Design of the N-Terminus Substituted Curvature-Sensing Peptides That Exhibit Highly Sensitive Detection Ability of Bacterial Extracellular Vesicles

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Extracellular vesicles (EVs) have emerged as important targets in biological and medical studies because they are involved in diverse human diseases and bacterial pathogenesis. Although antibodies targeting the surface biomarkers are widely used to detect EVs, peptide-based curvature sensors are currently attracting attention as a novel tool for marker-free EV detection techniques. We have previously created a curvature-sensing peptide, FAAV and applied it to develop a simple and rapid method for detection of bacterial EVs in cultured media. The method utilized the fluorescence/Förster resonance energy transfer (FRET) phenomenon to achieve the high sensitivity to changes in the EV amount. In the present study, to develop a practical and easy-to-use approach that can detect bacterial EVs by peptides alone, we designed novel curvature-sensing peptides, N-terminus-substituted FAAV (nFAAV) peptides. The nFAAV peptides exerted higher α-helix-stabilizing effects than FAAV upon binding to vesicles while maintaining a random coil structure in aqueous solution. One of the nFAAV peptides showed a superior binding affinity for bacterial EVs and detected changes in the EV amount with 5-fold higher sensitivity than FAAV even in the presence of the EV-secretory bacterial cells. We named nFAAV5, which exhibited the high ability to detect bacterial EVs, as an EV-sensing peptide. Our finding is that the coil–α-helix structural transition of the nFAAV peptides serve as a key structural factor for highly sensitive detection of bacterial EVs.

Key words bacterial extracellular vesicle; coil–α-helix transition; structure flexibility; dynamic conformation change; extracellular vesicle (EV)-sensing peptide

Introduction

Extracellular vesicles (EVs) are lipid nanoparticles, which are secreted from not only mammalian cells but also bacterial cells.1,2) Since EVs are thought to be involved in diverse human diseases and bacterial pathogenesis and found to contain biomarkers and signaling molecules,3,4) they have recently emerged as important therapeutic targets.5–7) Although methods of EV detection with antibodies targeting their surface biomarkers are widely used to detect EVs, peptide-based curvature sensors are currently attracting attention as a novel tool for marker-free EV detection techniques.8,9) We previously created a novel curvature-sensing peptide, FAAV,10) which derives from sorting nexin protein 11,12) of the Bin/Amphiphysin/Rvs (BAR) protein family.13) FAAV selectively binds to bacterial EVs even in the presence of the EV-secretory bacterial cells in a cultured medium. Taking advantage of this feature of the peptide, we succeeded in developing a simple and rapid method for in situ vesicle detection in cultured media without EV purification steps. This method utilized the fluorescence/Förster resonance energy transfer (FRET) phenomenon between the FRET donor (nitrobenzoxadiazole (NBD)-conjugated FAAV) and the FRET acceptor (a membrane stain, N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl-hexatrienyl) pyridinium dibromide (FM4-64)) by the synergistic effect to achieve the high sensitivity to changes in the EV amount.9) Our purpose of the present study is to develop a practical and easy-to-use approach that can detect bacterial EVs simply by adding peptides into the cultured media. To accomplish our goal, we need to design a novel curvature-sensing peptides with higher sensitivity than FAAV.

The conformational changes of FAAV from a random coil to an α-helix is important for strongly binding to vesicles because the peptides with a flexible structure in aqueous solution could easily approach the vesicle membrane covered with hydrophilic polysaccharide chains4) and insert the hydrophobic face into the lipid-packing defects.9,10) Based on this theory, we designed N-terminus-substituted FAAV (nFAAV) peptides that form a more stable α-helix structure than FAAV
upon binding to vesicles while maintaining a random coil structure in aqueous solution. Amino acid residues, such as Asn and Asp, located at the N-terminus are reported to induce α-helix formation of peptides in trifluoroethanol via end-capping effects,\textsuperscript{13,19} as their side chains adopt favorable rotamer conformations to form hydrogen bonds to free backbone NH groups and stabilize the α-helix.\textsuperscript{16,17} We expected that an α-helix stabilizing by the end-capping effect is particularly exerted upon binding of the peptides to the lipid membrane because the hydrogen bonding is considered to be facilitated under hydrophilic conditions.\textsuperscript{18} When the Gly-Ala-Gly sequence at the N-terminus of FAAV is substituted with an α-helix-inducing amino acids, the N-terminal region could facilitate α-helix formation of FAAV as the starting point.

In the present study, we used EVs released from a Gram-negative bacterium, *Shewanella vesiculosa* HM13, as a model because of its unique features, such as the large production of EVs with a single size of approximately 100 nm in diameter including a cargo protein with high purity.\textsuperscript{19} We found that one of the nFAAV peptides, which undergoes dynamic conformation changes upon binding to the vesicles, showed higher binding performance to bacterial EVs than the original peptide. The nFAAV peptide detected changes in the amount of bacterial EVs with 5-fold higher sensitivity than FAAV, even in the presence of the EV-secretory bacterial cells. On the basis of these results, nFAAV5 was demonstrated to exert as a novel EV-sensing peptide. Our finding indicates that the dynamic conformation changes of EV-sensing peptides are a key structural factor for highly sensitive detection of bacterial EVs.

### Experimental

**Peptide Synthesis**

Peptides were constructed on a Rink Amide resin on the basis of Fmoc solid-phase peptide chemistry. Peptide synthesizers, PSSM-8 (Shimadzu, Kyoto, Japan) and PurePep Chorus (Gyros Protein Technologies, Uppsala, Sweden) were used for the peptide synthesis. For coupling of amino acids, the reaction was performed at 25 °C for 1 h using a coupling system of 1-[bis(dimethylamino)methylene]-1H-benzotriazolium 3-oxide hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt)/N,N-diisopropylethylamine (DIEA). For evaluation of the vesicle binding, peptide chains bearing the GSGS linker segment and Lys(Mtt) (Mtt = 4-methyltryptyl) at the C-terminus were constructed. After peptide chain construction, N-termini were masked with tert-butyloxycarbonyl (Boc) groups using di-tert-butyl dicarbonate in the presence of N-methylmorpholine/dimethylformamide (1:49) (3 h at 25 °C). Then, the Mtt group was selectively removed on the resin by incubation with hexafluoroisopropanol/dichloromethane (1:4) for 3 h at 25 °C.\textsuperscript{20} Fluorescence labeling for the C-terminal Lys residue was performed using 4-fluoro-7-nitrobenzofurazan (NBD-F) (2.7 equiv (equiv.)) (Dojindo, Kumamoto, Japan) for 16 h at 25 °C.\textsuperscript{21} The final desorption of the peptides and the cleavage from the resin were conducted using trifluoroacetic acid/1,2-ethanediethyl (95:5) for 3 h at 25 °C. The peptides were purified by the reverse-phase (RP) HPLC on a COSMOSIL 5C\textsubscript{18}-AR-II column (4.6 mm I.D. × 150 mm) (Nacalai Tesque, Kyoto, Japan), and the masses of the products were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Bruker Daltonics Japan, Yokohama, Japan). The absorbance spectrum of the dye-labeled peptides was measured in pH-controlled phosphate-buffered saline [PBS(–): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na\textsubscript{2}HPO\textsubscript{4}, and 1.5 mM KH\textsubscript{2}PO\textsubscript{4} (pH 7.4)] by the UV-Vis spectrophotometer DU-640 (Beckman Coulter, Brea, CA, U.S.A.).

**Preparation of Liposomes**

Liposomes were prepared as follows; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), cholesterol, and 1-palmitoyl-2-oleoyl-sn-glyce ro-3-phospho-L-serine (POPS) were mixed with a molar ratio of 60: 15:15:10 in chloroform in an eggplant flask and evaporated for 30 min followed by vacuum drying overnight, finally yielding a lipid film on the inner surface of the flask. The lipid film was gently hydrated in Dulbecco’s phosphate-buffered saline (DBPS) (2.7 mM KCl, 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 200 mM NaCl, 8.1 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.5 mM MgCl\textsubscript{2} 6H\textsubscript{2}O, and 0.9 mM CaCl\textsubscript{2})\textsuperscript{22} for one hour at 25 °C with occasional vortexing to prepare a liposome solution. To control the size of the liposomes, a LypsoFast extrusion system (Avestin, Mannheim, Germany) and Whatman polycarbonate filters (GE Healthcare Japan, Tokyo, Japan) with pore sizes of 200 nm were used. Two polycarbonate filters were attached to the LypsoFast extrusion system, and the size of the liposomes was adjusted by passing 21 times through polycarbonate filters. The lipid concentrations of the liposomes were determined using LabAssay Phospholipid Kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) according to the manufacturer’s instructions. Particle sizes of the liposomes were determined using Zetasizer Nano ZS (Malvern Panalytical, Worcestershire, U.K.) with a scattering angle of 173° at 25 °C.

**Measurement of Circular Dichroism (CD) Spectra**

Non-labeled peptides without the linker segment and the NBD moiety were used to measure CD spectra. The peptide concentrations were estimated on the basis of their weight. Non-labeled peptides (80 µM) and liposomes (at a lipid concentration of 4 mM) were mixed together in DPBS and incubated for 10 min at 25 °C. Then, the CD spectra were obtained using a cuvette with a light path length of 1 mm and J-820-L circular dichroism spectrometers (JASCO, Tokyo, Japan).

**Preparation of the Bacterial EVs and Cells**

Rifampicin-resistant *S. vesiculosa* HM13-Rif\textsuperscript{\textsuperscript{a}} (denoted as HM13) and a mutant secreting bigger EVs than the HM13-Rif\textsuperscript{\textsuperscript{a}} strain (named as 6B) were aerobically grown to the early stationary phase in 5 mL of liquid lysogeny broth using a sample tube at 18 °C. The cells in the cultured medium were pelleted by centrifugation at 6800 × *g* and 4 °C for 10 min and subsequently washed with DPBS three times. EVs were collected from the cultured medium as previously described\textsuperscript{23} with slight modifications. Briefly, the cell-free supernatant was centrifuged at 13000 × *g* and 4 °C for 15 min, and then filtered through a 0.45-µm pore polyesulfone filter to remove the remaining debris. EVs were obtained by ultrafiltration of 9 mL of the filtrate at 100000 × *g* (average centrifugal force) and 4 °C for 2 h with an OptimaX centrifuge (Beckman Coulter). The pellet was resuspended in 900 µL of DPBS and used as EVs in the subsequent experiments. Particle sizes of the EVs were determined using Zetasizer Nano ZS (Malvern Panalytical) with a scattering angle of 173° at 25 °C. For quantification of the lipid concentration of the bacterial EVs and the cells, the EVs and the cells were stained with a lipophilic fluorescent
molecule, FM4-64 (Thermo Fisher Scientific, Waltham, MA, U.S.A.), according to the method described before. Briefly, the EVs and the cells were stained with FM4-64, and the lipid concentrations were calculated on the basis of the calibration curve of the lipid concentration-known liposomes stained with FM4-64.

**Evaluation of Vesicle Binding of Peptides** The NBD-labeled peptides (0.5 μM) and vesicles were mixed together in 150 μL/well DPBS on a 96-well assay plate with flat bottom (Iwaki, Shizuoka, Japan), and then the NBD fluorescence intensity was measured at 25°C using VICTOR Nivo Microplate Reader (PerkinElmer, Inc. Japan, Yokohama, Japan) with an excitation filter of 480/30 nm and an emission filter of 530/30 nm. The data were obtained under optimized experimental conditions (e.g., exposure time: 500 ms, and the Z-focus: 8.5 mm from the bottom). All the data show the fluorescence intensity after subtraction of the background. For correcting the light scattering effect by bacterial cells, the apparent fluorescence intensity of the control sample was measured as the parent fluorescent intensity of cell suspension at the same lipid concentration, not including the peptides, was measured as a control. The apparent fluorescence intensity of the control was subtracted from the intensity of cells including the NBD-labeled peptides to obtain the net intensity. The light scattering effect of the EVs was not observed under any conditions.

**Determination of $K_d$ Values** Under an assumption that the vesicle binding manner of the peptides follows the Langmuir isotherm model, the $K_d$ values for vesicles were determined using the following equation (1):

$$B_c = \frac{B_{\text{max}}}{1 + \frac{K_d}{c}}$$  

where $B_c$, $B_{\text{max}}$, and $c$ indicate the ratio of bound peptides to vesicles, the max level of the binding, and a given lipid concentration of vesicles, respectively.

**Results**

**Design of the nFAAV Peptides** The sequence of nFAAV1–nFAAV5 are shown in Table 1. To design peptides exhibiting higher $\alpha$-helicity, we focused on the N-terminal Gly-Ala-Gly sequence of FAAV. Since the Gly residue possesses considerable conformational flexibility, the Gly-Ala-Gly sequence would be entropically disadvantageous to be incorporated into the constrained $\alpha$-helical structure. Our strategy is to replace the relaxed N-terminal region of FAAV with Asn and Asp, of which the side chains could hydrogen bond to the free NH group at the N-terminus to contribute to $\alpha$-helix-stabilizing (Fig. 1A, as exemplified with nFAAV5). The Gly residue at the position 1 of FAAV was replaced with Asn and Asp in nFAAV1–nFAAV2 and nFAAV3–nFAAV5, respectively. The Gly residue at the position 3 located at the boundary area between the hydrophobic and hydrophilic faces was substituted with Ala or Aib to facilitate $\alpha$-helix formation of nFAAV1–nFAAV2 and nFAAV4–nFAAV5 (Fig. 1B, respectively). Lys was incorporated into the sequences of nFAAV3–nFAAV5 to maintain the same net charge (+2).

The peptide chains were constructed by Fmoc solid-phase peptide synthesis on a Rink amide resin. For vesicle binding assay, after peptide chain construction and N-terminal modification, a Lys residue at the C terminus of FAAV and nFAAV1–nFAAV5 was labeled via a GSGS linker with NBD (FAAV-NBD and nFAAV1–nFAAV5-NBD) (Table 1), a dye
that emits strong fluorescence in the hydrophobic environment, such as vesicle membranes. Peptide binding to vesicles was thus evaluated by an increase in NBD fluorescence intensity. For CD measurement, FAAV and nFAAV1–nFAAV5 without the linker segment and the NBD moiety (FAAV (CD) and nFAAV1–nFAAV5 (CD)) were synthesized (Fig. S1–S12).

**The Secondary Structure of the nFAAV Peptides** To investigate a change of the secondary structure of nFAAV peptides in the absence and presence of vesicles, we measured CD spectra using nFAAV1–nFAAV5 (CD) peptides and liposomes. The liposomes used as a model vesicle here following our previous reports were composed of POPC, POPE, cholesterol, and POPS at 60, 15, 15, and 10 mol%, respectively. The liposome pure system is ideal for evaluation of binding ability of the peptides for the following reasons: (i) no influence by the presence of surface proteins and sugar chains on vesicles; (ii) controllable size and lipid composition; and (iii) highly homogeneous condition in an experimental system. The diameter of the extruded liposomes was determined by dynamic light scattering to be 97.5 ± 0.6 nm (mean ± standard deviation (S.D.), n = 5). The lipid concentration of the extruded liposomes was calculated using LabAssay Phospholipid Kit. The molar ellipticity of the peptides at 222 nm ([θ]222) is used as an indicator of α-helicity. In the absence of the liposomes, all the peptides showed a random coil structure in solution, and the [θ]222 values of nFAAV3, nFAAV4, and nFAAV5 were higher and those of nFAAV1 and nFAAV2 were lower than that of the original peptide, FAAV (CD) (Fig. 2A). In the presence of the liposomes, all the peptides exhibited typical CD spectra reflecting an α-helical structure (Fig. 2B). The α-helix contents estimated on the basis of [θ]222 values are summarized for all the peptides in Table 1. To simply evaluate the dynamic conformation change of the peptides between vesicle-bound and -unbound states, in the present study, the ratio of the [θ]222 values (Rθ) was defined as an indicator of the conformation-change degree of the peptides between the presence and absence of vesicles (Table 1). For instance, the Rθ value of nFAAV5 (CD) was higher than that of FAAV (CD), which means that the peptide has more dynamic conformation change than FAAV (CD) when binding to the vesicles. Note that because the bacterial EVs contain a single major cargo protein P49, CD spectra of the peptides in the presence of the EVs can be influenced by that of P49. Herein, we used the Rθ values of the peptides in the presence of the liposomes instead. Since the Rθ value is an indicator showing the difference in the structural changes of the peptides between the vesicle-bound and -unbound states at the equilibrium, it can be diverted to the degree of the conformation change of the peptides when binding to the EVs.

**Binding Abilities of the nFAAV Peptides to Bacterial EVs and Cells** Induced fit of curvature-sensing peptides upon binding to vesicles is accompanied by the coil–α-helix transition of the peptides. Therefore, the binding affinity is fundamentally supposed to be related with the degree of conformational changes between vesicle-bound and -unbound states. To evaluate the relationship between the binding affinity of the peptides for the liposomes and the degree of conformational changes, the Kd values were plotted versus the Rθ values. If the Rθ value is larger, it means that the peptide has more structural flexibility and involves larger conformation changes upon binding to vesicles. The lipid concentration of the bacterial EVs was determined based on the calibration curve of the lipid concentration-known liposomes stained with a membrane stain, FM4-64 (cf. Experimental). In the present study, FM4-64 was used only for a purpose to calculate the lipid concentrations of the EVs or the bacterial cells, but not for the FRET measurement as described in our previous study. The Kd values of the peptides binding to the bacterial EVs with a diameter of 91.5 ± 9.7 nm (mean ± S.D., n = 5) were analyzed using the equation (1) based on the Langmuir isotherms model, as shown in Table 1 and Fig. 3A. An increase in the NBD fluorescence intensity depending on changes in the EV ratio is due to an increase in the total number of lipid-packing defects on the EVs, which provides the binding sites for the curvature-sensing peptides. In the comparison with FAAV-NBD, nFAAV5-NBD showed higher fluorescence intensity at the lipid concentration of the EVs less than 100 μM (Fig. 3A) and the binding affinity was improved to be 30 μM (Table 1). When the Kd values were plotted as a function of their Rθ values, the data exhibited a strong correlation (R = −0.94) (Fig. 3B). In contrast, when the Kd values were plotted as a function of their RP-HPLC retention time, the data showed a weak correlation (R = −0.20) (Fig. 3C). The retention time reflects the hydrophobicity of the peptides with an unstructured state under the RP-HPLC condition; thus, this hydrophobic parameter is supposed to be an independent

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**Fig. 2.** CD Spectra of FAAV and nFAAV Peptides Measured Alone (A) and in the Presence of the Liposomes with a Diameter of 98 nm (B)

The peptides without the GSGSK(NBD) moiety were used here. The concentrations of the vesicle lipids ([L]) and the peptides ([P]) were 4 mM and 80 μM, respectively. (Color figure can be accessed in the online version.)
indicator unaffected by the secondary structure. These data demonstrated that the binding affinity of the peptides for the EVs is highly correlated with their degree of conformational changes but not with their hydrophobicity.

We also evaluated the adsorptivity of peptides to the EV-secretory bacterial cells because the in-situ vesicle detection method requires high EV-selectivity without binding to the cells in complex environment such as cultured media. Note that the EVs and the EV-secretory cells show the same lipid A structures regarding the acyl substitution pattern, but FAAV can specifically recognize the EVs by binding to the near surface of the curved membrane without being trapped with polysaccharide chains. While the EVs with a globular shape has a size of approximately 100 nm in a diameter, the cells with an elliptical shape is approximately $500 \times 1000$ nm in the short and the long axes, respectively. It means that the former has highly curved membranes and the latter has flatter membranes. The curvature-recognizing ability of peptides is important property to distinguish the EVs from the cells in cultured media. As same as FAAV-NBD, no increase in the NBD fluorescence of nFAAV-NBD peptides was detected even 30 min after peptide addition to the cells (Fig. 3D).
time-course experiment revealed that the NBD fluorescence intensity for all the peptides topped out at 5 min following the addition of the peptides to the bacterial EVs (Fig. 3D), meaning that these properties make it possible to rapidly detect the bacterial EVs in cultured media. To confirm that the curvature sensitivity of the peptides contributes to their distinguishing ability between the EVs and the bacterial cells, we prepared two types of EVs with slightly different diameters (92 and 156 nm) and evaluated their sensitivity to the membrane curvature. We measured the fluorescence intensity of the peptides in the presence of EVs with a smaller diameter (92 nm) or a larger diameter (156 nm) at the same lipid concentration to obtain the ratio of fluorescence intensity of the former to the latter (ratio of fluorescence intensity \( \phi_{92 \text{nm}} / \phi_{156 \text{nm}} \)) (Fig. 3E). It was revealed that the peptides are highly sensitive to a small difference of the membrane curvature, and the curvature-sensing ability of newly designed peptides is maintained at a similar level comparable with FAAV and not significantly affected by the N-terminal substitution.

**Highly Sensitive Detection of Bacterial EVs in the Cultured Medium with nFAAV** Finally, to demonstrate the applicability of nFAAV5-NBD for the in-situ vesicle detection, we investigated its ability to detect changes in the EV amount in the presence of the bacterial cells, mimicking the condition of the cultured medium. In the previous work, we revealed that the amount of the secretory EVs varies in a range of 45–70 \( \mu \text{M} \) at the lipid concentration, when the bacterial cells are cultured in a 96-well plate.\(^9\) The lipid concentration of the cells was calculated based on the fluorescent intensity of FM4-64 under an identical method as determining the lipid concentration of the EVs (cf. *Experimental*). In the present study, to evaluate whether nFAAV has a sensitivity enough to cover the entire range of the EV-secretory amount described above, we prepared the amount of the EVs in a wider range of 6–96 \( \mu \text{M} \) at the lipid concentration. The amount of the cells was fixed at a lipid concentration of 300 \( \mu \text{M} \). When the NBD fluorescence intensity of each peptide at the EV lipid concentration of 6 \( \mu \text{M} \) was used as the standard, the relative fluorescence intensity of FAAV-NBD was 3.1-fold higher when the EV amount was increased 16-fold (Fig. 4A). In contrast, nFAAV5-NBD exhibited the 15.2-fold higher relative fluorescence intensity when the EV amount was increased 16-fold. Although the fluorescence intensity of FAAV-NBD and nFAAV5-NBD had a high linearity for changes in the EV amount (\( R = 0.98 \) and 0.99, respectively), nFAAV5-NBD detected changes in the EV amount with 5-fold higher sensitivity than FAAV-NBD. On the other hand, nFAAV1–4-NBD peptides with lower binding affinities for the EVs (Table 1) did not show any improvement of their...
EV sensitivity than FAAV-NBD (Fig. 4B). These results demonstrated that nFAAV5-NBD with high sensitivity to changes in the EV amount in the presence of the bacterial cells can be used as a novel EV-sensing peptide to quantify EV production in this range by simply adding the peptide into cultured media and measuring the fluorescence intensity without complicated treatments.

**Discussion**

In the previous work, we adapted the FRET method using FAAV-NBD and FM4–64 pairs to the EV detection to enhance the sensitivity by using the synergistic effect of the binding degree of FAAV-NBD and FM4–64. In the present study, we succeeded in designing a new peptide, nFAAV5-NBD, effective for highly sensitive detection of the EVs secreted from a Gram-negative bacterium, *S. vesiculosa* HM13, as a model (Fig. 4). The feature we especially want to emphasize is that nFAAV5-NBD alone, which is highly sensitive to changes in the EV amount, achieved a detection level comparable to the method using the FRET phenomenon. nFAAV5-NBD has a significant advantage over our previous method in the convenience for quantification of the EV amount by simply adding the peptides to the solution and subtracting the background signals of the NBD dye without complicated calculations.

We have previously proposed a model of an amphipathic peptide sensing high curvature membranes as following: i) the electrostatic interaction between the positively charged residues of the peptide and negatively charged lipid-heads of liposomes serves one of important factors for the peptide to approach the liposome membrane; ii) a curvature-sensing peptide adopts an amphipathic α-helical structure on the membrane surface and inserts the hydrophobic face into hydrophobic binding pockets of lipid-packing defects on highly curved membranes.

Our finding in this study is that the dynamic conformation changes of EV-sensing peptides from a random coil structure to an α-helical structure is a novel key factor for highly sensitive detection of the bacterial EVs. The structure flexibility of nFAAV5 plausibly makes it easy to approach the membrane surface of bacterial EVs covered with polysaccharide chains (Fig. 5), and its large conformational change upon binding to vesicles led to its superior detecting performance for the EVs over FAAV (Fig. 4). The importance of the structural change of membrane-curve sensors upon contact with the highly curved membrane has been recognized in the amphipathic lipid-packing sensor (ALPS) motif of Golgi-associated proteins ArfGAP1, GMAP-210, a sterol-binding protein Kes1p, and a human protein of the nuclear pore complex Nup133. Like ALPS motifs, it is also reported that the synaptic vesicle protein α-synuclein is unfolded in solution but forms an amphipathic helix accompanied by binding to small vesicles.

The difference in the detection sensitivity of the EVs between nFAAV5-NBD and the other peptides mainly attributed to the binding affinity to the bacterial EVs (Table 1). nFAAV5-NBD has a property that it highly responds to the EVs at a lower lipid concentration less than 100 µM, while it shows a maximum intensity comparable to that of FAAV (Fig. 3A). The environment-sensitive dye, NBD, which is conjugated to the peptides, strongly emits fluorescence in hydrophobic environments such as membranes or organic solvents, while it weakly exhibits fluorescence in an aqueous environment. This is due to the difference in the exposure degree to water molecules working as a quencher of the nitro group of the NBD moiety. The smaller EVs with highly curved membranes provides more binding sites (i.e., lipid-packing defects) to curvature-sensing peptides if the lipid concentration is the same. If the number of peptides that bind to the EVs increases, the total fluorescence intensity of NBD is increased in proportion to it. Therefore, the maximum fluorescence intensity could be considered to indicate a parameter of the relative occupancy ratio of the peptide binding sites on the EV surface. In comparison with nFAAV5-NBD, nFAAV1–3-NBD peptides showed the lower maximum fluorescence intensity (Fig. 3A), which could be a reason why no improvement of the *in-situ* EV detection was observed in these peptides (Fig. 4). In the case of nFAAV4-NBD, although the maximum fluorescence intensity was higher than FAAV-NBD and nFAAV5-NBD at a lipid concentration of 250 µM (Fig. 3A), it did not provide any improvement in the EV binding at the lower lipid concentration range of 6–96 µM (Fig. 4).

The highly sensitive detection ability of the EVs of nFAAV5-NBD results from the substitution of the three residues at the N-terminus. In general, the first residue has non-helical ϕ, ψ angles, but its carbonyl group starts to participate in the first backbone helical hydrogen bond. Elongation of helix from the N-terminus is thermodynamically favorable because the entropy loss of incorporating another residue into the helix is more than compensated by the energy from the new hydrogen bond. (However, the Gly-Xaa-Gly sequence is too conformationally flexible to incorporate the N-terminus into the relatively constrained α-helical structure.) Substitution of the amino acids at the N-terminus with Asn or Asp, of which the side chains stabilize α-helix formation by formation of hydrogen bond to free backbone NH groups, could facilitate the α-helicity of the peptides. We expected that the hydrogen bond forms especially under hydrophobic conditions such as membranes. For nFAAV5, the best position of the Lys and Aib residues could also contribute to the α-helix stabilization in addition to the N-capping effect by Asp. On the other hand, for nFAAV1 and nFAAV2, the substitution with the Asn residue at the N-terminus resulted in an improvement in α-helicity in the presence of the liposomes, but it also increased α-helicity in the absence of the liposomes (Fig. 2).
As a result, their $R_\theta$ values were not improved. For nFAAV3 and nFAAV4, the non-optimized amino acid sequences on the boundary area may have led to a decrease in $\alpha$-helicity in the presence of liposomes. Therefore, these results suggested that while it is certain that the N-capping effect of the first amino acid contributes to the structural stability of the peptides, the contribution of the position and type of the subsequent amino acids is also not negligible.

**Conclusion**

In the present study, we newly designed the N-terminus-substituted peptides and studied the relationship of their structural changes and binding affinities using liposomes and bacterial EVs. Especially, nFAAV5, which undergoes dynamic conformation changes upon binding to vesicles, showed high binding performance for bacterial EVs covered with hydrophilic polysaccharide chains. Moreover, it was demonstrated that nFAAV5 with high sensitivity for the bacterial EVs works as a novel EV-sensing peptide because it is applicable for quantification of changes in the amount of the EVs even in the presence of the EV-secretory bacterial cells. Our finding is that the dynamic conformation changes of EV-sensing peptides is a novel key factor for highly sensitive detection of bacterial EVs.

**Acknowledgments**

This work was financially supported by the Ueo-Chikuenkai Foundation, Kyoto, Japan (K. Kawano), the Takeda Science Foundation (K. Kawano), ICR Grants for Promoting Integrated Research, Kyoto University (K. Kawano and T.O.), JSPS KAKENHI (JP18K19178 to T.K. and JP20H04707 to S.F.), and Grant from the Institute for Fermentation, Osaka, Japan (L-2019-2-012 to T.K.).

**Conflict of Interest**

The authors declare no conflict of interest.

**Supplementary Materials**

The online version of this article contains supplementary materials.

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