**Characterization of the Structure of Lipodisq Nanoparticles in the presence of
KCNE1 by Dynamic Light Scattering and Transmission Electron Microscopy**

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Keywords: Lipodisq nanoparticles; KCNE1; DLS; TEM

Abstract

A recently developed membrane mimetic system called styrene maleic acid lipid particles (SMALPs) or lipodisq nanoparticles has shown to possess significant potential for biophysical studies of membrane proteins. This new nanoparticle system is composed of lipids encircled by SMA copolymers. Previous studies showed that SMA copolymers are capable of extracting membrane proteins directly from their native environments without the assistance of detergents. However, a full structural characterization of this promising membrane mimetic system is still lacking. In this study, the formation of lipodisq nanoparticles was characterized upon addition of the membrane protein KCNE1. Initially, multi-lamellar vesicles (MLVs) containing KCNE1 (KCNE1-MLVs) at a lipid to protein molar ratio of 500/1 were prepared using a standard dialysis method. SMA copolymers were then added to KCNE1-MLVs at a series of lipid to SMA weight ratios to observe the solubilizing property of SMA in the presence of the KCNE1 membrane protein. The solubilizing process of KCNE1-MLVs by SMA copolymers undergoes a transition phase at low SMA concentrations (samples with weight ratios of 1/0.25, 1/0.5, and 1/0.75). More lipodisq nanoparticles were formed at higher SMA concentrations (Samples with weight ratios of 1/1, 1/1.25, and 1/1.5) were directly observed in the corresponding TEM images. A single sharp DLS peak was observed from the sample at the weight ratio of 1/1.5, which indicated the complete solubilization of KCNE1-MLVs. Interestingly, the critical weight ratio for empty MLVs was found to be 1/1.25 previously, which suggested that the presence of KCNE1 makes it more difficult for the solubilizing process of the SMA copolymers. Also, a TEM image of the 1/1.5 sample showed the presence of silky aggregates of excess copolymers. Overall, this study demonstrated the

ability of SMA copolymers to form lipodisq nanoparticles in the presence of the membrane protein KCNE1.

1. Introduction

Membrane proteins are difficult to study due to their hydrophobic properties. Specific detergents are required to extract membrane proteins out of their native environment or to solubilize membrane proteins from inclusion bodies when expressed in a bacterial system^{1, 2}. Finding a detergent for the proper solubilization of membrane proteins can be cumbersome, and the structural properties of target membrane proteins are not guaranteed to be preserved in the detergent micelles owing to the lack of a proper lipid environment. Other membrane mimetics such as multi-lamellar vesicles (MLVs), bicelles, and most recently nanodiscs are thus commonly used to study membrane proteins in a lipid bilayer as opposed to a detergent micelle^{3, 4}. Past structural studies on membrane proteins have relied on the use of these membrane mimetics and tremendous progress has been achieved⁴⁻⁹. However, the sample preparation of membrane proteins for biophysical studies is still a challenging problem because of drawbacks that each of the membrane mimetics possess. MLVs are normally heterogeneous which are challenging for some spectroscopic studies, and the hydrophobic inside presents issues when a study of the cytoplasmic domain of a membrane protein is needed. Bicelles can be aligned in a magnetic field which is preferable for some magnetic resonance spectroscopic studies and homogenous samples are also obtainable. However, the lipid compositions that are able to form bicelles are limited which restricts the application of this membrane mimetic system for all proteins. Nanodiscs have emerged as an important membrane mimetic system over the last decade with the benefit of no restriction on the types of lipids that can be used⁸⁻¹⁰. It does require the use of a membrane scaffold protein which may potentially affect the optical studies of the target protein.

Recently, a new membrane mimetic system called SMALPS and/or lipodisq nanoparticles was developed and has shown great potential as a better membrane mimetic alternative compared to traditional membrane model systems¹¹⁻²⁶. Lipodisq nanoparticles are composed of 3:1 styrene maleic acid (SMA) copolymer and phospholipids. It is structurally similar to nanodiscs, but nanodiscs use a membrane scaffold protein for the peripheral of the lipid disk, while for the lipodisq nanoparticles the rim used is a SMA copolymer. The SMA copolymer is synthesized from the copolymerization of styrene and maleic anhydride monomer. It is beneficial to use the SMA copolymer because it won't interfere with optical studies of target membrane proteins as opposed to the membrane scaffold protein. More importantly, not only does it have all the advantages that nanodiscs can provide, it also possesses its own characteristic property in that it can extract the membrane protein out of its native environment in a detergent-free manner¹⁷. This is highly desirable because the structural and functional properties can be maintained which is not common for traditional membrane mimetics. Also, lipodisq nanoparticles are easy and quick to prepare.

Recent work in the Lorigan group has demonstrated the formation of lipodisq nanoparticles through mixing of 3:1 SMA copolymers and POPC/POPG MLVs with varying amounts of 3:1 SMA copolymers²⁰. That work gave insight into the formation of lipodisq nanoparticles from the size perspective using solid-state NMR (ssNMR) spectroscopy, dynamic light scattering (DLS), and transmission electron microscopy (TEM). However, in order to better establish the usage of lipodisq nanoparticles as a good membrane mimetic system, it is critical to study the lipodisq nanoparticles in the presence of a membrane protein. In this study, lipodisq nanoparticles containing the

membrane protein KCNE1 was formed from a mixture of 3:1 SMA copolymers and POPC MLVs containing KCNE1 (KCNE1-MLVs) at a lipid to protein molar ratio of 500/1. KCNE1 is one of the five members of KCNE subfamily. KCNE1 is a transmembrane protein consisting of 129 amino acids that modulates the function of a voltage gated potassium ion channel (K_v)^{1, 2}. This ion channel is essential to the cardiac action potential that mediates heartbeat and is also critical for potassium ion homeostasis in the inner ear. Dominant mutations in KCNE1 lead to congenital long-QT syndrome and congenital deafness. Besides the important physiological role of KCNE1, it is well expressed in bacterial system as well as excellent protein stability. Thus it serves as a good model protein for the study of this new membrane mimetic system.

DLS and TEM techniques were performed to characterize the size distributions of the lipodisq nanoparticles containing KCNE1 (KCNE1-lipodisq). This work further speculates that using this new membrane mimetic can potentially help to advance the structural study of membrane proteins, which is challenging with conventional biophysical methods.

2. Materials and Methods

2.1. Expression and purification of KCNE1

The expression and purification of the membrane protein KCNE1 was described previously^{1, 2, 27}. In brief, the His6-tag expression vectors (pET-16b) containing the cDNA for wild type KCNE1 was transformed into *E. coli* BL21-CodonPlus(DE3)-RP competent cell. A single colony was inoculated into 5 mL of LB (Luria-Bertani broth) containing 50 µg/mL ampicillin for overnight growth. The pre-culture was then transferred to 500 mL of LB containing 50 µg/ml ampicillin. The large culture was allowed to grow at 37 °C and 250 rpm. When the OD₆₀₀ reached 0.8 – 0.9, the culture was induced with 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were collected by centrifugation after overnight induction. The cell pellets were resuspended in 30 mL of lysis buffer (70 mM Tris-Cl, 300 mM NaCl, pH 8.0) and lysed with a sonic dismembrator (Fischer Scientific). The lysates were centrifuged at 40,000 g for 20 minutes. The resulting pellet was resuspended in the urea buffer (8 M urea, 20 mM Tris-Cl, 150 mM NaCl, 0.2% SDS, 2 mM β-ME, pH 8.0) and rotated overnight at room temperature. Overnight suspensions were again centrifuged at 40,000 g for 20 minutes. The resulting supernatant was mixed with 4 mL of Ni-NTA resin (Thermo Scientific) that had been previously equilibrated with the urea buffer. Samples were then rotated at room temperature for 2 hours. Excess protein was washed away by pouring the resin suspension into a 20 mL gravity flow column and rinsing with 6 volumes of TS/SDS buffer (20 mM Tris-Cl, 200 mM NaCl, 0.2% SDS, 2 mM β-ME, pH 8.0). Purified KCNE1 was eluted into 6 mL of elution buffer (250 mM imidazole, 0.2% SDS, 2 mM β-ME, pH7.0). The total concentration was obtained by measuring the

OD₂₈₀ with the extinction coefficient of 1.2 mg/mL per 1.0 absorbance value. The purity of the KCNE1 protein was confirmed by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) (see Figure S1).

2.2. Preparation of KCNE1 containing lipodisq nanoparticles

KCNE1-MLVs was subject to extrusion through a 400 nm filter membrane followed by a 200 nm filter membrane¹⁶. SMA copolymers were dissolved in buffer (100 mM NaCl, 20 mM HEPES, pH 7.0) to a final concentration of 5% (m/v) followed by 1 minute of water-bath sonication to a complete dissolution. Lipodisq nanoparticles with different lipid to copolymer weight ratios (1/0.25, 1/0.5, 1/0.75, 1/1, 1/1.25, and 1/1.5) were formed by adding SMA copolymers dropwise to KCNE1-MLVs. Samples were then mixed well by vortexing and two freeze/sonication cycles. Samples were allowed to equilibrate at room temperature overnight.

2.3. Dynamic light scattering (DLS)

DLS measurements were performed on a Zetasizer nano series (Malvern Instruments) at 25 °C in disposable 40 µl micro cuvettes. Data were collected for 20 seconds and averaged for 10 scans. The distance distribution is shown on a log scale using the Igor Pro software program (WaveMetrics).

2.4. Transmission electron microscopy (TEM)

One drop of control KCNE1-MLVs and KCNE1-lipodisq samples were placed onto 300 mesh carbon-coated copper grids for 10 seconds. Extra sample was removed with filter paper. Grids were then stained with two drops of 1.5% uranyl acetate for about 30 seconds, and extra stain was removed with filter paper. Grids were left for air dry, and the Images were recorded using a Joel-1200 electron microscope operated at 100 KeV.

3. Results and Discussion

Multi-lamellar vesicles containing KCNE1 (KCNE1-MLVs) at a lipid to protein molar ratio of 500/1 were obtained through extensive dialysis against buffer (25 mM HEPES, 150 mM NaCl, pH 7.0). 3:1 SMA copolymers were then added to form KCNE1-lipodisq nanoparticles at different lipid to SMA weight ratios, specifically, weight ratios of 1/0.25, 1/0.5, 1/0.75, 1/1, 1/1.25, and 1/1.5 were used. To examine the solubilizing ability of SMA copolymers on MLVs containing a membrane protein, DLS and TEM analysis was performed. Figure 1 shows DLS and TEM data from the control sample (KCNE1-MLVs without SMA copolymers). The size distribution from the DLS intensity profile was found to be wide and centered around 200 nm, and inhomogeneity is clearly observed in the TEM image. The size distribution of lipodisq nanoparticles may also be obtained by using other techniques such as analytical ultracentrifugation or static light scattering.

Figure 2 reveals DLS and TEM data of KCNE1-lipodisq nanoparticles at different lipid to SMA weight ratios. Figures 2(A)-2(C) represent data collected for samples with lower SMA copolymers with weight ratios of 1/0.25, 1/0.5, and 1/0.75. Figure 2(A) shows a single peak with a large size distribution and the corresponding image revealed the formation of vesicle aggregates upon addition of a small amount of SMA copolymers (weight ratio of 1/0.25). This extremely low amount of SMA copolymers was not able to completely solubilize KCNE1-MLVs and no KCNE1-lipodisq nanoparticles were observed. Instead, it indicated the presence of aggregated KCNE1-MLVs, which accounts for the large size distribution of this sample. Since the DLS data were plotted as an intensity profile, which is more sensitive to large particles than small particles. This

explains the single peak observed in the DLS data. Figures 2(B) and 2(C) show a broad DLS size distribution, and fragmentation of KCNE1-MLVs and small KCNE1-lipodisq nanoparticles were observed from the corresponding TEM images. Both DLS and TEM data indicate the existence of a transition phase for the solubilizing of KCNE1-MLVs at this higher amount of SMA copolymers (weight ratios of 1/0.5 and 1/0.75). SMA copolymers were shown to extract lipid discs out of the KCNE1-MLVs and partial solubilized KCNE1-MLVs were observed. Figures 2(D) - 2(F) show DLS and TEM data of samples with higher amounts of SMA copolymers (Weight ratios of 1/1, 1/1.25, and 1/1.5). DLS data were shown to present narrower DLS size distributions at the smaller size range, which indicated the formation of smaller KCNE1-lipodisq nanoparticles and a better solubilization. The corresponding TEM images supported the DLS data well. Interestingly, a single DLS peak with small size distribution indicating complete solubilization of vesicles was seen to be the sample with a weight ratio of 1/1.5, which is different from a weight ratio of 1/1.25 found previously in the absence of membrane proteins²⁰. This difference suggested that the presence of KCNE1 made it more difficult to solubilize the vesicles. Thus, more SMA copolymers are needed to fully solubilize the lipids that encapsulated the proteins. The reason could be that the presence of KCNE1 in the lipid complex interferes slightly with the solubilizing property of SMA copolymers.

The data indicated that it is possible to form lipodisq nanoparticles containing a membrane protein at similar amounts of SMA copolymers used when no proteins are present. However, more SMA copolymers might be needed to achieve full solubilization of the protein lipid complex. As seen from the TEM images of Figures 2(D) and 2(E), the majority of KCNE1-MLVs were solubilized and nanosized particles were formed,

however, very small amounts of partially solubilized KCNE1-MLVs and excess SMA copolymers were present, ultra-centrifugation or size-exclusion chromatography is hence recommended to remove either insolubilized MLVs or excess SMA copolymers or fractionation of species in order to isolate species with equal reactivity for further delicate biophysical experiments (see Table 1 for a detailed particle size listing).

To function properly under physiological conditions, membrane proteins require a proper matrix to maintain in a correct folding state. This membrane system possesses an obstacle for the research of membrane proteins. The most native environment that maintains the structure of membrane proteins is the cell membrane which the proteins reside in. However, cell membranes are an extremely complicated system which consists of numerous biological components. This could restrict functional studies of target proteins, especially with conventional biophysical tools. Thus, different membrane mimetics have been developed and extensively applied for decades, to significantly advance the ability to study the structural and functional properties of membrane proteins. However, they all have their own benefits and drawbacks. The newly developed lipodisq nanoparticles possess great potential over the conventional membrane mimetics with the following benefits: (1) homogenous samples are achieved; (2) samples are at a relatively small size which is suitable for common biophysical techniques; (3) the rim that holds the lipid discs with polymers will not interfere with optical studies of the target protein; (4) It could be used to extract membrane proteins from their native membrane environment in a detergent-free manner¹⁷. Previous biophysical studies from our lab and others have reported that there is no significant structural and functional perturbation on membrane proteins due to lipodisq nanoparticles formation^{12,16,20}. More structural and

characterization studies on this new membrane mimetic system are needed, especially in the presence of membrane proteins. Additional studies are needed to clearly understand whether membrane proteins and MLV complexes undergo fragmentation before being completely solubilized by SMA copolymers.

In this study, a titration experiment with varying amounts of SMA copolymers was performed to explore the formation of lipodisq nanoparticles in the presence of a membrane protein (KCNE1). An optimal lipid to SMA weight ratio of 1/1.5 is suggested for a complete solubilization of MLVs containing the membrane protein KCNE1. The observations from this study could assist the biophysical studies of other membrane proteins utilizing this new membrane mimetic system.

Control

Figure 1: DLS data (left) and TEM image (right) for control POPC MLVs containing KCNE1 at a lipid to protein molar ratio of 500/1.

Figure 2: DLS data (left) and TEM images (right) for lipodisq nanoparticles generated from different lipid to copolymer weight ratios: (A) lipodisq nanoparticles (1/0.25), (B) lipodisq nanoparticles (1/0.5), (C) lipodisq nanoparticles (1/0.75), (D) lipodisq nanoparticles (1/1), (E) lipodisq nanoparticles (1/1.25), (F) lipodisq nanoparticles (1/1.5).

Lipodisq nanoparticles	Major particle size distributions by DLS (nm)
Control	198
1/0.25	539
1/0.5	53 – 339
1/0.75	101
1/1	64
1/1.25	17
1/1.5	14

Table 1: Particle size distributions determined by DLS for lipodisq nanoparticles generated from different lipid to copolymer weight ratios.

ASSOCIATED CONTENT

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Notes

The authors declare no competing financial interests.

Supporting Information

SDS-PAGE with Coomassie staining following expression and purification of His₆-KCNE1 (Figure S1).

Acknowledgements

We thank the Center for Advanced Microscopy & Imaging at Miami University for the help of obtaining TEM images. This work was supported by National Institutes of Health Grant R01 GM108026 (to GAL) and National Science Foundation Grant CHE-1305664 (to GAL).

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