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Biophysical Studies of Styrene-maleic Acid Copolymer Stabilized Membrane Mimetics

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Biophysical Studies Of Styrene-Maleic Acid Copolymer Stabilized Membrane Mimetics

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Biophysical Studies Of Styrene-Maleic Acid Copolymer Stabilized Membrane Mimetics

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2013

Abstract

Traditional methods of isolating membrane proteins involve the use of detergents that destabilize the membrane environment. Biophysical studies of membrane proteins are challenged by the requirement of a stable membrane platform. Lipodisq® nano-particles are novel membrane mimetics that consist of 3:1 styrene-maleic acid copolymer encircling phospholipids to form discoidal species on the order of 10 nm in diameter. Bacteriorhodopsin was previously incorporated into Lipodisq® nano-particles from its native purple membrane without the addition of detergent. In this study, dynamic light scattering was used to investigate the stability of empty Lipodisq® nano-particles over time. These data show that Lipodisq® nano-particles are stable for up to 48 hours at both 20 and 30°C. At each temperature, Lipodisq® nano-particles consist of greater than 95% of the total volume of the sample. Therefore, it is reasonable to investigate the properties of lipids and membrane proteins in Lipodisq® nano-particles using experimental techniques that require up to 48 hours.
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**Abbreviations**

BR: bacteriorhodopsin  
CD: circular dichroism  
DEER: double electron electron resonance  
DLS: dynamic light scattering  
DMPC: 1,2-dimyristoyl-sn-glycero-3-phosphocholine  
EPR: electron paramagnetic resonance  
ESR: electron spin resonance  
Keps: kilo counts per second  
NMR: nuclear magnetic resonance  
NS-TEM: negative stain-transmission electron microscopy  
PdI: polydispersity index  
PM: purple membrane  
Tris: trisaminomethane
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Chapter I. Introduction

1.1 Biological Membranes and Membrane Proteins

Biological membranes of cells and organelles delineate environments for different functional purposes. The major molecular species composing the biological membrane include phospholipids, sterols, and proteins. The phospholipid bilayer is a two dimensional array of amphipathic lipids consisting of a charged, polar phosphate head group and two nonpolar acyl chains of varying degrees of carbon length and unsaturation. Common phospholipid head groups include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylglycerol. The acyl chains lengths are typically in the range of 16 to 22 carbons (1). However, longer and shorter chain lengths are not uncommon.

In general, lipids undergo a gel to liquid crystalline phase transition at a characteristic melting temperature. Below the melting temperature, lipid acyl chains are rigid and packed closely together in the gel phase. As the temperature is increased, lipids pass a pre-transition into a ripple phase where the acyl chains have greater movement. Finally, when the temperature reaches the main transition, the acyl chains adopt more entropically favorable conformations with the greater degrees of freedom. Points of unsaturation in the carbon backbone of acyl chains introduce rigid angles of rotation that ultimately increases the disorder of the bilayer(1).

Biological membranes contain integral membrane proteins and surrounding peripheral proteins. Integral membrane proteins traverse the phospholipid bilayer to interact with lipid acyl chains, while peripherally associated proteins face one side of the phospholipid bilayer to interact with lipid head groups and the aqueous environment.
Important interactions within the lipid bilayer occur between the transmembrane regions of the integral membrane protein and lipid acyl chains as well as between the extra-membranous portions of the protein and lipid head groups. Integral membrane proteins belong to multiple functional classes such as receptors, transporters, channels, and enzymes.

1.2 The purple membrane and bacteriorhodopsin

Expressed in the plasma membrane of *Halobacterium salinarum*, bacteriorhodopsin (BR) is an integral membrane protein that consists of seven transmembrane alpha helices. BR self-associates into trimers in the purple membrane (PM) as part of a hexagonal two-dimensional array (2). It has a chromophore, all-trans retinal that has a maximal absorbance at 568 nm and a molar extinction coefficient ($\varepsilon$) of 63,000 M$^{-1}$cm$^{-1}$ (3). Upon activation of its photocycle by green light, it generates an electrochemical gradient by pumping protons across the membrane. This gradient is used by ATP synthase to synthesize ATP in the cytoplasm (4). The structure of BR served as a model for other alpha helical membrane proteins prior to the determination of the crystal structure of rhodopsin, the archetypical G-protein coupled receptor(5).

1.3 Challenges in studying membrane proteins

It is important to study the physical properties of membrane proteins in a well-defined, native-like environment so that experimental observations reflect the underlying biology. The amphipathic nature and topological considerations of membrane proteins introduce additional challenges that are not considerations for studying soluble proteins
These challenges begin with limitations of obtaining sufficient quantities of protein for study. Different techniques require different amounts of protein. Obtaining membrane proteins in high yield is limited by low levels of membrane protein expressed in cells and further loss of protein in the purification process (6). Following this, membrane proteins must be reconstituted into well-defined model membrane systems that provide a stable environment for study. Traditionally, membrane proteins have been effectively isolated by detergents and studied as a solubilized species in the detergent micelle. Alternatively, they have been incorporated into membrane mimetic platforms (6). Both detergent and membrane mimetics have associated disadvantages, including dimensions, limited accessibility of the protein, and decreased protein stability that can inhibit effective membrane protein studies. Lipodisq® nano-particles are a model membrane system with an ability to overcome these disadvantages (6, 7).

1.4 Detergents

Detergents are amphipathic molecules that are commonly used to disrupt the membrane and remove membrane proteins directly from their native environment. Detergent solubilization progresses along a detergent concentration gradient as follows. First, detergent monomers partition into the membrane and begin to destabilize it. When the detergent concentration exceeds the critical micelle concentration the lipid bilayer is disrupted and fragments. Ultimately, at high detergent concentrations, detergent-lipid mixed micelles are generated (1). Although some proteins are sufficiently stable in the micelle to be studied despite removal from the membrane, some are not. For instance, the initial detergent partitioning into the native bilayer has been shown to destabilize
rhodopsin even at concentrations below the critical micelle concentration (8). Protein-lipid interactions in a detergent micelle differ vastly from the interactions involved in a two-dimensional bilayer. Thus, detergents cannot completely substitute for the stability of a bilayer and are unsuitable as membrane mimetic systems for many studies.

1.5 Membrane model systems

1.5.1 Liposomes

Membrane proteins may be studied in liposomes, lipid vesicles that provide a reconstituted bilayer of well-defined lipids. To form liposomes, hydrated lipid suspensions are mechanically agitated by sonication, successively freeze-dried in liquid nitrogen or extruded through a membrane filter of a particular size (9). Liposomes can be unilamellar or multilamellar and typically form heterogeneously with physical dimensions on the order of a few hundred nanometers in diameter. Only one side of membrane proteins is accessible for experiments when reconstituted in liposomes (6). Due to their large size, liposomes are unsuitable for spectrophotometric studies, because they scatter more light in the UV-Visible range than they transmit. This light scattering produces an exceptionally high absorbance signal that is outside the linear range, so that absorbance measurements at 280 nm are no longer directly proportional to protein concentration. Liposomes are also problematic for spectroscopy studies such as nuclear magnetic resonance spectroscopy (NMR) and circular dichroism (CD) on membrane proteins. Large molecules tumble slower with longer rotational correlation times, which ultimately contribute to spectral line broadening and limit the size of protein that can be
studied using NMR. Liposomes are also incompatible for CD because their large dimensions can scatter light of high enough intensity to interfere with the measurement.

1.5.2 Nanodiscs

Nanodiscs provide a valuable system in which to study membrane proteins. It is a membrane model system that consists of a planar, discoidal phospholipid bilayer with the hydrophobic core of the bilayer encircled by two belts of membrane scaffolding protein (MSP). Derived from lipoproteins, the membrane scaffolding protein length determines the diameter of the nanodisc, which may range from 10 nm to 12 nm or higher depending on the protein construct (10, 11). Nanodiscs are formed as follows. Detergent is added to solubilize membrane proteins from the native membrane. Detergent solubilized membrane proteins are mixed with MSP and phospholipids at an experimentally defined ratio (10-12). As detergent is removed from the system, the MSPs coil around phospholipids that surround the membrane protein in an energetically spontaneous process to form nanodiscs (12). One and two rhodopsins have been reconstituted into nanodiscs at specific protein to lipid ratios (13). Nanodiscs offer the advantages of smaller size, a bilayer environment, and access to both sides of the embedded protein. However, protein stability may be irreversibly affected prior to detergent removal. In addition, absorbance of the membrane scaffolding protein at 280 nm may interfere with UV-Vis spectroscopic studies (6).

1.6 Lipodisq® nano-particles
Amphipathic polymers have been developed to form nanoscale lipid-polymer assemblies for drug delivery purposes and to serve as membrane mimetics for biophysical studies of membrane proteins. As was the case for nanodiscs, preparation of membrane mimetics using many of these amphipathic polymers requires the addition of detergent (6, 7, 14). However, unlike other amphipathic polymers, 3:1 styrene-maleic acid copolymer has been used for the detergent-free preparation of Lipodisq® nano-particles (15-17).

The 3:1 styrene-maleic acid copolymer can be used at physiologically relevant temperatures and pH values (6). It is believed to hypercoil around phospholipids such as 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) to form discoidal lipid bilayer (6). Both BR, with seven transmembrane α-helices, and PagP, a β-barrel, are model membrane proteins that have been incorporated as single-molecules into Lipodisq® nanoparticles (15). Previous studies have characterized the average size of empty and BR-containing Lipodisq® nanoparticles to be smaller in diameter than MSP-bound nanodiscs (15-17). Transmission electron microscopy (TEM) studies have corroborated the results from dynamic light scattering (DLS) and support the model that Lipodisq® nanoparticles are discoidal structures that range between 5-13 nm in diameter (15, 16).
Lipodisq® nano-particles are a developing platform that may be used to study membrane proteins using various kinds of spectroscopy and single-molecule techniques. Lipodisq® nano-particles have similar advantages as nanodiscs without the disadvantage of absorbance at 280 nm of the membrane scaffolding proteins. Bacteriorhodopsin incorporated into Lipodisq® nano-particles have been studied with many spectroscopic techniques including NMR, ESR (DEER), CD and characterized by negative stain TEM and DLS (15-17).

1.7 Dynamic light scattering

Light scattering is a universal property of molecules that arises from the polarizability of the electron cloud at the atomic level. While static light scattering detects the time-averaged intensity of light by observing a large sample volume, dynamic light scattering detects the individual fluctuations in scattering intensity in a small sample volume (18). Dynamic light scattering is a noninvasive and highly sensitive biophysical technique that is capable of detecting a wide range of particle sizes. DLS can provide a qualitative analysis of the stability of a sample with respect to changes in the light scattering intensity profile (19, 20).

The technique utilizes a laser beam that provides a monochromatic and coherent light source to directly measure molecules undergoing Brownian motion (19-21). Brownian motion corresponds to the random, stochastic collisions between molecules. The instrument detects the minute time-dependent fluctuations of light scattering intensity from the sample and generates an autocorrelation function that decays over time (21).
The intensity of light scattered at any second in time during the measurement can be quantified as the photon count rate in kilo counts per second (kcps) (22). The count rate can provide information about the concentration of the sample. An increase in the sample concentration corresponds to an increase in count rate, while a decrease in sample concentration corresponds to a decrease in count rate (22). The time dependent trend of the count rate for the sample can indicate aggregation if the count rate increases over time or sedimentation if the count rate decreases over time (19).

The shape of the autocorrelation decay function provides qualitative information about the sample. Larger molecules scatter more light to the detector than smaller molecules and produce an autocorrelation function that decays over a longer time. The width of the autocorrelation function between the time of maximum and minimum correlation is a graphical indicator of the polydispersity of the sample (22). The autocorrelation decay can be fit with either a single exponential (known as Cumulants analysis) or a multiple exponential algorithm (22). A translational diffusion coefficient that describes a hard sphere with a hydrodynamic Stokes radius is calculated directly from the fit of the autocorrelation decay (21).

Cumulants analysis of the autocorrelation function calculates the average hydrodynamic diameter (Z-average diameter) and the polydispersity index (PdI) of the sample. The polydispersity index is a dimensionless scale between zero and one that quantifies the degree of polydispersity of a sample (22). Values closer to zero represent increasingly monodispersed sample composition, while values greater than 0.7 indicates that the sample may be too polydisperse for meaningful DLS analysis. The Z-average
diameter is a value that may be used to compare various unimodal intensity distributions with polydispersity values less than 0.3 (19, 22).

Size distributions can be plotted by assuming that the sample consists of a continuous distribution of hydrodynamic radii. A size distribution by intensity is generated directly from the autocorrelation fit by plotting the relative scattering intensity of species in the sample population against the size of each species (21). The intensity distribution is most suited for meaningful analysis when there is a single peak in the profile (19). Rayleigh scattering theory is appropriate for analysis of spherical particles that are much smaller in size than the wavelength of the incident light (21). Under the Rayleigh approximation, \( I \propto d^6 \), where \( I \) is the intensity of the scattered light and \( d \) is the diameter of the particle (21). Non-spherical particles, including discoidal particles, are analyzed as spherical particles and described by a hydrodynamic diameter equivalent to the size of a spherical particle with the same translational diffusion coefficient (21).

Under the conditions that there are multiple peaks, a size distribution by volume provides a more accurate depiction of the sample constituents. In this case, the Rayleigh light scattering approximation is no longer appropriate since part of the sample consists of particles sizes on the order of wavelength of the incident light (21). Application of Mie theory is necessary to generate analytical solutions using the refractive index of the sample and transforms the intensity distribution into the volume distribution (19-21).

1.8 Study design

This study investigate will the stability of Lipodisq® nano-particles consisting of only the SMA polymer and phospholipids (empty Lipodisq® nano-particles) and those
containing phospholipids and BR (BR Lipodisq® nano-particles) for up to two days. The phospholipid, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) with no unsaturated acyl chains (14:0 PC), will be used for all of the following experiments. Lipodisq® nanoparticles will be measured at 20 and 30°C bordering the lipid phase transition of DMPC ($T_m = 23°C$). For the purposes of this study, dynamic light scattering will be used to follow the intensity distribution of Lipodisq® nano-particles, the smallest species, to determine if there is loss of intensity over 48 hours of measurement. The migration of intensity from Lipodisq® nano-particles to larger species in the overall intensity distribution will be used as a qualitative measure of stability. The stability of these Lipodisq® nano-particles over time will provide a relative time frame for which experiments can be conducted on incorporated proteins. Bacteriorhodopsin will be used as a model protein for this study as it has been used in Lipodisq® nano-particles previously for comparison (15, 17).

Chapter II. Methods

2.1 Materials

Lipids were purchased from Avanti Polar Lipids, Inc. and provided by Dr. Nathan Alder (Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT). Dr. Anthony Watts (Department of Biochemistry, University of Oxford, UK) kindly provided the 3:1 styrene-maleic acid (SMA) polymer obtained from Malvern Cosmeceutics Ltd. Dr. Robert Birge from the (Department of Chemistry, University of Connecticut, Storrs, CT) kindly provided wild type purple membrane containing bacteriorhodopsin.
2.2 Formation of empty Lipodisq® nano-particles

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) in chloroform was dried down under a nitrogen stream for approximately 20 minutes and put in a vacuum desiccator overnight to remove remaining traces of organic solvent. The 3:1 styrene-maleic acid (SMA) polymer was prepared to a 2.5% (w/v) solution in 50 mM Tris buffer (pH 8). DMPC was hydrated to 20 mg/ml with the same buffer and sonicated in a water bath until opalescent. DMPC liposomes were made by extruding 17X through 0.4 micron membrane filters. An equimolar amount of 2.5% SMA polymer solution was added to DMPC liposomes post extrusion and equilibrated on a shaker for one hour. The solution (final volume: 500 µl) was centrifuged in a Beckman Coulter Optima™ ultracentrifuge (rotor: TLA100) at 20°C, 40,000 RCF, 5 acceleration/5 deacceleration for 40 minutes. The supernatant was then removed for dynamic light scattering measurements. Low speed centrifugation at 6238 RCF was carried out in a RevSpin 102 bench top centrifuge in an attempt to purify Lipodisq® nano-particles.

2.3 Bacteriorhodopsin reconstitution into Lipodisq® nano-particles

DMPC liposomes were prepared in 50mM Tris buffer (pH 8) with 300 mM NaCl. Wild type PM with BR was pelleted a Beckman Coulter Optima™ ultracentrifuge (rotor: TLA100) at 20°C, 40,000 RCF, 5 acceleration/5 deacceleration for 40 minutes. Purple membrane was resuspended in DMPC liposomes that were extruded to 400 nm at a 1:5 (wt:wt) ratio of PM:DMPC to reach a final protein to lipid ratio of approximately 1:172 (17). The sample was sonicated in a water bath without heating for approximately 30
minutes. An equivolume amount of 2.5% SMA polymer solution was added to the suspension and equilibrated on a shaker for one hour. The solution was centrifuged again under conditions for pelleting the PM for 40 minutes. The supernatant was then removed for dynamic light scattering measurements.

2.4 Dynamic light scattering experiments

Dynamic light scattering experiments were carried out on a Zetasizer Nano S (Malvern) to determine the intensity distribution of Lipodisq® nano-particles. Sets of ten experiments with ten seconds of data acquisition per experiment were carried out for each timed measurement at 15 minutes increments over the course of 48 hours. For the purposes of generating size distributions, the refractive index of DMPC was set to 1.371 (23, 24). The refractive index of the dispersant was automatically set to 1.331.

Chapter III. Results

3.1 Optimization of Lipodisq® nano-particle formation

DLS determinations were carried out to determine the size distribution of particles in the Lipodisq® preparation. Figure 1 is representative of the data obtained from experiments conducted at 20°C. These data indicate the smallest species exhibit mean diameters of approximately 6-8 nm and account for 95-100% of the total light scattering intensity. The polydispersity indices ranged from 0.10-0.25 for these intensity distributions, which is consistent with values for a monodispersed sample.
Empty Lipodisq® nano-particles, the smallest species, have been purified using size exclusion column chromatography (15, 17). We sought to simplify this procedure by using differential centrifugation. Lipodisq® nano-particles prepared by centrifugation of smaller total sample volumes and Lipodisq® nano-particles measured a week after preparation both exhibited polydisperse intensity distributions. Samples with polydisperse intensity distributions were subjected to centrifugation at 6238 RCF and 40,000 RCF to determine whether empty Lipodisq® nano-particles may be purified using centrifugation (Figure 2a). Lipodisq® nano-particles of approximately 5 nm in diameter consisted 38.5% of the intensity distribution (Figure 2a) and 99.9% of the volume distribution (Figure 2b). After the 40 minutes of centrifugation at 40,000 RCF, Lipodisq® nano-particles of 7 nm in diameter consisted 73.3% of the intensity distribution. One hundred percent of the volume distribution consisted of 4 nm diameter Lipodisq® nano-particles. Accordingly, the decrease in photon count rate from 358 kcps to 219 kcps corresponded to the sample having a higher PdI (0.77) before than after (PdI= 0.38) centrifugation. Samples with polydisperse intensity distributions were centrifuged 6238 RCF to determine if lower speeds would still produce significant recovery of intensity in Lipodisq® nano-particles (Figure 2c). Centrifugation at 40,000 RCF for 40 minutes showed approximately 26% increase in the intensity of Lipodisq® nano-particles, while
centrifugation at 6238 RCF produced only a slight difference in intensity. The latter change is probably within the bounds of experimental error.

![Graphs](image.png)

**Figure 2.** Relative size distribution before and after centrifugation of empty Lipodisq nano-particles at 20 degrees. (a) Size distribution by intensity and (b) size distribution by volume before (red) and after (blue) centrifugation at 40,000 RCF. (c) Bar graph of the percent intensity of Lipodisq nano-particles before and after centrifugation at 6238 RCF and 40,000 RCF.

### 3.2 Stability of empty Lipodisq® nano-particles

Polydisperse intensity distributions were observed after a week of preparation and led to further experiments to determine whether Lipodisq® nano-particles remain uniform in size or undergo significant aggregation into larger species. Since the size distribution by intensity weights large particles heavier than small particles, aggregates scatter enough light to disproportionately bias the intensity distribution. For this reason, the size distribution by volume was monitored to provide a more accurate depiction of the sample (Figure 3a-b). Although the intensity distribution of Lipodisq® nano-particles was observed in some of the experiments were polydisperse, DLS data indicates that Lipodisq® nano-particles contribute to greater than 99% of the sample volume (Figure 3c).
We were interested in identifying a general time frame in which Lipodisq® nanoparticles remain stable. This would be useful in experimental studies where these nanoparticles are used as a model membrane. The size distribution changes in Lipodisq® nano-particles were followed at two temperatures, because we were interested in the stability at temperatures bordering the DMPC phase transition at 23°C. Over the course of 48 hours, intensity distributions of empty Lipodisq® nano-particles at 20°C varied negligibly from initial measurements (Figure 4). Since Lipodisq® nano-particles had a more heterogeneous intensity distribution at 30°C (data not shown), the volume distribution was used to evaluate the stability (Figure 5). Over the course of 48 hours, the volume distribution still consists only of Lipodisq® nano-particles. These data indicated that Lipodisq® nano-particles were stable for at least 48 hours after preparation.
In Figure 6, the change in percent intensity of Lipodisq® nano-particles was followed for up to 48 hours at both 20°C and 30°C. Although empty Lipodisq® nano-particles had different initial intensities for experiments conducted at 20°C and consisted of more than 70% of the intensity distribution for at least 48 hours. (Figure 6a). Similarly, empty Lipodisq® nano-particles exhibited almost no change from initial contributions to the volume distributions in the experiments conducted at 30°C (Figure 6b). Lipodisq® nano-particles consisted of almost 100% of the volume distribution for the entire 48
hours. These data reflect the uniformity of the size distributions for 48 hours both below and above the melting temperature of DMPC in Figures 4 and 5.

![Figure 6. Change in size distribution of empty Lipodisq nano-particles for up to 48 hours. (a) Percent intensity is plotted in 6 hour increments. (b) Percent volume is plotted in 6 hour increments. Each symbol corresponds to one experimental time course.](image)

### 3.3 Optimization of bacteriorhodopsin containing Lipodisq® nano-particles

Wild type bacteriorhodopsin in the purple membrane was not incorporated into Lipodisq® nano-particles (Figure 7). Based on previous attempts to incorporate BR into Lipodisq® nano-particles for preliminary TEM studies (Figure 8), we expected to observe a hypsochromic shift in the absorbance spectra of BR due to incorporation of monomers instead of trimers (17). The expected shift in retinal absorbance peak from 568 nm to 555 nm is absent in the absorbance spectrum of bacteriorhodopsin-incorporated Lipodisq® nano-particles.
Chapter IV. Discussion

In previous studies, dynamic light scattering has been used to measure the diameter of empty Lipodisq® nano-particles to range between 5-12 nm in diameter (16). Similarly in this study, empty Lipodisq® nano-particles were the smallest species in the intensity distribution with Z-average diameters between 6 and 11 nm. Low polydispersity index values of 0.10-0.25 and unimodal intensity and volume distributions were observed for all measurements. These characteristics are consistent with the conclusion that empty Lipodisq® nano-particles are uniform in size and are the predominant species present in the volume distribution of the sample. As a result, Lipodisq® nano-particles are readily identifiable using DLS measurements and DLS can be used to confirm the presence of empty Lipodisq® nano-particles in solution.

Gel filtration chromatography has been used to separate BR Lipodisq® nano-particles from empty ones (15, 17). In general, column chromatography is a fairly high-resolution method for purifying and isolating analytes of different sizes from solution. In this study, we investigated differential centrifugation as a direct, low-resolution method
for purifying Lipodisq® nano-particles that displayed polydisperse intensity distributions after formation. Centrifugation of the sample at 40,000 RCF for 40 minutes achieved approximately a 20% enrichment of intensity in the smallest species, the empty Lipodisq® nano-particles (Figure 2c). Thus, empty Lipodisq® nano-particles can be purified by centrifugation with forces of at least 40,000 RCF. This has practical implications for the formation of Lipodisq® nano-particles. When Lipodisq® nanoparticles are formed and the sample is polydisperse, centrifugation can be used as a method of removing massive aggregates. The removal of aggregates effectively concentrates the Lipodisq® nano-particles into a more uniform population of sizes. Centrifugation may also be useful to remove aggregates after BR Lipodisq® nano-particles are purified on a column. This may be investigated further as a lower resolution method for purifying BR Lipodisq® nano-particles.

After establishing that empty Lipodisq® nano-particles may be purified using differential centrifugation, we focused our efforts on identifying a valid time frame to conduct experiments on proteins incorporated into Lipodisq® nano-particles. A 48-hour time course was carried out to determine the rate of change of the intensity distribution contributed by Lipodisq® nano-particles below and above the melting temperature of DMPC (23°C). At 20°C, intensity distributions were stable throughout each 12 hour period (Figure 4a-d) and the sample consists almost entirely of Lipodisq® nano-particles. At 30°C, intensity distributions were observed to vary between each 12 hour increment before settling into an equilibrium state of two peaks after the initial 12 hour period (data not shown). This may be due to the presence of larger species inducing a nucleation process that encourages formation of massive particles over time. However, aggregation
has minimal influence on the overall sample quality since Lipodisq® nano-particles still consist of the majority of the volume distribution (Figure 5a-d). We concluded that empty Lipodisq® nano-particles are stable over the course of 48 hours at temperatures below and above the melting temperature. This determined a time frame of at least 2 days within which empty Lipodisq® nano-particles are stable experimental platforms.

Bacteriorhodopsin was not incorporated for DLS studies using the experimental procedure described in the methods. We believe that the lack of incorporation is due to the sonication step in which no heat was applied to the sample. In previous literature, the purple membrane was sonicated with DMPC liposomes under gentle heating conditions (17).

Further data must be collected on BR Lipodisq® nano-particles for comparison. Differential centrifugation will most likely be poorly equipped for separating empty Lipodisq® nano-particles from BR Lipodisq® nano-particles. Sucrose gradient centrifugation, which separates analytes based on their relative densities, may be a viable alternative for separating empty Lipodisq® nano-particles from BR Lipodisq® nano-particles.

Even without additional data, these experiments with empty nano-particles indicate that incorporation of bacteriorhodopsin from the purple membrane should not significantly impact stability since bacteriorhodopsin in the purple membrane alone is extremely stable. DLS size distributions of bacteriorhodopsin-incorporated Lipodisq® nano-particles would most likely be consistent with results obtained for empty nano-particles since the hydrodynamic radius would not be expected to alter significantly with protein incorporation.
Lipodisq® nano-particles may also be used to incorporate other membrane proteins in different lipid environments. Varying lipid and protein type in these Lipodisq® nano-particles may limit the time frame in which experiments can be conducted. This will depend on the stability of the type of lipid or protein. Individual proteins may have properties that limit their stability when incorporated into Lipodisq® nano-particles. In addition, replacing saturated DMPC with other unsaturated lipids may impose a limit on the stability of Lipodisq® nano-particles since unsaturated lipids spontaneously oxidize over time.

In conclusion, studies using Lipodisq® nano-particles can be conducted for at least 48 hours with minimal formation of aggregate species. Samples can be stored at temperatures up to 30°C with little to no effect on uniformity for at least 48 hours. Beyond 48 hours, it is advisable to centrifuge at 40,000 RCF for 40 minutes to pellet massive aggregates and recover a more monodispersed sample of Lipodisq® nano-particles.
References


