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Model Phospholipid Liposomes to Study the β -Amyloid-Peptide-Induced Membrane Disruption

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Abstract

Model phospholipid liposomes have been utilized widely to study the molecular interactions between peptides and membrane bilayers. In the mechanistic study of Alzheimer's disease (AD), disruption of neuronal cell membranes has been considered as a major contribution for the β -amyloid (A β) peptides' neurotoxicity. However, clear interpretation of the A β -induced cellular membrane at high-resolution level is challenging because of the co-existence of multiple pathways. Here we present the generation of simplified model liposome systems that will facilitate the in-depth mechanistic studies. Protocols for the preparation of model liposomes and the characterization of individual membrane disruption effects will be described.

Keywords

β -Amyloid peptides; Phospholipid liposomes; Membrane disruption; Fibrillation; lipid mixing; Electrophysiology; Ion channels

1 Introduction

A major challenge that hinders the in-depth, high-resolution studies on the β -amyloid (A β)-peptide-induced membrane disruption process is that the A β -liposome systems are typically heterogeneous [1]. Such heterogeneity is mainly induced by the high tendency of aggregation for A β peptides in aqueous solution at the concentration of most biophysical studies (i.e., $\sim \mu\text{M}$ range) [2–4]. Even in the simplest phospholipid bilayers that contain only zwitterionic phosphatidylcholine (PC) and negatively charged phosphatidylglycerol (PG), interactions between A β and membranes may be initiated with monomeric, small oligomeric, and large oligomeric peptides in in vitro model systems. Distinct individual states of A β at the initial stage are structurally heterogeneous, and may lead to different downstream membrane disruption effects [5, 6]. The co-existence of these pathways greatly increases the complexity of model systems. For instance, two of the previously reported membrane-associated A β aggregation pathways, the fibrillation and the formation of Ca^{2+} -selected ion channel, involved membrane interactions of distinct A β species. The A β fibrillation pathway is initiated from monomeric peptides that were absorbed by the

membrane bilayer [5, 7]. In this case, the bilayer surface may serve as templates, which promote the formation of specific structures on pathway of the fibrillation [5]. Along with the fibrillation process, local membrane disruption may lead to membrane leakage and/or the uptake of lipids [5, 8]. On the other hand, the ion channel activities were detected in the presence of membrane-incorporated A β oligomers [9]. Molecular dynamic simulations suggest that the β -barrel-like oligomer structures are stabilized by the surrounding lipids [9–11].

This protocol describes the generation and biophysical characterization of three distinct A β -membrane model systems [12]. These systems are produced with well-controlled initial A β concentrations and peptide-to-lipid (P:L) molar ratio. Biophysical techniques are utilized to determine the dominant membrane-disruption effects in each system. These model systems, because of the reduction of heterogeneity, may be utilized in future works for high-resolution studies of the molecular mechanisms that contribute to A β -related toxicity.

2 Materials

Buffer solutions utilized in this protocol were prepared with deionized water. All organic solvents were analytical grade with the exception of HPLC-grade water and acetonitrile used for high-performance liquid chromatography (HPLC). Procedures for the manual peptide synthesis and purification are well established and considered as routine protocols, and therefore will not be described in details here.

2.1 Liposome Preparation

1. A β 40 peptides (*see* Note 1).
2. HFIP.
3. Phospholipids: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 25 mg/mL in chloroform (*see* Note 2).
4. Extruder.
5. Support filter paper.
6. 1.0 μ m or 100 nm pore-size polycarbonate membranes.
7. 10 mM phosphate buffer (10 mM Na₂HPO₄, 10 mM NaH₂PO₄, 0.01% m/v NaN₃) with pH adjusted to 7.4.
8. Vacuum desiccator.
9. *n*-Decane.

2.2 Circular Dichroism (CD) Spectroscopy

1. Circular Dichroism Spectrometer.

¹For the synthesis of 40-residue A β peptides, it is important to use resin with relatively low substitution because the primary sequence is highly hydrophobic. We found that ~0.3 mmol/g substitution works fine for our synthesis.

²The amount of POPC was calculated specifically for individual sample preparation based on the desired amount of A β and the P:L ratio.

2. Quartz cuvette with 1 mm path length (max. Volume 350 μ L).

2.3 Fluorescence Assays

1. A fluorimeter equipped with temperature control or a water bath.
2. Fluorescent phospholipid mixture: POPC containing 1 mol% NBD-phosphatidylethanolamine (NBD-PE, 0.2 mg/mL in chloroform) and 1 mol% Rhodamine-B-labeled phosphatidyl-ethanolamine (Rh-PE, 0.2 mg/mL in chloroform).
3. Thioflavin-T (ThT) buffer: 4 mM ThT in 10 mM phosphate buffer, pH = 7.4 (*see* Note 3).
4. Calcein-contained phosphate buffer: 5 mM calcein in 10 mM phosphate buffer (pH 7.4).
5. Quartz micro-scale fluorescent cuvette with 1 mm path length (max. Effective volume 120 μ L).

2.4 Fluorescence Imaging

1. Rhodamine-green-labeled A β (Anaspec, Part # AS-61134).
2. Rh-PE.
3. Phospholipid mixture for fluorescence imaging: POPC containing 1 mol% Rh-PE (0.2 mg/mL in chloroform).

2.5 Transmission Electron Microscopy (TEM)

1. Glow-discharged carbon film, 300-mesh carbon-coated copper grid.
2. Uranyl acetate.

2.6 Electrophysiological Measurements

1. Two-compartment electrophysiological chamber with a 200 μ m aperture.
2. Glutaraldehyde: ~10% v/v in deionized water.
3. HEPES buffer: 10 mM HEPES with 150 mM KCl and 1 mM magnesium gluconate, pH adjusted to 7.4.
4. n-Decane.

³The ThT solution was prepared by directly dissolving ThT powder into the deionized water, bath-sonicating for 5 min at ambient temperature and filtering through 0.22 μ m syringe filter unit. This solution should be prepared fresh.

3 Methods

3.1 Preparation and Characterization of the “External Addition” A β -Liposome Model System with Dominant Fibrillation Pathway

Figure 1 illustrates the A β evolution pathway within this model system. Briefly, A β peptides are absorbed to membrane surface, where the enhancement of local peptide concentration promotes the initial fibrillation. Chain elongation is then continued on the rapidly formed fibril nucleus. Membrane bilayers are locally disrupted by the initial nucleation process, which results in content leakage [7, 13, 14].

3.1.1 Liposome Preparation

1. Dry the desired amount of POPC in chloroform under N₂ flow.
2. Remove all organic solvent in a vacuum desiccator for at least 8 h.
3. Resuspend the dried lipid film in phosphate buffer (*see Note 4*).
4. Rotate the mixture for 1 h at room temperature.
5. Extrude the mixture using 100 nm pore-size polycarbonate membrane for at least 15 cycles at room temperature to ensure the homogeneity of vesicle size. The liposome solution can be stored at 4 °C, but extrusion should be done freshly before usage.

3.1.2 Addition of A β Peptides into Liposome

1. Dissolve the desired amount of A β peptide in 0.1 M NaOH and lyophilize to remove any pre-formed A β aggregates [6].
2. Dissolve the peptide powder in DMSO (2% v/v to the final volume of liposome solution) and transfer it into liposome solution (*see Note 5*).

3.1.3 Monitoring Fibrillation Using ThT Fluorescence (*see Note 6*).

1. Equilibrate the fluorimeter sample chamber to 37 °C and stabilized for 1 h before measurement. For example, one can use a fluorimeter that is equipped with a water-bath cuvette holder.
2. Mix a 100 μ L aliquot of the A β -liposome solution with 10 μ L of 4.0 mM ThT solution and transfer the mixture into the fluorescence cuvette (*see Note 3*) [15].
3. Cap the cuvette and seal it with parafilm.
4. Allow the sample to sit in the holder for 5 min before data collection.
5. Record the fluorescence signal with excitation and emission wavelengths at 440 nm and 490 nm, respectively, over ~14,400 s with 5 s dwell time.

⁴. Phosphate buffer was added so that the final concentration of A β was ~ 50 μ M for all our samples.

⁵. For practicality, it is better to dilute the concentrated DMSO/A β solution with phosphate buffer rather than adding the DMSO solution drop by drop into the buffer. This is to avoid instant aggregation of A β due to the slow dispersion of DMSO in water.

⁶. Technically, we have utilized ThT fluorescence to measure the initial stage of fibrillation instead of the elongation of A β fibrils.

6. Record control ThT fluorescence traces for liposomes without the addition of A β and subtract this trace from the sample trace. A typical fluorescence emission trace for this mode system was shown in Fig. 2a.

3.1.4 Monitoring of Fibrillation Using TEM

1. Prepare the sample as outlined in Subheadings 3.1.1 and 3.1.2, and incubate at 37 °C quiescently.
2. Remove a 10 μ L aliquot of the A β -liposome solution at the desired time point and dispense onto a glow-discharged carbon film supported by lacey carbon on a 300 mesh copper TEM grid.
3. After 2 min, blot the residual solution with tissue paper and rinse the grid twice with deionized water.
4. Stain the grid with 10 μ L 2% uranyl acetate for 35 s (*see* Note 7).
5. Blot the excess staining solution with tissue paper.
6. Dry the grid in air and tested using TEM. Representative TEM images with different incubation time was shown in Fig. 2b.

3.1.5 Monitoring of Liposome Leakage Using Calcein Fluorescence

1. Prepare the calcein-contained liposome using a protocol similar to Subheading 3.1.1, with the exception that the calcein- contained phosphate buffer is used for the resuspension of the dried lipid film in **step 3**.
2. The final concentration of calcein should be kept at 1 mM for the liposome solution (in 10 mM phosphate buffer).
3. Extrude the liposome mixture as in **step 5** Subheading 3.1.1.
4. Transfer the extruded liposome mixture to a snakeskin dialysis tube with 3.5 kDa molecular weight cutoff and dialyze against 1000 $\times$ bulk phosphate buffer (10 mM, pH 7.4).
5. Repeat the dialysis procedure three times with buffer exchange to ensure complete removal of uncaptured calcein (*see* Note 8).
6. After dialysis, add A β peptide to the liposome solution as in Subheading 3.1.2.
7. Transfer 110 μ L of A β -liposome (calcein contained) solution to the fluorescence cuvette and pre-warm the solution to 37 °C in the cuvette holder.
8. Record a kinetic fluorescence trace of the A β -liposome-calcein mixture as in Subheading 3.1.3, using excitation and emission wavelengths set to 495 nm and 515 nm, respectively.

⁷The staining time and the concentration of uranyl acetate has been optimized to provide best contrast for the TEM imaging for the A β -liposome samples. These parameters must be optimized for other samples.

⁸The first and second dialysis were performed at ambient temperature for 2 h, and the third round was done at 4 °C overnight. We observed that the green color (calcein) in the dialysis buffer faded over multiple rounds.

9. Record control calcein fluorescence traces for the liposomes without A β and subtract the control trace from the sample. A representative trace is shown in Fig. 2a above the ThT fluorescence.

3.1.6 Monitoring of the Global Secondary Structures of A β Using CD Spectroscopy

1. Incubate A β -liposome preparations at 37 °C quiescently.
2. Dilute a 12 μ L aliquot solution to 300 μ L using deionized water (*see* Note 9), and transfer it into a 1 mm path-length CD cuvette.
3. Record the CD spectrum from 190 nm to 260 nm wavelength range with at least a 1 nm/s scanning rate and 40-scan signal averaging.
4. Control CD spectrum was recorded with only POPC liposomes in the absence of A β , and subtracted from the spectrum for the POPC/A β samples. A representative CD spectrum with 20-h incubation time for this sample was shown in Fig. 2c.

3.2 Preparation and Characterization of the “External Addition” A β -Liposome Model System with Dominant Lipid Mixing Pathway

The main difference between the fibrillation pathways and lipid mixing, in terms of the sample preparation protocol, is the P:L molar ratio. The identification and characterization of these two competing pathways were described in our published papers [7,13, 14, 16]. For this protocol, we will focus on the fluorescence assay that has been utilized to characterize the vesicle fusion in the A β -liposome model system (sketched in Fig. 3a).

3.2.1 Liposome Preparation

1. Premix the fluorescent phospholipid mixture containing 80% *v/v* non-fluorophore-labeled liposomes (only POPC), 20% fluorophore-labeled liposomes (POPC with NBD-PE and Rh-PB) as shown in Subheading 2.3.
2. Dry the film in a similar fashion as described in **step 2** of Subheading 3.1.1.
3. Take up the mixture in 10 mM phosphate buffer and form liposomes using the same protocols in Subheading 3.1.1, **steps 4–5**.
4. Add A β at a concentration of 1:120 P:L ratio (*see* Note 10).

3.2.2 Measurements of Lipid Mixing Using Fluorescence

1. Warm up the fluorimeter to 37 °C and equilibrate for 1 h.
2. Transfer a 110 μ L aliquot of the A β -liposome mixture to the fluorescence cuvette.

⁹The dilution of A β -liposome solution was necessary for our CD spectrometer setup. Otherwise the driving voltage for our instrument tends to be too high. It may not be necessary for other instrumental setups, and should be optimized.

¹⁰This particular P:L ratio gives dominant vesicle fusion effect over the fibrillation, based on our published work [13].

3. Incubate the mixture in the cuvette holder for 5 min and record the fluorescence trace with the excitation and emission wavelengths at 480 nm and 585 nm, respectively.
4. Record control fluorescence traces for liposomes without the addition of A β and subtract them from the sample.
5. To quantify the percentage of vesicle fusion, after the fluorescence signal reaches plateau, add a 10 μ L aliquot of 20% SDS solution (SDS dissolved in deionized water) to the fluorescence cuvette.
6. Incubate the mixture for 5 min and record the fluorescence emission level. This value represents 100% lipid mixing [17, 18]. A representative fluorescence trace for this sample was shown in Fig. 3b.

3.3 Preparation and Characterization of the “Pre-incorporation” A β -Liposome Model System with Dominant Membrane Fragmentation Pathway

As shown in Fig. 4, using a different sample preparation protocol, named “pre-incorporation,” the model system adopted a completely different membrane disruption pathway. We have identified that membranes are largely fragmentized in this model system, resulting in an aggregation composed of A β -lipid complex [7, 12–14]. During this process, ion channels may be temporarily formed and stabilized within the membrane bilayer at low P:L ratio such as 1:120, but not at high ratio such as 1:30. In this protocol, we will describe the “pre-incorporation” sample preparation procedure and the characterization of the formation of A β -lipid complex and ion channels.

3.3.1 Co-dissolving of Lipids and A β

1. Dissolve A β in HFIP (*see* Note 11).
2. Mix the desired amount of lipids (in chloroform) and A β (in HFIP).
3. Remove the organic solvents using N₂ flow.
4. Dry the film by completely removing residual solvents in a vacuum desiccator for at least 8 h.

3.3.2 Liposome Preparation

1. Resuspend the dried film in 10 mM phosphate buffer. The A β concentration should be kept at 2.0 mg/mL.
2. Rotate the mixture at ambient temperature for 1 h.
3. Perform 10 freeze-thaw cycles on the mixture using liquid N₂ and bath sonicator (*see* Note 12).

¹¹The exact volume of HFIP was not critical here. We typically use 1.0 mL to ensure all A β peptides are dissolved.

¹²For POPC, the bath sonication was performed at ambient temperature, because this lipid has a phase transition temperature at ~ -2 °C. If other types of lipid with higher phase transition temperature are utilized. A heated water bath may be necessary.

4. Extrude the mixture using a 100 nm pore-size polycarbonate membrane (*see* Note 13). For “pre-incorporation” samples, the A β -contained liposome has to be prepared freshly before measurements and cannot be stored.
5. Incubate the pre-incorporation mixture at 37 °C quiescently for further testing, such as the CD and fluorescence assays described in previous sections.

3.3.3 Visualization of the Co-localization of Lipids and A β Peptides

1. Prepare the “pre-incorporation” A β -liposomes using the protocols in Subheadings 3.3.1 and 3.3.2 using rhodamine-green-labeled A β and 1 mol% Rh-PE (molar percentage to the total lipids), with the exception that a 1.0 μ m pore-size polycarbonate membrane was utilized for the extrusion (*see* Note 14).
2. Add 400 μ L of A β -liposome suspension to glass bottom confocal dish (MatTek Corporation).
3. Capture the liposome images using a laser scanning confocal microscope (Leica TCS SP2, Germany) with HCX PL APO 63 $\times$ 1.4 oil immersion objective by 4 time digital zoom-in, with 488 nm and 543 nm excitation and emission wavelengths. A representative fluorescence image obtained on the “preincorporation” sample with P:L 1:30 was provided in Fig. 5a.

3.3.4 Measurements of Ion Channel Activities

1. Dry 20 μ L, 25 mg/mL POPC in chloroform using N₂ flow.
2. Resuspend the lipid film in 75 μ L, 10 mM phosphate buffer with brief sonication.
3. Add 11.7 μ L, 2 mg/mL freshly dissolved A β in deionized water.
4. Perform 10 freeze-thaw cycles on the mixture using liquid N₂ and water bath sonicator for 10 cycles (*see* Note 15).
5. Sample preparation with cross-linking:
 - (a) Prepare the A β -liposome using **steps 1–4**. After freeze-thaw, add 15.7 μ L of glutaraldehyde (70% v/v in water) to the liposome solution to cross-link any membrane-associated A β oligomers [9, 19].
 - (b) React at ambient temperature for 20 min.
 - (c) Quench the reaction with 21.7 μ L Tris buffer (1.0 M, pH 7).
6. Black lipid membrane (BLM) for ion channel measurements:
 - (a) Dry a 50 μ L aliquot of POPC in chloroform with N₂ flow.
 - (b) Re-dissolve the lipid film in 75 μ L *n*-decane.

¹³For the “pre-incorporation” sample, we found it was necessary to do the extrusion with 1.0 μ m pore-size membrane followed by 100 nm pore-size membrane.

¹⁴In this case, the larger pore-size membrane was utilized because of the resolution obtained on our fluorescence imaging instrumental setup.

¹⁵Experimentally, we observed that the mixture becomes transparent after 10 cycles of freeze-thaw.

- (c) Fill the electrophysiological chamber with 10 mM HEPES buffer.
- (d) Deposit a 10 μ L aliquot POPC/decane solution onto the 200 μ m aperture between two compartments in the electrophysiological chamber to form the BLM (*see* Note 16).
- (e) Place the cathode and anode of an Ag/AgCl electrode in the inner and outer compartments of the electrophysiological chamber, respectively.
- (f) Allow the BLM to stabilize for 10 min, and record the baseline.
- (g) Add the A β liposomes into the outer compartment of the chambers (*see* Note 17).
- (h) Add 3 μ L, 100 mM CaCl₂ to both compartments to facilitate the fusion between liposomes and BLM [20].
- (i) Gently mix the solution with pipette tips and recording of current trace was continued for 30–90 min. Two representative electronic traces were shown in Fig. 5b.

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¹⁶. Since the aperture is submerged below the surface of buffer, it is critical to insert the pipette tip under the liquid surface. Let the tip hang slightly above the aperture and slowly deposit the POPC/decane, using a microscope to visualize the process. We found that it was necessary to let the dispersed decane cover the aperture for a few seconds to obtain the best BLM. In addition, the concentration of POPC in decane seems to be critical.

¹⁷. The amount of A β -liposome that has been added in our experiment was 60 μ L, and we found that it gave best results in the recorded electronic traces. This parameter should be optimized experimentally [21].

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Fig. 1.

Proposed Aβ fibrillation pathway in the presence of membrane. Step (1), absorption of Aβ to the membrane surface. Step (2), initial binding between Aβ and membrane bilayer, which induces local membrane disruption and cellular leakage. Step (3), initial nucleus of Aβ on membrane surface. Step (4), the fibril chain elongation

Fig. 2.

Biophysical characterization of the “external addition” sample with P:L ratio 1:30. **(a)** Fluorescence assays for fibrillation (gray) and calcein content leakage (black) plotted on the same time scale. **(b)** TEM images of A β -membrane system taken at different time points of incubation. The scale bar represents 100 nm. **(c)** CD spectrum of A β -membrane system taken at 20-h incubation time

Fig. 3.

Fluorescence measurements on vesicle fusion. **(a)** Fluorescence assay that has been utilized to measure the lipid mixing. The distances between fluorophore-labeled lipids (black spheres) and quenching lipids (gray spheres) become larger during vesicle fusion. **(b)** Representative fluorescence emission curve of lipid mixing for the “external addition” sample with P:L ratio 1:120

Fig. 4.

Proposal of membrane disruption for the “pre-incorporation” samples. **(a)** Absorption of Aβ oligomers by membrane (blue spheres, top view). **(b)** Incorporation of Aβ oligomers in the membrane bilayer forms ion channels at low P:L ratio. **(c)** Elution of Aβ oligomers from membrane bilayer results in Aβ-lipid complex and large aggregation

Fig. 5. Membrane fragmentation and ion channels induced by “pre-incorporation” A β in liposomes. **(a)** Confocal fluorescence images of A β -lipid aggregation (models presented in Fig. 4c) which showed the co-localization of lipid (red channel, Rh-PE) and peptides (green channel, Rh-green-labeled A β). **(b)** Representative electrophysiological current traces of samples prepared using protocol **steps 5–7** in Subheading 3.3