Self-assembly of Amphiphilic Peptide in Phospholipid Membrane

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An amphiphilic molecule \textbf{Lipid-(RADA)$_2$} consisting of a repeating RADA peptide sequence and alkyl chains appending a fluorescent nitrobenzoxadiazole (NBD) dye, was synthesized. In a fluorescence microscopic observation, localization of an aggregate containing \textbf{Lipid-(RADA)$_2$} at a bilayer membrane formed with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was observed.

\textbf{Keywords:} Peptide, Phospholipid, Self-assembly, Fluorescence microscopy, Vesicle

1. Introduction

In physiological conditions, cells control various biological functions by assembling biomolecules in a bilayer membrane [1–5]. For example, lipid raft, a type of membrane microdomain composed of self-assemblies of sphingolipids and cholesterol, accumulates membrane proteins, and plays important roles in signal transduction through the membrane, infection of bacteria and viruses, cell adhesion, vesicle transport, and intracellular polarity [1–3]. Inspired by the lipid raft formation in a biological membrane, a variety of synthetic methodologies of self-assembly in a bilayer membrane have been developed. For example, self-assemblies of nucleic acids [6], saccharides [7], synthetic polymers [8] and peptides [9] in phospholipid membranes have been reported.

Peptide-based self-assemblies in aqueous media are an attractive platform capable of imparting various bioactive functions by controlling the amino acid sequence. Indeed, a lot of biomaterials with superb functions have been realized by using self-assembling peptides [10–20]. In this work, to study self-assembly of peptides at a bilayer membrane, we designed an amphiphilic molecule \textbf{Lipid-(RADA)$_2$}. \textbf{Lipid-(RADA)$_2$} consists of a peptide headgroup having a repeating RADA sequence and alkyl chains attached at main-chain and side-chain amino groups of the N-terminal lysine residue, in which a fluorescent nitrobenzoxadiazole (NBD) dye was appended (Figure 1). \textbf{(RADA)$_2$} peptide has alternating hydrophilic (R and D) and hydrophobic (A) amino acid residues [21]. Such an alternating sequence between hydrophilic and hydrophobic amino acid residues is known to be favorable to form a $\beta$-sheet
Fig. 2. Synthetic scheme of Lipid-(RADA)$_2$. 
self-assembly [18–20]. The alkyl chains with a fluorescent group were attached to (RADA)$_2$ peptide as an anchoring unit into the phospholipid membranes and for direct observation under a fluorescence microscopy.

2. Methods

2.1. General

$^1$H nuclear magnetic resonance (NMR) spectra were recorded on a JEOL ECX-400 spectrometer. High-resolution electrospray ionization time-of-flight mass (HR ESI TOF MS) spectra were recorded on a Bruker micrOTOF-QII. Fluorescence and phase-contrast microscopic observations were performed with an Olympus IX-73 microscope.

2.2. Reagents

$N,N'$-Dimethylformamide (DMF), $\text{Et}_2\text{O}$, $N$-methyl-2-pyrrolidone (NMP), piperidine, and trifluoroacetic acid (TFA) were purchased from Kishida Chemical (Tokyo, Japan). $N,N'$-Disopropylethylamine (DIEA), hydrazine monohydrate and mineral oil were purchased from Watanabe Chemical Industries (Hiroshima, Japan). 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti (Alabama, U.S.A.). Ultrapure water (filtered through a 0.22 μm membrane filter, >18.2 MΩ cm) was purified in Purelab DV35 of ELGA (Buckinghamshire, UK).

2.3. Synthesis

2.3.1. Synthesis of 1

To an aqueous solution (4.5 mL) of NaHCO$_3$ (0.88 g, 10.5 mmol), a MeOH solution (25 mL) of 4-chlorobenzyl alcohol (1) (2.3 g, 10.5 mmol) was added at 50 °C and stirred for 10 min. To the resulting solution, a MeOH solution (25 mL) of 1H$_2$N-Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Dde)-OH, Fmoc-NH-SAL Resin, HATU, HBTU, and HOBT•H$_2$O were purchased from Avanti (Alabama, U.S.A.). Ultrapure water (filtered through a 0.22 μm membrane filter, >18.2 MΩ cm) was purified in Purelab DV35 of ELGA (Buckinghamshire, UK).

2.3.2. Synthesis of (RADA)$_2$ and Lipid-(RADA)$_2$

(RADA)$_2$ and Lipid-(RADA)$_2$ were synthesized by Fmoc solid-phase peptide synthesis as shown in Figure 2. A mixture of HBTU (3.05 g, 8.04 mmol) and HOBT•H$_2$O (1.25 g, 8.16 mmol) in DMF (16 mL) as condensation reagents, a mixture of DIEA (2.8 mL) and NMP (14.3 mL), and a mixture of TIS (62.5 μL), TFA (2.4 mL), and water (62.5 μL) as cleavage reagents were prepared just before the synthesis. Fmoc-NH-SAL Resin (0.1 mmol) was dispersed in DMF (2 mL) in a polypropylene tube and soaked over 3 h at 25 °C. After removing DMF by filtration, piperidine in DMF (20%, 2 mL) was added to the resin and mixed with a vortex device for 1 min. After removing the reaction solution, piperidine in DMF (20%, 2 mL) was added and the reaction mixture was shaken for 10 min at 25 °C. After removal of the solution phase of the reaction mixture, the resin was washed with DMF (2 mL, 5 times), CH$_2$Cl$_2$ (2 mL, 3 times) and DMF (2 mL, 3 times). Then, Fmoc-protected amino acid (0.3 mmol) dissolved in the mixture of condensation reagents (700 μL) and the mixture of DIEA and NMP (700 μL) was added to the resin. After shaking for 15 min at 25 °C, the solution phase of the mixture was removed by filtration and the resin was washed with DMF (2 mL, 5 times), CH$_2$Cl$_2$ (2 mL, 3 times) and DMF (2 mL, 3 times). The Fmoc deprotection reactions with piperidine and coupling reactions of Fmoc-protected amino acid were repeated following the designed sequence. For the synthesis of Lipid-(RADA)$_2$, after the deprotection of Fmoc group of 4, stearic acid (142.2 mg, 0.5 mmol) was coupled using HATU (186.5 mg, 0.49 mmol) and DIEA (0.17 mL, 1.00 mmol) in DMF (1.5 mL), which was then reacted with hydrazine monohydrate (30 μM) in DMF (1.5 mL) for deprotection of the Dde group to afford 5. 5 was coupled with 2 (189.2 mg, 0.50 mmol) using HATU (186.5 mg, 0.49 mmol) and DIEA (0.17 mL, 1.00 mmol) in DMF (1.5 mL) to afford 6. After removal of the solution phase of the reaction mixture, the resin was washed with DMF (2 mL, 5 times), CH$_2$Cl$_2$ (2 mL, 3 times) and DMF (2 mL, 3 times). The mixture of cleavage reagents (2.5 mL) was added to the resin and the reaction tube was left
to stand for 90 min at 25 °C with gentle shaking every 30 min. The solution was collected into a polypropylene centrifuge tube by filtration. The reaction tube was rinsed with TFA (500 μL, 3 times), which is also collected by filtration. To the centrifuge tube, Et₂O (40 mL) was added and the tube was mixed on a vortex device for 1 min followed by centrifugation at 4 °C (3500 × g, 5 min) and removal of the supernatant liquid. After repeating this process for 3 times, the residue was dried under vacuum over 2 h at 25 °C, dispersed in water and lyophilized.

HR ESI TOF MS: m/z calculated for C₁₂₂H₂₁₈N₂₁O₁₈²⁺ [Lipid-(RADA)₂ + 2H]²⁺ 799.9834; found 799.9855.

3. Results and discussion

To study self-assembly of Lipid-(RADA)₂ at a lipid bilayer membrane, DOPC giant unilamellar vesicles (GUVs) were prepared in the presence of Lipid-(RADA)₂. A 10 μL of CHCl₃/MeOH (2/1) solution of DOPC (2 mM) and a 10 μL of MeOH solution of Lipid-(RADA)₂ (2 μM) were mixed in a glass test tube, and the mixture was gently dried under N₂ flow to produce thin lipid film. The film was subsequently dried under vacuum over 3 h at 25 °C. 200 μL of mineral oil was added to the lipid film and sonicated for 1 h. For the preparation of an emulsion, a 75 μL of the resulting mineral oil solution was mixed with a 3 μL of sucrose aq. (200 mM). The remaining mineral oil solution (125 μL) was gently put on a 50 μL of glucose aq. (200 mM) and the mixture was centrifuged (1600 × g) for 1 min. After the centrifugation, a 50 μL of the emulsion was put on top of the resulting mixture and centrifuged (1600 × g) for 1 min. On the top of the resulting mixture after the centrifugation, a 50 μL of the emulsion was put on and centrifuged (1600 × g) for 1 min. After removing the upper layer composed of the mineral oil, the lower layer was collected for the microscopic observations.

Phase-contrast micrographic observation showed a ring image (Figure 3a), indicating the formation of a GUV. A ring image was also observed by fluorescence microscopy, indicating localization of Lipid-(RADA)₂ at the DOPC bilayer membrane. Interestingly, the fluorescence microscopic observation showed a bright spot with a strong fluorescence intensity at a part of the membrane (Figure 3b, yellow arrow), suggesting localization of an aggregate containing Lipid-(RADA)₂. It is also noteworthy to mention that, in the phase-contrast micrograph, intravesicular medium is relatively dark compared to the extravesicular medium. This is likely due to the different refractive index between glucose and sucrose solutions, and the sucrose solution was enclosed in the vesicle. Thus, it is likely that a mixture of DOPC and Lipid-(RADA)₂ ([DOPC]/[Lipid-(RADA)₂] = 1000/1) formed a stable bilayer membrane with a localization of an aggregate containing Lipid-(RADA)₂.

As shown in Figure 4, (RADA)₂ in water containing 2.2% trifluoroacetic acid formed dispersed precipitates indicating the self-assembling property. This result suggests that the self-assembling property of the (RADA)₂ unit likely contributes to the aggregate formation, at least partially, of Lipid-(RADA)₂ at the membrane. Detailed analyses of the self-assembling structures of Lipid-(RADA)₂ at the membrane and physicochemical properties of DOPC GUVs containing Lipid-(RADA)₂ are currently under investigation.

4. Conclusion

We synthesized Lipid-(RADA)₂, a lipid consisting of a peptide with a self-assembling property.
property and a fluorescent probe, and studied its localization at a bilayer membrane. Microscopic observations indicated that Lipid-(RADA)$_2$ was localized at a DOPC bilayer membrane, and an aggregate containing Lipid-(RADA)$_2$ was visualized at the membrane.

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