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RAPID PAPER

Minimum Amino Acid Residues of an α-Helical Peptide Leading to Lipid Nanodisc Formation

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Abstract: Nanodiscs are a relatively new class of nanoparticles composed of amphiphilic α-helical scaffold peptides and a phospholipid bilayer, and find potential applications in various fields. In order to identify the minimum number of amino acid residues of an amphiphilic α-helical peptide that leads to nanodisc formation, seven peptides differing in lengths (22-, 18-, 14-, 12-, 10-, 8-, and 6-mers) that mimic and modify the *C*-terminal domain of apoA-I (residues 220–241) were synthesized. At a concentration of 0.3 mM, the 6- and 8-mer peptides did not present any surface activity. In case of the 10-mer peptide, the aqueous surface tension initially decreased and reached a constant value of 51.9 mN/m with the 14-, 18-, and 22-mer peptides. Moreover, upon mixing the surface-active peptides (14-, 18-, and 22-mers) with dipalmitoylphosphatidylcholine (DMPC) liposomes (2.5:1, peptide : DMPC), the turbid DMPC liposome solution rapidly became transparent. Further analysis of this solution by negative-stain transmission electron microscopy (NS-TEM) indicated the presence of disk-like nanostructures. The average diameter of the nanodiscs formed was 9.5 ± 2.7 nm for the 22-mer, 8.1 ± 2.7 nm for the 18-mer, and 25.5 ± 8.5 nm for the 14-mer peptides. These results clearly demonstrate that the surface properties of peptides play a critical role in nanodisc formation. Furthermore, the minimum length of an amphiphilic peptide from the *C*-terminal of apoA-I protein that can lead to nanodisc formation is 14 amino acid residues.

Key words: nanodisc, amphiphilic α-helical peptide, phospholipid bilayer, surface tension, negative stain transmission electron microscopy (NS-TEM)

1 INTRODUCTION

Nanodiscs are self-assembling discoidal nanoparticles composed of amphiphilic α-helical scaffold proteins or peptides that wrap themselves around the circumference of a lipid bilayer in a belt-like manner (Fig. 1)[1, 2]. It is generally accepted that nanodiscs represent the simplest models of high density lipoprotein (HDL) particles, which play a crucial role in the process of reverse cholesterol transport (RCT). In RCT, HDL particles or lipoproteins such as apolipoprotein A-I (apoA-I) remove excess cholesterol from peripheral tissues and transport it to the liver for elimination[3, 4].

Although native HDLs are heterogeneous particles with distinct sizes and shapes (discoidal and spherical), nanodiscs that reconstitute HDLs homogeneously have attracted considerable attention particularly as a new class of potential therapeutic agents for enhancing the RCT pathway by promoting cholesterol efflux[5, 6]. More recently, the interest in nanodiscs has found a focus in applications

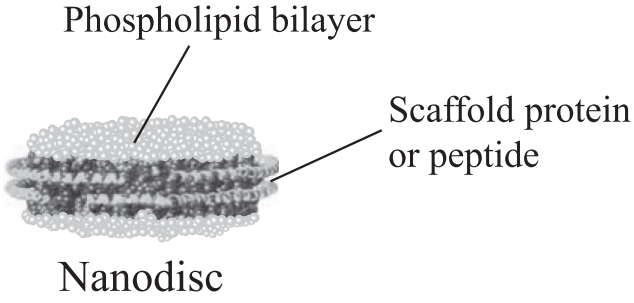

Fig. 1 Structure of nanodisc.

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beyond their physiological role in lipid metabolism. They serve not only as useful platforms capable of packaging membrane proteins such as bacteriorhodopsin (bR)[7, 8] and cytochrome P450s[9] in a native-like membrane environment, but also as useful vehicles for hydrophobic drugs[10] or biomolecules[11, 12]. In contrast to the typical bilayer structure of liposomes[13], nanodiscs are smaller and finer nanoparticles with high dispersion stability, and are thus expected to be potentially more useful than liposomes and may find applications in various fields.

Generally, the preparation of nanodiscs is accomplished by the surfactant dialysis method[1, 2, 14–17] in which scaffold α-helical proteins or peptides are mixed with a certain amount of phospholipids solubilized into micelles using a surfactant such as cholate. Dialysis against excess water initiates the self-assembly process by removal of surfactants, and the amphiphilic α-helices stabilize the edges of the phospholipid bilayers, thus leading to homogeneous lipid nanodisc formation. The use of surfactants is avoided wherever possible, because they are often reported to denature proteins or peptides and their complete removal is difficult.

Recently, we have succeeded in the artificial synthesis of an amphiphilic α-helical 22-mer peptide (NH_2-PVLESFKASFLSALEEWTKKLN-NH_2) that mimics and modifies helix 10 of the human apoA-I protein (residues 220–241)[18]. By promoting surface activity of the peptide and by a mere mixing of the peptide with phospholipid liposomes without using any additional surfactant, lipid nanodiscs could be produced easily and rapidly. It was also found that a unique amphiphilic structure of the α-helical peptide, one face of which localizes mainly hydrophilic amino acids and the other face mainly hydrophobic amino acids, provides surface properties such as reduced surface tension and ability to solubilize lipids. However, detailed structure-property relationships of amphiphilic α-helical peptides, especially the effect of the number of amino acid residues on the surface and nanodisc formation properties, still remain unclear. A peptide with a less number of amino acids is advantageous when the cost and variety of nanodisc applications is considered.

In this study, we synthesized seven different human apoA-I peptides of varying amino acid lengths (residues 220–241), which mimic and modify amphiphilic α-helical peptides (Table 1). The effects of amino acid residues on the surface properties of peptides were evaluated by surface tension measurements, dynamic light scattering (DLS), and negative stain transmission electron microscopy (NS-TEM). Finally, we ascertained the critical peptide length of the *C*-terminal apoA-I domain that leads to lipid nanodisc formation.

2 EXPERIMENTAL PROCEDURE

2.1 Peptide synthesis

Peptides were synthesized using standard Fmoc chemistry with a Syro I peptide synthesizer (Biotage). A typical synthesis was carried out using a stepwise solid phase peptide synthesis (SPPS) procedure on a Rink amide MBHA resin (0.09 mmol scale using 0.4 mmol g^{-1}). Standard side chain protecting groups included Asn (Trt), Lys (Boc), Thr (tBu), Trp (Boc), Glu (tOBu), and Ser (tBu). Couplings were performed using 1-[Bis (dimethylamino) methylene] -1*H*- benzotriazolium 3-oxide hexafluorophosphate (HBTU) in DMF over 90 min. Fmoc groups were removed using 40% piperidine in DMF. The peptide was cleaved from the resin, with a concomitant side chain deprotection, by agitation in a solution of 95:2.5:2.5 trifluoroacetic acid (TFA)/ triisopropylsilane (TIS)/water for 3 h. The crude peptide was precipitated with diethyl ether, centrifuged, washed three times with diethyl ether, and then purified by a pre-

Table 1 Peptide sequence and measured mass based on MALDI-TOFMS.

	Sequence *N*-terminal → *C*-terminal	Measured Mass [M + H]$^+$	Calculated Mass [M + H]$^+$
22-mer	H_2N-PVLESFKASFLSALEEWTKKLN-NH_2	2537.14	2536.37
18-mer	H_2N-SFKASFLSALEEWTKKLN-NH_2	2097.76	2098.13
14-mer	H_2N-SFLSALEEWTKKLN-NH_2	1662.57	1664.89
12-mer	H_2N-LSALEEWTKKLN-NH_2	1430.91	1430.79
10-mer	H_2N-ALEEWTKKLN-NH_2	1230.83	1230.68
8-mer	H_2N-EEWTKKLN-NH_2	1045.78	1046.56
6-mer	H_2N-WTKKLN-NH_2	788.71	788.47

parative reverse-phase (RP)-HPLC on a C18 column. Purity was confirmed by analytical RP-HPLC. Binary gradients comprising solvent A (99% H_2O, 0.9% acetonitrile, 0.1% TFA) and solvent B (90% acetonitrile, 9.9% H_2O, 0.07% TFA) were employed for HPLC. The purified peptide was characterized by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, where sinapinic acid (SA) was used as a matrix. Reagents used in this study are mentioned in supporting information.

2.2 Surface tension measurement

The surface tension of the peptide, at 25℃, was determined by the pendant drop method, performed using an apparatus consisting of an automatic interfacial tensiometer (DM500, Kyowa Interface Science) and the Drop Shape Analysis software of FAMAS ver. 2.01[19]. A setscrew was used to press the solution out of a syringe and form a drop at its tip. Drop shape analysis was performed as follows: a drop profile was extracted from the image of a drop following which a curve fitting program was used to compare the experimental and theoretical drop profiles (Young-Laplace method), which provided the corresponding surface tension value. The evolution of the drop surface tension was followed over 10 min for each concentration of the purified peptide solution. Peptide concentration was determined by a V-560 UV-Vis spectrometer (JASCO).

2.3 Peptide-lipid nanodisc preparation

Before preparation of the nanodiscs, multilamellar vesicles (MLVs) of dipalmitoyl phosphatidylcholine (DMPC) were prepared[20]. For this purpose, DMPC was dissolved in chloroform in a test tube and the solvent was removed by blowing nitrogen gas and by a subsequent overnight storage at room temperature under vacuum to leave a thin lipid film on the test tube wall. Milli-Q ultrapure water (18.3 MΩ cm), used to avoid interference from salts, was added to this lipid film and the test tube was shaken vigorously on a vortex mixer to yield an aqueous solution of MLVs. The formation of MLVs and their sizes (several micrometers) were confirmed using an optical microscope. The conversion of MLVs to nanodiscs was performed by addition of a peptide solution in a 1:2.5 peptide : lipid weight ratio (w/w), followed by vortexing for 30 min, and subsequent incubation for 24 h at 25℃[5].

2.4 Transmission electron microscopy (TEM)[21]

A freshly glow-discharged copper grid (200 mesh) coated with carbon (Excel support film, 200 mesh, Nisshin EM Co.) was inverted, with its carbon surface facing downwards, onto a 2 μL droplet of the sample solution placed on Parafilm®. The sample was blotted using a filter paper after 30 s incubation, following which the grid surface was touched onto a 20 μL water drop. Water was blotted out with filter paper. After repeating the treatment with a second drop of water, the grid was immediately placed onto a 25 μL droplet of aqueous 0.75% phosphotungstic acid at 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 at 120 kV on an H-7650 transmission electron microscope (Hitachi High-Technologies).

2.5 Dynamic light scattering (DLS)

The size distribution of the nanodiscs was measured using dynamic light scattering with a DLS-7000 (Otsuka Electronics Co., Japan) using a 488 nm wavelength 75 mW Ar laser as a light source at 25℃. The time-dependent correlation function of the scattered light intensity was measured at a scattering angle of 90°. Particle size distributions were determined using the software provided with the instrument.

3 RESULTS AND DISCUSSION

For designing the 22-mer peptide (NH_2-PVLESFKASF LSALEEWTKKLN-NH_2), we focused on the *C*-terminal domain of human apoA-I protein (residues 220–241), known to be critical for lipid binding and cholesterol efflux in the native protein[22]. The domain was altered by introducing two conservative amino acid substitutions viz., Ala to Val at position 227 to increase amphiphilicity and Trp to Tyr at position 236 to improve spectrophotometric analysis. In a previous study, this peptide was found to behave like general surfactants, and its estimated critical association concentration (CAC) and surface tension at CAC were 2.7×10^{-5} M and 51.2 $mN \cdot m^{-1}$, respectively.

In order to identify the minimum number of amino acid residues of an amphiphilic α-helical peptide that leads to nanodisc formation, seven peptides differing in lengths (22-, 18-, 14-, 12-, 10-, 8-, and 6-mers) were synthesized by decreasing the number of amino acid residues by SPPS. Sequences of the peptides are shown in Table 1. The length of the peptides was gradually reduced from 22 to 6 amino acid residues towards the *C*-terminal domain of human apoA-I protein

The synthesized peptides were assigned by MALDI-TOFMS, and their measured mass ($[M+H]^+$), together with calculated mass, is listed in Table 1. The measured mass of each peptide was in good agreement to the calculated mass, indicating successive synthesis of the designed peptides. Purity of the peptides was analyzed by analytical RP-HPLC after their purification with preparative RP-HPLC (Supporting information).

Surface activity of the peptides and surface tensions of the aqueous peptide solutions were measured by the pendant drop method. Figure 2 shows the relationship between the surface tension and number of amino acid

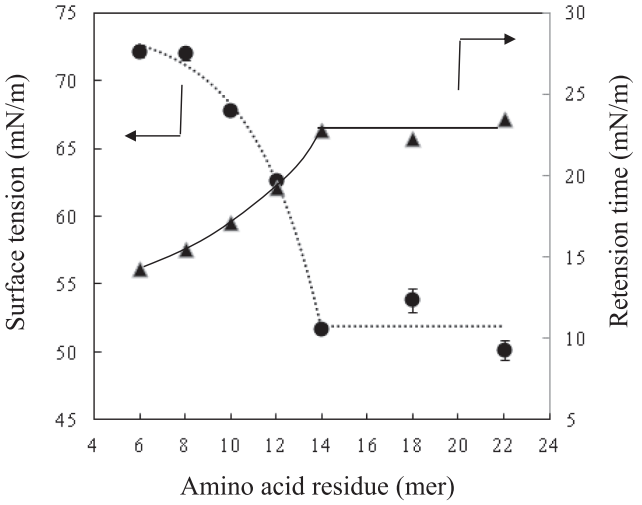

Fig. 2 Relationship between surface tension, RP-HPLC retention time, and the number of amino acid residue of the peptide.

residues of the peptides at a concentration of 0.3 mM. The 6-mer or 8-mer peptides, which included only two hydrophobic amino acids (Leu and Trp), did not show any surface activity. The 10-mer peptides, which included two additional hydrophobic amino acids (Leu and Ala), showed surface tension-lowering activity. Surface tension decreased further upon introduction of hydrophobic (Leu and Phe) or hydrophilic (Ser) amino acids, and reached a constant value when using the 14-mer peptide. This suggested that 14 amino acid residues from the *C*-terminal of human apoA-I protein play an important role in its amphiphilicity. These results, together with our earlier result that showed that surface activity is critical for nanodisc formation with phospholipids, predict that the peptides possessing more than 14 amino acid residues would lead to lipid nanodisc formation. The RP-HPLC retention times of each peptide, Fig. 2 (right axis), serve as hydrophobicity indicators. Interestingly, the retention times, which increased with an increase in the length of peptides, became constant for peptides possessing 14 or more than 14 amino acid residues.

Based on our previous work[18], 0.3 mM peptide solutions were mixed with various amounts of DMPC liposomes, (2.5:1, peptide : DMPC), to confirm the formation of nanodiscs with phospholipids. Although we previously demonstrated that CAC values of peptides are important for nanodisc formation[18], the minimum number of amino acid resides for effective nanodisc formation was investigated at a constant peptide concentration in this study. Vortexing of the peptides (22-, 18-, or 14-mers) possessing more than 14 amino acids rapidly cleared turbid DMPC liposome solutions while solutions remained largely turbid or became slightly transparent when peptides with less than 12 amino acids (12-, 10-, 8-, or 6-mers) were used,

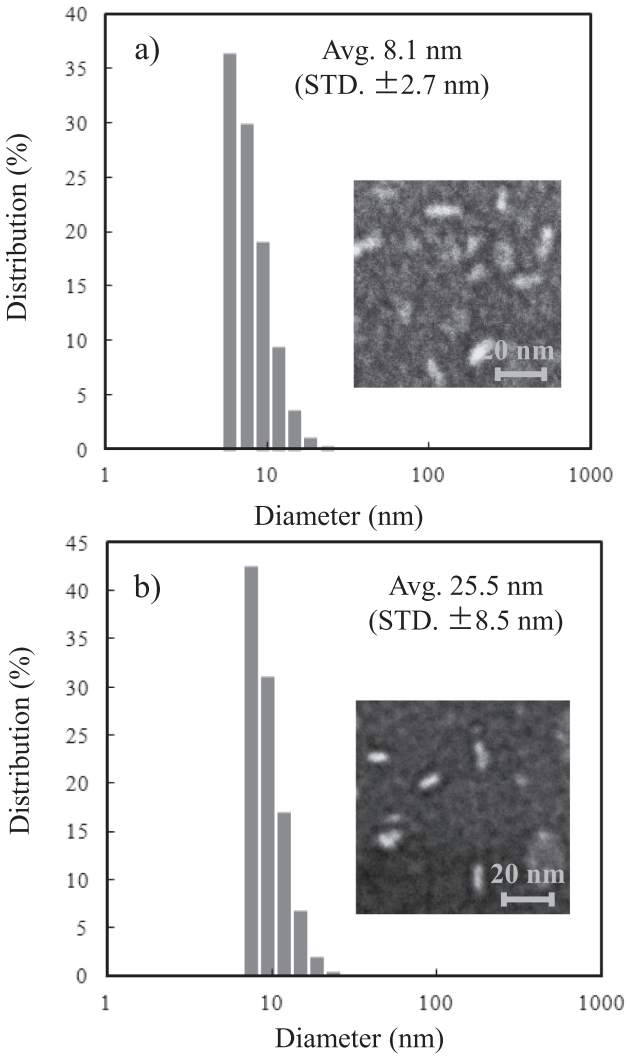

Fig. 3 Size distribution and NS-TEM images of nanodiscs produced from (a) 18-mer, and (b) 14-mer peptides.

suggesting nanodisc formation with the former peptides.

The size distribution of peptide/DMPC complexes analyzed by DLS is shown in Fig. 3. This figure clearly indicated that the 18- and 14-mer peptides gave rise to nanoparticles that were 8.1 ± 2.7 nm and 25.5 ± 8.5 nm in diameter, respectively. This suggested that formation of nanodiscs proceeded as predicted from surface tension measurements. The 22-mer peptide was already reported to form 9.5 ± 2.7 nm diameter nanodiscs. Since the 22-, 18-, and 14-mer peptides also exhibited approximately the same hydrophobicity (based on RP-HPLC), the difference in nanodisc sizes may be caused by specific interactions arising between peptides possessing different sequences and DMPC, and not due to hydrophobic interactions. Peptides with less than 12 amino acids (12-, 10-, 8-, and 6-mers) gave rise to peptide/DMPC complexes that were

beyond the upper limit of DLS detection (2 μm), indicating the presence of DMPC liposomes even after mixing of peptides.

Negative stain TEM images of the nanoparticles produced using the 18- and 14-mer peptides are shown in Fig. 3. Circular and rod-like objects observed in TEM correspond to top- and side-view of the nanodiscs. Particles stacked into a rouleaux formation observed in the image can be attributed to a phenomenon known to be an artifact of negative-stain TEM protocols[23−25]. Further, it is known that DLS measurements often fail to detect nanometer-sized small aggregates, which coexist with micrometer-sized particles such as liposomes. This suggests the possibility of nanodisc formation also with shorter peptides (12-, 10-, 8-, and 6-mer) albeit at lower yields. Our DLS and TEM measurements demonstrated effective nanodisc formation with 22-, 18-, and 14-mer peptides under the tested conditions.

To our knowledge, this is the first observation that difference in surface activity of peptides, induced by a decrease in the number of amino acid residues, is closely related to their ability to form lipid nanodiscs. The minimum peptide length of the *C*-terminal of apoA-I leading to nanodisc formation was concluded to be 14 amino acids. The hydrophile-lipophile balance (HLB) is well known to be an important concept for general surfactants possessing hydrophilic and hydrophobic groups. However, the amphiphilic helical peptide used in this study possesses hydrophilic and hydrophobic "faces" instead of "groups". It seems that a progressive concept is necessary to handle amphiphilic peptides with hydrophilic and hydrophobic "faces". These findings will greatly contribute not only to the fundamental understanding of HDLs but also to practical applications of nanodiscs in various fields.

4 CONCLUSION

Seven peptides differing in lengths (22-, 18-, 14-, 12-, 10-, 8-, and 6-mers) that mimic and modify the *C*-terminal domain (residues 220–241) of the apoA-I protein were synthesized by SPPS. Although the 6- and 8-mer peptides did not show any surface activity, the surface tension of water initially decreased with the 10-mer peptide, and became saturated at 51.2 mN m^{-1} for the 14-, 18-, and 22-mer peptides. Upon mixing the surface-active peptides (14-, 18-, and 22-mer) with DMPC liposomes (2.5:1, peptide : DMPC), turbid DMPC liposome solutions rapidly became transparent, and several disk-like nanostructures could be directly observed by negative-stain TEM. These results clearly demonstrate that the surface properties of peptides play an important role in nanodisc formation, and 14 amino acids from the *C*-terminal of apoA-I is the minimum number within a peptide that leads to nanodisc formation.

These findings will greatly contribute not only to the fundamental understanding of HDLs but also to practical applications of nanodiscs in various fields.

Supporting Information Available

This material is available free of charge via the Internet at http://dx.doi.org/jos.63.10.5650/jos.ess.14172.

References

1) Bayburt, T. H.; Grinkova, Y. V.; Sligar, S. G. Self-Assembly of Discoidal Phospholipid Bilyaer Nanoparticles with Membrane Scaffold Proteins. *Nano Lett.* **2**, 853-856 (2002).
2) Shih, A. Y.; Freddolino, P. L.; Sligar, S. G.; Schulten, K. Disassembly of Nanodiscs with Cholate. *Nano Lett.* **7**, 1692-1696 (2007).
3) Natarajan, P.; Ray, K. K.; Cannon, C. P. High-Density Lipoprotein and Coronary Heart Disease. *J. Am. Coll. Cariol.* **55**, 1283-1299 (2010).
4) Hara, H.; Yokoyama, S. Interaction of Free Apolipoproteins with Macropharges -Formation of High-Density Lipoprotein-Like Lipoproteins and Reduction of Cellular Cholesterol. *J. Biol. Chem.* **266**, 3080-3086 (1991).
5) Zhao, Y.; Imura, T.; Leman, L. J.; Curtiss, L. K.; Maryanoff, B. E.; Ghadiri, M. R. Mimicry of High-Density Lipoprotein: Functional Peptide-Lipid Nanoparticles Based on Multivalent Peptide Constructs. *J. Am. Chem. Soc.* **135**, 13414-13424 (2013).
6) Nissen, S. E.; Tsunoda, T.; Tuzcu, E. M.; Schoenhagen, P.; Cooper, C. J.; Yasin, M.; Eaton, G. M.; Lauer, M. A.; Sheldon, W. S.; Grines, C. L.; Halpem, S.; Crowe, T.; Blankenship, J. C.; Kerensky, R. Effect of Recombinant ApoA-I Milano on Coronary Atherosclerosis in Patients with Acute Coronary Syndromes. *JAMA* **290**, 2292-2300 (2003).
7) Bayburt, T. H.; Grinkova, Y. V.; Sligar, S. G. Assembly of Single Bacteriorhodopsin Trimers in Bilyaer Nanodiscs. *Arch. Biochem. Biophys.* **450**, 215-222 (2006).
8) Blanchette, C. D.; Cappuccio, J. A.; Kuhn, E. A.; Segelke, B. W.; Benner, W. H.; Chromy, B. A.; Coleman, M. A.; Bench, G.; Hoeprich, P. D.; Sulchek, T. A. Atomic Force Microscopy Differentiates Discrete Size Distributions Between Membrane Protein Containing and Empty Nanolipoprotein Particles. *Biochim. Biophys. Acta* **1788**, 724-731 (2009).
9) Baas, B. J.; Denisov, I. G.; Sligar, S. G. Homotropic Cooperativity of Monomeric Cytochrome P450 3A4 in a Nanoscale Native Bilayer Environment. *Arch. Biochem. Biophys.* **430**, 218-228 (2004).
10) Lacko, A. G.; Nair, M.; Paranjape, S.; Johnson, S.; Mc-

Conathy, W. J. High Density Lipoprotein Complexes as Delivery Vehicles for Anticancer Drugs. *Anticancer Res.* **22**, 2045-2049(2002).
11) Oda, M. N.; Hargreaves, P. L.; Beckstead, J. A.; Redmond, K. A.; van Antwerpen, R.; Ryan, R. O. Reconstituted High Density Lipoprotein Enriched with The Polyene Antibiotic Amphotericin B. *J. Lipid Res.* **47**, 260-267(2006).
12) Redmond, K. A.; Nguyen, T. S.; Ryan, R. O. All-Trans-Retinoic Acid Nanodisks. *Int. J. Pharm.* **339**, 246-250 (2007).
13) Sharma, A.; Sharma, U. S. Liposomes in Drug Delivery: Progress and Limitations. *Int. J. Pharm.* **154**, 123-140 (1997).
14) Jonas, A. Reconstitution of High-Density-Lipoproteins. *Methods Enzymol.* **128**, 553-582(1986).
15) Denisov, I. G.; Grinkova, Y. V.; Lazarides, A. A.; Sligar, S. G. Directed Self-Assembly of Monodisperse Phospholipid Bilayer Nanodiscs with Controlled Size. *J. Am. Chem. Soc.* **126**, 3477-3487(2004).
16) Marty, M. T.; Wilcox, K. C.; Klein, W. L.; Sliger, S. G. Nanodisc-Solubilized Membrane Protein Library Reflects The Membrane Proteome. *Anal. Bioanal. Chem.* **405**, 4009-4016(2013).
17) Cuellar, L. A.; Prieto, E. D.; Cabaleiro, L. V.; Garda, H. A. Apolipoprotein A-I configuration and cell cholesterol efflux activity of discoidal lipoproteins depend on the reconstitution process. *Biochim. Biophys. Acta* **1841**, 180-189(2014).
18) Imura, T.; Tsukui, Y.; Taira, T.; Aburai, K.; Sakai, H.; Abe, M.; Kitamoto, D. Surfactant-like Properties of an Amphiphilic α-Helical Peptide Leading to Lipid Nanodisc Formation. *Langmuir* **30**, 4752-4759(2014).
19) Imura, T.; Masuda, Y.; Minamikawa, H.; Fukuoka, T.; Konishi, M.; Morita, T.; Sakai, H.; Abe, M.; Kitamoto, D. Enzymatic Conversion of Diacetylated Sophoroselipid into Acetylated Glucoselipid: Surface-Active Properties of novel Bolaform Biosurfactants. *J. Oleo Sci.* **59**, 495-501(2010).
20) Otake, K.; Imura, T.; Sakai, H.; Abe, M. Development of a New Preparation Method of Liposomes Using Supercritical Carbon Dioxide. *Langmuir* **17**, 3898-3901 (2001).
21) Duong, P. T.; Collins, H. L.; Nichel, M.; Lund-Katz, L.; Rothblat, G. H.; Phillips, M. C. Characterization of Nascent HDL Particles and Microparticles Formed by ABCA1-Mediated Efflux of Cellular Lipids to ApoA-I. *J. Lipid Res.* **47**, 832-843(2006).
22) Kiss, R. S; Kay, C. M.; Ryan, R. O. Amphipathic α-Helix Bundle Organization of Lipid-Free Chicken Apolipoprotein A-I. *Biochemistry* **38**, 4327-4334(1999).
23) Zhang, L.; Song, J.; Cavigiolio, G.; Ishida, B. Y.; Zhang, S.; Kane, J. P.; Weisgraber, K. H.; Oda, M. N.; Rye, K.-A.; Pownall, H. J.; Ren, G. Morphology and Structure of Lipoproteins Revealed by an Optimized Negative-Staining Protocol of Electron Microscopy. *J. Lipid Res.* **52**, 175-184(2011).
24) Schneeweis, L. A.; Koppaka, V.; Lund-Katz, S.; Phillips, M.C.; Axelsen, P. H. Structual Analysis of Lipoprotein E Particles. *Biochemistry* **44**, 12525-12534(2005).
25) Raussens, V.; Drury, J.; Forte, T. M.; Choy, N.; Goormaghtigh, E.; Ruysschaert, J. M.; Narayanaswami, V. Orientation and Mode of Lipid-Binding Interaction of Human Apolipoprotein E C-terminal Domain. *Biochem. J.* **387**, 747-754(2005).