Lipid Nanodisc Formation using Pxt-5 Peptide Isolated from Amphibian (Xenopus tropicalis) Skin, and its Altered Form, Modify-Pxt-5

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Abstract: Nanodiscs are self-assembled discoidal nanoparticles composed of amphiphilic α-helical scaffold proteins or peptides that accumulate around the circumference of a lipid bilayer. In this study, Pxt-5, which is an antimicrobial peptide isolated from the skin of Xenopus tropicalis, and its modified peptide (Modify-Pxt-5) were synthesized by solid-phase peptide synthesis (SPPS). Their surface properties, which are an important factor in inducing nanodisc formation, were investigated by circular dichroism (CD) spectroscopy, surface tension measurement, phospholipid vesicle clearance assay, and negative-staining transmission electron microscopy (NS-TEM). The α-helicity of Pxt-5 (8.4%) improved drastically to 45.6% by four amino-acid substitutions (Modify-Pxt-5). Both the peptides, having hydrophobic and hydrophilic faces, behaved like general surfactants, and the surface activity of Modify-Pxt-5 (CAC: 9.5×10⁻⁵ M, γCAC: 30.3 mN⋅m⁻¹) was much higher than that of Pxt-5 (CAC: 7.9×10⁻⁵ M, γCAC: 38.1 mN⋅m⁻¹). A turbid L-α-dimyristoylphosphatidylcholine (DMPC) vesicle solution (T = 0.3%) quickly turned transparent upon addition of Pxt-5 or Modify-Pxt-5. After twelve hours, the transmittance of vesicle solution with Modify-Pxt-5 (T = 96.2%) was found to be higher than that of vesicle solution with Pxt-5 (T = 83.5%), and then the micro-solubilized solutions were observed by NS-TEM. Interestingly, nanodisc structures were found in the vicinity of DMPC vesicles in both the images, and the average diameter of the nanodiscs was 11.2 ± 6.0 nm for those containing Pxt-5 and 10.8 ± 5.8 nm for those containing Modify-Pxt-5. It was also found that Modify-Pxt-5 effectively self-assembles into nanodiscs compared to Pxt-5 without any substitutions.

Key words: nanodisc, antimicrobial peptide, amphibian skin, surface activity, NS-TEM

1 INTRODUCTION

Nanodiscs are unique discoidal nanoparticles whose diameters range from 8 to 17 nm1,2. They are generally composed of apolipoprotein A-I (apoA-I) or membrane scaffold proteins (MSP) that wrap themselves around the circumference of lipid bilayers in a belt-like manner as shown in Fig. 1.3,4. It is already known that nanodiscs are the simplest models of high-density lipoprotein (HDL) particles, which play an important role in reverse cholesterol transport (RCT) in the body5,6. Thus, reconstituted nanodiscs have attracted considerable attention as a relatively new class of therapeutic agents for enhancing the RCT pathway7,8.

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New-found interest in nanodiscs has been focused on applications beyond their physiological role, and they are found to serve not only as a useful platform for membrane proteins in a native membrane-like environment, but also as potentially useful vehicles for drug delivery, cosmetic, and food applications.

Nanodiscs formed with apoA-I or MSP are generally prepared by the surfactant dialysis or BioBeads removal method, where the nanodisc formation is accomplished by the removal of the surfactants after mixing apoA-I or MSP with a certain amount of phospholipids solubilized into surfactant micelles. However, this method is not always appropriate when considering a wide variety of potential nanodisc applications, because the dialysis process is time- and energy-consuming, and the complete removal of the surfactants is often difficult.

By using surfactant-like α-helical peptides, we previously succeeded in the preparation of nanodiscs in a single step without the use of any additives such as surfactants. Surfactant-like properties of amphiphilic α-helical structures, that mimic helix 10 of apoA-I (residue 220-241), allowed us to produce nanodiscs quickly just by mixing the peptides with phospholipid vesicles, and the obtained nanodiscs were stable for at least six months.

It is well known that many membrane-active peptides, such as magainins, are isolated from the African clawed frog, *Xenopus laevis*, which show broad-spectrum antimicrobial activity. In order to further explore the nanodisc-forming peptide candidates in this study, we focused on antimicrobial peptides isolated from amphibian skin because they have an amphiphilic α-helical structure, which leads to interaction with phospholipid bilayer membranes and formation of supramolecular assemblies.

In cell-membrane research, the lipid-raft hypothesis is one of the most significant concepts, which proposes that lipid rafts are tightly packed aggregates of cholesterol and sphingolipids that float in a sea of phospholipids. One of the most significant concepts, which proposes that lipid rafts are tightly packed aggregates of cholesterol and sphingolipids that float in a sea of phospholipids, is the idea that lipid rafts play a key role in membrane trafficking and signal transduction. Lipid rafts are dynamic structures that can be disrupted by various stimuli, including cholesterol depletion or sphingomyelinase treatment.

In this study, the amino acid sequence of Pxt-5 (FIGALLGPLLNLLK-NH₂) was modified to improve helicity and surface activity. Pxt-5 and Modify-Pxt-5 (FIGALLQWLLELLK-NH₂) were then synthesized by solid-phase peptide synthesis (SPPS). Their surface activity and self-assembling properties, which induce nanodisc formation, were then investigated by circular dichroism (CD) spectroscopy, surface tension measurement, phospholipid vesicle clearance assay, negative-staining transmission electron microscopy (NS-TEM).

### 2 MATERIALS AND METHODS

#### 2.1 Materials

1. [Bis(dimethylamino)methylene]-1H-benzotriazolium 3-oxide hexafluorophosphate (HBTU, 99.3%) and 9-fluorenylmethylloxycarbonyl (Fmoc)-protected amino acids (98.0%) were purchased from Peptide Institute Inc. (Osaka, Japan). 1-Hydroxy-1H-benzotriazole (HOBt, 99.0%) was purchased from Dojindo Laboratories Co., Ltd. (Kumamoto, Japan). Rink amide 4-Methylbenzhydrolamine (MBHA) resin was purchased from Peptides International Inc. (Osaka, Japan). Trifluoroacetic acid (TFA, 99.0%), diisopropylethylamine (DIEA, 99.0%), pipericidine (99.0%), triisopropylsilane (TIS, 99.0%), and high performance liquid chromatography (HPLC) grade acetonitrile (CH₃CN) were purchased from Wako Pure Chemical Industries (Osaka, Japan). L-α-dimyristoylphosphatidylcholine (DMPC, 99.0%) was purchased from NOF Corp. (Tokyo, Japan).

#### 2.2 Peptide Synthesis

The peptides Pxt-5 (FIGALLGPLLNLLK-NH₂) and Modify-Pxt-5 (FIGALLQWLLELLK-NH₂) were synthesized using standard Fmoc chemistry with a Syro I peptide synthesizer (Biotech). A typical synthesis was conducted via a stepwise SSSP on Rink amide MBHA resin. Standard side-chain protecting groups including Trp (Boc), Asn (Trt), Lys (Boc), Glu (Obut), and Gln (Trt). Couplings were performed for a period of 90 min using HBTU in DMF. Fmoc groups were then removed using 40% piperidine in DMF. The peptides were cleaved from the resin with concomitant side-chain deprotection by agitation in a 95:2.5:2.5 TFA:TIS:water solution for 3 h. The crude peptides were precipitated using diethyl ether and centrifuged, then washed three times with diethyl ether, and then purified by preparative reverse-phase (RP) HPLC on a C18 column. The purity was confirmed by analytical RP-HPLC. Binary gradients of solvent A (99% H₂O, 0.9% CH₃CN, and 0.1% TFA) and solvent B (90% CH₃CN, 9.93% H₂O, and 0.07% TFA) were employed for HPLC. The purified peptides were characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF, Bruker Daltonics Inc.) mass spectrometry, where sinapinic acid (SA) was used as a matrix.
2.3 Circular Dichroism (CD) spectrometry

CD spectra were recorded at 25°C on a J-710 spectrophotometer (JASCO Corp.) using a quartz cell with 0.1 cm path-length. Spectra (20 scans) were obtained with a 3 nm bandwidth and a scan speed of 1 nm/s. All CD spectra were reported in terms of ellipticity units per mole of peptide residues \([\theta]_{222}\) which was calculated using the equation

\[ [\theta]_{222} = \theta_{obs} / (10 \text{lcm}) \]

where \(\theta_{obs}\) is the ellipticity in millidegrees, \(l\) is the path length in centimeters, \(c\) is the peptide concentration in moles per liter, and \(n\) is the number of amino-acid residues. The percentage helical content of the peptides (\(\alpha\)-helicity) was estimated on the basis of the \([\theta]_{222}\) value, using the equation

\[ \alpha\text{-helicity} = \left( 1 - \frac{[\theta]_{222} + 3000}{36000 + 3000} \right) \times 100 \]

2.4 Surface Tension Measurements

The surface tension of the peptides was determined by the pendant drop method at 25°C, which was performed using an automatic interfacial tensiometer (DM500, Kyowa Interface Science) and the drop-shape was analyzed using the software FAMAS (InterFace Measurement & Analysis System) version 2.01. A drop was formed at the tip of the syringe by pressing the solution out by means of a screw-thread. A drop-profile was extracted from the drop image; then, a curve-fitting program compared the extracted drop-profile with a theoretical one (the Young-Laplace method), and gave the corresponding surface tension value. For each concentration of the purified peptide solution, the drop surface tension was observed for a period of 10 min.

2.5 Peptide-Lipid Nanodisc Preparation

Before preparation of the nanodiscs, DMPC multilamellar vesicles (MLVs) were prepared as follows. DMPC was dissolved in chloroform in a test tube. The solvent was then removed, first by blowing nitrogen gas into the test tube, and then by overnight storage at room temperature under vacuum, creating a thin lipid film on the test-tube wall. Ultrapure water, used to avoid interference from salts, was added to this lipid film at 25°C, and the test tube was shaken vigorously in a vortex mixer to yield the MLV aqueous solution (4 mM). The formation of MLVs whose diameters are in several micrometers was then confirmed by an optical microscope. We performed the direct conversion from an MLV to a nanodisc by adding each peptide solution at a 1:7 peptide:phospholipid molar ratio, vortexing the sample for 30 min, and subsequently incubating the sample for 24 h at 25°C.

2.6 Negative-Staining Transmission Electron Microscopy (NS-TEM)

A glow-discharged copper grid (200 mesh) coated with carbon (Excel support film, Nissin EM Co.) was immediately inverted, carbon surface down, onto a 2 μL droplet of the sample solution placed on Parafilm M. After 30 s, the sample was blotted using filter paper, and the grid surface was touched to a 20-μL water drop, again followed by blotting with filter paper. The surface was treated again with a second drop of water. The grid was immediately placed onto a 25-μL droplet of aqueous solution of 0.75% phosphotungstic acid (pH 6.8) and then treated again with a second drop of phosphotungstic acid. After 20 s, the excess stain was removed and the grid was allowed to dry thoroughly. Images were taken on an H-7650 transmission electron microscope (Hitachi High-Technologies) at 120 kV.

3 RESULTS AND DISCUSSION

Helical-wheel diagrams for the peptides Pxt-5 (FIGALL-GPILLNLLK-NH₂) and Modify-Pxt-5 (FIGALLQWLELLK-NH₂) are illustrated in Fig. 2. The diagrams show that both Pxt-5 (Fig. 2(a)) and Modify-Pxt-5 (Fig. 2(b)) display distinct amphiphilicity, wherein hydrophilic amino acids are localized on one face and hydrophobic amino acids are localized on the other face. This configuration could exhibit a surface-activity similar to typical amphiphilic molecules such as surfactants.

Modify-Pxt-5, composed of the 14 amino acids, was designed to improve the \(\alpha\)-helicity and surface activity of Pxt-5, and was altered from Pxt-5 by introducing four amino acid substitutions: glutamine (Q) for glycine (G)-3,-7 and tryptophan (W) for proline (P)-8, glutamic acid (E) for asparagine (N)-11 according to Chou and Fasman algorithm\(^{27,28}\). Thereby, glycine (G), proline (P), and asparagine (N) known as helix-breakers were replaced by glutamine (Q), tryptophan (W), and glutamic acid (E) which are known as helix-formers.

The peptides were synthesized and purified by following the standard solid-phase protocols and characterized by RP-HPLC and MALDI-TOF MS. Figure 3 shows MALDI-TOF mass spectra for Pxt-5 (Fig. 3(a)) and for Modify-Pxt-5 (Fig. 3(b)). Obtained molecular ion[M+H]⁺ peaks

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**Fig.2** Helical wheel diagrams for the peptides used in this study, illustrating its amphiphilic nature: ○, non-polar (hydrophobic); ●, acidic; ★, basic; □, polar uncharged (hydrophilic).
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were 1481.41 (1480.91 calculated) for Pxt-5 and 1728.23 (1727.17 calculated) for Modify-Pxt-5. The observed values are comparable to the calculated values, indicating that Pxt-5 and Modify-Pxt-5 were successfully synthesized.

The secondary structures of Pxt-5 and Modify-Pxt-5 were then analyzed by CD spectroscopy. The CD spectra for the peptides in ultrapure water are shown in Fig. 4. The peptide concentration was 0.8 mM. Typical α-helical CD bands can be observed clearly in the aqueous solution for Modify-Pxt-5, with two negative peaks at 208 and 222 nm. Then the α-helicity was estimated using the equation in Section 2.3. The estimated α-helicity of Modify-Pxt-5 was 45.6%, which is more than five times higher than that of Pxt-5 (8.4%). This indicates that, as predicted, α-helicity greatly improved by the substitution of the amino acids. The lower α-helicity of Pxt-5 without substitution was attributed to the fact that the antimicrobial peptide such as magainin, isolated from amphibian skin, is highly flexible and unstructured in aqueous solution, but its α-helicity is known to be drastically improved in the model membrane environments such as trifluoroethanol/water solutions.

We then assessed the surface tension of the peptides, because we have previously revealed that the surface activity of the peptides is an important property for spontaneous nanodisc formation. The surface tensions of aqueous peptide solutions at various concentrations were measured by the pendant drop method at 25°C. Figure 5 shows the relationship between surface tension and peptide concentration. For both the peptides, having hydrophobic and hydrophilic faces, the surface tension decreased due to the adsorption of the peptide at the air/water interface, and became constant at the adsorption equilibrium, suggesting α-helix formation of Pxt-5 at the air/water interface unlike in bulk solutions.

The intersection point of the two fitted lines is defined as the critical association concentration (CAC). The surface activity of Modify-Pxt-5 (CAC: 9.5 × 10⁻⁵ M, γCAC: 30.3 mN·m⁻¹) was found to be much higher than that of Pxt-5 (CAC: 7.9 × 10⁻⁵ M, γCAC: 38.1 mN·m⁻¹), which results from a higher α-helicity of Modify-Pxt-5. This strong surface activity could allow the peptides to micro-solubilize phospholipid vesicles and form nanodiscs.

As previously reported, based on a belt-like model of the nanodisc, we can also assume their composition using following equations:

\[ ML = 2\pi R_L \]
\[ NS = \pi R_S^2 \]
\[ R_L - R_S = r \]

where \( M \) and \( N \) are the number of phospholipids and peptides in one leaflet of the bilayers, respectively, \( L \) and \( r \) are the length and diameter of a helical peptide, respectively, \( R_L \) is the radius of nanodiscs occupied with phospholipids, \( R_S \) is the radius of nanodiscs, and \( S \) is the average surface area per phospholipid.

The length and diameter of a typical helix, composed of
Antimicrobial peptides leading to lipid nanodisc formation

After twelve hours, the transmittance of vesicle with the addition of Pxt-5 or Modify-Pxt-5 above their CAC. The turbid DMPC vesicle solution with a diameter of 10 nm. The surface activity of Modify-Pxt-5 was found to be higher than that of vesicle with Pxt-5(T = 83.5%), indicating a higher micro-solubilizing ability of Modify-Pxt-5 (Fig. 6).

We then performed DMPC vesicle clearance assay by the addition of either Pxt-5 or Modify-Pxt-5. The peptide:DMPC molar ratio was 1:7. The turbid DMPC vesicle solution(T = 0.3%) quickly turned transparent by the addition of Pxt-5 or Modify-Pxt-5 above their CAC. After twelve hours, the transmittance of vesicle with Modify-Pxt-5(T = 96.2%) was higher than that of vesicle with Pxt-5(T = 83.5%), indicating a higher micro-solubilizing ability of Modify-Pxt-5 (Fig. 6).

To confirm nanodisc formation, NS-TEM observation was performed for the solutions after micro-solubilization by Pxt-5 (Fig. 7(a)) or Modify-Pxt-5 (Fig. 7(b)). The TEM observation indicated rod-like objects (e.g., arrows) which correspond to the side-view of the nanodiscs in the vicinity of relatively large circular vesicles. Some nanodiscs also stacked into a rouleaux formation in the image, a phenomenon well known to be an artifact of negative-stain TEM protocols during nanodisc observation. The rouleaux formation of nanodiscs formed after micro-solubilization by Modify-Pxt-5 (Fig. 7(b)) were more remarkable than that of nanodiscs formed after micro-solubilization by Pxt-5 (Fig. 7(a)). The average diameter of the Pxt-5 nanodiscs estimated from the TEM images (Fig. 7(a)) was 11.2 ± 6.0 nm in the vicinity of relatively large vesicles with a diameter of 35.5 ± 14.0 nm. The average diameter of the Modify-Pxt-5 nanodiscs (Fig. 7(b)) was 10.8 ± 5.8 nm in the vicinity of vesicles with a diameter of 29.5 ± 5.5 nm. Based on the clearance assay and NS-TEM observation, Modify-Pxt-5 is likely to be useful for effective nanodisc formation.

These results clearly demonstrated the formation of nanodiscs from antimicrobial peptides isolated from the skin of Xenopus tropicalis, diploid frogs.

4 CONCLUSION

In this study, Pxt-5, an antimicrobial peptide isolated from the skin of Xenopus tropicalis, and its modified version (Modify-Pxt-5) were synthesized by the solid-phase peptide synthesis (SPPS), and their surface properties were investigated.

The α-helicity of Pxt-5(8.4%) improved drastically to 45.6%(Modify-Pxt-5) by the substitution of four amino acids. The surface activity of Modify-Pxt-5(CAC: 9.5 × 10⁻⁵ M, γcac: 30.3 mN·m⁻¹) was much higher than that of Pxt-5 (CAC: 7.9 × 10⁻⁵ M, γcac: 38.1 mN·m⁻¹), and then the turbid DMPC vesicle solution(T = 0.3%) quickly turned transparent by the addition of Pxt-5 or Modify-Pxt-5. After twelve hours, the transmittance of nanodiscs formed from Modify-Pxt-5(T = 96.2%) was found to be higher than that of those formed by Pxt-5(T = 83.5%).

Interestingly, nanodiscs were found surrounding DMPC vesicles in both the NS-TEM images, and the average nanodisc diameter estimated was 11.2 ± 6.0 nm for those containing Pxt-5 and 10.8 ± 5.8 nm for those containing Modify-Pxt-5. It was also found that Modify-Pxt-5 effectively self-assembled into nanodiscs compared to Pxt-5 without any substitution.

These results clearly demonstrated the formation of nanodiscs from antimicrobial peptides isolated from the skin of Xenopus tropicalis, diploid frogs. This finding could greatly contribute to the application of nanodiscs as vehicles in drug delivery, cosmetic, and food applications.

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