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Ligand-Printed Ion Pore Composed of Polypeptide Assembly in a Lipid Bilayer Membrane

Masahiro Higuchi,[1,4*] Tomoyuki Koga[2], Takatoshi Kinoshita,[2,4] Yoshiaki Kobuke,[3,4] Masami Kawaguchi[1]

[1*]Department of Chemistry for Materials Faculty of Engineering, Mie University,
1515, Kamihama-cho, Tsu, Mie 514-8507, Japan
Fax: 81-59-231-9431, e-mail: higuchi@chem.mie-u.ac.jp
[2]Department of Materials Science and Technology, Nagoya Institute of Technology,
Gokiso-cho, Showa-ku, Nagoya 466-8555, Japan
[3]Graduate School of Materials Science, Nara Institute of Science and Technology
8916-5, Takayama, Ikoma, Nara 630-0101, Japan
[4]CREST (Japan Science and Technology)

A simple and novel approach for the preparation of a synthetic ligand-gated ion channel having multi-recognition ability was investigated. It involves the ligand-induced formation of an amphiphilic polypeptide assembly that acts as an ion channel in lipid bilayer membrane. Various functional groups, that bind to the specific site on the target ligand(X), were introduced at the amino terminal of α-helical polypeptide. The interaction between the sites of the ligand(X) and the terminal functional groups of the polypeptides could induce the specific location of the α-helical polypeptide rods , *i.e.*, the formation and/or reorientation of the polypeptide assembly in the lipid bilayer. After that the removing of the ligand(X) provided the ligand-printed ion pore in the membrane. It is also confirmed that re-binding of the ligand(X) could close the pore, thus confirming the gating function of the ion pore. In addition, a ligand(Y) was inactive to the ligand(X)-printed ion pore, *i.e.*, it did not close the channel. This ligand-printed polypeptide assembly may permit a novel and easier production of the ligand-gated ion channel, which will give a novel approach for the construction of signal transduction molecular devices.

Key words: polypeptide assembly; recognition structure; substrate-induced rearrangement; ligand gated ion channel

1. INTRODUCTION

Life phenomena, such as signal transduction and chemical balance in neural systems, are determined by proper and exquisitely timed changes in ion channel mediated conductance and/or membrane potential. Acetylcholine receptor is the most thoroughly studied ligand-gated ion channel.[1)] Binding of acetylcholine to the receptor induces a reorientation of five amphiphilic α-helical segments, M2, that form pore in the membrane, which results in the transition from a closed to an open state of the channel.[2)] Studies on mimicking signal reception and transduction mechanism of biological membrane by synthetic systems, may be important not only to the understanding of a simple and/or essential mechanism for transferring information through biological membrane, but they also may provide the basis of molecular devices such as bio-sensor in which sensed information can be converted to electrical signals. Recently, Vogel *et al* reported[3)] that a synthetic ligand-gated ion channel that comprised a ligand-binding region, which is recognized by a specific monoclonal antibody, and a branched channel-forming polypeptide having a mellitin sequence converted the information of ligand binding to electrical signals. On the other hand, we have reported[4)] that ligand-induced polypeptide assemblies composed of α-helical polypeptides bearing various functional groups at the amino terminals, which are formed by an interaction between the ligand and the amino terminal functional groups of the polypeptides in a lipid membrane, exhibited a specific ability to recognize the corresponding ligand.

In this paper, we report a novel approach to the preparation of a synthetic ligand-gated ion channel system, which can convert the chemo-information of the ligand(X) to the membrane potential change via on/off switching the ion pore with production of a photochemical response of a membrane potential sensitive dye in the membrane. That is, the recognition of the ligand(X) by the polypeptide assembly, that is to say, a blocking of the ligand(X)-printed ion permeable pore by itself, induced fluorescence intensity changes of the membrane potential sensitive dye, which was introduced in the membrane beforehand. The chemical signal of ligand(Y) could not be transmitted to the photochemical event in the ligand(X)-printed ion pore system.

2. EXPERIMENTAL

2.1 Materials

Poly(γ-methyl L-glutamate) (PMG) was obtained by the polymerization[5)] of the *N*-carboxyanhydride of the L-glutamic acid γ-methyl ester in dimethylformamide (DMF) solution with 2-aminoethane-*S*-trityl-thiol as the initiator. The molar ratio of anhydride to the initiator was 30, and the polymerization occurred at room temperature over 24 h. A number average degree of polymerization of 22 was estimated from the molar ratio of the trityl moiety to the γ-methyl L-glutamate residues of PMG.

PMG that had Fmoc-L-Asp(OBut) at the amino terminal was obtained from the coupling reaction between PMG and Fmoc-L-Asp(OBut) in the DMF solution for 24 h with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride and *N*-hydroxy-benzotriazole.[6)] The amino terminal Fmoc protecting

group of the polypeptide was removed in the DMF solution containing 20 vol % piperidine for 30 min. We obtained the polypeptide that had the Asp residue at the amino terminal (PMG(Asp)), by removing the *t*-butyl protecting group of the amino terminal Asp residue in a trifluoroacetic acid solution containing 2.5 vol % water and 2.5 vol % 1,2-ethanedithiol, at room temperature for 1.5 h. Polypeptides that had Tyr, Trp, and Ser residues at the amino terminal (PMG(Tyr), PMG(Trp), and PMG(Ser)) were obtained in a similar manner as above, using Fmoc-L-Tyr(But), Fmoc-L-Trp, and Fmoc-L-Ser(But), respectively. The degrees of introduction of Asp, Tyr, Trp and Ser residues to the amino terminal of the polypeptide were determined to be 75, 71, 70, and 76%, respectively, from the fluorescence intensity of the Fmoc protecting group at 310 nm in the DMF solution.

Amphiphilic poly[(γ-methyl-L-glutamate)-*co*-(L-glutamicacid)]s with 5 mol % of L-glutamic acid residues that had Asp, Tyr, Trp, and Ser residues at the amino terminal (PMG/GA(Asp), PMG/GA(Tyr), PMG/GA(Trp), and PMG/GA(Ser)) were prepared by saponification[7] of PMG(Asp), PMG(Tyr), PMG(Trp), and PMG(Ser), respectively.

2.2 Vesicles.

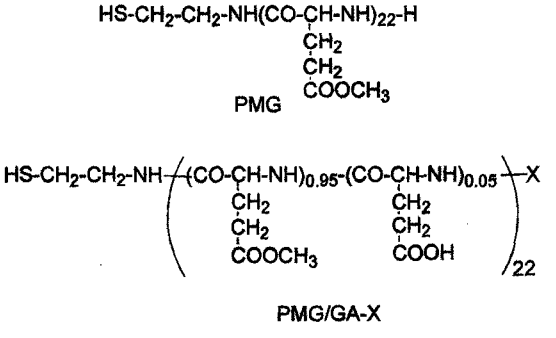
Dipalmitoylphosphatidylcholine (DPPC) and polypeptides were each dissolved in DMF. The solutions were mixed and poured into a glass flask, and then a thin film was formed in the inner surface of the flask by evaporating the solvent. Buffer solution (50 mM Tris-HEPES, pH 6.8) containing 0.2 M potassium gluconate was added to this flask, and it was sonicated to prepare the vesicle. The concentration of DPPC was 0.2 mg / mL. The molar ratio of the polypeptide to DPPC was 0.01. The molar ratio of PMG/GA(Asp), PMG/GA(Tyr), PMG/GA(Trp), PMG/GA(Ser), and PMG was fixed at 1:1:1:1:4. Buffer solution containing ligand, acetylcholine (ACh) or γ-aminobutyric acid (GABA) (final concentration of the ligands were fixed to 0.1 mM), was added to the vesicle at 60 °C above the phase transition temperature of the vesicle, 40 °C, and then incubated for 10 min to form the ligand-induced polypeptide assemblies having ligand recognition ability. The vesicle solution thus obtained was rapidly quenched to 0 °C to fix the structure of the polypeptide assembly in the lipid bilayer membrane. And then it was passed through an Econo-Pac 10DG (Bio-Rad Lab., bed volume 10 mL) gel filtration column by using 0.2 M sodium gluconate in 50 mM Tris-HEPES buffer, pH 6.8, as an eluting buffer. to remove the ligand and external potassium gluconate. The fraction containing DPPC vesicles was collected and added to the buffer solution containing 0.2 M sodium gluconate. The resulting vesicles had an ionic concentration gradient between the interior (0.2 M potassium ion) and exterior (0.2 M sodium ion) under isotonic conditions and the ligand free ligand-printed polypeptide assembly.

2.4 Fluorescence Measurements.

Membrane potential changes of the vesicle induced by the gating of the ligand-printed ion pore was investigated by a fluorescence technique using 3,3'-dipropylthiadicarbocyanine (di-*S*-C3(5)) as a probe. 3 μL of 0.25 mM di-*S*-C3(5) ethanol solution was added to 2.5 mL of 50 mM Tris-HEPES buffer containing 0.2 M sodium gluconate, and the solution was briefly stirred in a quart cuvette at 20 °C. 100 μL of DPPC vesicle suspension obtained by the gel filtration, which had 0.2 M potassium gluconate in the interior and 0.2 M sodium gluconate in the exterior, was added to the di-*S*-C3(5) solution. Fluorescent intensity changes at 670 nm of the dye after the addition of 10 μL stock solution of ligands, ACh and GABA, and gramicidin, respectively, were recorded with excitation at 622 nm. During the measurements, the solution was gently stirred. The ligands and gramicidin were added from stock solution in 50 mM Tris-HEPES buffer, pH 6.8, containing 0.2 M sodium gluconate and trifluoroethanol, respectively. The concentrations of the stock solutions were 1.5 mM for ligands and 25 μM for gramicidin.

3. RESULTS AND DISCUSSION

Figure 1 shows the ligand-gated ion channel model polypeptides, consisting of amphiphilic α-helical polypeptides having various functional groups at the amino terminals. The functional groups for the ligand-binding site in this work are Asp, Tyr, and Trp[8] for nicotine acetylcholine receptor (nAChR), and Ser and Tyr[9] for γ-aminobutyric acid receptor (GAGAR). They were attached to amino terminals of the amphiphilic α-helical polypeptides by condensation reaction between carboxylic groups of those amino acids and amino terminal groups of the polypeptides, respectively. We have chosen a poly[(γ-methyl L-glutamate)-*co*-(L-glutamic acid)] with 5mol% L-glutamic acid as an ion permeable pore-forming region, which was obtained by the saponification of poly(γ-methyl L-glutamate). The secondary structure of these polypeptides in trifluoroethanol solution was estimated from circular dichroism (CD) spectra. The CD spectrum of these polypeptides showed a double minimum profile at 208 nm and 222 nm, typical of a



nAChR model peptides		GABAR model peptides	
X ; Asp	PMG/GA-Asp	X ; Ser	PMG/GA-Ser
Tyr	PMG/GA-Tyr	Tyr	PMG/GA-Tyr
Trp	PMG/GA-Trp		

Fig.1 Chemical structure of nAChR model polypeptides and GABAR model polypeptides.

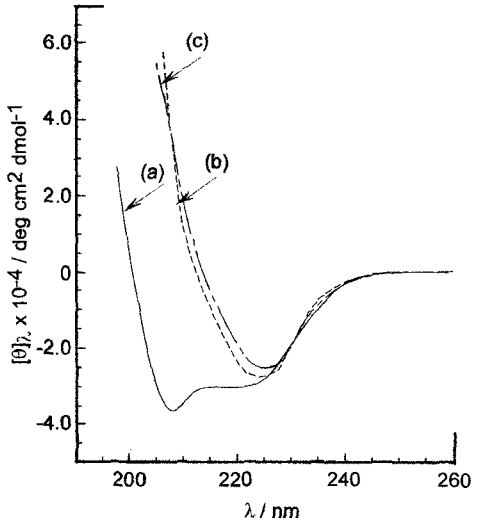


Fig.2 CD spectra of PMG/GA(Asp), PMG/GA(Tyr), PMG/GA(Trp), PMG/GA(Ser), and PMG in (a) trifluoroethanol solution, in (b) DPPC vesicle after incubation with ACh, and in (c) DPPC vesicle after incubation with GABA. The concentration of the polypeptide was 6.9 X 10^{-4} res.M in trifluoroethanol solution and 6.3 X 10^{-5} res.M in DPPC vesicle, respectively. The concentration of DPPC was 0.2 mg / mL. The molar ratio of the polypeptides to the DPPC was 0.01. The molar ratio of PMG/GA(Asp), PMG/GA(Tyr), PMG/GA(Trp), PMG/GA(Ser), and PMG was 1:1:1:1:4.

stable right-handed α-helix[10] (Figure 2(a)). The helical content was estimated to be 85% from the value of the molar ellipticity at 222 nm.

The structure of the ligand-printed amphiphilic polypeptide assemblies in the lipid bilayer membrane was estimated by CD spectra. The spectra of these polypeptides after being incubated with ligand in DPPC vesicle showed a remarkable variation, *i. e.*, the spectrum was distorted with red shifting of a 222 nm band toward 227 nm and decreases of a 208 nm band (Figure 2(b) and 2(c)). The same distortions of the spectra have been shown with aggregated α-helical polypeptides.[11] Thus, the polypeptides have an α-helical conformation, and several amphiphilic α-helical rods self-associated to form the bundles, which were induced by the interaction between the ligand and terminal amino acids of the amphiphilic polypeptides in the vesicle. The ligand recognition abilities of the ligand-printed polypeptide assemblies were reported in a previous study[4]. The Ach-printed polypeptide assembly, which was formed by the interaction between the ACh and the terminal amino acids (Trp, Tyr, and Asp) of the polypeptides showed a specific binding ability to ACh rather than GABA. The binding constant, K, of ACh (1.54 x 10^5 M^{-1}) was 4.3 times larger than that of GABA (3.62 x 10^4 M^{-1}). On the other hand, the polypeptide assembly induced by the incubation with GABA, GABA-printed assembly, exhibited specific binding ability to GABA (K = 7.58 x 10^4 M^{-1}) compared with ACh (K = 4.06 x 10^4 M^{-1}).

We have already reported[12] that the transmembrane bundles composed of amphiphilic polypeptide assemblies in a lipid bilayer membrane acted as ion permeable pores. The gating of the ion permeable pore composed of the ligand-printed amphiphilic polypeptide assembly induced by re-binding of the ligand was investigated by membrane potential changes of the vesicle membrane. It is well known that the membrane potential across the vesiclar bilayer can be monitored by the fluorescence intensity of the membrane potential sensitive dye such as di-*S*-C3(5) . This dye is a membrane permeant cation, and the distribution of the free dye in the vesicle suspension therefore varies with membrane potential, with the dye uptake into the vesicle increasing as the membrane potential becomes more negative. When di-*S*-C3(5) binds to the vesicle the fluorescent intensity is decreased owing to the concentration quenching.[13] In our case, the vesicles, which have potassium in the interior and sodium in the exterior, show a negative membrane potential because of the difference of the diffusion potentials between the interior potassium ion and exterior sodium ion. Ion permeation through the polypeptide assemblies reduces the negative membrane potential, resulting in the increase in fluorescent intensity of di-*S*-C3(5). So the membrane potential changes resulting from the ion pore blocking by the ligand were monitored by fluorescence intensity changes of di-*S*-C3(5) at 20 °C. Figure 3

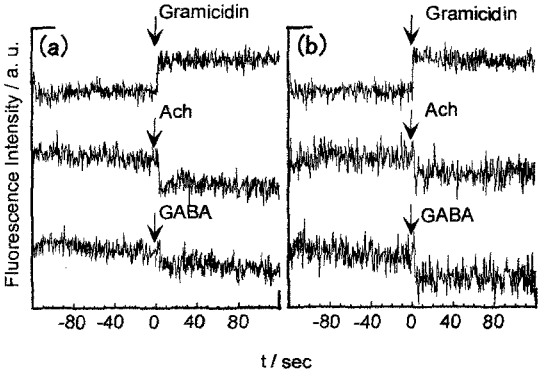


Fig. 3 Fluorescent intensity changes of 0.3 μM di-*S*-C_3(5) in DPPC vesicle suspension containing (a) ACh-induced polypeptide assembly and (b) GABA-induced polypeptide assembly, which were prepared by incubation with corresponding ligands on addition of ACh and GABA, respectively. The final concentration of the added ligands was 6 μM. Fluorescent intensity changes of the dye in pure DPPC vesicle on addition of 0.1 μM gramicidin are also shown in these figures.

shows fluorescent intensity changes of di-S-C3(5) in the vesicle suspension solution containing ligand-induced polypeptide assemblies after the addition of the ligands. In this figure, the fluorescent intensity changes of the dye in the pure DPPC vesicle suspension induced by the addition of Gramicidin for comparison are also shown. Gramicidin forms pore in the lipid membrane, which confer a large permeability to potassium and sodium ions. The effect of Gramicidin was to increase markedly the fluorescent intensity when added to the vesicle suspension. The addition of ACh to the vesicle containing the ACh-induced polypeptide assembly induced a remarkable decrease of the fluorescent intensity of di-*S*-C3(5). In contrast, GABA addition to

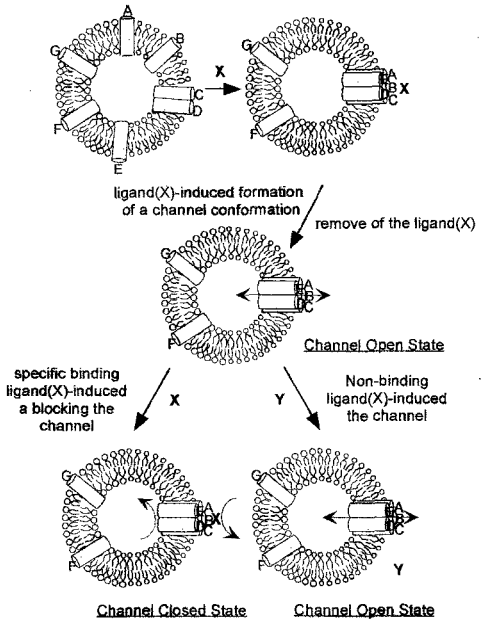


Fig.4 Schematic view of a synthetic ligand-gated ion channel having multi-sensing ability in a lipid bilayer. The channels were formed by the ligand-induced rearrangement of the amphiphilic polypeptides resulting from the interaction between the ligand and amino terminal functional groups of the polypeptide. The gating of the ion channel by the binding of the corresponding ligand molecules is schematically illustrated. The amino terminal functional groups are denoted as A, B, C, D, E, F, and G, and ligands are denoted as X and Y. Ligand(X) induced the rearrangement of amphiphilic polypeptides having the specific functional groups, A, B, C, and D, to form the bundle, which acted as an ion permeable pore in the membrane. Re-binding of ligand(X) to the polypeptide assembly blocked the ion permeable pore. On the other hand, ligand(Y) did not block the ion permeable pore, because of the non-specific interaction between ligand(Y) and the polypeptide assembly.

the vesicle suspension did not produce significant changes of the fluorescent intensity of the dye. On the other hand, the fluorescent intensity of di-*S*-$C_3(5)$ in DPPC vesicle containing the polypeptide assembly, which was formed by GABA-induced rearrangement of the polypeptides, exhibited a significant decrease by the addition of GABA compared with the addition of ACh to the vesicle suspension. These results suggest that the ion permeable pore formed by the amphiphilic polypeptide assembly as a result of the interaction between the ligand and amino terminal functional groups of the polypeptides in the bilayer membrane of the vesicle was blocked by the re-binding of the corresponding ligand. Figure 4 shows the proposed channel gating mechanism of the ligand-induced reorientation of the amphiphilic polypeptide assembly. In this system, the chemical information, recognition of ligands to the ligand-induced polypeptide assemblies, was converted to photochemical signals, that is, fluorescent intensity changes via the membrane potential changes caused by the channel gating. This system has the advantage that it permits easy production of a ligand-gated ion channel having multi-sensing ability through the use of amphiphilic polypeptides having various functional groups at the terminals, because the molecular recognition structures are induced by the interaction between the ligands and the terminal functional groups. This system may be employed in signal transduction devices.

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