Current Topics

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Review

Bio-inspired Multiblock Molecules for Membrane Functionalization

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A multipass transmembrane (MTM) structure is prevalent in membrane proteins for a wide range of functions. Typically, the MTM structure is constructed of bundled multiple α-helices spanning the membrane which are connected by flexible domains. One characteristic feature of MTM proteins is dynamic functions such as stimuli responses and conformational changes. In this review, the development of synthetic molecules forming an MTM structure in membranes is highlighted. The MTM folded structure is developed using an amphiphilic molecular design with a multiblock strategy between rigid hydrophobic components and flexible hydrophilic units. Such synthetic amphiphiles not only form the MTM structure by folding but also self-assemble to construct supramolecular ion channels. An elaborated molecular design of the MTM structure with a ligand-binding pocket allows for ligand-gated regulation of ion transport. Light-triggered membrane deformation for vesicle budding is also demonstrated.

Key words membrane protein; multipass transmembrane structure; ion channel; vesicle budding; foldamer; self-assembly

1. INTRODUCTION

Membrane proteins are the central molecules that functionalize biological membranes. The transport of molecules, ions, and signals is an important vital function performed by membrane proteins. The barrel-type structure of membrane proteins is an effective architecture for the transport. A family of porins forms a nanometer-scale pore composed of a β-barrel structure, which performs efficient transport of water. A multipass transmembrane (MTM) structure is also prevalent among membrane proteins, as seen in rhodopsin, adrenergic receptors, sodium channels, ATPase, etc. Multiple α-helices span the membrane to form a bundle, where the α-helices are connected by flexible domains. A characteristic property of membrane proteins with the MTM structure is dynamic functions. Rhodopsin, a seven-transmembrane protein, is photosensitive, in which the light-triggered isomerization of the retinal co-factor prompts a conformational change in the molecule. It is also well known that their orientation in the membrane lead to a large conformational change in the molecule. In this review, the development of synthetic mimics of membrane proteins, especially MTM proteins, and characterization of their functions are highlighted.

2. SYNTHETIC MOLECULES FORMING MTM STRUCTURES

The MTM structure seen in membrane proteins is constructed by multiple membrane-spanning α-helices connected with flexible chains, where the α-helices assemble with each other to form a bundle. The rigidity of the α-helices likely allows spanning of the bilayer membrane, and changes in their orientation in the membrane lead to a large conformational change in the molecule. It is also well known that the inside of the bilayer membrane is hydrophobic, while the outside is hydrophilic. Thus, to reconstruct the MTM structure, a multiblock design with two repeating domains, one a hydrophobic, rigid domain as the membrane-spanning unit and the other a hydrophilic, flexible domain as the extramembranous part, is likely effective. On the basis of this basic concept, multiblock amphiphiles 1 and 2 consisting of fluorescent bis(phenylethynyl benzene) (BPEB) as the hydrophobic rigid aromatic units connected with the hydrophilic, flexible oligo(ethylene glycol) chains were developed, where 1 and 2 contain one and four BPEB units, respectively. Amphiphile 1 in tetrahydrofuran (THF) fluoresces at 367 and 384 nm upon excitation at 313 nm, showing a bathochromic shift in fluorescence upon self-assembly, where monomeric BPEB emits fluorescence at 390 nm upon excitation at 320 nm and BPEB in aggregated states such as in a crystal shows 440-nm fluorescence. Amphiphile 1 in tetrahydrofuran (THF) fluoresces at 367 and 384 nm upon excitation at 313 nm, showing a bathochromic shift upon the addition of water (426 and 451 nm in a THF–water (1/9) mixture). Meanwhile, 1 in THF shows a hypsochromic shift in the absorption spectrum upon the addition of water. Amphiphile 2 also exhibits similar absorption

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and fluorescence changes depending on the solvent polarity increase. Dynamic light scattering analyses of 1 and 2 in a THF–water (1/9) mixture show the formation of 100-nm-scale aggregates. Thus, in aqueous media, 1 and 2 form cofacial planar H-aggregates at the aromatic unit.

Amphiphiles 1 and 2 localize in a bilayer membrane comprising phospholipids, which can be directly observed by optical microscopy. Phase-contrast microscopic observation allows the visualization of the formation of giant unilamellar vesicles (GUVs) of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) containing 1 or 2 prepared by the gentle hydration method, and the GUVs also display fluorescent images under conditions exciting the BPEB unit (Fig. 2). The fluorescence depolarizing assay using spin-labeled phospholipids (SLPs) allows for detailed investigation of the location and orientation of 1 and 2. SLPs bearing spin probes at the 12- and 16-posi-

Fig. 1. Molecular Structures of 1 and 2

Fig. 2. (A, B) Phase-Contrast and (C, D) Fluorescent Micrographs of DOPC GUVs Containing (A, C) 1 and (B, D) 2 at 20°C

Scale bars: 10 μm. [DOPC]=200 μM, [1]=[2]=20 μM. Excitation wavelength: 330–385 nm, observation wavelength: >420 nm. Adapted from Muraoka et al. with permission from the Royal Society of Chemistry.
tions of an oleoyl chain of DOPC quench the fluorescence of the BPEB units of 1 and 2 in a comparable manner and more efficiently than an SLP bearing a spin probe at the 5-position. These results indicate that the BPEB unit of 1 and 2 is located in the middle of the lipid bilayer with an orientation nearly parallel to the oleoyl tails. Thus, it is likely that the BPEB unit of 1 and 2 spans the bilayer.

Liposomes consisting of DOPC and 1 show emission peaks at 366 and 384 nm with a profile similar to that of 1 in THF ([1]/[DOPC] = 0.00050, excited at 315 nm). An increase in the concentration of 1 to [1]/[DOPC] = 0.10 prompts enhancement of the broad emission band at >400 nm with a decrement in the intensity of the original emission, showing the maximum emission at 426 nm (Fig. 3A). Hence, 1 assembles in the bilayer membrane forming face-to-face stacking at the BPEB unit upon an increase in the concentration. In sharp contrast to 1, 2 in the bilayer shows emission peaks at 428 and 448 nm even at [2]/[DOPC] = 0.00050. Importantly, a 200-fold increase in the concentration of 2 causes only a slight change in the spectral profile ([2]/[DOPC] = 0.10), and therefore the fluorescence of 2 is affected only slightly by the concentration in the bilayer (Fig. 3B). Thus, the BPEB units of 2 likely form intramolecular face-to-face stacking, i.e., by adopting a folded structure analogous to MTM proteins.

3. ION TRANSPORT BY SUPRAMOLECULAR ION CHANNEL FORMATION

One of the representative functions performed by MTM proteins is the transport of ions and molecules, and it is interesting to determine whether the synthetic MTM foldamers are capable of transport. The detailed investigations described below revealed that 2 transports ions by the formation of a supramolecular ion channel, while 1 is incapable of their transport.

Real-time conductance measurement of a planar DOPC

![Fluorescence Spectra of DOPC GUVs Containing (A) 1 and (B) 2 in Water at 20°C](image1)

![Conductance Recording of a DOPC Bilayer Membrane Containing 1 at the Applied Voltage of 80 mV in HEPES Buffer at 20°C (20 mM, Containing 50 mM KCl, pH 7.5)](image2)
bilateral membrane containing 1 shows little flow of current, regardless of the concentration of 1. In contrast, a planar DOPC bilayer membrane containing 2 displays discontinuous current signals corresponding to potassium ion flow with constant intensities at 5.6 ± 0.5 pA and its double at the applied voltage of 80 mV with the mean lifetime of each current signal τ = 2.6 ms, suggesting ion flow through ion channels (Fig. 4). Analysis using the Hille equation based on the conductance profile revealed the cross-sectional area of the channel S = 0.76 nm². Large unilamellar vesicles (LUVs) prepared with DOPC and 2 encapsulating the fluorescent pH indicator 8-hydroxyxpyrene-1,3,6-trisulfonate (HPTS[1]) shows pH changes in the intravesicular medium due to the ion influx/efflux through the ion channels of 2, which allows for the concentration-dependency analysis of ion transport. Sigmoidal dependency between the concentration of 2 and the ion transport rate is analyzed by the Hille equation, revealing the Hille coefficient n = 3.8 ± 0.1, suggesting that the major working channel is likely a tetramer. The discontinuous conductivity observed in Fig. 4 indicates that the channel has a dynamic feature undergoing opening and closing motions. Taking into account the folded conformation of 2 in the bilayer membrane forming a plate-like architecture, it is considered that the channel has a rhombic architecture consisting of the four self-assembled molecules of 2, and the geometry of the molecules changes between the closed and open states. Among the alkali metal cations, 2 transports smaller cations faster, following Eisenman sequence XI. Eisenman sequence XI indicates metal cations, transports smaller cations faster, following 2 changes between the closed and open states. Among the alkali 2 assembled molecules of other, and the geometry of the molecules channel has a rhombic architecture consisting of the four self- 2 in the bilayer membrane accounting the folded conformation of 2, which allows for the concentration-dependency analysis of ion transport. Sigmoidal dependency between the concentration of 2 and the ion transport rate is analyzed by the Hille equation, revealing the Hille coefficient n = 3.8 ± 0.1, suggesting that the major working channel is likely a tetramer. The discontinuous conductivity observed in Fig. 4 indicates that the channel has a dynamic feature undergoing opening and closing motions. Taking into account the folded conformation of 2 in the bilayer membrane forming a plate-like architecture, it is considered that the channel has a rhombic architecture consisting of the four self-assembled molecules of 2, and the geometry of the molecules changes between the closed and open states. 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4. LIGAND-GATED ION TRANSPORTATION

Switching of ion transport in response to signals, such as a ligand, light, and mechanical force, is widely found in ion channels, which regulates signal transduction and sensing. Such sophisticated molecular systems seen in membrane proteins provide hints for the design of dynamic synthetic molecular devices for switchable ion transport. Light-responsive synthetic and semi-synthetic ion channels have been developed so far. However, in spite of their universality in nature, synthetic ligand-gated ion channels have not been widely developed with only a few examples reported including systems with irreversibility and the use of an inorganic ligand.

To lead to the development of a reversible synthetic ligand-gated ion channel, a receptor 3 was designed (Fig. 5A). 3 has iteratively aligned hydrophobic and hydrophilic sequences consisting of a chiral hydrophobic core bearing two diphenylacetylene (DPA) units and hydrophilic octaethylene glycol (OEG) chains connected via a phosphoric ester linkage. The termini of the OEG chains are capped with hydrophobic triisopropyl (TIPS) groups. It is expected that 3 interacts with aromatic amines via hydrophobic and electrostatic interactions at the DPA and phosphoric ester parts located close to each other.

Spectroscopic studies revealed the detailed conformation of 3 in solution and in the bilayer membrane. In 1H-NMR spectroscopic measurements, the addition of D2O (up to 50% v/v) to the THF-d8 solution of 3 induces an upfield shift of the signals assigned to the aromatic protons (δ = 7.33, 7.34, 7.47, and 7.50 ppm) to 7.25, 7.28, 7.33, and 7.36 ppm, respectively, [3] = 5.0 mM). This spectral change induced by the increase in solvent polarity is considered to be due to the enhanced magnetic shielding by the aromatic groups. Diffusion-ordered NMR spectroscopy measurements allow for the evaluation of the hydrodynamic diameter dh of 3 at 20°C in THF-d8 (dh = 1.35 nm) and in a 1:1 mixture of THF-d8 and D2O (dh = 1.41 nm), which are almost comparable with each other and consistent with the calculated molecular size.
of 3 (1.40 nm), indicating that 3 hardly aggregates under these conditions. These NMR experiments indicated that, in the aqueous condition, 3 adopts a bent conformation, where the two DPA units are in proximity, likely due to hydrophobic and aromatic interactions.

The conformational change in 3 in response to the increase in solvent polarity is visualized by circular dichroism (CD) spectroscopy monitoring the chiral geometry of the two DPA units (Fig. 6A). 3 in THF exhibits a weak Cotton effect, and the addition of water induces intensification of the CD signals of 3 ([3]=3.4 μM). Importantly, the CD spectra of 3 in water show little concentration dependency between [3]=0.034 μM and 3.4 μM below the critical aggregation concentration, suggesting that the CD signals of 3 essentially originate from the chiral molecular geometry. Thus, it is likely that the conformation of 3 is relatively flexible in THF and becomes more rigid with the formation of a bent structure upon the addition of water.

3 localizes in the phospholipid bilayer membrane. Phase-contrast microscopy displays the formation of GUVs composed of DOPC and 3 ([DOPC]=0.18 mM, [3]=0.020 mM). The GUVs are also visualized under a fluorescence microscope monitoring the fluorescence of the DPA units (λex=330–385 nm), indicating the incorporation of 3 into the bilayer membrane. Investigation of the localization and orientation of 3 in the bilayer membrane using the fluorescence depth-quenching method revealed that the DPA units of 3 likely locate inside the bilayer and are nearly in parallel with the alkyl chains of DOPC. Importantly, DOPC-3-LUVs exhibit a CD spectrum similar to that of 3 in water. Thus, it is suggested that 3 embedded in the DOPC bilayer membrane adopts a bent conformation forming intramolecular stacking of the DPA units (Fig. 5B). The bent conformation likely fits in the DOPC monolayer in the liposomal membrane on the basis of the length of the hydrophobic parts of 3 (1.54 nm). This feature allows for the measurement of the isotherm of the Langmuir–Blodgett monolayer to evaluate the area per molecule, and the estimated area per molecule of 3 in the DOPC monolayer is 1.0 nm². This value is close to the sum of the calculated sectional areas of two DPA units and two TIPS groups (1.11 nm²), indicating that the two DPA units as well as the two TIPS groups are embedded in the hydrophobic layer of the DOPC monolayer (Fig. 5B).

It is expected that the bent foldamer with the anionic phosphodiester groups and aromatic DPA units in proximity is advantageous for binding with amine-bearing aromatic groups, such as phenethylamine (PA), through aromatic and electrostatic interactions. The addition of PA to the DOPC-3-LUV suspension in water elicits spectral changes in the UV-Vis absorption and CD ([DOPC]=0.18 mM, [3]=0.020 mM, 20°C). UV-Vis absorption spectroscopic measurement shows a red shift of the absorption bands corresponding to the DPA units. CD spectroscopic measurement shows a significant spectral change with an isodichroic point, which levels off at [PA]/[3]>1000 (Fig. 6B). Thus, it is indicated that 3 interacts with PA in the DOPC bilayer membrane and changes the conformation around the DPA units. A Job plot of 3 with PA shows the maxima at the molar fraction of 0.5, suggesting a 1:1 complexation of 3 and PA. On the basis of the CD spectral change, the dissociation constant Kd between PA and 3 in the DOPC bilayer is estimated to be 443 μM. The Biacore surface plasmon resonance assay also showed that the Kd value is 370 μM, consistent with the CD spectroscopic assay estimation.

The ion transport activity of 3 in the DOPC bilayer can be studied with real-time conductance measurement. The planar bilayer membrane of a mixture of DOPC and 3 dispersed in n-decane is prepared on an orifice containing 3 uniformly on both sides, and the membrane is sandwiched between two chambers ([3]/[DOPC]=7.9×10⁻⁵). The bilayer shows little current flow in the absence of PA. The addition of PA to the upper chamber has almost no effect on the current profile ([PA]=0.20 μM). Interestingly, subsequent addition of PA to the lower chamber triggers a significant increase in the current flow, where pulsed currents with average flows of 4.9 and 10.8 pA are observed at the voltage of 80 mV, likely corresponding to singly and doubly formed ion channels, respectively (Fig. 7). β-Cyclodextrin (βCD) is known to interact with PA in aqueous media with the dissociation constant Kd=41.0 mM. The addition of βCD to the upper chamber to detach PA from 3 significantly reduces the frequency of the current flow, and the subsequent addition of βCD to the lower chamber returns to the impermeable state of 3 ([βCD]=5.0 mM). Rebinding of PA to 3 prompts the enhancement of the current flow. Hence, 3 in the DOPC bilayer shows reversible ligand-gated ion transport. Ion transport analysis...
using vesicles containing a pH-sensitive fluorescent probe, HPTS, in the internal aqueous phase allows investigation of the concentration dependency of the ion transport by 3. Nonlinear dependency of the ion transport rate of 3 on its concentration is observed, and analysis with the Hille equation reveals the Hille coefficient to be $n = 3.1$ ($R^2 = 0.995$). Therefore it is likely that the two half-channels of the trimeric assembly of the 3·PA complex stack with each other to form a supramolecular ion channel. It should be noted that removal of the terminal TIPS groups of 3 deactivates the ion transport function regardless of the complexation with PA. The two TIPS groups of 3 embedded in the hydrophobic alkyl layer of the bilayer membrane possibly play an anchoring role in the membrane, which is likely advantageous for the formation of channels.

5. LIGHT-TRIGGERED VESICLE BUDDING

Deformation of membranes such as vesicle fission or budding is an important function in a living cell for vesicle trafficking and endocytosis. One of the mechanisms of biological membrane deformation is the attachment of a coatomer—adaptor protein complex onto a cargo receptor, triggering budding. Artificial membrane-deformation methodologies, on the other hand, have also been actively developed. For example, vesicle formation procedures from undefined lipid aggregates by electroformation, gentle hydration, water/oil emulsion, microfluidic devices, and IR irradiation are representative examples. In the course of research on synthetic ion channels by membrane-spanning amphiphiles, a cyclic amphiphile 4 consisting of two fluorescent BPEB units and four tetraethylene glycol chains, which are connected via methyl benzoate and trans-azo benzene units, was developed (Fig. 8), and it was discovered that 4 shows vesicle budding triggered by UV and visible light irradiation.

An organic solution of DOPC and 4 in a mixture of MeOH and CHCl$_3$ is evaporated to prepare a uniform thin film at the bottom of a glass test tube ([DOPC] = 2.0 mM, [4] = 0.40 mM). After hydration of the film obtained with an aqueous solu-
tion of glucose and sucrose, shapeless particles are observed in the aqueous medium ([glucose]=100 mM, [sucrose]=100 mM, [DOPC]=200 µM, [4]=40 µM). Interestingly, UV light irradiation prompts micrometer-scale vesicle formation from the particle surface (Fig. 9). Continuous irradiation allows for separation of the generated vesicles from the particles. Neither heating to 80°C in the dark nor irradiation with an IR laser (0.29 mW, 1064 nm) induce vesicle budding, suggesting that the effect of heat is negligible in vesicle formation from the particles. Taking advantage of light use, single vesicle formation from the micrometer-scale area on the particles is possible upon irradiation with a 405-nm laser with a focus diameter of 1 µm. Control studies indicated that the BPEB unit is essential for light-triggered vesicle formation and that the azobenzene unit enhances the efficiency. The amphiphilic structure of 4 is also likely important for blending with the lipid. Transmission electron microscopic (TEM) and microtomographic observations of the nonirradiated particles display membrane architectures at the surface and inside ([DOPC]=200 µM, [4]=40 µM). The thickness of the membranes is 21–37 nm, and thus they are likely multilayered. In the TEM observation of a photoirradiated particle, a vesicle attached to the surface with a 4.7-nm thick membrane, corresponding to a unilamellar membrane, is seen. After further light irradiation, the surface membranes disappear and only fragmentary membranes are observed. Hence, it is considered that the surface membranes of the particle become micrometer-scale unilamellar vesicles.

6. CONCLUSION

Amphiphiles with multiblock structures between rigid hydrophobic components and flexible hydrophilic units are developed, inspired by membrane proteins forming MTM structures. Aromatic groups such as BPEB are used as the hydrophobic components, allowing not only membrane spanning but also visualization by fluorescent microscopic observation and spectroscopic measurement for conformational analysis, and the aromatic groups are connected to hydrophilic oligo(ethylene glycol) chains. Spectroscopic analyses indicate that the aromatic groups span the bilayer membrane and self-assemble intramolecularly, and thus it is suggested that the multiblock amphiphiles form folded structures analogous to the MTM architecture in the bilayer membrane. Interestingly, the folded amphiphiles transport ions. Detailed analyses suggest that the ion transport is conducted by self-assembly of the folded amphiphiles forming supramolecular ion channels. On the basis of the supramolecular ion channel formation by the multiblock amphiphiles, an amphiphilic receptor was developed consisting of iteratively aligned hydrophobic and hydrophilic building blocks such as a chiral hydrophobic core bearing two aromatic DPA units and hydrophilic OEG chains connected via phosphoric ester linkages. The receptor embedded in the bilayer membrane interacts with an aromatic amine, likely by hydrophobic and electrostatic interactions at the DPA and phosphoric ester parts, respectively. The receptor shows reversible ligand-gated ion transport by forming a supramolecular channel upon complexation with the ligand. The multiblock amphiphiles functionalize the membrane not only for transport but also for deformation. A cyclic amphiphile consisting of a multiblock formula with two BPEB units and four tetraethylene glycol chains connected via methyl benzoate and trans-azobenzene units was also developed, which allows for vesicle budding of phospholipid bilayer membrane triggered by UV and visible light irradiation. Thus, the multi-

Fig. 9. Time–Course Phase-Contrast Microscopic Observation of Light-Triggered Vesicle Formation from a Shapeless Particle Consisting of DOPC and 4 in an Aqueous Solution of Glucose and Sucrose ([DOPC]=200 µM, [3]=40 µM, [Glucose]=[Sucrose]=100 mM)

Photomicrographs taken after irradiation (330–385 nm) at 20°C for (A) 0, (B) 10, (C) 25, and (D) 175 s are shown. White arrows indicate the micrometer-scale vesicles generated. Scale bars: 10 µm. Adapted from Shima et al.51 with permission from the American Chemical Society.
block amphiphiles are versatile molecules for membrane functionalization and are expected to lead to varied bio-related applications.

**Conflict of Interest** The author declares no conflict of interest.

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