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Construction of a pH-Responsive Artificial Membrane Fusion System by Using Designed Coiled-Coil Polypeptides

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Abstract: In many viruses, pH-responsive coiled-coil domains in the specific fusion proteins play important roles in membrane fusion and the infection of viruses into host cells. To investigate the relationship between the conformational change of the coiled coil and the fusion process, we have introduced a de novo designed polypeptide as a model system of the coiled-coil domain. This system enables the systematic study of the dynamics of pH-responsive coiled-coil polypeptide–membrane interactions. First, we designed and synthesized pH-responsive isoleucine-zipper triple-stranded coiled-coil polypeptides. Then the relationship between the pH-induced conformational change of the polypeptide and the membrane's interactive properties was studied by physicochemical methods. Structural changes in the designed polypeptides were examined by means of circular dichroism measurements. And finally, the behavior of the membrane fusion was investigated by leakage of liposomal contents, turbidity analysis, dynamic light scattering, and lipid mixing experiments. Our data show that coiled-coil formation under acidic pH conditions enhances polypeptide-induced membrane fusion. The results in this study demonstrate that an artificial membrane fusion system can be constructed on a molecular level by the use of a pH-responsive isoleucine-zipper triple-stranded coiled-coil polypeptide.

Keywords: de novo design · isoleucine zipper · liposomes · membrane fusion · peptides · pH-responsive coiled coil

Introduction

Membrane fusion is a crucial event in the biological function of living organisms. Fertilization involves membrane fusion of a sperm with an egg, and cell division requires membrane fusion to re-seal plasma membranes after the cell divides. Membrane fusion is also observed in endocytosis, exocytosis, and cellular membrane traffic.[1–9] However, spontaneous membrane fusion reactions are not achieved because of large energetic barriers in biological membranes. The energetic barriers are caused by strong hydration, as well as electrostatic and steric repulsions.[10–14] In living organisms, membrane fusion proteins are believed to overcome these various barriers; up to now many proteins associated with the membrane fusion process have been identified.[15–22] Among these fusion proteins, the viral spike glycoproteins responsible for the penetration of enveloped viruses into their host cells have been well characterized.[23–25] Their mechanistic and structural role in the fusion process occurring between the envelopes of viruses and the membranes of host cells has been studied extensively, both with intact viral fusion proteins and with synthetic analogues of the fusion protein domains. Influenza virus fusion, mediated by influenza hemagglutinin (HA), has been a particularly prominent model and has served as the paradigm for studying viral and nonviral membrane fusion mechanisms. HA is a homotrimer of identical subunits, each containing two disulphide-linked polypeptides, HA1 and HA2. The HA1 subunit contains the binding site for sialic acid on the membrane of the host cells. The HA2 subunit contains fusion peptide (a highly conserved hydrophobic sequence of about 20 amino acids), which facilitates the fusion event. The fusion is triggered by the mildly acidic pH that prevails in the endosome. Remarkably, the structure of HA completely changes in response to a pH change. In particular, under acidic pH conditions the amino terminus of the HA2 subunit changes from a helical hairpin structure to an extended triple-stranded coiled-coil

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structure, thereby causing the buried fusion peptide to appear on the outside.[26–30] To investigate the relationship between the structure of the polypeptide and membrane fusion, we designed synthetic coiled-coil polypeptides that change their conformation to the membrane in response to pH. We then studied the liposomal membrane fusion properties using these synthetic polypeptides under both neutral and acidic pH conditions. The use of synthetic fusion polypeptides is valuable for further investigation of possible roles for the HA2 region in fusion processes and also in the development of novel membrane fusion agents.

	defgabc	*defgabc*	*defgabc*	*defgabc*	
IZ	YGG IEKKIEA	IEKKIEA	IEKKIEA	IEKKIEA	
IZ-E1-St	--- -------	-------	----E--	-------	GGK*
IZ-E2-St	--- -------	-------	----E--	----E--	GGK*
IZ-E3-St	--- -------	----E--	----E--	----E--	GGK*
W-IZ-E3-St	W-- -------	----E--	----E--	----E--	GGK*
WI_3-IZ-E3-St	WIII-- -------	----E--	----E--	----E--	GGK*

Figure 1. Amino acid sequences used for a pH-induced trimeric coiled coil. Only amino acids different from the IZ sequence are indicated. The bar indicates the same amino acid as used in IZ. The heptad repeats are preceded by the YGG sequence for the peptide quantitation. K* indicates lysine incorporated with stearic acid at the side-chain amino group.

Results and Discussion

Design of peptide with pH-dependent conformational change to membrane: The α-helical coiled coil is characterized by heptad repeats of seven amino acid residues, denoted *a* to *g*, with hydrophobic residues at the *a* and *d* positions.[31,32] Two to five right-handed α helices wind around one another with amino acids at the *a* and *d* position facing the hydrophobic core in a left-handed supercoil. We previously constructed a nativelike triple-stranded coiled-coil peptide, IZ, which consists of 31 amino acid residues containing four repeats of the heptad, IEKKIEA (*defgabc*).[33] We showed that the IZ derivative, IZ-2aE, which has one Glu residue at the *a* position, changes its structure from a random coil at pH 7.0 to the coiled coil at pH 5.0.[34] In macrophage scavenger receptors, a Glu residue at the hydrophobic core has been known to play an important role in pH-dependent conformational change. The pH-dependent conformational change observed in IZ-2aE is similar to macrophage scavenger receptors. Based on IZ-2aE showing a pH-dependent conformational change, we designed several IZ-2aE derivatives to anchor a membrane; to anchor the peptide in the lipid bilayer we used stearic acid.

Hydrophobic residues attached to the peptide sometimes contribute to the aggregation of the peptide.[35] Therefore, we analyzed the effect of a number of Glu residues incorporated at the *a* position of IZ (Figure 1). The IZ-E1-St has one Glu residue at the 3*a* position, IZ-E2-St has two Glu residues at the 3*a* and the 4*a*, and IZ-E3-St has three Glu residues at the 2*a*, the 3*a*, and the 4*a* positions, respectively. The structure of the designed peptides was analyzed by using CD spectroscopy. IZ-E1-St showed an α-helical coiled coil at both pH 4.3 and pH 7.3 as indicated by the negative peaks at $\lambda = 208$ and 222 nm (Figure 2a). Additionally, the oligomerization state of IZ-E3-St was analyzed by gel filtration at both pH values by using a Sephadex G-50 column. The polypeptide was eluted at the same position at which the coiled-coil trimer from the standard IZ polypeptide was eluted, indicating that IZ-E1-St formed a triple-stranded coiled coil (Figure 3a). We speculate that this unexpected coiled-coil formation of IZ-E1-St at pH 7.3 would be caused by the higher coherency of the hydrophobic stearic acid moiety. To decrease the stability of the

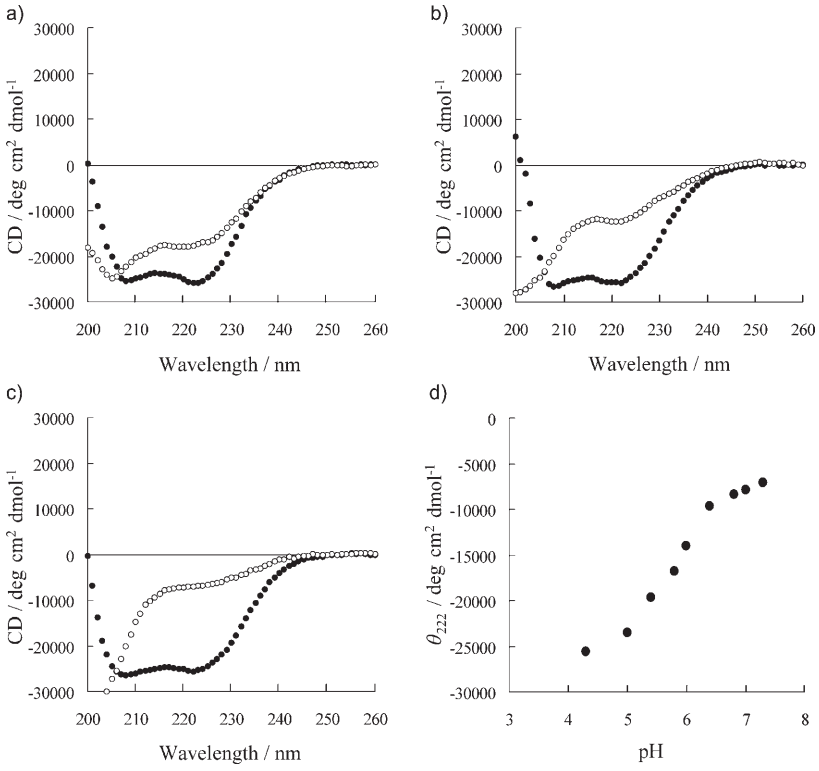


Figure 2. Circular dichroism spectra of a) IZ-E1-St, b) IZ-E2-St, and c) IZ-E3-St peptide at pH 7.3 (○) and 4.3 (●). d) pH-dependence of the IZ-E3-St peptide. The measurements were performed in a citric acid/phosphoric acid buffer (10 mM) containing 0.1 M NaCl at various pH conditions. The peptide concentrations were 40 μM.

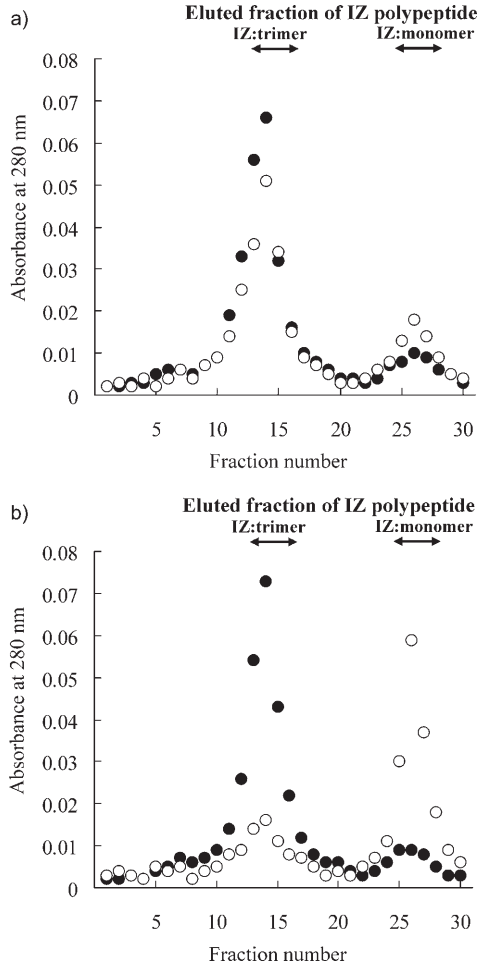


Figure 3. Analysis of the eluted fraction of a) IZ-E1-St and b) IZ-E3-St at pH 7.3 (○) and 4.3 (●) using a Sephadex G-50 column. The elution was performed in a citric acid/phosphoric acid buffer (10 mM) containing 0.1 M NaCl at 20 °C. The arrows indicate the eluted position of the standards, IZ in 10 mM Tris-HCl buffer (trimer) and IZ in 6 M guanidine hydrochloride solution (monomer).

coiled-coil structure, we increased the number of Glu at the hydrophobic positions. IZ-E2-St still exhibited an α-helical structure at pH 7.3, although the α-helical content decreased with the small negative peak at 222 nm (Figure 2c). IZ-E3-St formed a random coil at pH 7.3, as indicated by the negative peak below 200 nm, whereas it formed an α-helical structure at pH 4.3 (Figure 2c). Thus, IZ-E3-St exhibited a similar conformational change to that of hemagglutinin. pH titration was carried out with IZ-E3-St (Figure 2d). IZ-E3-St formed a random coil at neutral pH and an α-helical structure under acidic pH conditions, with a midpoint of transition of pH 5.7. The aggregation degree of IZ-E3-St was analyzed by gel filtration at pH 4.3 by using a Sephadex G-50 column. IZ-E3-St was observed to have formed a triple-stranded coiled coil (Figure 3b).

To create the artificial membrane fusion system promoted by the pH-responsive polypeptide, we prepared the EggPC liposome containing the W-IZ-E3-St polypeptide. This designed polypeptide has a Trp residue at the N terminus to enable the investigation of lipid–polypeptide interactions by Trp fluorescence experiments. Multilamellar vesicles were prepared by evaporation of a solution of EggPC and W-IZ-E3-St in chloroform, followed by hydration in an aqueous buffer. The suspension was submitted to five freeze–thaw cycles for equilibration. Small unilamellar vesicles (SUVs) for this experiment were prepared by extrusion across a polycarbonate unipore membrane (100 nm pore size) using a Mini-Extruder Set. The mixture was separated by using a Sephadex G-50 column to remove the unbound peptide from the liposome. The structure of the peptide on the EggPC liposome was subjected to CD spectra measurements (Figure 4a). Due to the presence of the lipid, CD spectra could not be measured below 202 nm. It showed the minima at 208 and 222 nm at pH 4.3, thereby indicating the α-helical structure. However, a prominent minimum around 220 nm was not observed at pH 7.3. Thus, the peptide formed the helical structure at pH 4.3 and a random structure at pH 7.3, as in the case of the peptide in solution. The pH titration curve is also consistent with that of the peptide in solution (Figure 4b). A similar conformational change was found in solution and on the liposome. Thus we could obtain a pep-

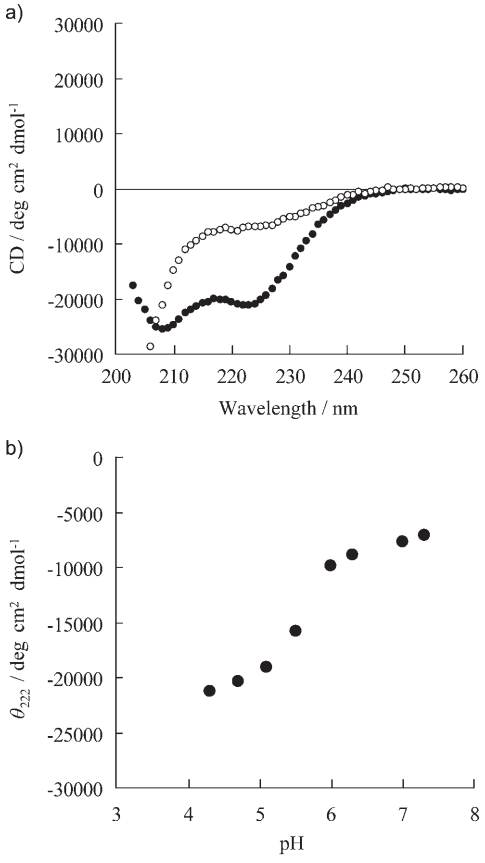


Figure 4. a) Circular dichroism spectra of W-IZ-E3-St peptide in EggPC liposome at pH 7.3 (○) and 4.3 (●). b) pH-dependence of W-IZ-E3-St peptide. Measurement was performed in a citric acid/phosphoric acid buffer (10 mM) containing 0.1 M NaCl using W-IZ-E3-St including EggPC liposome.

tide that mimics the conformational change of that of hemagglutinin on the membrane.

Analysis of the membrane fusion process: To analyze whether the membrane fusion actually occurs, we first measured the turbidity of the EggPC solution by monitoring the absorbance at 600 nm (Figure 5). Without the peptide, the

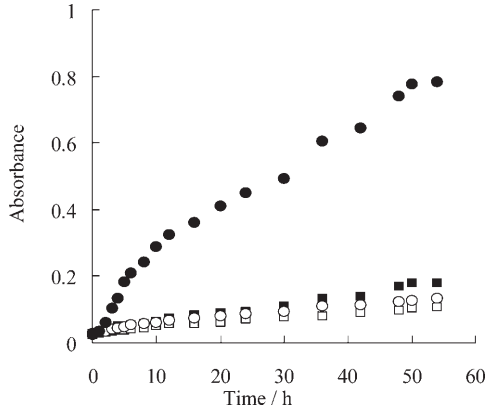


Figure 5. Time course for the turbidity analysis of EggPC liposome in the absence (squares) and presence (circles) of W-IZ-E3-St. The measurements were performed in a citric acid/phosphoric acid buffer (10 mM) containing 0.1 M NaCl at pH 7.3 (open symbols) and 4.3 (closed symbols).

turbidity was only slightly increased at both pH 7.3 and pH 4.3. Addition of W-IZ-E3-St to the EggPC solution affects the slight increase in turbidity at pH 7.3. On the other hand, at pH 4.3, the turbidity was increased by more than ten times. These results indicated that W-IZ-E3-St has no effect at pH 7.3, whereas it increased the turbidity of the EggPC solution at pH 4.3. This result together with the CD results described before indicates that triple-stranded coiled-coil formation of W-IZ-E3-St promoted the intervesicular interactions including fusion or aggregation of the liposome.

The influence of W-IZ-E3-St on liposomal stability and membrane fusion was also investigated by a leakage experiment of calcein inserted into the membrane.[36] In general, following membrane fusion, some contents encapsulated in lipids are released into the medium. SUVs of EggPC containing W-IZ-E3-St and calcein were prepared by the same method as above, and free calcein molecules were removed by using gel filtration chromatography. In this study, encapsulated calcein is in high concentration (75 mM) where fluorescence is quenched so that an increase in fluorescence signal corresponds to the release of calcein in compartments of greater volume where fluorescence is no longer quenched. Figure 6 demonstrates the results of our calcein leakage experiments. In the liposome containing W-IZ-E3-St, a remarkable increase of leakage was observed only at an acidic pH (pH 4.3). Similar leakage of liposome contents due to the turbulence of the lipid membranes was observed in the past using the synthetic polypeptide from influenza HA protein[29] or coiled-coil peptides.[37] On the other hand, the control liposomes without the W-IZ-E3-St polypeptide

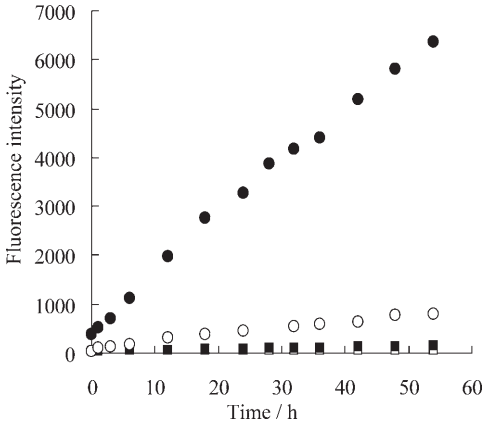


Figure 6. Time course for the calcein release from EggPC liposome in the absence (squares) and presence (circles) of W-IZ-E3-St. The measurements were performed in a citric acid/phosphoric acid buffer (10 mM) containing 0.1 M NaCl at 20 °C. The peptide concentrations were 40 μM. In the presence of WI_3-IZ-E3-St, the calcein releases are shown at pH 7.3 (open symbols) and 4.3 (closed symbols).

were not destabilized in either acidic or neutral pH. This higher activity of the W-IZ-E3-St at an acidic pH is related to the conformational change of the polypeptide. In other words, these results indicate that the formation of a coiled-coil trimer by the polypeptide allows the liposomal phospholipids to interact directly.

Membrane fusion is accompanied by an increase in the size of the liposome. Hence, variation in the size of the liposome by membrane fusion was studied by using dynamic light scattering. The measured average sizes for the EggPC liposome without W-IZ-E3-St just after extrusion were (113 ± 31) nm (pH 7.3) and (137 ± 38) nm (pH 4.3). For the liposome containing W-IZ-E3-St, the corresponding values were (20 ± 36) nm (pH 7.3) and (141 ± 31) nm (pH 4.3) (Figure 7). In the absence of W-IZ-E3-St, liposome size distributions were almost constant under both neutral and acidic pH conditions during a period of 54 h. Furthermore,

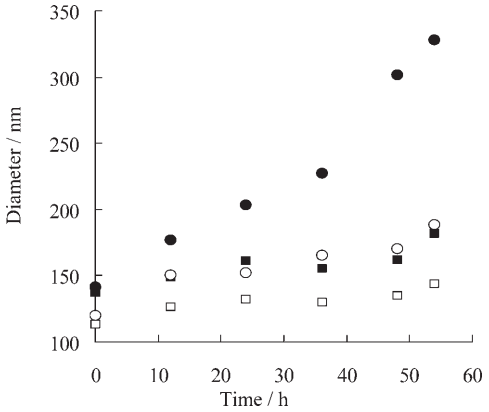


Figure 7. Time course for the variation in the size of the EggPC liposome in the absence (squares) and presence (circles) of W-IZ-E3-St. The measurements were performed in a citric acid/phosphoric acid buffer (10 mM) containing 0.1 M NaCl at pH 7.3 (open symbols) and 4.3 (closed symbols).

the polypeptide did not bring a definite change to the liposome size at pH 7.3. On the other hand, the liposome containing W-IZ-E3-St under acidic conditions showed a remarkable growth in particle size. These results also supported the contribution of W-IZ-E3-St at an acidic pH to intervesicular interactions.

We cannot distinguish between membrane fusion and aggregation of the membrane from the experiments mentioned above. Therefore, we further analyzed whether membrane fusion is promoted by the W-IZ-E3-St peptide. It is known that energy transfer occurs between two fluorescent compounds if they are settled within Förster length. We prepared EggPC containing both NBD and rhodamine, and excitation at $\lambda = 460$ nm of NBD gave the fluorescence at 590 nm of rhodamine.[38] It is expected that if membrane fusion occurs, the concentration of the two fluorophores will decrease, and, accordingly, the intensity of the fluorescence at 590 nm will decrease. Actually, upon the addition of polyethylene glycol (PEG) 2000, a well-known promoter of the membrane fusion,[19] the fluorescence at 590 nm decreased, which indicated that membrane fusion occurred (data not shown). Then the EggPC containing W-IZ-E3-St was added either at pH 7.3 or pH 4.3 (Figure 8a, b). At pH 7.3, the fluorescence spectrum was almost the same as that without W-IZ-E3-St, and remained unchanged even after 24 h (Figure 8b). On the other hand, at pH 4.3, the intensity of the fluorescence at 590 nm decreased as time passed (Figure 8a), and this remarkable spectral change was not observed in the absence of W-IZ-E3-St, which suggests that the polypeptide-induced membrane fusion did occur. The results reveal that the membrane tends to aggregate under acidic pH conditions, and the coiled-coil form of W-IZ-E3-St at an acidic pH plays a critical role in lipid mixing.

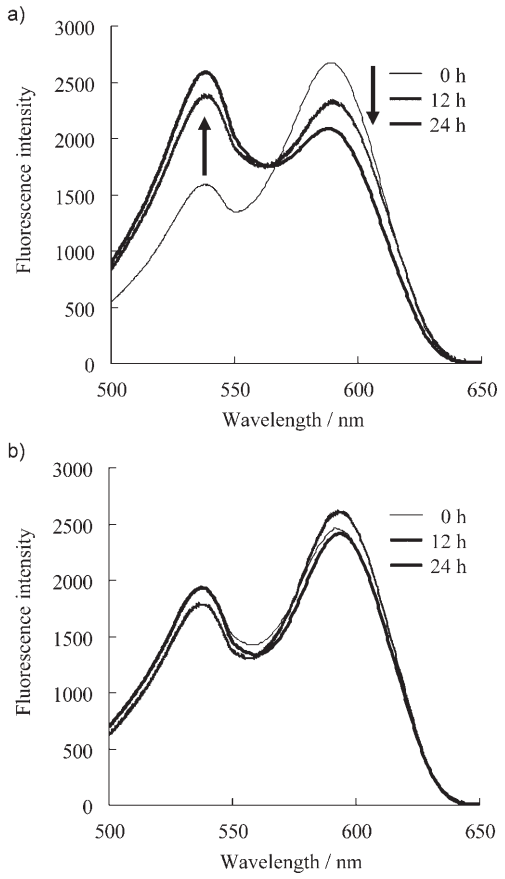


Figure 8. Fluorescence spectra of W-IZ-E3-St and NBD-PE/Rh-PE containing liposome at a) pH 4.3 and b) pH 7.3. The measurements were performed in a citric acid/phosphoric acid buffer (10 mM) containing 0.1 M NaCl at 20 °C.

In addition, to establish that the results obtained from the lipid mixing assay do support membrane fusion, a contents mixing assay was performed to present this system as a novel model for membrane fusion. Contents mixing was determined by a Tb^{3+}/dipicolinic acid (DPA) assay.[39] This fusion assay was started with two populations of SUVs, one (the EggPC liposome containing W-IZ-E3-St) encapsulating DPA and the other (the bare EggPC liposome) containing Tb^{3+} ions. Fusion and mixing of the aqueous contents led to an increase in fluorescence intensity of the Tb^{3+}/DPA complex. At pH 4.3, a mixing of internal aqueous contents, shown by the marked increase in fluorescence emission of the Tb^{3+}/DPA complex, was observed (Figure 9a, b). On the other hand, the increase of fluorescence intensity due to contents mixing was not observed at pH 7.3 (Figure 9b). It appears, then, that membrane fusion observed by a dilution of fluorescent lipid probes was confirmed by the assay of contents mixing. The contents mixing assay was conducted regularly for the purpose of confirming full membrane fusion.

Proposed mechanism for polypeptide-induced membrane fusion: Based on the results mentioned above, the designed polypeptide, W-IZ-E3-St, is able to act as a pH-responsive membrane activation agent. As shown in Figure 7, a remarkable increase in vesicle diameters in the presence of the liposome containing W-IZ-E3-St was observed at an acidic pH. This change in the vesicle size is larger than the value expected, which is obtained by calculating the fusion of two vesicles. The results obtained from DLS measurements may support the membrane aggregation phenomena rather than the membrane fusion phenomena. However, the results from the lipid mixing assay (Figure 8) and the contents mixing assay (Figure 9) strongly support that polypeptide-induced membrane fusion occurs in this system. This polypeptide-induced fusion is only observed when W-IZ-E3-St formed the coiled-coil structure under acidic conditions.

To address the mechanism of the membrane fusion, we analyzed the Trp fluorescence because the Trp residue is sensitive to the environment of the indole side chain. The Trp residue showed fluorescence emission maxima between 327 and 332 nm in a hydrophobic environment and at 354 nm in an aqueous environment.[40] The fluorescence maxima and intensity of Trp are the same at pH 4.3 and pH 7.3. We analyzed the fluorescence of the Trp residue when W-IZ-E3-St was bound to the liposome (Figure 10). At pH 7.3, where the peptide exhibited a random structure,

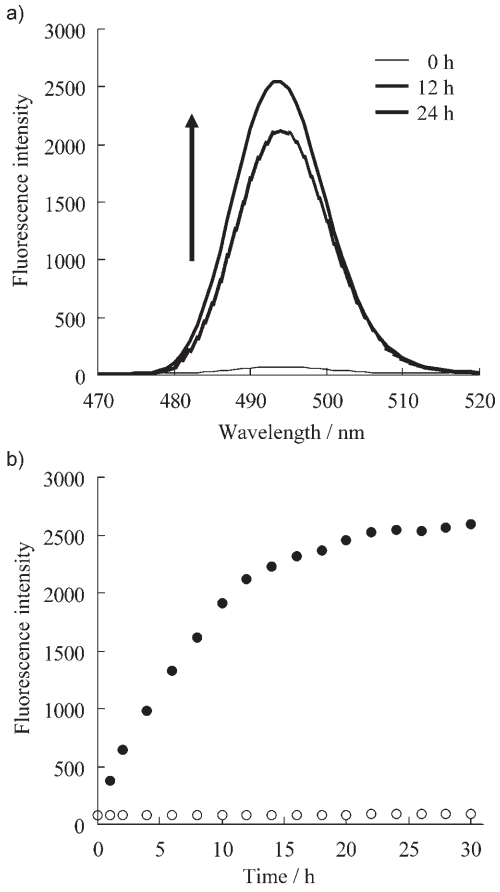


Figure 9. a) Fluorescence spectra of a 1:1 mixture of the liposome containing W-IZ-E3-St encapsulating DPA and the bare liposome encapsulating $TbCl_3$ at pH 4.3. b) Time course for contents mixing assay at pH 4.3 (●) and 7.3 (○). The measurements were performed in a citric acid/phosphoric acid buffer (10 mM) containing 0.1 M NaCl and 1 mM EDTA at 20 °C.

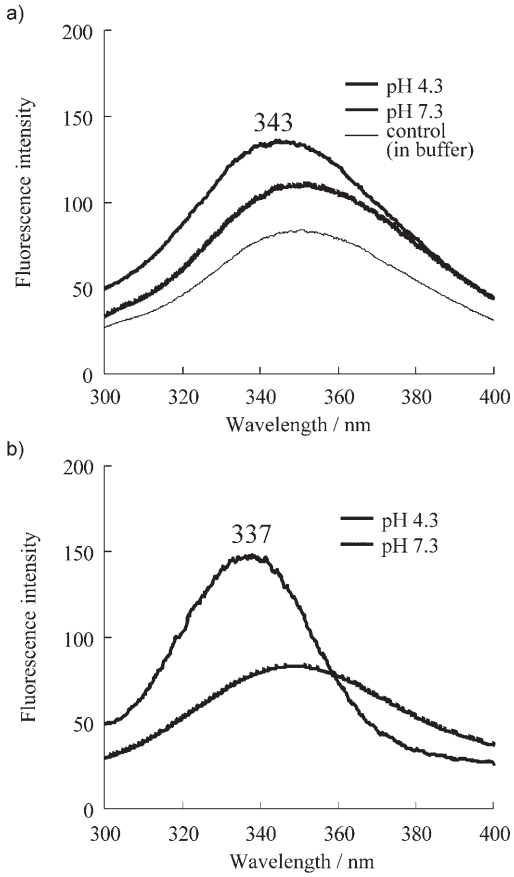
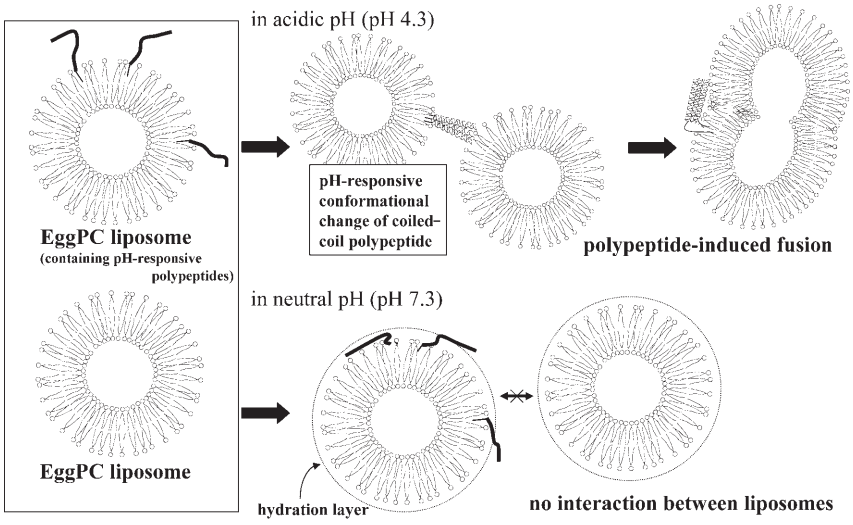


Figure 10. Fluorescence spectra of the Trp moiety in a) W-IZ-E3-St and b) WI_3-IZ-E3-St settled in liposome at pH 4.3 and 7.3. The measurements were performed in a citric acid/phosphoric acid buffer (10 mM) containing 0.1 M NaCl at 20 °C.

the fluorescence maximum was 355 nm, which indicates that the Trp residue was exposed to the hydrophilic environment. At pH 4.3, where the peptide exhibited the helical structure, the fluorescence decreased to 343 nm, which indicates that Trp was buried in the hydrophobic environment. These results suggest that the peptide aggregated on the membrane as well as in the buffer solution, and the three Trp residues were more buried in the hydrophobic position. We speculate the following conformational change depicted in Scheme 1. The peptide exhibits the extended form along the liposome surface with the Lys residues facing toward the liposome to interact with the phospholipid, and the Trp residue is assumed to be buried in the hydration layer of its own liposome. At pH 4.3, W-IZ-E3-St forms the coiled-coil structure, and thereby the N-terminal moiety of the liposome-bound polypeptide is allowed



Scheme 1. Schematic description of the pH-responsive membrane fusion based on our results.

to escape from the hydration layer and is exposed to the target membrane. Next, compatibility between the indole group of the N-terminal Trp residue and phospholipids are brought into contact with the target membrane. Finally, the amphiphilicity of the Trp residue leads to the insertion into the other (target) liposomal membrane. This mechanism is similar to that of the fusion of the influenza virus into host cells by using HA.

Improvement of the membrane fusion: The fusion peptide of HA from influenza has a hydrophobic sequence of about 20 amino acids. Hence, the higher hydrophobicity of the N terminus of W-IZ-E3-St should promote membrane fusion. We designed a new candidate. WI_3-IZ-E3-St has an even more hydrophobic Trp-Ile-Ile-Ile sequence at the N terminus (Figure 1). Therefore, WI_3-IZ-E3-St seems to be able to penetrate deep into the target membrane. We analyzed the membrane fusion by using the same procedures as those carried out with W-IZ-E3-St. Based on calcein leakage and DLS measurements, WI_3-IZ-E3-St-induced membrane fusion was observed only under acidic conditions, as in the case of W-IZ-E3-St. Remarkably, the data showed higher membrane fusion activity of WI_3-IZ-E3-St, which inserted itself into the target more easily. Further blueshift to 337 nm was observed in the presence of the newly designed liposome containing WI_3-IZ-E3-St under acidic conditions (Figure 10b). Through the WI_3-IZ-E3-St-induced membrane fusion process, the Trp residues are settled in a more hydrophobic environment.

It has been reported that membrane fusion occurs within 30 min in the artificial fusion systems.[29,41–43] However, it took quite a long time in our membrane fusion system. We can solve this problem by using the sequence of a membrane-binding polypeptide such as Magainin 2. Additionally, composition of the phospholipids in the liposomal membrane may control fusion behavior. In any case, this system is very significant because it is the first observed example of a membrane fusion phenomena driven by pH-responsive conformational change of de novo designed polypeptides.

Conclusion

The results demonstrated herein indicate that an artificial membrane fusion system can be constructed on a molecular level by the use of pH-responsive coiled-coil polypeptides, which could eventually be extended to a pH-responsive drug delivery system and gene transfer system. Such programmable membrane fusion systems further provide an attractive approach to the design and construction of lipid vesicle arrays.

Experimental Section

Peptide synthesis: All polypeptides used in this study were synthesized by the solid-phase synthesis method on Rink amide resin using *N*-Fmoc-protected α-amino acids (Fmoc = 9-fluorenylmethoxycarbonyl), *O*-(benzotriazol-1-yl)tetramethyluronium hexafluorophosphate (HBTU), and 1-hydroxybenzotriazole (HOBt). The side-chain protection groups were Glu(O*t*Bu), Lys(Boc) (Boc = *tert*-butoxycarbonyl), and Tyr(*t*Bu). When stearic acid was attached to the C terminus of peptides, Fmoc-Lys(Mtt) (Mtt = 4-methyltrityl) was attached to Rink amide resin using HBTU/HOBt activation. The Mtt group was removed by 1% trifluoroacetic acid (TFA) in CH_2Cl_2 for 30 min. After confirmation of removal of the Mtt group by a ninhydrin test, stearic acid was condensed to the side-chain amino group of Lys using HBTU/HOBt. The reaction was confirmed by a ninhydrin test. Then the amino acid was elongated by using the standard Fmoc strategy. Deprotection and cleavage were performed by treatment with TFA/ethanedithiol/anisole/ethylmethylsulfide (93:1:3:3 v/v) for 2 h. Purification was carried out by reverse-phase HPLC on a YMC-Pack ODS-A column (10 mm (i.d.) × 250 mm, 5 μm, YMC Inc., Japan) eluted at 4 $cm^3 min^{-1}$ with linear acetonitrile/water gradients containing 0.1% (v/v) TFA over the course of 30 min. The final products were characterized by using analytical HPLC and MALDI-TOF mass spectrometry: *m*/*z* calcd for IZ: 3538; found: 3538; *m*/*z* calcd for W-IZ-E3-St: 4118; found: 4118; *m*/*z* calcd for WI_3-IZ-E3-St: 4457; found: 4457.

Liposome preparation and introduction of polypeptide: Small unilamellar vesicles (SUVs) were prepared by evaporation of a solution of EggPC in chloroform in a round-bottomed flask, followed by hydration in aqueous buffers (3 mg lipid, 1 cm^3). The suspension was submitted to five freeze–thaw cycles for equilibration. Then the SUVs for this experiment were prepared by extrusion of the suspension across a 100 nm polycarbonate unipore membrane (Whatman) twice by using a Mini-Extruder Set (Avanti). To create EggPC containing the peptide, the W-IZ-E3-St or WI_3-IZ-E3-St (0.15 mg) was added and frozen and thawed. The EggPC liposome containing the peptide was prepared in the same way as described above.

Circular dichroism (CD) measurements: All CD measurements were performed by using a Jasco J-820 spectropolarimeter and a 2 mm pathlength cuvette at 20 °C. The polypeptide concentration was determined by the absorbance at 275 nm in a guanidine hydrochloride solution (6 M).[44] The mean residue ellipticity, $[\theta]$, is given in deg cm^2 $dmol^{-1}$. CD spectra were obtained in a citric acid/phosphoric acid buffer (10 mM) containing NaCl (0.1 M). The effect of the pH on the α-helical content was determined to monitor $[\theta]_{222}$ as a function of pH from 4.3 to 7.3.

Polypeptide-induced leakage of liposomal contents: The SUVs were prepared by the same procedures as described in the liposome preparation except that calcein (75 mM) was added to the EggPC film. After the SUVs containing calcein were prepared, free calcein outside of the liposomes was removed by means of Sephadex G-25 gel filtration chromatography. Leakage of calcein to the external medium was monitored by the increase in fluorescence. Fluorescence measurements for these leakage experiments were carried out by using a HITACHI F-2500 spectrofluorometer with the excitation wavelength at 488 nm.

Turbidity analysis: The turbidity of liposome solutions was analyzed by using a SHIMADZU UV-2200A spectrophotometer. The turbidity of the samples was judged from the increment of the absorbance at 600 nm. The SUVs were prepared by the same procedures as described in the liposome preparation.

Liposome size distribution determination: Dynamic light scattering (DLS) of liposome suspensions was studied by using an N5 Plus autocorrelator (Beckman-Coulter) equipped with a 632.8 nm He–Ne laser light source. Scattering was detected at 15.7, 23.0, 30.2, and 62.6°. Particle size distributions at each angle were calculated from autocorrelation data by the CONTIN program.[45] The average liposome size was calculated to be the y intercept at the zero angle of the measured average particle size values versus $\sin 2\theta$. All buffer solutions used were filtered with a 0.22 μm filter just before liposome preparation. The collection times for the autocorrelation data were 1–4 min.

Lipid mixing experiments by using fluorescence resonance energy transfer (FRET): The SUVs containing NBD-PE and Rh-PE were prepared according to the same procedures as described above. The concentrations of the NBD-PE and Rh-PE were 1 mol% against the EggPC. The mixing of phospholipids was followed by using the FRET method.[36] In this

work, we used equal concentrations of unlabeled and labeled vesicles diluted into a buffer solution at the appropriate pH, and monitored the fluorescence of 533 nm from NBD (donor) and 590 nm of Rhodamine (Rh) (acceptor). Experimental data were acquired by the use of a HITACHI F-2500 spectrofluorometer.

Fusion assay by contents mixing: EggPC liposomes containing DPA (50 mM) and containing $TbCl_3$ (5 mM) were prepared and untrapped probes were removed by using Sephadex G-25 gel filtration chromatography equilibrated with assay buffer (10 mM citric acid/phosphoric acid buffer containing 0.1 M NaCl and 1 mM EDTA). Vesicle fusion resulted in the formation of the fluorescent Tb^{3+}/DPA complexes. Fluorescence of the complexes is detected at 493 nm with excitation at 276 nm. The fluorescence measurements for contents mixing experiments were carried out by using a HITACHI F-2500 spectrofluorometer.

Tryptophan fluorescence spectroscopy: Tryptophan fluorescence measurements were carried out at 20 °C by using a HITACHI F-2500 spectrofluorometer with the excitation wavelength at 278 nm.

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