Peptid-tethered bilayer lipid membranes and their interaction with Amyloid ß-peptide

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The Amyloid peptide (Aß), a normal constituent of neuronal and non-neuronal cells, has been shown to be a major component of the extracellular plaque of Alzheimer disease (AD). The interaction of Aß peptides with the lipid matrix of neuronal cell membranes plays an important role in the pathogenesis of AD. In this study, we have developed peptide-tethered artificial lipid membranes by the Langmuir–Blodgett and Langmuir–Schaefer methods. Anti-Aß40-mAb labeled with a fluorophore was used to probe Aß40 binding to these model membranes. Systematic studies on the antibody or Aß-membrane interactions were carried out by surface plasmon field-enhanced fluorescence spectroscopy. It was found that the Aß adsorption is critically depending on the lipid composition of the membranes, with Aß specifically binding to membranes containing sphingomyelin. Further, this preferential adsorption was markedly amplified by the addition of sterols (cholesterol or 25-OH-Chol). © *2007 American Vacuum Society.* [DOI: 10.1116/1.2804746]

I. INTRODUCTION

Amyloid ß-peptide (Aß), a prominent constituent of brain plaques characteristic of Alzheimer disease (AD), is a 40- or 42-residues polypeptide and is derived by the proteolytic cleavage of the Aß precursor protein. Although it remains unclear as to what role exactly Aß plays in AD, the prevalent hypothesis is that the neurotoxicity of Aß results from its direct interaction with membranes. It was reported that Aß has the ability to form ion channels in model membranes composed of phophatidyserine.[1] It was further demonstrated that the formation of ion channels in planar lipid membranes was closely related to the membrane lipid composition. Additionally, other membrane components promoted the formation of ß sheets of Aß, which is thought to be a striking feature of the Aß toxicity.[2,3] For example, the surface binding of Aß to negatively charged phospholipids has been shown to result in an induction of ß structure in Aß.[4] In recent years, evidence was reported that "lipid rafts" are the preferential sites for the formation of pathological forms of Aß,[5] as described in the case of the interactions of the Aß peptide with ganglioside-containing raft-like membranes made from cholesterol and sphingomyelin, or with sphingolipid-containing domains.[6–8] However, it is not clear whether the complexes of sphingomyelin and cholesterol are also potential binding sites for Aß. Systematic studies on the interaction between Aß and membranes especially composed of zwitterionic lipids can hardly be found, and thus, the interpretation of experimental results from different types of model systems is sometimes controversial. Moreover, much of the knowledge concerning the interaction between Aß and membranes is obtained from monolayer studies at the air-water interface[8,9] or from vesicle/liposome model systems,[6,7] which are all relatively simple membranes. Hence, it is necessary to perform investigations in a model membrane system, which is more complex and structurally closer to a cell membrane.

In order to approach these problems, we developed a peptide-tethered lipid membrane system on gold substrates prepared by the Langmuir–Blodgett (LB) and/or the Langmuir–Schaefer (LS) method. The advantages of the LB and LS techniques originate from the fact that the transfer of lipid layers is carried out under controlled surface pressure conditions and that well-defined layers can be deposited. In our case, each layer was transferred onto the substrate always at a surface pressure of $\pi = 30$ mN/m, which is the pressure of a biological membrane. In addition, the LB and LS methods allow for the construction of architectures containing a random composition of lipids at will. For our model membranes, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) always constitutes the proximal layer resembling the lipid at the inner side of a cell membrane. However, the lipid components of the distal layers are variable. A peptide sequence used as a spacer was covalently linked on one side to the gold surface and on the other end to a DMPE molecule as a part of the proximal monolayer of a tethered membrane. This tethering leads to a stable and robust coupling of the bilayer to the sensor surface and at the same time decouples the membranes sufficiently from the substrate to allow for the lipid matrix to exist in a fluid state as it is required for a number of membrane proteins (receptors, channels, carriers, etc.) for their proper functions; e.g., the translocation of alkali-ions mediated by valinomycin which requires the ion shuttle to be in a (locally) fluid lipid environment has been demonstrated to differentiate also in these tBLMs between Na^+ and K^+ ions, thus exhibiting the four orders of magnitude difference in conductance increase as originally observed in experiments with BLMs.[10]

Zwitterionic lipids [1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) and sphingomyelin], principal components of the outer monolayer of a plasma membrane, con-

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stitute the distal layers of the model systems. The importance of cholesterol in the Aß binding in model membranes has recently received considerable attention.[9,11] Hence, we incorporated cholesterol in POPC or sphingomyelin layers in order to study the role of these important membrane components. Cholesterol is rather susceptible to oxidation leading to the formation of cholesterol oxidation products. Oxysterols, oxidative derivates of cholesterol, are thought to potentially result from oxidative stress implicated in the central neuronal loss in AD.[12] It was shown by cell-culture studies that most neuronal cells were very sensitive to 25-hydroxycholesterol (25-OH-Chol) toxicity.[13] However, many questions concerning the importance of oxysterols in AD development remain to be answered. Since cholesterol is a vital component of many membranes, we are very interested in the effects of substituting oxysterols for cholesterol on the Aß binding to the model membranes. For this reason we also used a peptide-tethered membrane containing 25-OH-Chol as a model to assess whether the oxysterol influences Aß adsorption, which may provide further insight into some aspects of AD.

We monitored the interaction of Aß with model membranes using surface plasmon field-enhanced fluorescence spectroscopy (SPFS), which is the combination of surface plasmon and fluorescence spectroscopy for sensor applications.[14,15] Anti-Aß-mAb labeled with a fluorophore was used to detect Aß adsorbed on membranes. Our results show that Aß binding is critically determined by the type and composition of the lipids in the model membranes.

These studies with the tethered bilayer lipid membranes were complemented by a few experiments with Langmuir monolayers applying fluorescence microscopy in order to confirm the occurrence of phase separated lipid domains also in the case of the lipid mixtures used in our studies.

II. MATERIALS AND METHODS

The synthetic Aß40 peptide was purchased from Biopeptide Co., LLC; its purity (>95%) was analyzed by high performance liquid chromatography. Unless otherwise stated, Aß refers to Aß40 in the following text. The peptide (1 mg) was initially dissolved in 1 ml of 1,1,1,3,3,3-hexafluoro-2-propanol in order to avoid self-aggregation[16] and then stored in 30 μl aliquots at −20 °C until use. After removal of the solvent by nitrogen gas, the peptide was redissolved in phosphate buffered saline (PBS) solution at the desired concentration.

The 19-mer peptide (P19) derived from the α-laminin subunit was obtained from American Peptide Co., Inc. *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide (EDC) and monoclonal anti-ß-Amyloid protein (epitope: 1–17) were purchased from Sigma-Aldrich. *N*-hydroxysuccinimide (NHS) was obtained from Perbio Science Deutschland GmbH. EDC and NHS were each dissolved in 10 ml Milli-Q water at the concentration of 75 and 11.5mg/ml, respectively, and stored in 500 μl aliquots at −20 °C until use.

All lipids, POPC, cholesterol, DMPE, 25-hydroxycholesterol, sphingomyelin (SM) from bovine brain were all purchased from Avanti Polar Lipids, Inc. *N*-octyl-ß-*D*-glucopyranoside was purchased from Merck Biosciences GmbH.

Alexa Fluor 647 monoclonal antibody kit was purchased from Molecular Probes Europe. B.V.

A. Fluorescence labeling of monoclonal antibodies

The procedures for the labeling were described in detail in the product information provided by Molecular Probes. Briefly, Alexa Fluro 647 was used as a label for the monoclonal antibodies sensitive to an epitope within amino acid sequence 1–17 of the Aß40. The solution of the antibody to be labeled (final concentration of 1 mg/ml) was mixed with reactive dye (Alexa Fluro 647) and gently inverted a few times to fully dissolve the dye. After 1 h of incubation at room temperature, free dye molecules were separated from the labeled protein preparation by running the solution through the purification resin column. The degree of labeling was determined by UV-visible absorbance spectroscopy. Four moles of Alexa Fluro 647 were bound to one mole of antibody.

B. Construction of peptide-tethered bilayer lipid membranes

LaSFN9 glass slides were carefully cleaned and coated with about 2 nm chromium and 50 nm gold on top by evaporation under vacuum in a commercial instrument (Edwards). For the peptide (P19) modification, a fresh planar gold surface was incubated with the peptide at a concentration of 40 μg/ml in 0.02% ammonia solution for at least 24 h. A stable monomolecular peptide layer was formed by the strong gold-sulfur interaction of the *N*-terminal cysteine moiety. The excess of unbound peptide was removed by extensive rinsing with Milli-Q water and the resulting optical thickness was monitored by surface plasmon resonance spectroscopy (SPS). The carboxyl groups of the peptide (P19) layer were activated with EDC/NHS for the subsequent reaction with the amines of the polar head of DMPE. EDC and NHS were mixed in equal amounts in a volume of 1 ml (50 mM NHS, 200 mM EDC) just before the immediate use and applied to the peptide-modified gold surface for less then 10 min.

For the LB transfer, pure DMPE monolayers were spread at room temperature from a chloroform solution (0.5 mg/ml) at the air/water interface in a Teflon trough with a dipping well. The surface pressure was monitored by a Whilhelmy plate. DMPE monolayers were transferred from the air/water interface of the Langmuir trough to the activated peptide-covered surface at a vertical withdrawal speed of 2 mm/min at a constant surface pressure of π=30 mN/m which was maintained using a computer feedback loop. Transfer ratios were approximately 1.

The distal lipid monolayer was obtained by Langmuir–Schaefer transfer. The substrate with the bilayer is then collected in a dish, avoiding any subsequent exposure to air. The proximal lipid monolayer in all cases was pure DMPE. The Langmuir–Schaefer transfer allows for any change in the

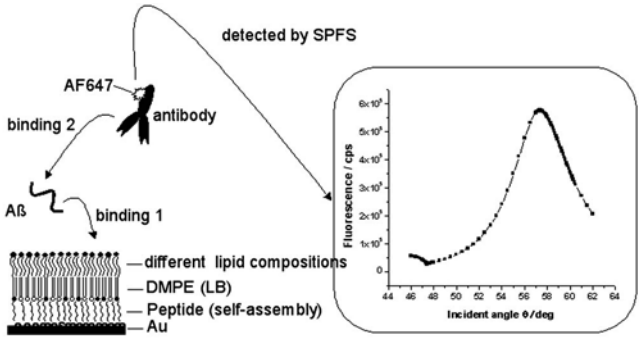

FIG. 1. Schematic illustration of the detection of Aß-membrane interactions by an antibody labeled with a fluorophore using SPFS.

lipid composition of the distal lipid monolayers. In this study, six artificial model systems with different composition of the distal lipid monolayers were built up.

C. Surface plasmon resonance spectroscopy and SPFS

The construction of the peptide-tethered lipid bilayers was monitored by surface plasmon resonance spectroscopy. The shift of the plasmon angular scan obtained was used to characterize the optical thickness of the thin films. Surface plasmons can be resonantly excited at a metal/dielectric interface by *p*-polarized light (from a HeNe laser at $\lambda = 633$ nm in our case) only at a well-defined angle of incidence (resonance angle), which is strongly dependent on the refractive index (or dielectric constant) profile of the samples within the optical evanescent field. For molecules adsorbing to the sensor surface, thereby changing the local refractive index, the shift of the resonance angle is proportional to the surface concentration of the molecules.

SPFS was applied for the detection of the interactions between Aß40 and the lipid bilayers by the recognition reactions of fluorophore-labeled anti-Aß-mAb from solution to surface-adsorbed Aß. The main advantage of SPFS is the increase in sensitivity for monitoring binding reactions of an analyte from the aqueous phase to the recognition sites at a functionalized interface, especially for small analytes. The principles and applications of SPFS have been described in detail elsewhere.[14,15] Upon binding to Aß40 adsorbed to the artificial lipid membranes, the fluorescence intensity of the dye chemically bound to the antibody was recorded. Hence, the interactions of Aß40 and lipid membranes can indirectly be investigated by detecting the fluorescence signals of fluorophores coupled to the antibody.

D. Binding specificity of Aß

The interactions of Aß and the membranes were probed by the detection of the antibody labeled with a flurophore bound to Aß at the membranes using SPFS (Fig. 1). Before the binding experiments, repeated rinsing with PBS ensured a proper environment for the protein binding. The Aß solutions were prepared as described earlier to a final concentration of 4.6 μM. The matrices were rinsed with PBS before the solutions of the antibody labeled with Alexa Fluor 647 dye at a final concentration of 45 nM were injected into the flow cell at a flow rate of 36 μl/min. After incubation for 20 min, the unbound peptide was rinsed out with PBS. The solutions of the antibody were injected and interacted with Aß at the membranes in the flow cell. After incubation for 30 min, the surface was rinsed with PBS for 20 min until equilibrium was reached in order to remove free and loosely associated dye molecules on the membranes. Then, SPS/SPFS scans were performed in order to obtain the fluorescence signals of the antibody binding to the lipid surface. Binding specificity of Aß to the model membranes was determined by the fluorescence intensity of the antibody bound to Aß at the membranes. This approach has common features with the widely used ELISA assay for antigen sensing: the chromophore-labeled detection antibody has a fixed stoichiometry to the Aß protein in our case. Hence, the measured fluorescence is a linear function of the (interfacial) analyte (Aß) concentration.

E. Fluorescence imaging

The phase behavior of Langmuir monolayers was monitored using epifluorescence microscopy with an attached charge coupled device camera mounted above the Langmuir trough (Nima 601). The apparatus allowed for the recording of pressure-area compression isotherms and fluorescence imaging simultaneously. A small amount (0.6%) of a fluorescent dye, NBD-PC, which is preferentially excluded from the ordered phase, is used to provide contrast between coexisting phases. Lipids were spread from a 1 mg/ml chloroform solution at the air/water interface of a 10 cm $\times$ 28 cm Teflon trough that had movable barriers to vary the surface pressure. After spreading the film, 30 min were allowed for solvent evaporation. The subphase contained Milli-Q water. All experiments were performed at room temperature.

III. RESULTS AND DISCUSSIONS

A. Preparation of peptide-tethered lipid bilayers

The establishment of the model system was motivated by the fact that plasma membranes of cells are asymmetric, that is, phosphatidylcholine and sphingomyelin have been found on the external leaflet of the plasma membrane whereas the aminophospholipids phosphatidylserine and phosphatidylethanolamine are predominantly on the inner side. This is in line with our model membrane system for which DMPE always resides in the proximal layer facing the substrate. The lipids mainly comprising cellular membranes were chosen as the distal layers of the model systems that interact with Aß. In this study, a gold substrate was used as a solid support allowing for the lipid lamellas to be characterized by surface plasmon resonance (SPR) and SPFS measurements. A hydrophilic peptide was employed as spacer molecule to tether the proximal lipid layer to the gold surface thus providing good stability and preserving the native (liquid-crystalline) state of the bilayer on the support.

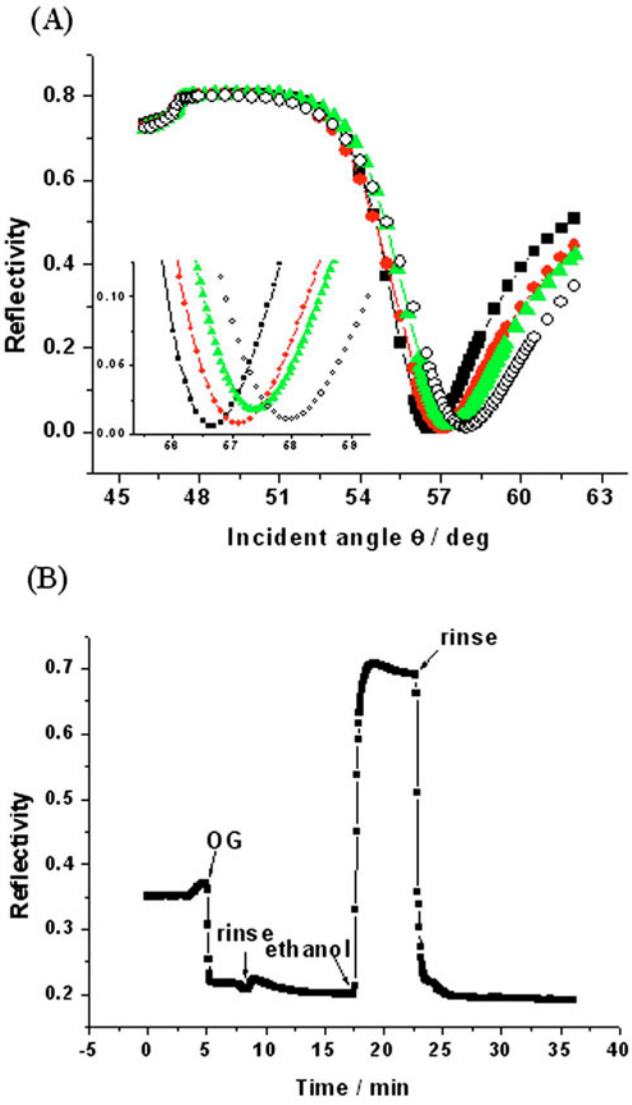

FIG. 2. (a) Assembly of a peptide-tethered lipid membrane on a gold surface. Angular scans of the reflected intensities for different interfacial architectures: the reference reflectivity curve (■) was measured for the Au surface in contact with water. The peptide monolayer resulted in an angular shift of the reflectivity curve (●); the lipid bilayer composed of DMPE and POPC was obtained by LB and LS transfer as described in the methods resulting in an additional angle shift (○). The layer of the remaining lipid molecules (covalently bound to the surface) obtained after treatment with detergent and ethanol results in an angular scan shifted to smaller angles (▲). The blow-up of the reflectivity curves is shown in the inset. (b) Kinetic protocol (reflectivity at theta=55.06 deg) of the treatment of the peptide-tethered bilayers with a detergent solution and ethanol, respectively.

SPR was used to demonstrate the formation of peptide-tethered lipid bilayers. Figure 2 shows the quantitative evaluation of the surface modification via the stepwise attachment of peptide and lipid bilayers onto the gold substrate. A full angular scan was recorded for every single layer after its surface attachment. All respective angular shifts were quantitatively analyzed. From Fresnel's equations, one can calculate the optical thickness of the coating (assuming for the peptide a refractive index of $n=1.41$, and for the lipids a refractive index of $n=1.5$).[17,18] The idea for using a 19 mer peptide as a spacer between the gold surface and the lipid bilayers originated from the following consideration: (1) the peptide, which contains 11 polar amino acids, creates a soft, hydrated cushion that acts as both a lubricating surface and a spacer; (2) the presence of a cysteine residue at the amino-terminus of the peptide allows for its covalent binding to the gold surface via a strong Au-thiolate bond, forming a rigid molecular layer with the orientation of the *C*-terminus of the peptide away from the gold surface; and (3) the carboxyl groups then allow for the covalent coupling to the primary amines at the polar head of DMPE. The optical thickness of the peptide layers derived from SPS measurements was about 2.2 nm, close to the 2.8 nm of the theoretical length of a 19 mer peptide molecule in the alpha helical conformation. In line with earlier studies[19] this indicated the formation of a dense monomolecular layer of the peptide on the gold surface. The complete coverage of the gold surface and the resulting hydrophilic character were further demonstrated by contact angle measurements (about $\theta=35°$), which was sufficiently hydrophilic to allow for LB transfer.

The formation of bilayers deposited on the peptide was also checked by SPR. For the bilayers composed of POPC and DMPE, the thickness of 4.2 nm thus obtained is quite reasonable compared to a DPPC bilayer thickness of 4.6 nm obtained by x-ray diffraction. It is well known that the membrane thickness decreases as the degree of unsaturated lipids increases. Thus, well-defined lipid bilayers can be obtained in the way described in the method section. The model systems applied in the present work were in all cases constructed by the LB and the LS techniques at high surface pressures (30 mN/m) that closely mimic the situation of a biomembrane.[20] The tethered bilayers were stable enough under continuous flow of buffer solution at a flow rate of 1 ml/min for at least 24 h.

Finally, the stability of the surface-tethered anchor lipids was checked by SPR [Fig. 2(b)]. Rinsing the tethered bilayer in the flow cell with detergent (1-*O*-octyl-*β*-*D*-glucopyranoside) and subsequently with ethanol removed all but the lipid molecules covalently bound to the surface Fig. 2(b). The average thickness of the layer remaining after rinsing with the detergent and ethanol accounted for about 20%–30% of the theoretical surface coverage of a lipid monolayer. Contact angle measurements showed a large increase in hydrophobicity of the surface from $\theta=35°$ for the pure peptide to $\theta=62°$ originating from contribution of the aliphatic chains of DMPE. Therefore, successful coupling of DMPE to the peptide surface was concluded. Taken together, it could be documented that a model system mimicking several relevant features of a cell membrane was successfully constructed.

B. Binding specificity of Aß to model membranes

In this work, anti-Aß40-mAb labeled with a fluorophore was used as a probe to detect Aß adsorbed to the model membranes. Different model membranes (POPC, POPC/sterols, POPC/SM, and POPC/SM/sterols) were established in order to investigate the interactions between lipid bilayers

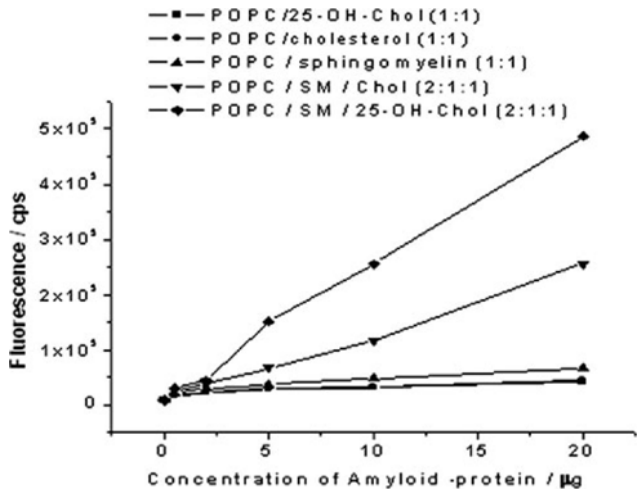

FIG. 3. Effects of lipid composition on Aß adsorption. By stepwise increasing the solution concentration of Aß, the fluorescence intensity of each concentration was obtained at the peak of the scan curves of fluorescence at the incident angle of theta=57.3 deg. The specific binding of Aß to the membranes containing the sterols and SM gives a clear increase in fluorescence signals as a function of the concentration of Aß. The steepest linear curve was obtained in the tertiary mixtures of POPC/SM/Oxy; the curves form the membranes composed of binary mixtures are almost identical to the background.

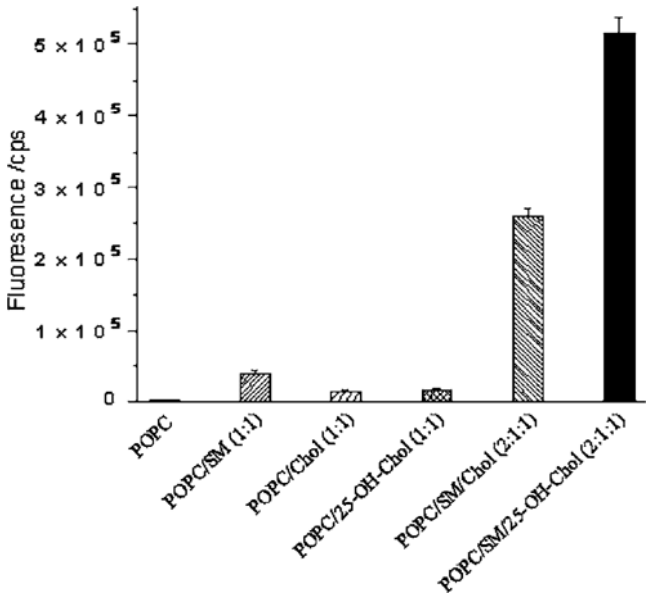

FIG. 4. Effects of lipid composition on Aß adsorption. Fluorescence intensities (*cps*) were taken at the maximum points of angular scan curves of fluorescence (cf. Fig. 1). The fluorescence signal from pure POPC was the lowest and the one from the mixture of SM/POPC increased, indicating the stronger association of Aß with SM. The fluorescence signal was significantly increased in the presence of SM and sterols. The strongest adsorption of Aß was found in the tertiary systems containing 25-hydroxycholesterol (25-OH-Chol).

of different composition and Aß. For the monolayers with more than two components, POPC accounted for 50 mol % of the lipids in the distal layers of the model membranes in order to resist nonspecific antibody adsorption. PC which is resistant to protein adsorption has been extensively studied in the fields of biomedical engineering.[21,22] Strong evidence has been given that a large fraction of free water of PCs renders the surface extremely hydrophilic, so that proteins are repelled from that surface.[23] It was also concluded that it is this strong hydration layer that allows for the antibody to contact the surface in a conformation similar to the native state.[24] Therefore, there is almost no nonspecific adsorption of the antibody to our model membranes in the absence of Aß. Hence, the fluorescence signals are thought to contain contributions only from the antibody binding to Aß at the membrane surfaces. We monitored the binding of Aß to the membranes by the detection of fluorescently labeled anti-Aß-mAb bound to Aß at the surfaces using SPFS. Hence, the interaction of Aß and the membranes was deduced from the fluorescent intensities of the bound antibodies.

Figure 3 demonstrates the effect of the lipid composition on the Aß adsorption. By stepwise increasing the solution concentration of Aß the (interfacial) fluorescence intensity measured by SPFS at each concentration as the peak intensity of an angular fluorescence intensity scan after the bound Aß was "decorated" with the chromophore labeled antibodies increased linearly. This indicates that the amount of Aß bound to the model membranes is proportional to the increase in the concentration of the peptide. This is a strong hint that binding occurs specifically and that, indeed, major nonspecific contributions can be neglected. The degree of the specific binding of Aß to the tethered bilayers of different composition depends critically on the membrane constituents: the steepest linear curve was obtained for the tertiary mixtures of POPC/SM/Oxy, whereas the curves form the membranes composed of only binary mixtures are almost identical to the background. These experiments, hence, support the notion that Aß specifically recognizes the SM/sterols and the absence of either one of them results in a substantial decrease in Aß adsorption, implying the selective binding of Aß to the special domains composed of SM and sterols. We should mention that we monitor here only the low concentration range of the peptide binding to the distal lipid layer, where saturation effects do not play a role yet. We are currently recording more detailed data covering also the high concentration range. These data will then allow us also to analyze the binding behavior in terms of affinity constants K_A. These results will be reported later.

Figure 4(a) summarizes these results. The graph shows that the amount of antibody bound to Aß at pure POPC membrane is the lowest among the four lipid systems and their mixtures studied, indicating that Aß has the weakest interaction with POPC. The addition of 50 mol % cholesterol (Chol) or 25-hydroxycholesterol (25-OH-Chol) to POPC increased the fluorescence signal, i.e., the Aß adsorption. These results are indicative of the role played by the sterols in Aß-membrane interaction, similar to the results that have been obtained by other methods.[11,25] It was reported that the sterols could facilitate Aß incorporation into the PC membranes if the content of the sterols exceeded 30 mol %.

For binary equimolar mixtures of POPC and sphingomyelin a slightly enhanced adsorption Aß was observed. This

preferential binding is possibly due to the specific recognition of Aß by sphingomyelin. This assumption is confirmed by the fact that sphingolipid-binding domains have been identified in Alzheimer, Prion, and HIV proteins.[8] Compared to sphingomyelin, however, the earlier results do not show that the sterols contribute considerably to the Aß adsorption even in the presence of 50 mol % sterols in the systems.

In recent years, lipid rafts mainly composed of sphingolipid and cholesterol have received attention in the pathogenesis of AD. Studies from many laboratories showed that the production and adsorption of Aß occurred at raft-like domains.[7,26] It was reported[6,7] that Aß showed a significantly higher affinity for gangliosides within rafts of cholesterol and sphingomyelin. Our results, indeed, demonstrate that the adsorption of Aß to mixed lipid membranes is remarkably amplified only if both components, sterols and SM, coexist in the membranes.

Obviously, the strongly enhanced driving force for Aß-membrane interaction is related to the sterol-SM association. The most significant increase in the fluorescence signal was observed in the system POPC/SM/25-OH-Chol (2:1:1), followed by the system POPC/SM/Chol (2:1:1). This is suggesting that 25-OH-Chol may also be more tightly associated with SM than with POPC like cholesterol. These data implicate that the role of 25-OH-Chol could be of a certain importance for AD development. A recent study has pointed to the presence of oxidation products of cholesterol that easily occur during the aging process.[27]

The existence of microdomains or lipid rafts rich in cholesterol and sphingomyelin has been demonstrated in artificial membranes by using diverse biophysical techniques, although doubts about their presence in living cells still exist.[28] However, it is not clear whether 25-OH-Chol can form microdomains with sphingomyelin. Therefore, epifluorescence images of Langmuir monolayers were recorded (pictures taken not shown). By doping the monolayers with a fluorescent phospholipid analog, lipid phase separation can be clearly visualized. In order to test the partitioning preference of the probe in different phases, Langmuir monolayers of DPPC with their well-studied phase behavior were imaged first. The fluorescently labeled lipid was excluded from the micro-domains as it is characteristic of DPPC monolayers at the air/water interface in the liquid expanded/liquid condensed phase coexistence region. We studied also monolayers composed of POPC/SM/Chol (2:1:1) and of POPC/SM/25-OH-Chol (2:1:1) to further validate that approach. The coexistence of two phases could be seen in the monolayers very clearly.

Moreover, the two-phase region of the monolayers was found also above the surface pressure of 30 mN/m, at which the second monolayer was deposited to the tethered monolayer from the air-water interface by the Langmuir–Schaefer technique to complete the bilayer. Thus, we believe that the microdomains of the distal layers are preserved upon the formation of a tethered membrane. The specificity of Aß binding to the model system should be closely related to the microdomains mainly composed of SM and sterols.

The present results seem to be in line with the observation that Aß enriches in the detergent-insoluble membrane compartment of human neuroblastoma cells[26] It is assumed that secreted Aß is internalized into the plasma membrane by the specific interaction with the distinct membrane domains. It was further reported[8] that Aß with its sphingolipid-binding motif specifically interacted with SM at the air-water interface. In our case, Aß also prefers SM over POPC, and this preference can be markedly amplified by the addition of sterols. A plausible explanation is that the strong interaction of the sterols (cholesterol or 25-OH-Chol) with SM results in the exposure of more recognition sites to Aß due to the formation of the ordered phase. This hypothesis is supported by other reports that the sphingomyelin undergoes a conformational change during the phase transition.[29] In other words, the conformation of SM in the ordered phase facilitates Aß to interact with the binding sites on SM more than that in the disordered phase.

Our findings may also correlate with a recent report in which it was shown that the level of SM is increased in the brain of Alzheimer patients.[30] High levels of SM in the central nervous system also give a hint as to its potential role in AD development. Of particular interest is the observation that the SM content increases with age in the cerebral cortex.[31] The present work also clearly demonstrates an important role of SM in Aß-membrane interaction. The results of our studies with SPFS suggest that 25-OH-Chol should have a stronger interaction with SM than will cholesterol. Studies in Chinese hamster ovary cells indicate that 25-OH-Chol stimulates the synthesis of SM. These observations implicate the possibly cooperative role of SM and the sterols in AD.

C. Microphase separation in mixed Langmuir monolayers

The existence of microdomains or lipid rafts rich in cholesterol and sphingomyelin has been demonstrated in artificial membranes by using diverse biophysical techniques, although doubts about their presence in living cells still exist.[28] However, it is not clear whether 25-OH-Chol can form microdomains with sphingomyelin. Therefore, epifluorescence images of Langmuir monolayers were recorded. By doping the monolayers with a fluorescent phospholipid analog (NBD-PC), any lipid phase separation can be visualized. In order to test the partitioning preference of the probe in different phases, Langmuir monolayers of DPPC with their well-studied phase behavior were imaged first (not shown). It was found that NBD-PC is largely excluded from microdomains with a propeller shape, which is characteristic of DPPC monolayers at the air/water interface in the liquid expanded/liquid condensed phase coexistence region. We employed also monolayers composed of POPC/SM/Chol (2:1:1) to further validate that approach. The coexistence of two phases was seen in the monolayers (also not shown) in agreement with other work.[32]

25-hydroxycholesterol, which closely resembles cholesterol, presumably also has the tendency to form ordered

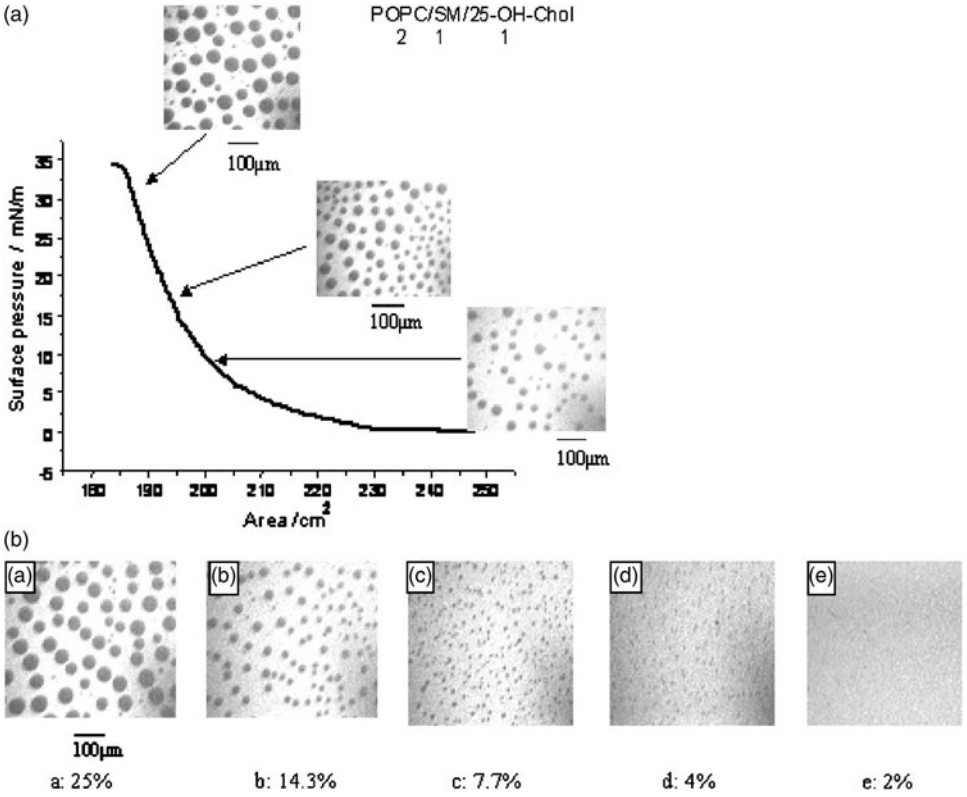

FIG. 5. Effects of 25-hydroxycholesterol on the formation of microdomains. (a) The monolayers composed of POPC/sphingomyelin/25-hydroxycholesterol doped with 0.6 mol % NBD-PC form microdomains at the above surface pressure of 20 mN/m. (b) The fluorescence images of the microdomains markedly changed as the concentration of 25-hydroxycholesterol in the tertiary mixture decrease and microdomains could not be observed at the concentration of 2 mol %.

phases with SM. In order to address this question, the phase behavior of monolayers of POPC/SM/25-OH-Chol (2:1:1) was investigated at the same conditions as before. Figure 5(a) clearly shows the microdomain formation despite the replacement of cholesterol with 25-OH-Chol in this tertiary mixture. These observations suggest that 25-OH-Chol could also form ordered phases with SM as does cholesterol. This conclusion was further strengthened by the fact that the imaging of the microdomains was critically dependent upon the 25-OH-Chol concentration [Fig. 5(b)], which indicated that 25-OH-Chol played a key role in the microdomain formation. More important is the fact that the two-phase region of the monolayers was found above the surface pressure of 30 mN/m, at which a well-defined second monolayer was deposited from the air-water interface by the Langmuir–Schaefer technique. Thus, it is believed that the microdomains of the distal layers are preserved upon the formation of tethered lipid bilayers. The specificity of Aß binding to the model membrane should be closely related to the microdomains mainly composed of SM and sterols.

IV. CONCLUSIONS

We successfully constructed a stable and well-defined assay system with which the Aß-lipid interactions could be studied. Another important point is that the model membranes have the same packing density of lipids as a cell membrane. Therefore, the adsorption behavior of Aß studied in our model system should be closely related to that in biomembranes. The combination of SPFS and peptide-tethered artificial membranes provides an ideal platform for investigations on AD.

In experiments comparing different lipid compositions, selective adsorption of Aß was observed. The simultaneous existence of SM and sterols in the model systems significantly enhanced Aß adsorption. Fluorescence imaging showed the formation of microdomains composed of SM and sterols at the conditions used for the buildup of the model membranes. Hence, the microdomains are thought to be the binding sites of Aß in bilayer systems. Sphingomyelin should receive much more attention in AD research than so far, although cholesterol is prevalently thought to be an important factor in AD, as well. In this work, an interesting phenomenon is that the sterols used (Chol and 25-OH-Chol) cannot increase Aß adsorption significantly unless SM is present in the model membranes. It can be assumed that the presence of SM modulates the interaction of Aß with the membranes. Among the model membranes used, the highest propensity of Aß to adsorb to the microdomains containing 25-OH-Chol suggests that oxidation of cholesterol may relate to some aspects of AD.

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