds can be formed between hydrophilic residues with phospholipid's carbonyl oxygen and phosphate oxygen groups. In addition, the hydrophobic C (28-42 residues) of the peptide can position into the nonpolar interior of lipid bilayers by hydrophobic interaction. Aβ-42 subsequently undergoes a transition from α-helix-enriched to β-sheet-enriched structures which initiate the nucleus formation and fibril growth [28]. Because lipid bilayers composed of unsaturated DOPC exists in fluid phase, Aβ-42 may insert deeply into the bilayer, by which the progressive aggregation of the peptide is affected [23].

In this chapter, I sought to investigate how cholesterol and 7keto affect membrane-mediated aggregation of Aβ-42. I found that the presence of cholesterol in pure DOPC vesicles inhibited the nucleation from monomers, but it considerably accelerated the nuclei to form fibrils (Table 5.1, Figures 5.1 and 5.3). The nucleation in lag phase of Aβ polymerization process depends on peptide concentration [3]. My previous results showed that cholesterol can reduce the association of Aβ with model membranes due to its ability to condense membrane phospholipids and render DOPC membranes more rigid (discussed in chapter 2) [32]. I proposed that the decreased amount of Aβ-42 monomers in association with

cholesterol-containing membranes can account for an inhibited nucleation. Moreover, a molecular dynamics study pointed out that cholesterol had a higher hydrogen bonding affinity with Aβ than headgroup of phospholipids. This sterol is able to compete with peptide-peptide binding during nucleation by forming hydrogen bonds with the peptide [28]. Therefore, the formation of nucleus was slowed down. However, when nuclei were already formed, its elongation and fibril formation were enhanced. As discussed previously, Chol membranes are more rigid than DOPC membranes, thus Aβ-42 was not able to penetrate deeply into the bilayer of cholesterol-containing membranes, and preferentially adsorbed to the surface. As a result, the rate of fibril elongation may be increased [34]. The enhancement of already-formed Aβ-42 aggregation by cholesterol was also reported previously [28]. These results contribute to further understanding of cholesterol's impact on the aggregation of Aβ. In particular, in addition to promoting Aβ/GM1 interaction which seeds the nucleus of Aβ aggregation [21], cholesterol is able to increase the fibrillation process by influencing the interaction of the peptide with lipid bilayer of membranes.

The impact of cholesterol-containing lipid vesicles on Aβ-42 fibrillation was significantly changed by a partial substitution of cholesterol with 7keto. 7keto-containing vesicels slightly increased the formation of Aβ-42 nuclei and remarkably inhibited fibril elongation in membranes (Figure 5.1, 5.3, and 5.4). I previously demonstrated that 7keto renders lipid bilayer less condensed and more fluid than cholesterol, thus accelerating Aβ-42 association with the bilayer (chapters 2 and 3) [32]. The higher amount of the peptide in membranes may results in an increased nucleation rate. On the other hand, Aβ-42 can insert deeply into the bilayer [32,37], thus the further elongation of the peptide is hindered.

The effects of cholesterol and 7keto on model membrane-mediated aggregation of Aβ-42 were strikingly different, demonstrated by kinetic analysis, AFM measurement, and scanning microscopic observation. Cholesterol accelerated amyloid nuclei to assemble into fibrils which had a low ability to localize in cell membranes, while 7keto maintained the peptide in protofibrillar aggregates which are highly able to associate with membranes. These findings add in understanding of cholesterol's impact on membrane-mediated Aβ-42 aggregation. Cholesterol is able to increase the fibrillation process by influencing the interaction of the peptide with lipid bilayer of membranes. However, its effect is disrupted by its oxidative derivatives, 7keto. Since protofibrils have been considered to be more toxic than fibrils, I proposed that cholesterol and 7keto are able to mediate Aβ-induced toxicity to membranes through influencing the peptide aggregation on membranes. In particular,

cholesterol is a protective factor of cells from the cytotoxicity of Aβ because it facilitates the peptide to form less harmful fibrillar species. Conversely, 7keto is a risk agent in Aβ-induced toxicity due to its ability to hinder the aggregation from more toxic protofibrils to fibrils.

5.5. Conclusions

In conclusion, I have shown that DOPC lipid vesicles facilitated amyloid nucleation and inhibited fibril elongation of Aβ-42 aggregation process compared to the aggregation without vesicles. They also influenced the morphology and localization of Aβ-42 aggregates in Jurkat T cells. The fibrils formed during Aβ-42 aggregation with DOPC vesicles had a lower height and higher ability to associate with cells compared to typical fibrils. I have clearly demonstrated that cholesterol and 7keto have strikingly different effect on membrane-mediated Aβ-42 aggregation. Briefly, the presence of cholesterol in lipid vesicles moderately inhibited the kinetics of nucleus formation and considerably accelerated fibrillar Aβ-42 growth. The partial substitution of membrane cholesterol with 7keto slightly increased the formation of nuclei from monomers and remarkably inhibited fibril elongation. Moreover, cholesterol-containing vesicles induced a faster formation of fibrils which has a considerable propensity to cells, while 7keto-containing vesicles inhibited the formation of fibrils, maintained the peptide in protofibrillar aggregates which were highly able to localize in cells. These results suggested that cholesterol and 7keto can modulate interaction of Aβ-42 with cell membranes by influencing the aggregation of the peptide. They are useful for understanding the impact of cholesterol and its oxidation in Aβ-induced Alzheimer's neurotoxicity.

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Chapter 6

General conclusions

6.1. General conclusions

This dissertation demonstrates the role of membrane lipids, which are cholesterol and its oxidized derivatives, in modulating the interaction between Alzheimer's amyloid beta and membranes. Cholesterol and oxysterols influenced not only the localization of Aβ with model and cell membranes but also membrane-mediated aggregation of the peptide. In general, cholesterol inhibited Aβ/membrane interaction and accelerated the formation of Aβ fibrils which are less harmful to cells than other aggregate species. Conversely, oxysterols enhanced the interaction and hindered Aβ fibrillation, thereby maintaining the existence of Aβ protofibrils, widely reported to be a harmful species.

In chapter 1, the current understandings of cholesterol, oxysterols, and the pathogenesis of AD were reviewed. The impact of Aβ/membrane interaction and Aβ aggregation as key events in the cytotoxicity of the peptide was shown. The controversial role of cholesterol and potential effect of oxysterols on Aβ/membrane interaction and Aβ fibrillation were highlighted. Based on this background, I pointed out the need for further investigation on these subjects and proposed the objectives of the dissertation.

In chapter 2, the impact of cholesterol and its oxidized derivatives in the interaction between Aβ and homogeneous membranes has been characterized. I have shown that oxysterols rendered homogeneous membranes more interactive with Aβ, in contrast to role of cholesterol in inhibiting Aβ/membranes interaction. I have demonstrated that two oxysterols had different impacts owing to distinct positions of the additional oxygen group in their structures. 7-ketocholesterol (7keto)-containing cell-sized liposomes exhibited a high propensity toward association with Aβ, while 25-hydroxycholesterol (25OH) systems were more capable of morphological changes in response to the peptide. Furthermore, Aβ-42 protofibrils had higher association with membranes, and caused membrane fluctuation faster than Aβ-40 species. These findings suggest the inhibitory effect of cholesterol and

enhancing influence of oxysterols on the interaction of Aβ with the lipid bilayer of membranes.

In chapter 3, the interaction of Aβ-42 protofibrils with heterogeneous model membranes, which retain the lateral lipid organization of cell membranes, under the effect of cholesterol and 7keto has been investigated. I have shown that cholesterol decreased the localization of Aβ-42 protofibrils in solid-ordered domains and increased that in liquid-ordered domains. The sterol changed the amount of Aβ associating with liquid-disordered (Ld) phase in different tendencies depending on the composition of heterogeneous membrane systems. These effects were associated with cholesterol's ability to alter the fluidity of lipid phases. In addition, 7-keto mainly enhanced the fluidity and interaction of protofibrillar Aβ-42 with Ld phase. These results demonstrate the impact of cholesterol in directly modulating Aβ interaction with lipid domains of membranes in addition to its effect on Aβ/GM1 binding as reported previously. They also indicate the harmful impact of cholesterol oxidized derivatives which promotes Aβ association with heterogeneous membranes.

In chapter 4, the Jurkat T cells, a kind of white blood cell, were used to assess the effect of cholesterol and 7keto on protofibrillar Aβ-42/cell-membrane interaction. The results have demonstrated that loss of membrane cholesterol strongly enhanced the interaction of Aβ-42 protofibrils with Jurkat T cells, decreased the viability the cells exposed to the protofibrils compared to cells with basal cholesterol content, while the increase in cholesterol content did not significantly change these processes. On the other hand, 7keto had a high ability to enhance the localization of Aβ-42 protofibrils in Jurkat T cell membranes and increase the effects of the peptide which reduce cell viability and increase cytosolic Ca^{2+} content of the cells. These results suggest that cholesterol has the beneficial role in Aβ-induced toxicity to T cells, in agreement with previous studies on neuronal cells, while 7keto may be a harmful factor in this process.

In chapter 5, the strikingly different effects of cholesterol and 7keto on Aβ-42 aggregation mediated by homogeneous model membranes has been demonstrated. I have shown that the presence of cholesterol in DOPC vesicles moderately inhibited the kinetics of nuclei formation and considerably accelerated fibrillar Aβ-42 growth. The partial substitution of membrane cholesterol with 7keto slightly increased the formation of nuclei from monomers and remarkably inhibited fibril elongation. Moreover, cholesterol-

containing vesicles induced a faster formation of fibrils which has a low propensity to cells, while 7keto-containing vesicles inhibited the formation of fibrils, maintain the peptide in protofibrillar aggregates which were highly able to localize in cells. Since the cytotoxicity of Aβ remarkably depends on the aggregated state, these results suggested that cholesterol hinders Aβ cytotoxicity to cells by accelerating the formation of fibrils, while 7keto mediates Aβ cytotoxicity by inhibiting the conversion of protofibrils to mature fibrils.

Using both model membrane systems and living cells, I have shown that cholesterol has a protective role and oxysterols, in particular 7keto, are risk factors in Aβ-induced cytotoxicity. The effect of cholesterol and oxysterols is associated with their ability to alter interaction of Aβ with membranes and fibrillation of the peptide mediated by membranes. As far as I am aware, this dissertation is the first systematic study about the effect of cholesterol oxidized derivatives on Aβ/membrane interaction. The findings of this dissertation are useful to clarify the impact of oxidative stress in cytotoxicity and neroinflammation induced by Aβ in AD pathology. They also strongly suggest that prevention and/or repair of oxidative stress by antioxidants and reduction of ROS generation may be a potential approach in the treatment of Alzheimer's disease.

6.2. Prospects of dissertation

From the findings obtained in this doctoral study, I propose some prospective studies on amyloid-induced toxicity in AD and other neurodegenerative diseases.

1. Clarifying the effect of 7keto and other oxysterols on Aβ-induced toxicity to neuronal cells. My studies have clearly shown the mediating role of oxysterols in Aβ interaction with model membranes and Aβ-induced toxicity to Jurkat T cells. However, it is widely known that neuronal cells are the main targets of Aβ during the pathogenesis of AD. Therefore, studies on neuronal cells could offer more important information to help unravel the link between oxysterols with Aβ-induced toxicity.
2. Investigating the ability of antioxidants such as naturally polyphenolic compounds to protect oxidized membranes from Aβ-induced toxicity. Antioxidants have been considered as potential candidates for therapy of AD. However, the efficiency and

mechanism by which antioxidants prevent, halt, or reverse the disease has not been fully understood [1].

3. Unravelling the impact of oxysterols, especially the compounds generated from autooxidation of cholesterol, in the pathogenesis of other neurodegenerative disease such as Parkinson's disease (PD). This work has demonstrated that oxysterols significantly influence the fibrillation and interaction of Alzheimer's amyloid beta. To date, more than 20 plasma proteins have been found to form amyloid peptides which are implicated in various neurodegenerative illnesses in human [2]. PD is one of common diseases associating with toxicity of α-synuclein to dopaminergic neurons [3]. Some studies have recently demonstrated the link of some oxysterols, mainly 24OH and 27OH with α-synuclein cytotoxicity [4,5]. However, the impact of oxysterols in the pathogenesis of PD remains an important subject that need to be investigated.

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List of publications

Journals

1. **Huong T.T. Phan**, Takahiro Hata, Masamune Morita, Tsuyoshi Yoda, Tsutomu Hamada, Mun'delanji C. Vestergaard, Masahiro Takagi (2013) The effect of oxysterols on the interaction of Alzheimer's amyloid beta with model membranes. Biochim. Biophys. Acta, Biomembr. 1828(11), 2487–2495.

2. **Huong T.T. Phan**, Tsuyoshi Yoda, Bindu Chahal, Masamune Morita, Masahiro Takagi, Mun'delanji C. Vestergaard (2014) Structure-dependent interactions of polyphenols with a biomimetic membrane system. Biochim. Biophys. Acta, Biomembr. 1838(10), 2670-2677.

3. **Huong T.T. Phan**, Mun'delanji C. Vestergaard, KeangOk Baek, Tsutomu Hamada, Naofumi Shimokawa, Masahiro Takagi (2014) Localization of amyloid beta (Aβ1-42) protofibrils in membrane lateral compartments: role of cholesterol and 7-Ketocholesterol. FEBS Lett, doi: 10.1016/j.febslet.2014.08.007 (In press).

4. **Huong T.T. Phan**, Mun'delanji C. Vestergaard, Naofumi Shimokawa, Masahiro Takagi. Effect of cholesterol and 7-Ketocholesterol on lipid bilayer-mediated aggregation of amyloid beta (Aβ1-42). (In preparation)

5. Tsuyoshi Yoda, **Huong T.T. Phan**, Naofumi Shimokawa, Mun'delanji C. Vestergaard, Tsutomu Hamada, Masahiro Takagi, Thermotropic liquid crystals formed by oxidized cholesterols. (Chem. Phys. Lipids, in revision)

6. Shafiul Alam, **Huong T.T. Phan**, Mio Okazaki, Masahiro Takagi, Kozo Kawahara, Toshifumi Tsukahara, Hitoshi Suzuki. Computational extraction of a neural molecular network through alternative splicing. (Submitted to BMC Research Notes)

Presentation

<u>International conferences</u>

1. **Huong T.T. Phan**, Takahiro Hata, Masamune Morita, Mun'delanji C. Vestergaard, Tsutomu Hamada, Masahiro Takagi. Dynamics of oxidized-cholesterols-containing membrane induced by Alzheimer’s amyloid beta, International symposium on

Biotechnology for Green Growth Program, Oct. 24–26, 2012, Kobe, Japan. (Oral presentation)

2. **Huong T.T. Phan**, Takahiro Hata, Masamune Morita, Mun'delanji C. Vestergaard, Tsutomu Hamada, Masahiro Takagi. The effect of oxidized cholesterols on the membrane dynamics induced by Alzheimer's amyloid beta, The first International Symposium on Biofunctional Chemistry 2012, Nov. 28–30, 2012, Tokyo, Japan. (Poster presentation)

3. **Huong T.T. Phan**, Takahiro Hata, Masamune Morita, Tsuyoshi Yoda, Tsutomu Hamada, Mun'delanji C. Vestergaard, Masahiro Takagi. Interaction of Alzheimer's amyloid beta with oxysterol-containing membranes, International Symposium of the German Society for Biochemstry and Molecular Biology: Molecular Life Sciences, Frankfurt, Germany, Oct. 3–6, 2013, Frankfurt, Germany. (Poster presentation)

4. Tsuyoshi Yoda, **Huong T.T. Phan**, Mun'delanji C. Vestergaard, Tsutomu Hamada, Masahiro Takagi. Thermo-induced Dynamics of Membranes and Liquid Crystals Containing Cholesterol Derivatives, IEEE Conference of Micro-Nano Mehatronics and Human Science, Nov. 4–7, 2012, Nagoya, Japan. (Oral presentation)

5. Tsuyoshi Yoda, Wataru Inui, **Huong T. T. Phan**, Naofumi Shimokawa, Mun' delanji C. Vestergaard, Tsutomu Hamada, Masahiro Takagi. Effects of lipid oxidation products on phase separation of biomimetic membrane, Soft Condensed Matter Physics Gordon Research Seminar: Complexity in Soft Matter, Aug. 18–23, 2013, Colby-Sawyer College New London, NH USA. (Poster presentation)

6. Tsuyoshi Yoda, **Huong T.T. Phan**, Mun'delanji C. Vestergaard, Tsutomu Hamada and Masahiro Takagi, Thermo-responsive Dynamics of Membranes and Liquid Crystals Containing Cholesterol Derivatives, IGER International Symposium on Cell Surface Structures and Functions , Sep. 1–3, 2013, Nagoya, Japan. (Poster presentation)

<u>Domestic conferences</u>

7. **Huong T.T Phan**, Masamune Morita, Tsuyoshi Yoda, Naofumi Shimokawa, Mun'delanji Vestergaard, and Masahiro Takagi. Effect of cholesterol and 7-ketocholesterol on localization of Alzheimer's amyloid beta (Aβ_42) in membrane domains, The 51th

annual meeting of the Biophysical Society of Japan, Oct. 28 – 30, 2013, Kyoto, Japan (Poster presentation).

8. **Huong Thi Thanh Phan**, Masamune Morita, Tsuyoshi Yoda, Naofumi Shimokawa, Mun'delanji Vestergaard, and Masahiro Takagi. Localization of Alzheimer's amyloid beta (Aβ_42) in membrane domains: role of cholesterol and its oxidation, Hokuriku regional conference in 2013. Nov. 22, 2013, Ishikawa, Japan. (Poster presentation)