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REVIEW ARTICLE

Atomic force microscopy to study molecular mechanisms of amyloid fibril formation and toxicity in Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a devastating neurodegenerative disease characterized by dementia and memory loss for which no cure or effective prevention is currently available. Neurodegeneration in AD is linked to formation of amyloid plaques found in brain tissues of Alzheimer's patients during post-mortem examination. Amyloid plaques are composed of amyloid fibrils and small oligomers – insoluble protein aggregates. Although amyloid plaques are found on the neuronal cell surfaces, the mechanism of amyloid toxicity is still not well understood. Currently, it is believed that the cytotoxicity is a result of the nonspecific interaction of small soluble amyloid oligomers (rather than longer fibrils) with the plasma membrane. In recent years, nanotechnology has contributed significantly to understanding the structure and function of lipid membranes and to the study of the molecular mechanisms of membrane-associated diseases. We review the current state of research, including applications of the latest nanotechnology approaches, on the interaction of lipid membranes with the amyloid-β (Aβ) peptide in relation to amyloid toxicity. We discuss the interactions of Aβ with model lipid membranes with a focus to demonstrate that composition, charge and phase of the lipid membrane, as well as lipid domains and rafts, affect the binding of Aβ to the membrane and contribute to toxicity. Understanding the role of the lipid membrane in AD at the nanoscale and molecular level will contribute to the understanding of the molecular mechanism of amyloid toxicity and may aid into the development of novel preventive strategies to combat AD.

Keywords

Alzheimer's disease, amyloid, amyloid toxicity, atomic force microscopy, cholesterol, lipid membrane, molecular mechanism, nanotechnology

History

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that results in progressive cognitive impairment, including dementia, personality changes, judgment, language skills and memory loss, eventually resulting in the death of the individual. AD is a member of the family of protein misfolding diseases. The 27 protein misfolding diseases identified to date all have an implicated protein that misfolds and aggregates to cause a specific pathology (Sipe et al., 2010; Stefani, 2012). AD is associated with the amyloid-β (Aβ) peptide. Other diseases in the protein misfolding disease family include Parkinson's disease (α-synuclein), Creutzfeldt-Jacobs (PrPc), Pulmonary Alveolar Proteinosis (SP-C) and diabetes (insulin) (Sipe et al., 2010).

The symptoms associated with AD were first identified by Alois Alzheimer in 1907 (Alzheimer, 1907). A significant period of time elapsed between Dr. Alzheimer's identification of the disease and significant progress in the study of AD; it was not until the mid to late 1980s that any real progress on AD was made, with the discovery that the Aβ peptide is correlated with AD symptoms (Masters et al., 1985; Tanzi et al., 1988) and the advancement of the amyloid hypothesis (Selkoe, 1991). Pathologically, AD is identified by the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles (NFT) in the brain tissues during post-mortem examination. It has been shown that Aβ is the primary constituent of these amyloid plaques, and that hyper-phosphorolated tau protein is the primary constituent in NFT's (Selkoe, 1991). Considerable effort has been put into characterizing these plaques in order to elucidate the molecular pathways of Aβ aggregation, with a shared goal of identifying therapeutic approaches to slow the onset of AD. At the micro- and nanoscales, the neuronal plaques consist of amyloid fibrils, which are protein structures with cross-linked beta-strands stacked together. Mature fibrils are a series of protofibrils that are twisted around one another and reach lengths of up to several micrometers, with a diameter of roughly 5–10 nm (Antzutkin et al., 2000). In the past, these fibrils were believed to be causal to AD. However, recent research has demonstrated that these fibrils are relatively inert compared to amyloid oligomers, which are much smaller than the fibrils (Ono et al., 2009). Whether these oligomers are a

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precursor to the amyloid fibril or lie along an off-pathway reaction coordinate is a matter of contemporary debate (Yamaguchi et al., 2010). By the turn of the last century, research results began to show that neuronal synapses were most affected by amyloid neurotoxicity by impairing potentiation as a result of the interaction between amyloid oligomers and the neuronal synapse (Walsh et al., 2002; Walsh & Selkoe, 2004). Concurrently, various groups reported that the misfolding of amyloid proteins was not an abnormal occurrence; rather, it is an intrinsic property of the backbone of any polypeptide chain (Dobson & Karplus, 1999). In addition, it was shown that cytotoxicity is a generic effect of all amyloid oligomers (Bucciantini et al., 2002) and is associated with the initial misfolding of oligomers setting off the amyloid cascade (Kayed et al., 2003; Stefani, 2012).

Post-mortem examinations of patients with Alzheimer's symptoms have shown a reduction in the physical size of the temporal and frontal lobes, hippocampus and amygdala – the regions involved in memory and learning process. This cerebral atrophy is the direct result of neuronal apoptosis and synaptic atrophy appearing concurrently with the presence of amyloid plaques and tau tangles (Mattson, 2004). In addition, inflammatory cytokines formed by degenerating neurons and activated microglia around the amyloid plaques may contribute to the symptoms associated with AD (Mattson, 2004).

Despite extensive research into AD and Aβ, no clear mechanism of action has been uniformly accepted, and little progress has been made in developing pharmaceuticals that eliminate, prevent or even significantly slow the devastation caused by AD. In the past, the presence of amyloid plaques in the brain, which showed apple green birefringence when stained with Congo Red, was the definitive post-mortem diagnoses for AD (Serrano-Pozo et al., 2011). Many cadavers display post-mortem cerebral amyloid plaques even though the living person never presented any Alzheimer's symptoms. Conversely, some people diagnosed with AD do not have any amyloid plaques post-mortem (Davinelli et al., 2011). This inconsistency can be caused by either false diagnoses or poor specificity of the classical Alzheimer's signs post-mortem. Currently, the clinical diagnosis for AD is based on accepted mental and cognitive testing or magnetic resonance imaging when large areas of the brain are already damaged. About 90% of patients diagnosed with AD are found to have AD on autopsy (Knopman et al., 2001). Recent advances have shown promise in making an AD diagnosis using cerebrospinal fluid or blood for patients who have not yet presented with AD symptoms (De Meyer et al., 2010). Some groups are now suggesting that large fibril plaques could play a protective role (Esparza et al., 2013; Yang et al., 2013). Therefore, more effort in elucidating the molecular mechanism of AD and amyloid toxicity is highly desired, considering that the number of AD patients is constantly growing with the elderly population.

Although the exact mechanism by which Aβ produces neurodegenerative symptoms remains unclear, amyloid fibril formation has been studied extensively as a cause and a signature of AD. The proposed mechanism of amyloid fibril formation involves cleavage of the trans-membrane amyloid precursor protein (APP) (O'Brien & Wong, 2011). When APP fragments (such as Aβ35, Aβ40 and Aβ42) become exposed to cellular fluids, they misfold into amyloid fibrils – thin insoluble fibers made up of the Aβ peptide arranged in a cross beta sheet structure (Berhanu & Hansmann, 2012). Fibrils are formed through the sequence of aggregation processes from monomers and oligomers. Whether oligomers are precursors to fibrils or form along a separate pathway is a subject of current research and academic debate.

Most early research on fibrillogenesis has been devoted to characterizing the formation of oligomers and fibrils in aqueous solution, where the effect of temperature, pH and other factors were studied (Kusomoto et al., 1998; Stine et al., 2002). Recent research indicated that fibril plaque formation and neurotoxicity are associated with biological membranes *in vivo*. Aβ neurotoxicity was first identified in the 1980s with the discovery that Aβ is the prime constituent in amyloid plaques (Allsop et al., 1983; Glenner & Wong, 1984; Masters et al., 1985; Hardy & Allsop, 1991). Neurotoxicity is associated with Aβ interactions with neuron cells and results in neuronal cell death. The exact mechanism of this neurotoxicity is still under debate and several mechanisms have been discussed in the literature: (1) the inflammatory effect of Aβ on the cell membrane (Verdier & Penke, 2004); (2) oxidative stress (Mutisya et al., 1994); (3) effect of metals (Sayre et al., 1999); (4) specific and nonspecific interactions with cellular and lipid membranes (Kayed et al., 2003; Lin et al., 2001; Sokolov et al., 2006; Wang et al., 2004) and (5) the damaging effect of Aβ on DNA (Geng et al., 2010).

It has been shown that sequence of Aβ also contributes to toxicity (Buchsteiner, 2012). Even though there is only a 2 amino acid difference in the primary structure of Aβ (1–40) and Aβ (1–42), there is a distinct aggregation pathway (Bitan et al., 2003) and toxicity (Dahlgren et al., 2002) between the two isoforms. Aβ (1–42) has a higher aggregation rate, higher propensity to bind metal ions and higher toxicity levels than Aβ (1–40) (Bitan et al., 2003; Bush & Tanzi, 2008).

When considering the role of cell membrane in amyloid toxicity, it is important to note that amyloid deposits are known to affect the synapse and are not uniform in the cerebral parenchyma, but Aβ is uniformly expressed and Aβ (1–42) is a normal constituent of all cerebrospinal fluid and is present in non-AD patients. Second, amyloid deposition increases with age, yet amyloid production does not. It appears that processes that clear amyloid deposits are diminished with age, as are mechanisms to protect against redox effects (Bush & Tanzi, 2008). A difference between Aβ production and Aβ clearance is likely an underlying factor in the AD process (Greenough et al., 2012).

Research by Lal's group indicates that oligomeric Aβ can form ion channels in lipid membranes, resulting in higher levels of intracellular calcium (Lin et al., 2001). Perturbations in membrane fluidity have been also suggested by Kremer et al. (2000) and Muller et al. (1998). Free radical production has been identified by Butterfield et al. (1999) and changes in lipid metabolism have been demonstrated by Koudinov et al. (1998). Other examples of cellular damage by amyloid include changing of membrane permeability (Abramov et al., 2011), induction of cell apoptosis (Loo et al., 1993), the formation of ion channels in cell membrane altering ion homeostasis (Arispe et al., 1993), the production of toxic

levels of hydrogen peroxide (Behl et al., 1994) and the thinning of the membrane (Sokolov et al., 2006).

These studies indicated that the mechanism of membrane toxicity induced by Aβ is very complex and still poorly understood (Berthelot et al., 2013). These studies also demonstrate the need to elucidate the role of the lipid membrane surface in the mechanism of amyloid fibril formation and toxicity at the nanoscale and molecular level. High-resolution imaging techniques such as atomic force microscopy (AFM) provide a powerful nanotechnology method with which to approach this challenging task.

Nanotechnology and AFM

Nanotechnology has contributed to the progress of modern medicine, contributing significantly to understanding the molecular mechanisms of disease and the development of novel methods for diagnostics and cure, which has resulted in the development of the modern discipline of nanomedicine. We focus specifically on one nanotechnology method – AFM. The AFM is a member of the scanning probe microscopy (SPM) family, which includes more than 20 scanning probe methods, such as scanning tunneling microscopy, Kelvin probe force microscopy (KPFM), near-field scanning optical probe microscopy and many other methods that appeared with the development of nanotechnologies. SPM has proven to be a remarkable tool for advancing Alzheimer's research (Connolly & Smith, 2010). It has also provided definitive nanoscale insight into the structure of amyloid fibrils, oligomers and their interactions with lipid membrane and each other (Lal & Arnsdorf, 2010). AFM and related SPM methods employ a sharp scanning probe (AFM tip) which is rastered over the surface of the sample. The probe is attached to a flexible beam (cantilever) which interacts with the sample through various forces (van der Waals, electrostatic). These forces are detected at each point following the deflection of the cantilever (Figure 1) and are used to produce a nanoscale 3D image with single molecule level resolution (Binning et al., 1986). The resolution of these methods is not limited by the diffraction of light as in optical microscopy, but is instead limited by the sharpness of the scanning probe, typically 2–10 nm. However, even atomic resolution can be achieved with an appropriate tip. With AFM, high-resolution three-dimensional images of biomolecules in physiologically relevant conditions can be obtained. In addition to imaging, AFM can be used in force spectroscopy mode, which allows the measurement of forces between the tip and the sample as the probe is brought repeatedly to and from the sample surface. This method allows for the studying of important mechano-elastic properties of the sample (including live cells) such as Young's modulus and adhesion (Burke et al., 2013). Because these physical properties of the cell are related to cellular function, AFM can thus be used to study biomechanical processes of the cell in order to understand not only cell morphology, but also cellular processes such as migration and division (Radmacher, 2007). Work by Burke et al. (2013) demonstrated that amyloid proteins decreased localized Young's modulus and adhesive properties in lipid membranes. When the molecules of interest are attached to the probe, binding forces between two individual molecules can be measured (Benoit et al., 2000; Hinterdorfer & Dufrene, 2006; Hane et al., 2013). AFM has been widely used to study various biological samples such as lipid membranes, cells, DNA and proteins (Hansma & Hoh, 1994).

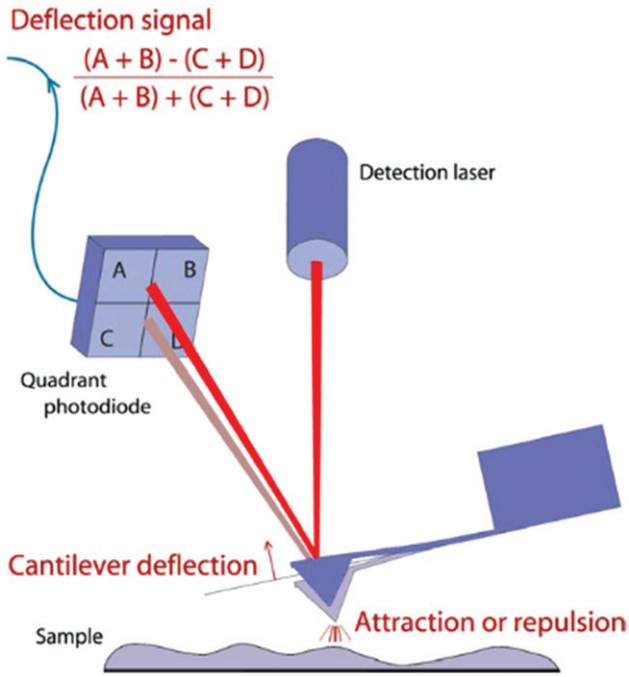

Figure 1. Schematics on AFM. Adapted from JPK Instruments AFM Handbook, 2005 (Image reproduced with permission from JPK Instruments, http://www.jpk.com).

AFM to study amyloid fibril formation and toxicity in AD

In this section, we review our recent work and other AFM studies in relation to AD and amyloid toxicity.AFM Imaging of fibrils and oligomers.

AFM has provided researchers with the nanoscale morphology of amyloid structures, revealing truly remarkable details into the etiology of neurodegenerative protein misfolding diseases. Much research has been done to characterize these fibrils and small oligomers with AFM imaging, beginning with work done by Krafft's group comparing fibrils imaged by EM to fibrils imaged by AFM (Stine et al., 1996). The authors were able to show the axial periodicity of the fibril structure, confirming structures obtained using electron microscopy techniques and confirming the fibril diameter to be 5–12 nm (Stine et al., 1996). With the increased resolution provided by AFM, many other groups were also able to obtain higher quality images of amyloid fibrils and oligomers (for example Adamcik et al., 2010; Campioni et al., 2010; Goldsbury et al., 1999; Harper et al., 1997; Irwin et al., 2013; Mastrangelo et al., 2006; Moores et al., 2011; Norlin et al., 2012; Ridgley & Barone, 2013; Segers-Nolten et al., 2007; Wang et al., 2003).

Amyloid fibril formation has been studied extensively in solution where the interaction between peptide molecules is mainly considered. According to the amyloid hypothesis (Chiti & Dobson, 2006; Petkova at al., 2005), interactions between amyloidogenic peptides in solution results in the

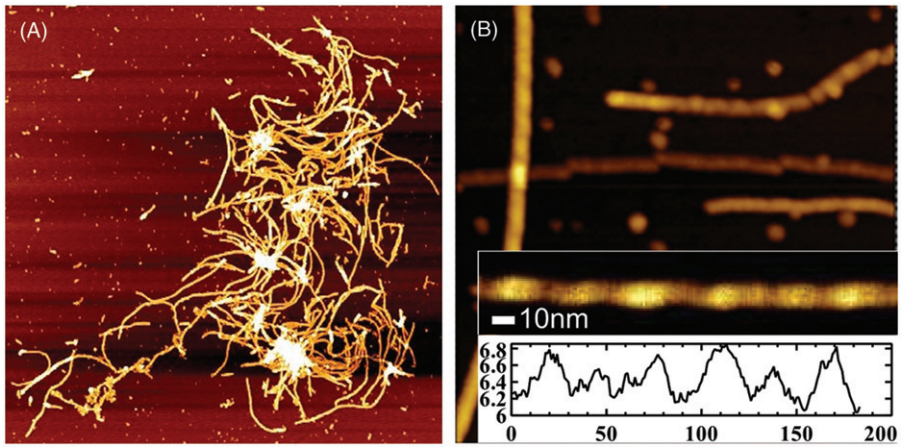

Figure 2. Amyloid peptide Aβ (1–42) incubated in solution for 24 hours and then deposited on mica and imaged in air. A 5×5 μm scan area (A) of the surface shows many fibrils and large fibril clusters, ranging from 100 nm to several μm. A high-resolution image (B) shows that the fibrils are tightly wound together forming helices, scan size is 500×500 nm. (B, adapted from Moores B, Drolle E, Attwood SJ, et al. (2011). Effect of surfaces on amyloid fibril formation. PLoS ONE 6:e25954).

formation of small oligomers and long fibrils with twisted morphology (Blackley et al., 1999; Chiti & Dobson, 2006; Lansbury, 1997; Moores et al., 2011), which elongate by attaching monomer units to the end of growing protofibrils. A left-handed helical twist in the fibril structure and a periodicity of 43 nm was reported (Lansbury, 1997). Interestingly, the structures of fibrils formed in solution are different from the protofibrils formed on surfaces (Blackley et al., 2000; Rocha et al., 2005; Zhu et al. 2002). Mastrangelo et al. (2006) used AFM to track the growth of amyloid oligomers on substrates, demonstrating that fibrils form as self-dimerizing monomers stack upon one another. We showed that after 24 h of incubation in solution, Aβ (1–42) forms fibrils that are long, continuous and twisted together into helices (Figure 2) with a continual increase in the length and height of amyloid fibrils both as a function of time and of peptide concentration in solution. The AFM images demonstrate that in addition to the large fibrils, small oligomers are also present in solution. Lansbury and colleagues suggested that there may be different pathways for fibril and oligomer formation, prompted by their observations that only certain prefibrillar species, when added to monomeric solution, ''seed'' the solution and accelerate aggregation time by having the solution skip the lag phase of aggregation. Importantly, it was reported that fibrils formed in solution are identical in structure to fibrils isolated from AD cerebral plaques (Lansbury, 1997).

A number of factors have been identified that alter the kinetics of amyloid fibril formation in solution: lowered pH, increased temperature and agitation as well as increased concentration of amyloid have all been shown to accelerate amyloid aggregation (Harper & Lansbury, 1997; Buell et al., 2012; Crespo et al., 2012). It has also been shown that the toxicity of amyloid species is highly dependent on the structural flexibility and exposure of hydrophobic residues of the peptide (Campioni et al., 2010).

Effect of surfaces on amyloid fibril formation

It has been emphasized that amyloid peptides may interact with the surfaces of lipid membranes. This theory has initiated numerous studies aiming to elucidate the effect of surfaces on amyloid fibril formation.

A growing number of recent research contributions emphasized the importance of cell membrane surfaces (Ambroggio et al., 2005; Choucair et al., 2007; Cordy et al., 2006; Dobson, 2001; Ege et al., 2005; Kayed et al., 2004; Lin et al., 2001; Quist et al., 2005; Sharp et al., 2002; Terzi et al., 1995; Yip et al., 2001) for amyloid toxicity and in particular the role of electrostatic interactions between the lipid membrane and amyloid-forming proteins (Choo-Smith et al., 1997; Kakio et al., 2002; Yanagisawa et al., 1995). While cell membrane surfaces are extremely important in amyloid toxicity, interpreting results from heterogeneous and complex systems like plasma membranes is often very difficult. Chemically modified surfaces with well-defined functionality can serve as initial simplified models to elucidate the effect of surfaces on amyloid binding and fibril formation, including electrostatic interactions.

Surfaces play a crucial role in amyloid fibril formation for many amyloidogenic peptides. The size and shape of amyloid aggregates and fibrils, formed on surfaces, as well as the kinetics of their formation, are affected by the physicochemical nature of the surface. A number of surfaces including nanoparticles (Linse et al., 2007; Pronchik et al., 2010), graphite (Brown et al., 2002) and mica (Zhu et al., 2002) have been studied to determine their effect on fibril formation. Surfaces have been shown to significantly accelerate the formation of amyloid fibrils for a variety of peptides compared to incubation in solution (Powers & Kelly, 2001; Zhu et al., 2002). This phenomenon may be attributed to increased local concentrations caused by surface interactions forcing peptide diffusion into a 2D plane (Shen et al., 2012). In addition to accelerating fibril formation, several authors reported that surfaces have an effect on the structure of fibrils (Blackley et al., 1999, 2000; Goldsbury et al., 1999; Qin et al., 2007; Zhu et al., 2002). For many peptides, fibrils formed on surfaces lack the twisted and intertwined morphology which is characteristic of fibrils grown in solution (Blackley et al., 2000), α-sinuclein and SMA (Zhu et al., 2002). The mechanism of fibril formation on surfaces includes nucleation and elongation (Blackley et al., 1999, 2000; Kowalewski & Holtzman, 1999; Zhu et al., 2002), where protofibrils elongate in various directions radiating from the central core, resulting in branched structures (Blackley et al., 2000; Moores et al., 2011; Zhu et al., 2002).

Multiple studies have illustrated the important role that surfaces play in amyloid fibril formation; however, these data are difficult to compare due to different research groups

conducting their studies under varying experimental conditions, such as pH, temperature, surface type and peptide type (Giacomelli & Norde, 2005; Hammarström et al., 2008; McMasters et al., 2005; Qin et al., 2007; Uversky et al., 2001; Yokoyama & Welchons, 2007). Along with experimental conditions, limited surface types studied (Powers & Kelly, 2001) make comparisons for the same type of amyloid proteins difficult (Hammarström et al., 2008; Zhu et al., 2002).

Electrostatic interactions with surfaces are of high importance, considering that Aβ is a charged (−3), polar molecule at physiological pH (pI = 5.5; Rauk, 2009). This is especially relevant as many membrane surfaces are charged and may therefore influence amyloid aggregation through electrostatic forces (Choo-Smith et al., 1997; Kakio et al., 2002; Yanagisawa et al., 1995).

Using AFM, we studied the electrostatic and hydrophobic interaction of Aβ (1–42) with three different surfaces: hydrophobic (CH_3); negatively charged (COOH); and positively charged (NH_2) surfaces at pH 7.8, at 37 °C, in order to provide a direct comparison on the effect of these surfaces (Moores et al., 2011). A progressive accumulation of Aβ deposits with time was observed on all three surfaces (Moores et al., 2011). We showed that the surface charge and hydrophobicity affects the structure, amount and surface coverage of Aβ deposits: hydrophobic CH_3 surfaces promoted the formation of amorphous aggregates while the charged NH_2 and COOH surfaces promoted small oligomers and small protofibrils. Interestingly, the structures of fibrils formed in solution are different from the protofibrils formed on surfaces (Blackley et al., 2000; Moores et al., 2011; Rocha et al., 2005; Zhu et al., 2002). Instead of characteristic twisted morphologies observed for fibrils formed in solution (Figure 2B), we observed the formation of small oligomers, which covered the surface of substrate completely and formed a monolayer of densely packed oligomers (Figure 3). The presence of small oligomeric units on surfaces and in solutions was also reported by others (Blackley et al., 1999, 2000, Rocha et al., 2005) for Aβ (1–40). Stable oligomers for both Aβ (1–40) and Aβ (1–42) were isolated also from brain and synthetic amyloid material. Dimeric (9 kDa) and trimeric (13.5 kDa) forms (Blackley et al., 1999; Roher et al., 1996) of Aβ have been previously observed by size-exclusion chromatography, whereas incubation of monomeric Aβ has led to the separation of 4-, 19- and 46-kDa fragments (Dyrks et al., 1993). There have also been reports from other groups of the

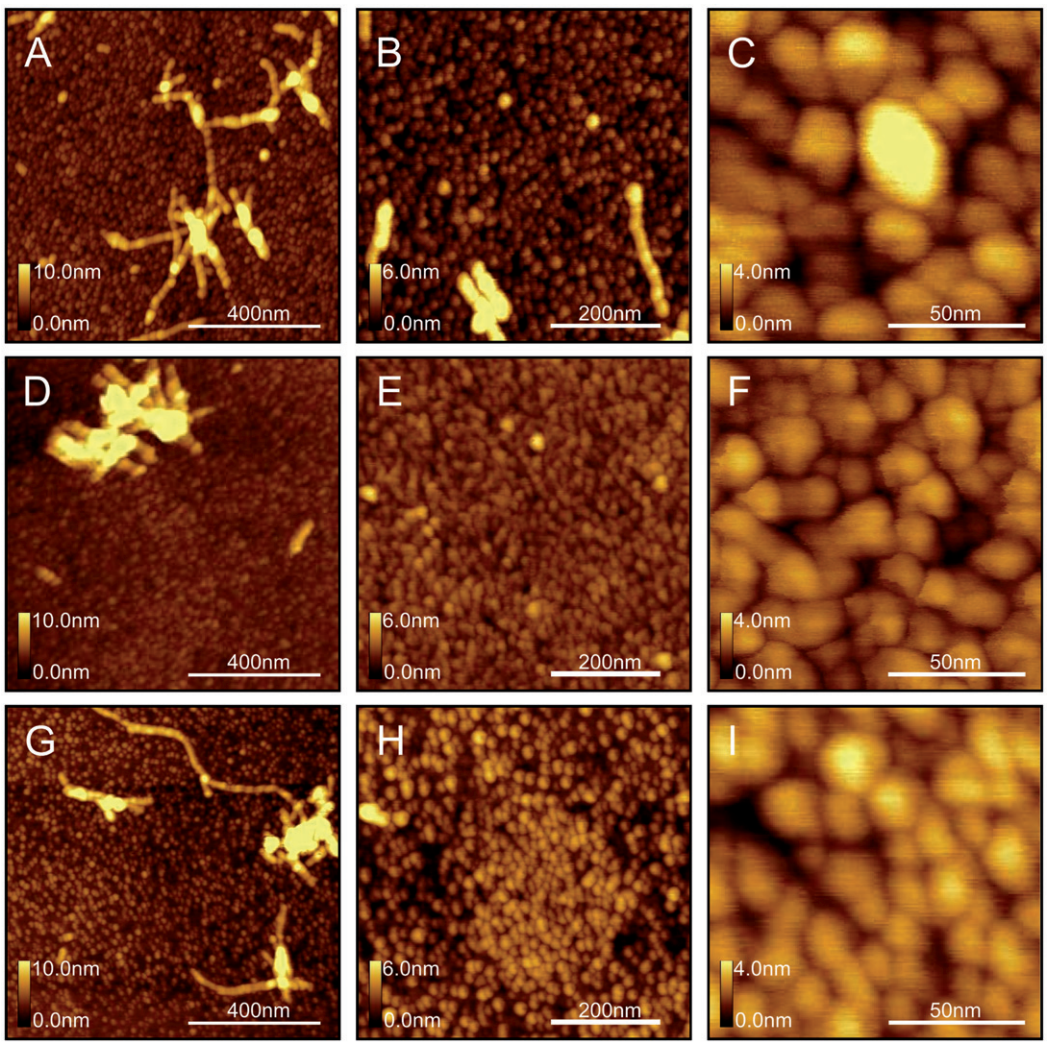

Figure 3. Small scan size images of AFM topography of Aβ aggregates formed on modified surfaces: CH_3– (A–C), COOH– (D–F), and NH_2– (G–I) modified surfaces, after incubation with Aβ 1–42 solution (500 μg/ml) for 1 h at 37 °C (Image reproduced from Moores B, Drolle E, Attwood SJ, et al. (2011). Effect of surfaces on amyloid fibril formation. PLoS ONE 6:e25954).

presence of oligomer units (Kuo et al., 1996; Levine, 1995; Roher et al., 1996). The deposition of small oligomers on all surface types was observed; however, the surface type influenced the shape and the size of the oligomers present. Interactions with the hydrophobic surface resulted in spherical oligomers with broader size distribution, while Aβ oligomers on charged surfaces were of triangular shape with a more narrow size distribution. This indicates that electrostatic interactions with the surfaces may affect the oligomer folding, and therefore the shape of the smaller building blocks. Triangular-shaped oligomers were proposed by simulations of trimers by Paravastu et al. (2008). As well, Wang et al. (2010) used molecular dynamics simulations to show that Aβ packing is affected by electrostatic interactions with surfaces; Aβ is relatively free to move at the CH_3 surfaces and may result in a more disordered surface than Aβ interaction with charged COOH and NH_2 surfaces, in which stronger electrostatic interactions of the Aβ may result in more ordered appearance of the Aβ (Wang et al., 2010). Building of protofibrils later in time occurs by adding small spherical building blocks to the surface bound oligomers (Figure 3A), which result to elongation in both directions and formation of fibrils with branched morphologies (Blackley et al., 2000; Moores et al., 2011; Zhu et al., 2002).

At longer times of incubation (22 h), the density of larger amyloid clusters was observed to be highest on CH_3-modified surface, lower on COOH-modified surfaces and lowest on NH_2-modified surfaces (Moores et al., 2011) (Figure 3).

It is clear that electrostatic interactions with surfaces directly influence Aβ fibril formation, which can be explained by the complex charge distribution in the Aβ peptide and its secondary structure dependence. The peptide has a net charge of −3, with six negatively charged residues and three positively charged residues, and an isoelectric point of approximately 5.5 (Rauk, 2009). We showed that a complex charge distribution develops in oligomers (dimers and pentamers) as compared to monomers (Moores et al., 2011). According to our analysis of electrostatic potential distribution (Moores et al., 2011), Aβ monomer, dimer or larger oligomers all differ in their surface charge distribution. In a monomeric α-helix structure, the charge is fairly evenly distributed to prevent a dipole from forming. A larger collective polarity, which induces stronger electrostatic surface interactions, is observed in oligomers in β-sheet conformations, as well as preferential ordering of the oligomers on the surfaces. This may be the driving force for oligomer binding and the reason for the more ordered and fibril-like structures that are seen on charged surfaces, compared to hydrophobic CH_3-modified surfaces. This may have an effect on binding of Aβ to charged or polar lipid molecules, which compose biomembranes.

Therefore, based on this analysis, we conclude that α-helical peptide clusters preferentially form on the hydrophobic CH_3 surface, and β-sheet clusters of various sizes form on the negatively charged COOH and positively charged NH_2 surfaces. This correlates with findings by other authors: McMasters et al. (2005) investigated amyloid fibril formation of the Aβ peptide on chemically modified mica bearing positively or negatively charged, or hydrophobic functional groups at pH 11.5 and showed that all surfaces cause adsorption/deposition of Aβ (10–35) peptide. Deposits were composed of peptides in β-sheet, β-turn, random coil and α-helical conformation. The equilibrium on hydrophobic monolayer was shifted toward an α-helix form. This is consistent also with findings by Giacomelli & Norde (2005), who showed that conformation of the Aβ (1–40) peptide (at pH 7 and 10, at 25 °C) strongly depends on the hydrophobicity of the surface. They observed intramolecular α-helix formation due hydrophobic interactions on the Teflon surfaces in contrast to intermolecular β-sheet formation due to electrostatic interactions on the mica surfaces (Giacomelli & Norde, 2005).

Effect of lipid membrane on amyloid fibril formation

AFM along with other studies confirmed the proposed mechanism of amyloid fibril formation and demonstrated the formation of Aβ oligomers and fibrils from monomeric solution of Aβ. The structure of both oligomers and fibrils formed in solution and on surfaces has been extensively studied at the nanoscale and at a great level of detail. Yet, the mechanism of amyloid toxicity still is not well understood.

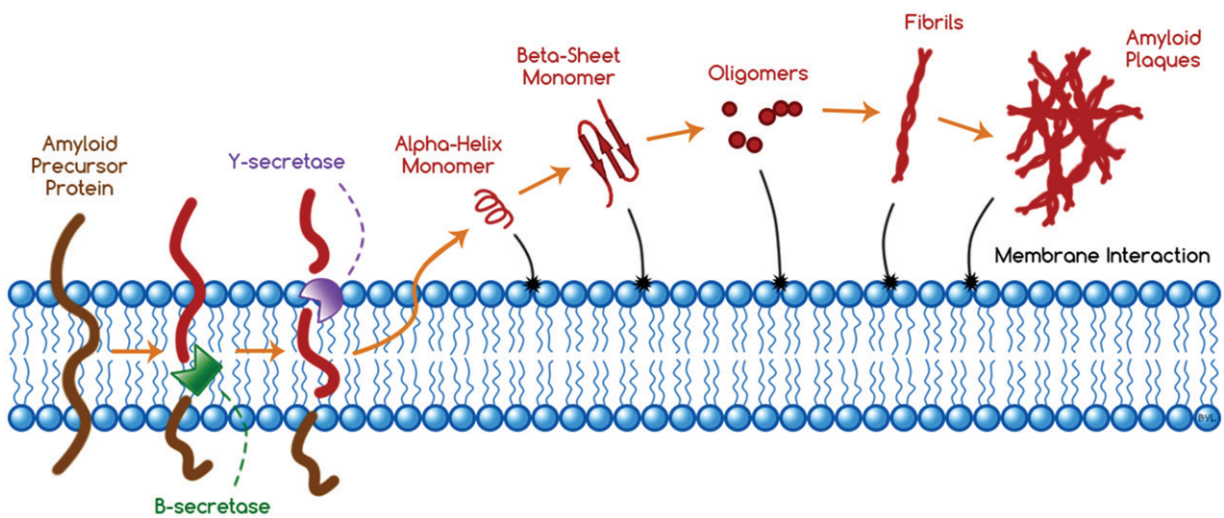

Figure 4. Schematic showing amyloid origin and the mechanism of the amyloid fibril formation and toxicity.

Amyloid fibril formation is a multiple state process, which starts with the cleavage of the amyloid fragments from the transmembrane APP, misfolding of Aβ monomers that form various structures, such as unfolded clusters, beta-sheet oligomers, larger fibrils and amyloid plaques. Due to the close proximity to the cell surfaces, these amyloid aggregates may interact strongly with the membrane (Figure 4). Increasing evidence supports the hypothesis that the state of the cell membrane (composition, morphology and other physicochemical properties) plays an important role in amyloid toxicity and AD.

To account for role of the cellular membrane, whose composition is known to change with age and in AD (Bartzokis, 2004; Farooqui et al., 1995, 1988; Selkoe, 1980; Wells et al., 1995), it has been hypothesized that within the cellular membrane itself, raft domains, enriched in cholesterol and sphingolipids, may constitute the environment in which amyloid-forming proteins cluster, thus increasing amyloid aggregation and toxicity.

A growing number of recent research contributions suggest the importance of the lipid membrane for amyloid fibril formation and toxicity (Abramov et al., 2011; Cordy et al., 2006; Ege et al., 2005; Kinnunen, 2009; Sasahara et al., 2013; Sharp et al., 2002; Tofoleanu & Buchete, 2012; Yip et al., 2001).

These studies emphasize the effect of charged lipids, pH, metal cations and cholesterol on the effectiveness of fibrillogenesis, suggesting that the interactions between the lipid membrane and amyloid are important. The stronger interaction of amyloid oligomers with cell membranes may induce the higher toxicity and thus disruption of the membrane function. Therefore, by controlling membrane properties, one may prevent the amyloid toxic effect and disease initiation. It is necessary to continue systematic studies in order to understand the physico-chemical properties of the cell and model membrane and the role they play in the amyloid toxicity.

Biological membranes are very complex structures, mainly composed of phospholipids and enriched with multiple transmembrane proteins and sterols assembled in rafts. Due to this complexity, lipid bilayers and monolayers are widely used to mimic biological membranes (Ohvo-Rekila et al., 2002) in order to study their structure and interaction with biomolecules, including amyloid peptides, nanoparticles and drugs (Bonn et al., 2004; Choucair et al., 2007; Hane et al., 2011; Yip et al., 2001).

Electrostatic and hydrophobic interactions between amyloid oligomers and lipid membranes play an important role during initial amyloid pathogenesis. Several groups have focused their efforts on elucidating the role of electrostatic and hydrophobic interactions of Aβ peptides with model lipid membranes and monolayers. Using a combination of ^{31}P MAS NMR (magic angle spinning nuclear magnetic resonance) and circular dichroism (CD) spectroscopy, Bokvist et al. suggested fundamental differences in the functional organization of supramolecular Aβ (1–40) membrane assemblies for two different scenarios with potential implication in AD: Aβ peptide can either be firmly anchored in a membrane upon proteolytic cleavage, preventing release and aggregation, or it can have fundamentally adverse effects when bound to membrane surfaces by undergoing accelerated aggregation, causing neuronal cell death (Bokvist et al., 2004). Their results suggested that two different molecular mechanisms of peptide membrane assemblies are involved in Aβ pathophysiology with the finely balanced type of Aβ–lipid interactions against release of Aβ from neuronal membranes and production of toxic β-structured aggregates. Therefore, pathological interactions of Aβ with neuronal membranes might not only depend on the oligomerization state of the peptide, but also on the type and nature of the supramolecular Aβ–membrane assemblies (Bokvist et al., 2004). Bokvist et al. proposed that Aβ interacts with lipid membranes by binding and insertion of the peptide in its monomeric form into the lipid membrane. It was hypothesized that these processes are driven by the interplay between electrostatic and hydrophobic interactions. This finding correlates with previous research on lipid monolayers showing that electrostatic interactions with negatively charged lipids increase peptide adsorption onto the monolayer at the air–water interface (Maltseva et al., 2005). It has also been proposed that the strong electrostatic interaction between the peptide and negatively charged lipids drives the peptide deep into the monolayer (Ege et al., 2005). Other authors (Jang et al., 2010, 2011; Lin et al., 2001; Quist et al., 2007) proposed that membrane can be damaged due to the creation of ion channels or pores, which induce cell malfunction by an unregulated toxic ion current (Abramov et al., 2011). In summary, the mechanism of Aβ toxicity is still not well understood, showing a discrepancy between suggested non-specific membrane alteration (Williams et al., 2011) and ion-channel formation suggested on the base of membrane reconstituted Aβ (Jang et al., 2011; Lin et al., 2001), and contradictory non-ion channel but membrane perforation alteration mechanisms (Sepulveda et al., 2010). In addition, molecular dynamics simulations by Jang et al. suggested various steps of membrane disorder due to the binding of Aβ peptides (Jang et al., 2013). Summarizing these studies, we propose that the molecular mechanism of Aβ toxicity involves several pathways of Aβ interactions with the lipid membrane: (1) binding of Aβ monomers and oligomers to the surface of lipid membrane and accumulation of Aβ deposits of the surface of lipid membrane without destroying membrane structure or integrity (Figure 5A); (2) penetration of Aβ into the membrane, creating disorder in the membrane structure and inducing unstructured defects and perforation (Figure 5B); and (3) induction of ion channels by Aβ (Figure 5C). We postulate that the prevalence of one pathway over another and the significance of the induced damage may strongly depend on the physical and chemical properties of the lipid membrane itself. Thus, the membrane structure and integrity may be important factors to take into account while considering strategies to overcome AD and related neurodegenerative disease.

Although other methods have been employed to approach this challenging task, AFM has been especially useful as it provided the possibility to directly visualize interactions of Aβ with the lipid membrane at the nanoscale and in physiologically relevant conditions.

Using AFM, it has been demonstrated that lipid composition, the phase of lipid membrane and presence of cholesterol-induced domains (rafts) affect amyloid aggregation

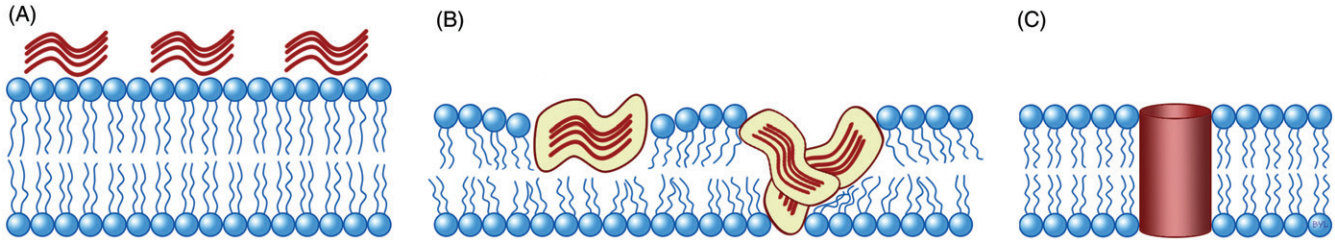

Figure 5. Schematic showing proposed mechanisms of amyloid interactions with lipid membrane. A – adsorption of Aβ on the surface of lipid membrane, building deposits on the top of membrane, B – partially penetrating inside the membrane, inducing membrane disorder and irregular pores, C – ion channel formation.

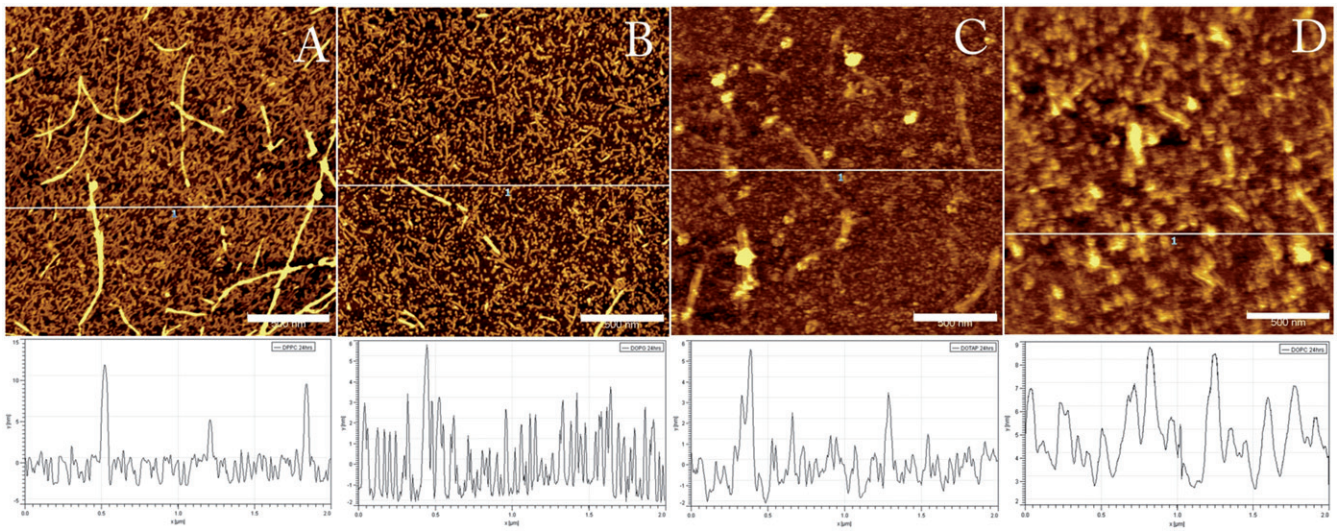

Figure 6. Amyloid β incubated on zwitterionic DPPC (A), anionic DOPG (B), cationic DOTAP (C) and zwitterionic DOPG (D). Incubation times pictured were 24 h (Image reprinted from Hane F, Drolle E, Gaikwad R, et al. (2011). Amyloid-beta aggregation on model lipid membranes: An AFM study. J Alz Dis 26:485–494, with permission from IOS Press).

(Choucair et al., 2007; Drolle et al., 2012; Goldsbury et al., 1999; Gorbenko & Kinnunen, 2006; Hane et al., 2011; Sheikh et al., 2012; Yip et al., 2001).

Lipid composition

Previously, we used AFM to investigate how time and membrane composition affected the aggregation of Aβ-42 on model phospholipid membranes, in particular looking at the effects of lipid head group charge and lipid membrane phase. AFM helped to directly resolve the nanoscale structure of amyloid oligomers and fibrils as well as the structure of planar supported lipid bilayers before and after incubation with Aβ-42. In order to study how amyloid binding is affected by lipid charge and phase, we used the following: neutral, gel phase, dipalmitoylphosphatidylcholine (DPPC); neutral, fluid phase 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC); anionic, 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(3-lysyl(1-glycerol)) (DOPG) and cationic, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). We showed that interactions of the Aβ oligomers with the membrane surface were affected by the charge on the lipid head group, changing the rate of adsorption and causing membrane disruption (Figure 5). We observed that the accumulations of amyloid aggregates are larger on the surface of DPPC membrane than that on DOPG and DOTAP membranes, where the oligomers and short fibrils are well resolved on the surface of supported membrane by AFM. On the positively charged DOTAP, small oligomers show reduced height on the surface of the membrane and disturb its surface, likely penetrating into the membrane. AFM topography images of a supported lipid membrane with Aβ are shown in Figure 6.

Our data showed that Aβ accumulates progressively on the surface of the zwitterionic DPPC and anionic DOPG membranes, according to the schematic representation in Figure 5(A). Amyloid aggregates incubated on cationic DOTAP and zwitterionic DOPC permeate into the membrane to a greater extent than DPPC and DOPG (Figure 6C and D). This observation correlates with the scenario proposed in Figure 5(B). Furthermore, we observed more membrane disruptions on the DOTAP and DOPC membranes compared to the DPPC and DOPG membranes. We attributed this difference to the electrostatic interactions between the lipid membranes and the Aβ and also to the difference in the phase of the lipid membranes. Both DPPC and DOPC have identical charge and type of head group but exist in different phases at room temperature: DPPC is in a gel phase, characterized by higher order in lipid tails, and DOPC is in fluid phase, characterized by lesser order lipid tails and large dynamics of lipid molecules (Ohvo-Rekila et al., 2002).

Our data correlate with findings by other authors, including Sabaté et al. (2012) and Bokvist et al. (2004), who observed stronger stabilizing interactions with negatively charged lipid models. Molecular dynamics simulations by Poojari et al. (2013) also demonstrated that zwitter ionic and anionic lipid surfaces promote Aβ stability in the bilayer. The affinity of Aβ for anionic lipids has been shown to affect the amyloidosis process (Maltseva et al., 2005). Ege et al. (2005) reported that cationic lipids can bind Aβ just as readily as anionic lipids. This correlates with our data that Aβ binds to both positively and negatively charged surfaces (Moores et al., 2011) as well to positively and negatively charged membranes (Hane et al., 2011).

In solution, Aβ reaches a reversible equilibrium of monomers and unstructured aggregates in random coils and oligomeric β sheet structures. Research by Terzi et al. (1995) has demonstrated that in the presence of negatively charged membrane lipids, this random coil and β sheet equilibrium shifts almost completely toward the β sheet conformation. It was hypothesized by Terzi et al. (1995) that this equilibrium shift is a result of the positively charged part of the Aβ peptide being attracted to the anionic lipids in the membrane forming a higher local surface concentration of the peptide on the surface. A combination of increased surface concentration and specific alignment of the peptide creates ideal conditions for the formation of amyloid fibrils. This process may act as a catalyst, reducing the activation energy required to correctly position the peptide chains, or the presence of the membrane may shift the thermodynamic equilibrium to favor the β sheet conformation (Terzi et al., 1995). We showed that the charge distribution in Aβ42 changes upon oligomer formation, making it a more pronounced dipole or multipole with growing oligomer size (Moores et al., 2011). This makes interactions with either cationic or anionic lipid membrane more preferable.

In addition, we demonstrated that membranes in the fluid phase, both cationic DOTAP and zwitterionic DOPC, bind Aβ and show larger penetration of Aβ into the membrane, compared to gel phase membranes, producing larger defects in the membrane. This is illustrated in the schematic in Figure 5(B). In addition, Kremer et al. (2000) demonstrated that Aβ changes membrane fluidity upon binding equally among different lipids. We and others showed that the lipid phase appears to be influential in the ability of Aβ to bind and penetrate the lipid bilayer (Ahyayauch et al., 2013; Choucair et al., 2007; Hane et al., 2011).

The fragment of peptide itself is important for the interactions of Aβ with lipid membrane. Ionov et al. (2010) found that Aβ (1–28) preferentially interacts with the hydrophilic part of the model membranes, while Aβ (25–40) locates itself in the hydrophobic core of the bilayer where it reduces the order of phospholipid packing.

In summary, the charge and the phase of the lipid membrane as well as the structure of Aβ itself are important factors for Aβ interactions with lipid membrane (Ahyayauch et al., 2013; Choucair et al., 2007; Ege et al., 2005; Hane et al., 2011; Ionov et al., 2010; Maltseva et al., 2005), which is an important initial step of amyloid fibril formation in the AD brain (Yanagisawa et al., 1995) and *in vitro* (Choo-Smith et al., 1997; Terzi et al., 1995).

Effect of cholesterol

Studies of Aβ interactions with membranes are of great interest to researchers, because the plasma membrane serves as the site of the accumulation of amyloid plaques. Earlier, we reviewed the data where model membranes of simple composition were studied in this relation, focusing on lipid charge and phase. However, because of the great complexity of membranes, the presence of lipid rafts and sterols may have an important effect on amyloid binding and toxicity. Cholesterol is an essential component for the formation of rafts within the membrane, and therefore, its effect on the interactions of amyloid with the membrane could be a key component of the molecular mechanism of amyloid toxicity. Although amyloid fibril plaque accumulation occurs on the surface of plasma membranes *in vivo*, the role of cell membrane rafts and their composition on Aβ fibril formation and toxicity are still not well understood.

Cholesterol is known to regulate various important functions of the membrane and is involved in signaling, molecule assembly, membrane fluidity and membrane protein trafficking (Giordani et al., 2008; Lingwood & Simons, 2010). The effect of cholesterol on the membrane is very complex and is still a matter of debate, mainly because of various factors that influence its effect, including the type and phase of membrane lipids (Ohvo-Rekila et al., 2002) and the concentration of cholesterol (Lipowski, 1995). Previous works (Bonn et al., 2004; Cadenhead, 1985; Demel & De Kruyff, 1976; McMullen et al., 2004; Ohvo-Rekila et al., 2002) have demonstrated that cholesterol plays a key role in controlling the fluidity, permeability and mechanical strength of the lipid membrane, as well as interfering with lipid phase transitions. These effects are also dependent on cholesterol concentration.

It has been hypothesized that lipid raft domains in cell membranes, enriched with cholesterol and sphingolipids, could provide an environment which promotes Aβ binding and amyloid formation. It has been shown that soluble Aβ aggregates bind to raft microdomains enriched by clusters of gangliosides (Williamson et al., 2008) and mediate membrane oxidative damage (Zampagni et al., 2010). A growing number of recent research contributions suggest the importance of the lipid rafts in amyloid fibril formation and toxicity (Cordy et al., 2006; Ege et al., 2005; Sharp et al., 2002; Yip et al., 2001). These studies emphasize the effect of charged lipids, pH, metal cations and cholesterol on the effectiveness of fibrillogenesis, suggesting that electrostatic interactions between the lipid membrane and amyloid proteins are important. The role of electrostatic interactions and detailed understanding of lipid electrostatic non-homogeneity is of particular interest in this regard. A recent study by Sheikh et al. (2012) also suggests that membrane interactions with Aβ monomers in the membrane greatly depend on cholesterol content, as well as lipid composition and phase state. In this study, cholesterol's presence in systems of DOPC and DPPC caused an increase in the initial kinetics of the area coverage of amyloid-β on the lipid bilayer surface by as much as two orders of magnitude, which suggests an increased membrane affinity for amyloid when cholesterol is present (Sheikh et al., 2012). Indeed, a recent molecular dynamics simulation study

has shown that cholesterol can act as a promoter for membrane–amyloid interactions, because its presence makes the binding process of amyloid to the membrane more energetically favorable (Yu et al., 2012).

Although cholesterol is an important constituent of lipid rafts (Zampagni et al., 2010), the role of cholesterol is not clear in the molecular mechanism of amyloid toxicity. In fact, the effect of cholesterol on amyloid toxicity in relation to AD is controversial. On one hand, research has shown an adverse effect of cholesterol on amyloid toxicity: membranes containing sterols, such as cholesterol, have been shown to activate the fibrillogenesis of Aβ suggesting a specific sterol binding site that accelerates amyloid aggregation (Refolo et al., 2000; Ryan et al., 2012). In addition, lipid membranes can catalyze the conversion of monomeric Aβ into toxic amyloid aggregates (Relini et al., 2009). Interactions with the cell membrane, especially in areas containing cholesterol-rich lipid rafts, are considered important for neurotoxicity (Kakio et al., 2003). Fahrenholz and colleagues among others have shown that cholesterol increases the cleavage of Aβ from APP (Fassbender et al., 2001; Kojro et al., 2001). Cholesterol has been shown to influence the fluidity of total brain extract and the extent of Aβ fibrillogenesis (Yip et al., 2001). These data showed a correlation between membrane cholesterol level and Aβ cell surface binding leading to cell death (Yip et al., 2001). Furthermore, Abramov et al. (2011) reported that addition of Aβ to lipid bilayers caused a calcium ion conductance that was significantly higher in membranes containing cholesterol. They concluded that increasing membrane cholesterol significantly increased Aβ-induced neuronal and astrocytic cell death. On the other hand, however, cholesterol has been reported to have a neuroprotective effect against Aβ (Arispe & Doh, 2002; Yip et al., 2001) and inhibit the formation of amyloid-induced ion channels (Kagan et al., 2002). Similarly, studies with cholesterol and amyloid plaques in human neuroblastoma cells have shown the possibility that increased cholesterol content reduces membrane perturbance by the amyloid plaques (Cecchi et al., 2009).

Cholesterol has been reported to alter the penetration of Aβ into the lipid bilayer (Dante et al., 2006) and monolayers (Ji et al., 2002). Notably, 20% of cholesterol completely inhibits monomeric Aβ (25–35) membrane insertion (Dante et al., 2006). The mechanism by which cholesterol attracts Aβ to the cell membrane is believed to be by increasing hydrogen bonding between Aβ and DPPC accelerating incorporation into the cell membrane (Abramov et al., 2011). Once cholesterol is present, cholesterol–Aβ interactions compete with inter-protein Aβ interactions and do not accelerate Aβ aggregation (Zhao et al., 2012). MD simulations indicate that Aβ40 prefers to reside on the surface of the cell membrane as opposed to inserting into cholesterol-depleted membranes. In cholesterol-rich membranes, Aβ (1–40) inserts only partially (Qiu et al., 2011). Amyloid insertion and ion channel formation will be further expanded upon in the next section of this review.

As mentioned previously, the role of electrostatic interactions within lipid membranes and a detailed understanding of lipid electrostatic non-homogeneity are of great interest in studying the interaction of charged species such as Aβ with the membrane. We recently reported that cholesterol has an interesting electrostatic effect on model lipid monolayers (Drolle et al., 2012) and in Bovine Lipid Extract Surfactant (BLES) surfactant, a complex lipid–protein mixture (Finot et al., 2010; Gunasekara et al., 2005). We demonstrated that cholesterol induces electrostatic domains in supported lipid-protein films of BLES surfactant (Finot et al., 2010), which results in surfactant failure. These intriguing electrostatic properties of cholesterol on membrane structure may be crucial in understanding the interaction of the plasma membrane with amyloid forming peptides.

In order to study the role of cholesterol-induced domains on Aβ–membrane interactions, we used high-resolution AFM, and model lipid membranes comprised of either pure DOPC or DOPC enriched with 20% cholesterol. We also used Frequency Modulated Kelvin Probe Force Microscopy (FM-KPFM) (Moores et al., 2011; Zerweck, et al., 2005), a modification of AFM, to resolve electrical surface potential maps in DOPC monolayer with and without cholesterol in order to elucidate the role of electrostatic domains.

Supported bilayers of pure DOPC or cholesterol-enriched DOPC were deposited on mica from vesicle solution and imaged by AFM in a liquid cell. Both samples were then incubated with Aβ-42 solution in buffer and then rinsed with water and imaged in water. As shown in Figure 7, on the pure DOPC bilayer, Aβ deposits were small, spherical and spread across the lipid membrane surface in a uniform distribution with no discernible preferential binding sites or clustering (Figure 7a). On the DOPC membrane with 20% cholesterol, amyloid fibril formation was no longer uniform. After 1 h of incubation, nanoscale islands or domains were observed with lateral dimensions from 30 to 400 nm and up to 1 μm that were loaded with amyloid deposits and surrounded by smooth areas of lipid bilayer void of Aβ deposits. These islands seemingly were formed as a result of the amyloid deposits binding to the membrane in a non-uniform and rather selective manner (Figure 7b). The amyloid deposits on the DOPC/cholesterol membrane were more of a fibril nature than resembling the spherical oligomers observed on a pure DOPC bilayer. The single fibrils protruding from the clustered domains were clearly visible, with an average height of 2.1 ± 0.3 nm above the membrane surface, and varying from 40 to 300 nm in length. This indicates that cholesterol promotes much stronger preferential binding of Aβ. Our data correlate well with the previously mentioned recent study by Sheikh et al. (2012), who reported that binding of Aβ to the membrane was increased two orders of magnitude when cholesterol was present in the membrane, and a recent molecular dynamics simulation study, which has shown that membrane cholesterol makes the binding process of amyloid to the membrane more energetically favorable (Yu & Zheng, 2012).

We evaluated the roughness parameters of the surfaces for both the DOPC and DOPC with cholesterol samples to estimate the damaging effects of Aβ deposits on the membranes. Cross sections for the pure DOPC membrane show a relatively flat surface due to uniform distribution of the deposits, with Aβ sinking slightly into the membrane (Figure 7a and 7c). This correlates with our data on amyloid binding to pure lipid bilayers (Hane et al., 2011). On the DOPC bilayer enriched with cholesterol, this distribution was

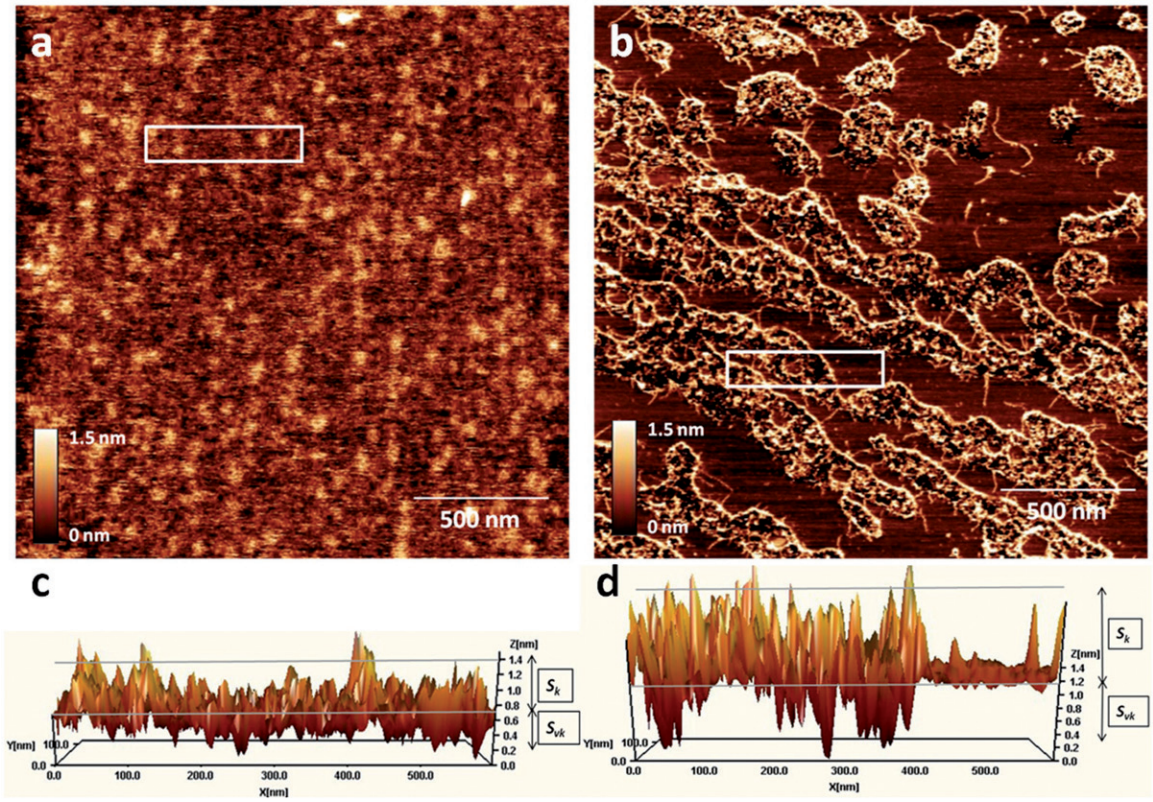

Figure 7. AFM topography images of Aβ binding to the lipid membrane: (a) pure DOPC membrane after 1 h incubation with Aβ (1–42), (b) DOPC membrane with 20% cholesterol after 1 h incubation with Aβ (1–42), 3D AFM images demonstrating the surface roughness of the pure DOPC membrane with Aβ deposits (c), (rectangular area marked in Figure 1A); and of the cholesterol-enriched DOPC membrane with Aβ deposits (d) (rectangular area marked in Figure 1B). (Image reproduced from Drolle E, Gaikwad R, Leonenko Z. (2012). Nanoscale electrostatic domains in cholesterol-laden lipid membranes create a target for amyloid binding. Biophys J 103:L27–L29, with permission from Elsevier).

no longer uniform. The domains of clustered Aβ deposits were formed (Figure 7b), which showed higher roughness on the cross section corresponding to the domains saturated with Aβ deposits and smooth areas corresponding to the pure membrane, void of any deposits (Figure 7d). The roughness of amyloid-saturated domains in DOPC/cholesterol membrane was 5 times larger compared to the pure DOPC membrane. This indicates that Aβ deposits produce much higher damage in the membrane domains saturated with cholesterol (both larger clusters on the top of the membrane and deeper holes inside the membrane, Figure 7b and 7d).

To look specifically at the role of the electrostatic effect of cholesterol, we used FM-KPFM. FM-KPFM is a powerful technique which, as we demonstrated earlier, is the only technique suitable of mapping the local electrical surface potential of complex self-assembled biological films with a lateral resolution of a few nanometers and sensitivity of a few millivolts in air at normal humidity (Moores et al., 2011; Zerweck et al., 2005). In addition to high sensitivity and high resolution, FM-KPFM also has the advantage of allowing both the topography and surface potential to be collected simultaneously on the same location of the same samples, allowing for easy comparisons.

Figure 8 shows AFM topography (A and B) and FM-KPFM surface potential images (C and D) of pure DOPC monolayer (A and C) and with 20% cholesterol (B and D). The pure DOPC lipid monolayer shows smooth topography (Figure 8a) and corresponding surface potential images that are featureless and uniform (Figure 8c). In contrast to this, in the DOPC lipid monolayer with 20% cholesterol, domains are observed in both topography (Figure 8b) and surface potential (Figure 8d). These domains shown in the surface potential map in Figure 8(d) indicate specifically the electrostatic effect of cholesterol and differ in surface potential about 61 ± 8 mV. Considering the charged nature of Aβ and complex nature of electrical charge distribution in oligomers (Moores et al., 2011), we are confident that electrostatic domains created in the DOPC lipid membrane by cholesterol attract the Aβ peptide, thus inducing the formation of the non-uniform islands or domains that are heavily loaded with amyloid deposits.

As shown earlier, cholesterol produces similar nanoscale electrostatic domains in lung surfactant, which leads to surfactant failure (Finot et al., 2010; Leonenko et al., 2006). These electrostatic domains create a distinct electrical potential, which affects the interaction of the surfactant film with charged species (Finot et al., 2010). We demonstrated that this previously unknown electrostatic effect of cholesterol is not limited to only lung surfactant films but extends to model lipid monolayers and lipid membranes and can greatly affect the interaction of Aβ peptides with the surface of lipid membrane.

We propose that this electrostatic effect of cholesterol on the monolayer has an important role in cholesterol–lipid systems and may be present in other, more complex systems as well. This earlier unknown electrostatic effect of

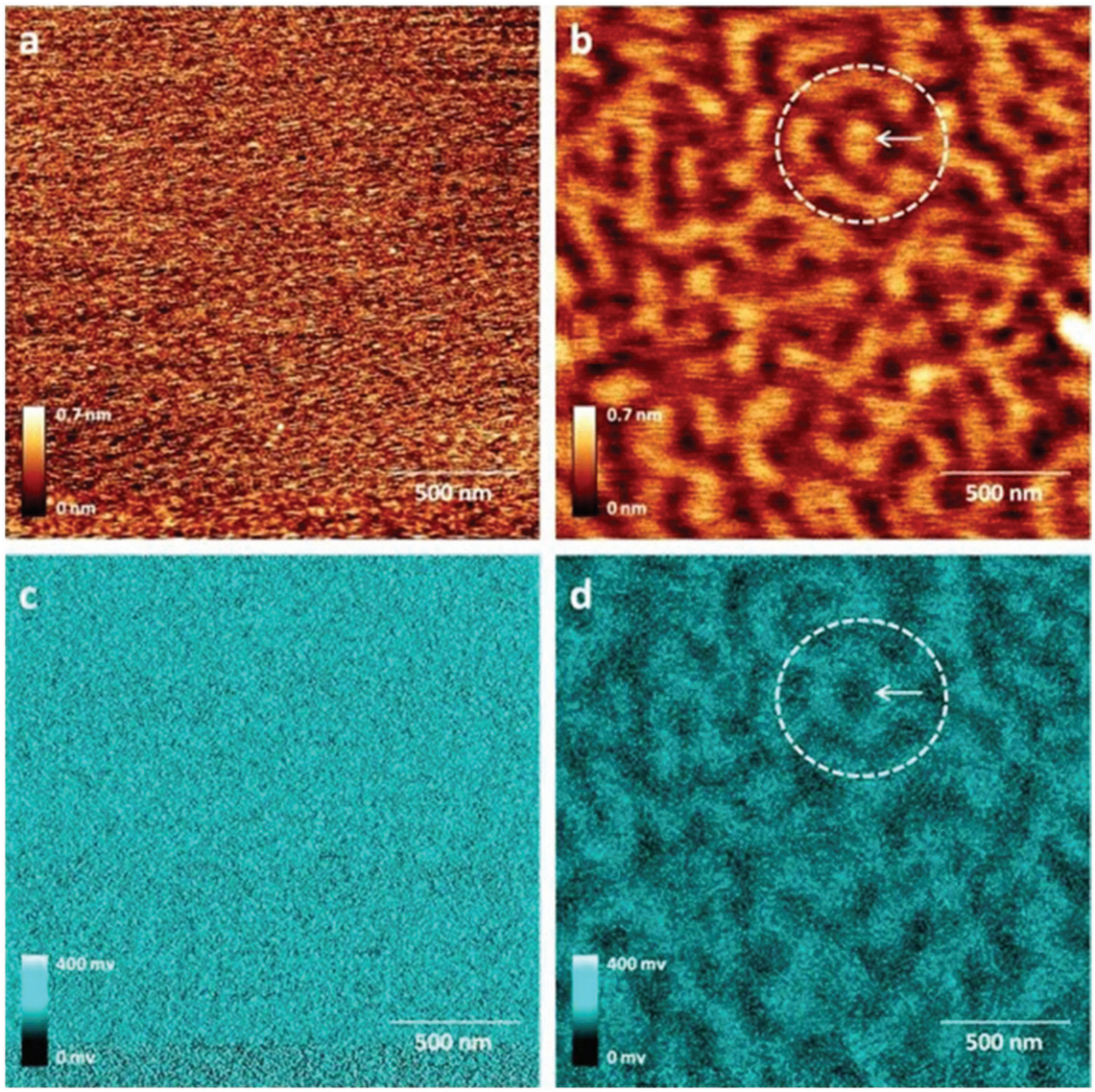

Figure 8. AFM and corresponding FM-KPFM images of lipid monolayers in the presence and absence of cholesterol. AFM images depicting the topography of pure DOPC monolayer (a), and DOPC monolayer with 20% cholesterol (b) and the corresponding FM-KPFM images depicting surface potential distribution of pure DOPC monolayer (c), and DOPC monolayer with 20% cholesterol (d). AFM and FM-KPFM images were collected with SmartSPM (AIST-NT). (Image reproduced from Drolle E, Gaikwad R, Leonenko Z. (2012). Nanoscale electrostatic domains in cholesterol-laden lipid membranes create a target for amyloid binding. Biophys J 103:L27–L29, with permission from Elsevier).

cholesterol may serve as a driving force for amyloid targeted attack on lipid membranes and therefore may be involved in the mechanism of amyloid toxicity.

Ion channels

In addition to binding and penetrating into the membrane, Aβ may interact with membrane forming ion channels, shown schematically in Figure 5(C). These ion channels destabilize cellular ionic homeostasis and induce cell pathophysiology and degeneration, characteristic of amyloid diseases.

Lal and colleagues provided strong evidence supporting this hypothesis, first proposed by Arispe (2004), that cellular dyshomeostasis caused by ion channels was responsible for at least some of the symptoms associated with AD. Quist used AFM to image tetrametic and hexomeric oligomeric structures formed by Aβ in reconstituted membranes, which resemble the ion channels in cell membranes. By using AFM, CD, gel electrophoresis and electrophysiological recordings, they showed that the presence of ion channels in lipid membranes is a common feature of the proteins implicated in neurodegenerative disorders such as AD, Parkinson's disease and frontotemporal dementia (Quist et al., 2005). Amyloid molecules, including Aβ-40, alpha-synuclein, ABri, ADan, serum amyloid A and amylin undergo supramolecular conformational change, and in reconstituted membranes, they form morphologically compatible ion-channel-like structures, which elicit single ion-channel currents. Kayed et al. (2009) referred to such ion-like structures as annular protofibrils (APF), a distinct off-pathway structure. Interestingly enough, even though APFs are structurally similar, they have shown that these structures are not nearly as membrane permeable as the fibrillar precursors termed prefibrillar oligomers.

Using the unique combination of AFM and electrical recording, Lal's group resolved their structures with AFM and showed that these ion channels, induced by assembly of Aβ in

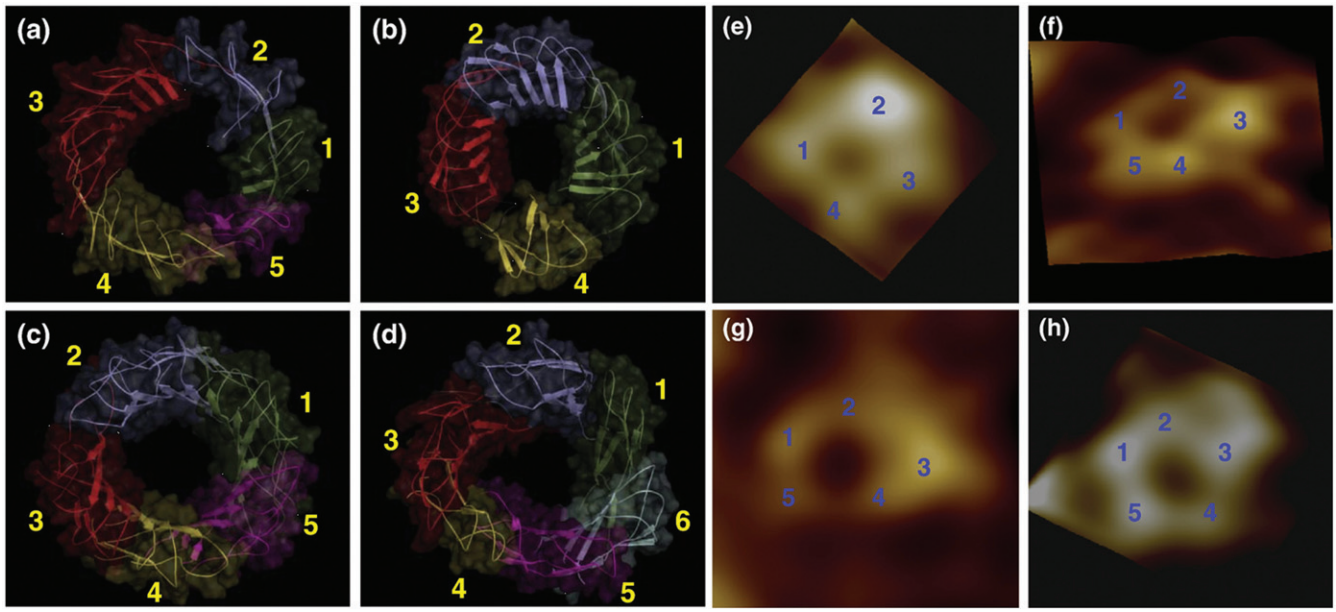

Figure 9. Molecular dynamics simulation and AFM images of Aβ induced ion channels. Side-by-side comparison between the computational barrels and the experimental channels. The simulated barrel structures with highlighted subunits for the 20-mer p3 (a), p3h (b), N9 (c), and N9h (d) barrels. The averaged barrels in the surface representation are shown in the view along the membrane normal. AFM images of p3 (e and f) and N9 (g and h) barrels show four or five subunits, consistent with the simulated barrels. Image sizes are 15×15 and 23×23 nm^2, respectively. (Figure reproduced from Jang H, Arce FT, Ramachandran S, et al. (2010). β-Barrel topology of Alzheimer's β-amyloid ion channels. J Mol Biol 404:917–934 with permission to use).

the membrane, have a distinct ion current activity when an electric potential is applied (Quist et al., 2007). For these experiments, Lal's group designed a special AFM liquid cell with two compartments separated by a silicon membrane with microfabricated nanopores of diameter 70–100 nm, on which the lipid bilayer can be deposited, while both compartments are immersed in buffer. Two electrodes are positioned in each compartment in order to apply a defined potential to measure membrane permeability or ion current induced in the membrane by Aβ. AFM imaging and ion current recording can be done on the same single ion pore with this approach (Quist et al., 2007).

Later, the Lal and Nussinov groups provided more evidence supporting the ion channel mechanism for AD (Jang et al., 2010). Using solid-state NMR, AFM imaging and molecular dynamics simulations, they demonstrated that small Aβ oligomers penetrate into the cell membrane, forming toxic ion channels and destabilizing the cellular ionic homeostasis. Using MD simulations, Jang modeled a β-barrel-like organization for these channels. β-Barrels are common in transmembrane toxin pores, typically consisting of a monomeric chain forming a pore, organized in a single-layered β-sheet with antiparallel β-strands and a right-handed twist. Their MD data in comparison with AFM images support a β-barrel channel organization for Aβ channels. Different from the transmembrane β-barrels where the monomers are folded into a circular β-sheet with antiparallel β-strands stabilized by the connecting loops, these Aβ barrels consist of multimeric chains forming double β-sheets with parallel β-strands, where the strands of each monomer are connected by a turn (Figure 9). The Aβ barrels adopt the right-handed β-sheet twist but still break into heterogeneous, loosely attached mobile subunits, in good agreement with AFM images, which allows for unregulated, hence toxic, ion flux.

The β-barrel channel structure reported by Jang et al. (2010) is rare in eukaryotes but is found in the mitochondrial (which is believed to be a bacterial relic) outer membrane and is known as a voltage-dependent anionic channel (Bay & Court, 2002; Casadio et al., 2002; Thinnes et al., 1989). Such anionic channels have a two-state voltage-dependent gating mechanism opening and closing the pore, inducing cytotoxicity and apoptosis (Abu-Hamad et al., 2009; Shulga et al., 2009). These studies are in agreement with electrophysiology data by Abramov et al. (2011) who reported that the addition of Aβ to lipid bilayers causes ion conductance, which was significantly higher in membranes containing cholesterol. They concluded that incorporation of Aβ into membranes and the ion channels created by Aβ are promoted by membrane cholesterol, which significantly increased Aβ-induced [Ca^{2+}] current, leading to neuronal and astrocytic cell death. Other theoretical work (Pannuzzo et al., 2013) reports significant change in membrane curvature due to amyloid binding and formation of nonchannel defects, which resemble conical holes inside the membrane. Such defects can also result to increased membrane permeability and ion currents. This case corresponds to the membrane damage shown in Figure 6(B).

These studies show that Aβ ion channels can serve as candidates for understanding the neurotoxicity of AD and second, they highlight the possibility of drug design targeting these amyloid ion channels. The formation of these channels and their activity are dependent on the properties of lipid membrane and in particular on the presence of membrane cholesterol.

Summary

In summary, we reviewed the major aspects of Aβ interactions with lipid membrane in relation to amyloid toxicity and AD, with a specific focus on AFM studies, which provide

nanoscale insight into the molecular mechanism of these interactions. We have shown that it is now increasingly accepted that Aβ interacts with the membrane in a complex mechanism where several pathways are possible: accumulation of the surface of lipid membrane (Figure 5A), penetration into the membrane, causing disorder in lipid organization (Figure 5B) and formation of ion pores and ion channels, which leads to unregulated ion leakage and, ultimately, cell death (Figure 5C).

Recently, it has been shown that Aβ peptides have a lot of similarities with antimicrobial peptides (AMP). AMPs are small peptides with a strong antibiotic activity against a wide range of microorganisms, killing bacteria by interaction with the bacterial plasma membrane through a complex mechanism. This mechanism includes binding to the membrane (carpeting mechanism), disordering of membrane integrity, formation of ion channels and large pores and the eventual disintegration of the membrane (Jang et al., 2011). AMP and Aβ both form fibrils in solution and both interact with lipid membranes, as well as being capable of forming ion channels (Jang et al., 2011). In addition, *in vitro* studies suggest that Aβ is an unrecognized AMP that may normally function in the innate immune system, as Aβ exerts antimicrobial activity against eight common and clinically relevant microorganisms with a potency equivalent to, and in some cases greater than AMP (Soscia et al., 2010). AMPs are known to be very specific in recognizing the structure of bacterial membranes through electrostatic interactions. Similarly, Aβ may have some specificity, allowing Aβ to recognize changes in membrane structure and integrity and subsequently induce toxic effects and cell death. Therefore, the role of the lipid membrane in the molecular mechanism of amyloid toxicity is very important in the pathogenesis of AD and may provide avenues for the development of preventive and treatment strategies to combat AD and other amyloid-induced neurodegenerative disorders.

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Declaration of interest

The authors report no declarations of interest.

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