Polymer–Lipid Complexes

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Detergent-Free Formation and Physicochemical Characterization of Nanosized Lipid–Polymer Complexes: Lipodisq**

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A major challenge for biophysical studies of membrane proteins is obtaining stable, homogenous samples. Traditional detergent solubilization and liposome-based methods of reconstitution may lead to protein inactivation, heterogeneous and polydisperse sized particles, and sample aggregation.[1] While membrane scaffold protein (MSP) stabilized nanodiscs have facilitated the formation of monodisperse protein samples,[2] a drawback is the detergent-based preparation method. Here we present a physicochemical characterization of polymer-stabilized lipid particles termed Lipodisq, a novel nanosized lipid-based platform capable of incorporating membrane proteins.[3] The polymers used in the Lipodisq technology can solubilize commonly used lipids such as dimyristoylphosphatidylcholine (DMPC) without the use of detergents. The small size of Lipodisq (diameter of around 9–10 nm at pH 7.4) renders them potentially suitable for many biophysical methodologies, including electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) spectroscopies, electron microscopy (EM), and circular dichroism (CD) spectroscopy.

DMPC and a polymer formed from a molar styrene to maleic acid ratio of 3:1 (termed 3:1 SMA, see Figure S1 in the Supporting Information) were used as a model system to generate Lipodisq particles. Their formation using 3:1 SMA and DMPC at a weight ratio of 1.25:1.0 was followed by dynamic light scattering (DLS) at 550 nm and 25 °C (Figure 1), resulting in solution clarification within a minute. Higher SMA to DMPC ratios of 3:1 can also be used, though the excess polymer may increase the total solution viscosity, while decreased amounts results in sub-optimal DMPC solubilization. The dynamic light scattering data indicate a monodisperse distribution of Lipodisq particles with an average diameter of 9 nm as previously reported,[3b] which is confirmed by negative stain transmission electron microscopy (TEM, Figure 1C).

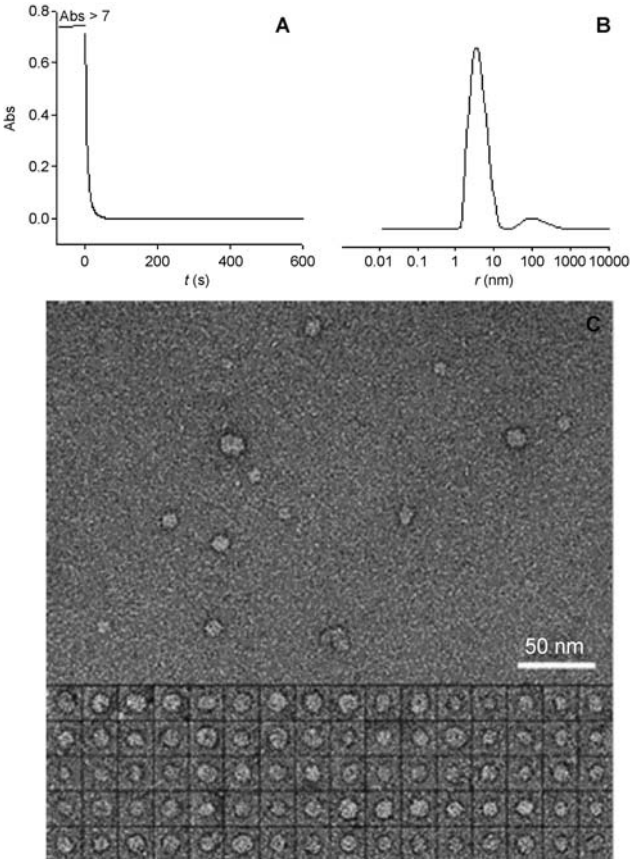

Figure 1. Light scattering data and size of Lipodisq particles. Lipodisq particle formation was observed by measuring the light scattering of the resulting polymer–lipid solution at 550 nm (A; Abs = absorption). DLS data (B) suggest that the Lipodisq formed have an average diameter of 9 nm, whereas TEM images (C) reveal that the Lipodisq diameter varies between 5–15 nm.

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^{1}H solid-state NMR (ssNMR) spectroscopy was used to characterize the interaction between the 3:1 SMA polymer and DMPC in a Lipodisq solution (Figure 2). Cross-peaks are observed by ^{1}H magic-angle spinning (MAS) nuclear Over-

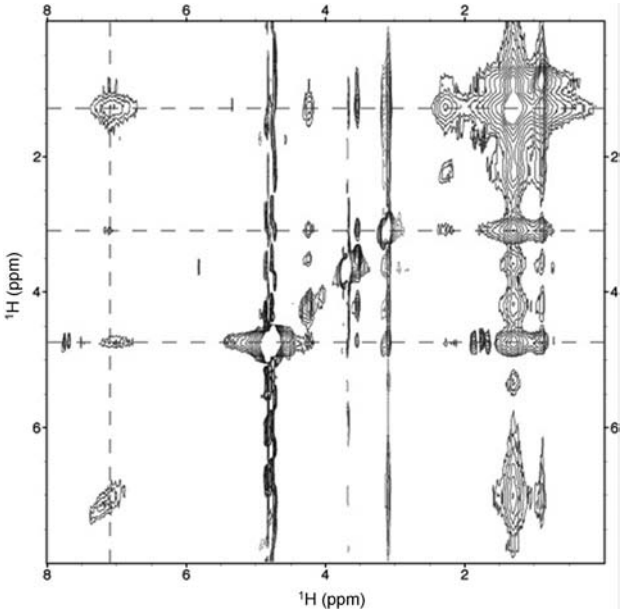

Figure 2. ^{1}H NOESY spectrum of DMPC-Lipodisq with cross-correlations indicated by dashed lines. The DMPC chemical shift assignments are indicated in Figure S1 in the Supporting Information and the 1D ^{1}H NMR spectrum of the pure polymer is shown in Figure S2 in the Supporting Information.

hauser effect spectroscopy (NOESY) between the styrene and lipid acyl chain protons, suggesting that these groups are in close proximity. The expected resonance for the choline headgroup $(\delta\text{-}CH)_3$ protons at 3.3 ppm (Figure S1 in the Supporting Information) is split and broadened compared to free lipid, consistent with the lipids at the edge of the Lipodisq particle forming an ionic interaction with the SMA maleic acid groups, whereas those in the center of the Lipodisq particle are unaffected.

To understand whether the presence of polymer increases the lateral pressure on the DMPC acyl chains, partitioning experiments with 2,2,6,6-tertramethylpiperidine-*N*-oxyl (TEMPO), a stable nitroxide spin label, were performed.[4] At 4 °C, TEMPO does not partition into the DMPC dispersion (Figure 3 A). Increasing the temperature above the DMPC dispersion main transition temperature at around 23 °C results in TEMPO partitioning into the liquid-crystalline DMPC dispersion bilayer as characterized by the appearance of the high-field peak H (Figure 3 A).[4a] Similarly, at 4 °C, no TEMPO partitions into the Lipodisq solution (Figure 3 B).

However, increasing the temperature to 32 °C results in a level of TEMPO partitioning that is approximately one third that observed for the DMPC dispersion (Figure 3 A) at the same temperature. This suggests lipids incorporated into Lipodisq particles do not undergo a highly cooperative phase transition from gel to liquid-crystalline phase, likely due to

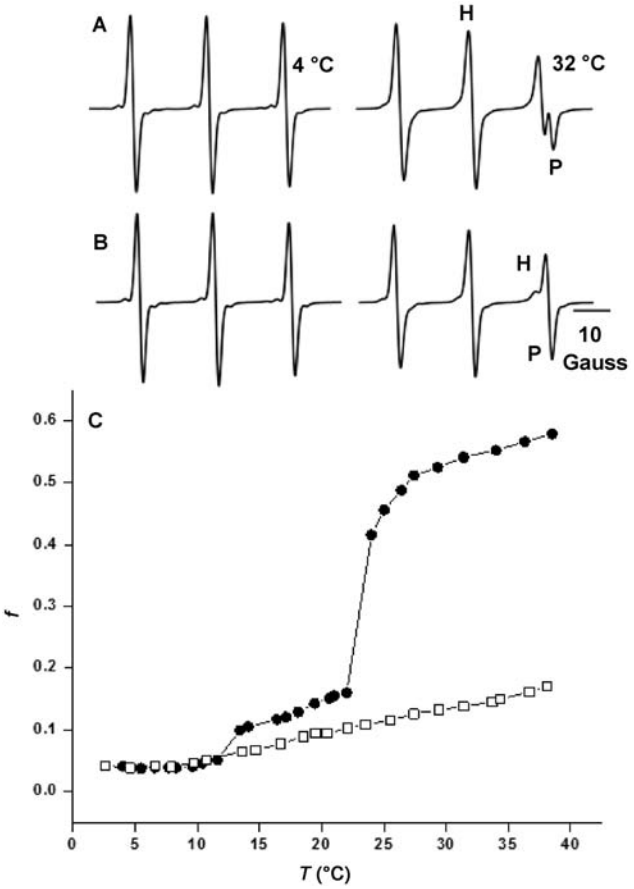

Figure 3. cw EPR spectra of TEMPO A) in a DMPC dispersion and B) in a Lipodisq solution at 4 and 32 °C. H and P are indicated. A partitioning parameter, *f*, defined as $f = H/(H+P)$, where H and P are the two high-field lines that arise when TEMPO is in either an aqueous (H) or hydrophobic (P) environment, can be calculated. C) *f* is shown as a function of temperature for the DMPC dispersion (●) and for the Lipodisq solution (□).

increased lateral pressure resulting from the polymer–lipid interactions. This is contrasted by experiments with MSP nanodiscs that suggest a phase transition from the gel phase to the liquid-crystalline phase in MSP nanodiscs.[5] The gradual increase in the partitioning factor, *f*, for the Lipodisq solution compared with the much greater increase in *f* for the DMPC dispersion with temperature (Figure 3 C) results from the decreased number of lipids undergoing a cooperative phase transition.

Probing the DMPC acyl chain ordering using EPR experiments with spin-labeled stearic acid[6] reveals that lipid–polymer interactions in Lipodisq preparations decrease lipid chain *trans–gauche* isomerizations compared to DMPC dispersions. The increased A_{II} splitting in the EPR spectra recorded for stearic acid spin-labeled at position 11 (11-SASL) incorporated into Lipodisq particles (Figure 4 A,B (upper spectra)) when compared to the DMPC dispersion (Figure 4 A,B (lower spectra)) illustrates the increased ordering both above and below the DMPC dispersion main-transition temperature. The effective lipid ordering by the polymer can be further quantified by the calculated apparent

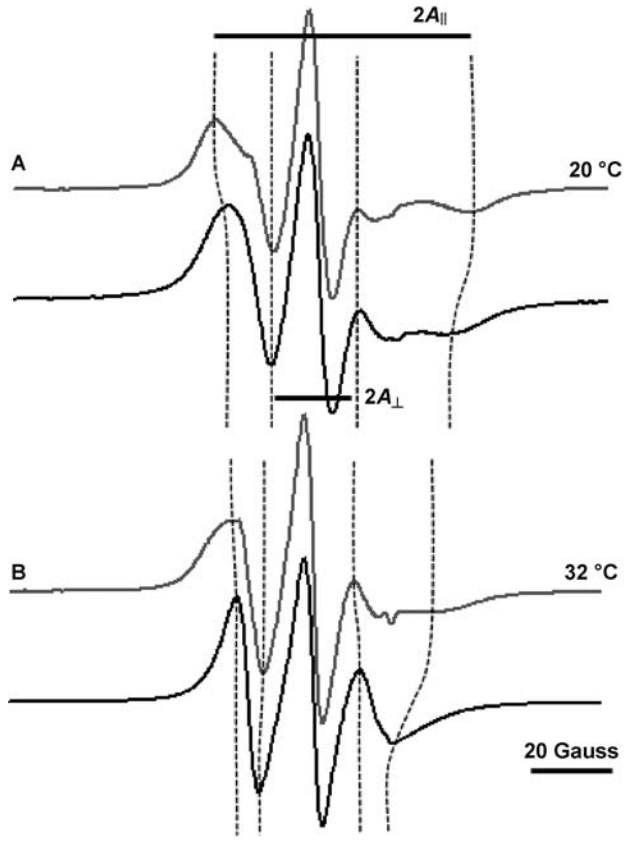

Figure 4. EPR spectra for 11-SASL incorporated into Lipodisq particles (upper spectra) and in a DMPC dispersion (lower spectra) at A) 20 °C and B) 32 °C. $2A_{\parallel}$ and $2A_{\perp}$ are indicated by dashed lines.[7] S_{app} can be calculated from $S_{app}=(A_{\parallel}-A_{\perp})/(A_{zz}-A_{xx})$, which is the ratio of the observed hyperfine anisotropy to the 25 Gauss maximum corresponding to rigid order, and defined by $0>|S_{app}|>1$ where $S_{app}=1$ is rigid and $S_{app}=0$ is isotropically disordered.[8]

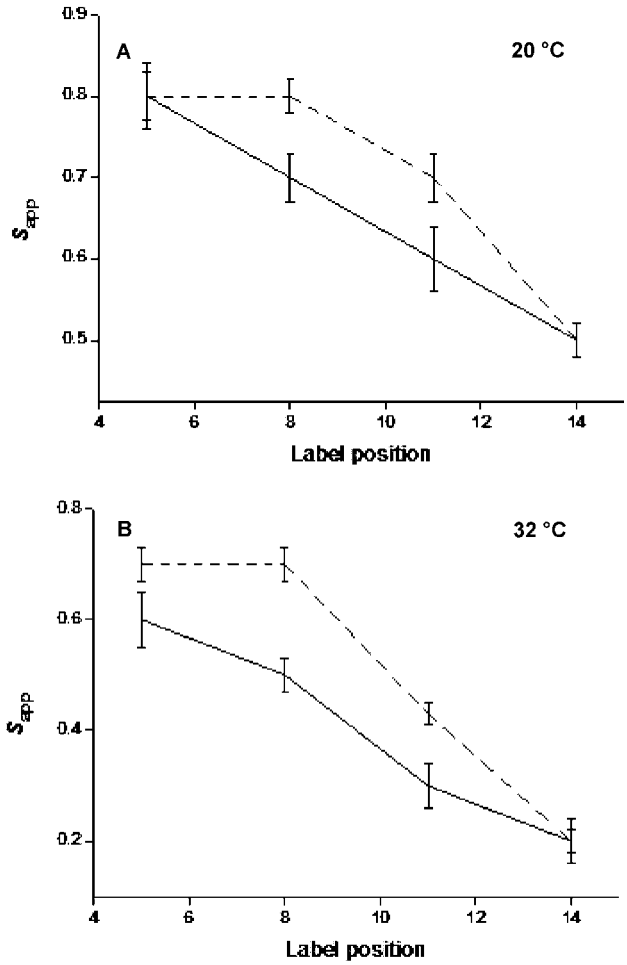

Figure 5. S_{app} (see Figure 4) determined for spin-labeled stearic acid incorporated into Lipodisq (dashed lines) and DMPC dispersions (solid lines) at A) 20 °C and B) 32 °C.

order parameter, S_{app}, for stearic acid spin-labeled at different positions (Figure 5).[6–7]

Stearic acids spin-labeled at positions 5 and 8, as for 11-SASL, also indicate a decreased motional freedom in the presence of 3:1 SMA polymer (Figure S1 in the Supporting Information) at both 20 and 32 °C (Figure 5). However, 14-SASL does not show an increased S_{app} upon addition of polymer, indicating that the motion of the lipid chains in the center of the Lipodisq particles is very similar to that in DMPC dispersions (Figure 5). The spin-labeled stearic acid EPR spectra indicate a single motionally averaged component, even though an estimated half of the lipids is interacting with the polymer. Through spectral anisotropy averaging, conventional spin-label EPR spectroscopy is sensitive to fast motions ($\tau_c < 10^{-9}$ s). The rotational correlation time, τ_r, for Lipodisq particles as determined by the Stokes–Einstein–Debye equation, is approximately 10^{-7} s assuming a discoidal morphology with a height of approximately 4 nm and a radius of 5 nm. Thus, the spin-label EPR method is therefore insensitive to the overall Lipodisq motion, but rather sensitive to intramolecular chain motion, suggesting differential perturbations by the polymer–lipid interactions where the Lipodisq lipids are more ordered compared to DMPC bilayers between around positions C8 and C12, but unperturbed at around C5 and around C14.

Differential scanning calorimetry (DSC) was used to characterize the Lipodisq solution enthalpic and lipid cooperativity properties. The DMPC lipid dispersion is characterized by a pretransition at 12 °C, and a highly cooperative gel-to-liquid crystalline phase transition at 23 °C.[9] In contrast, a single broad transition over a range of nearly 25 °C is observed in the Lipodisq DSC thermogram, with the main transition at around 15 °C (Figure 6). Similarly, DSC data for MSP nanodiscs displays a gradual and broad phase transition that spans over around 20 °C, though the transition temperature for MSP nanodiscs occurs at around 28 °C, which is 13 °C higher than the transition observed for Lipodisq.[5]

The calorimetric enthalpy (H_{cal}) is reduced from (5.26 ± 0.06) kcal mol^{-1} for the DMPC dispersion to (3.36 ± 0.002) kcal mol^{-1} for the Lipodisq solution (Figure 6). Further, the cooperativity of the phase transition, as calculated by the ratio of the van't Hoff energy (H_v) over H_{cal}, is reduced fifteen-fold from around 300 lipids molecules in the main transition of the DMPC dispersion to around 20 lipids in the Lipodisq solution (Figure 6). This is supported by the

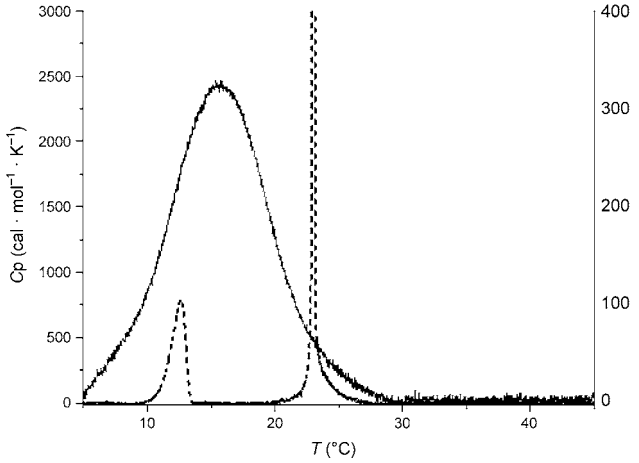

Figure 6. Differential scanning calorimetry thermogram for a DMPC dispersion (dashed line, *Y* Axis, left) and Lipodisq (solid line, *Y* axis, right). c_p is the specific heat at constant pressure. The DMPC dispersion is characterized by two phase transitions, the pre-transition at 12 °C, and the highly cooperative main transition at 23 °C. The Lipodisq solution is characterized by a single, low-cooperativity phase transition centered around 16 °C. The calorimetric enthalpy (H_{cal}) and the van't Hoff enthalpy (H_v) are reduced from (5.26 ± 0.06) kcal mol^{-1} and 1650 kcal mol^{-1}, respectively, for the DMPC dispersion to (3.36 ± 0.002) kcal mol^{-1} and 68 kcal mol^{-1} for Lipodisq particles.

TEMPO partitioning data (Figure 3), and is similar to the values reported for nanodiscs.[5] This is attributed to the smaller size of Lipodisq particles compared to the DMPC dispersions, and the presence of lipids that are likely not to participate in the cooperative phase transition, resulting in a much smaller cooperative unit and hence phase transition broadening. Indeed, if 90 DMPC lipids are estimated in each leaflet in a Lipodisq particle, then approximately 40 DMPC molecules per leaflet would interact with the polymer, similar to that previously suggested for MSP nanodiscs, and Apo-AI and Apo-AII discoidal particles.[2a,10] Thus, about one half of the total DMPC lipids in Lipodisq may be excluded from the cooperative phase transition because of their increased ordering compared with DMPC in dispersions.

This study demonstrates that the Lipodisq system is a potential detergent-free alternative to MSP nanodiscs. These data here show that Lipodisq particles show a broad, non-cooperative thermotropic phase transition, and the 3:1 SMA polymer exerts lateral pressure on the lipid tails, but does not perturb the center (terminal methyl groups). In a wider perspective, the further detailed analysis of the structural and physical chemical properties of the Lipodisq system will be crucial towards their application in biophysical studies with membrane proteins, and as delivery vehicles for hydrophobic compounds.

Experimental Section

DMPC was purchased from Avanti Polar lipids (Birmingham, Alabama, USA) and the 3:1 SMA polymer (approximate molecular weight of 9500 kDA) was provided by Malvern Cosmeceutics. TEMPO was purchased from Sigma and the spin-labeled stearic acids were either purchased from Sigma, or synthesized in-house. A DMPC dispersion was prepared by hydration of a lipid film in buffer (50 mM Tris buffer, pH 7.4) at a final concentration of 30 mM (20 mg mL^{-1}). To form Lipodisq particles, the DMPC dispersion underwent 10 freeze–thaw cycles and extrusion through a 400 nm filter. A 2.5 % w/v SMA-polymer solution in the same buffer as above was added at an equal volume to the DMPC MLV solution and allowed to equilibrate for 1 hour. DLS was carried out on a Viscotek 801 particle sizer (Malvern Instruments) at 25 °C. Teh data represent the average of ten 20 s experiments. The intensity distribution was generated using OmniSize 3.0.

For TEM measurements, a Lipodisq solution was adsorbed for 10 s to parlodion carbon-coated grids rendered hydrophilic by glow discharge at low pressure in air. Grids were washed with four drops of water and stained with two drops of 2 % uranyl acetate. Images were recorded by a Gatan Ultrascan CCD camera using an FEI TF20 electron microscope operated at 200 kV.

ssNMR experiments were carried out on a Varian Infinityplus 500 MHz spectrometer equipped with a 4 mm MAS HXY probe at 20 °C. All samples were prepared at final concentrations of 10 mg mL^{-1} in deuterated buffer. For NOESY experiments, samples were prepared with DMPC. ^{1}H NOESY experiments performed at 500 MHz were acquired with TPPI phase cycling with 90° pulses of 3 μs. 512 complex data points were collected in the indirect dimension, and a 4 s pulse delay was used. Samples were rotated at 8 kHz MAS and at 20 °C. Spectra were referenced to the terminal methyl group of DMPC at 0.89 ppm.

For continuous-wave (cw) spin-label EPR experiments, DMPC dispersions were prepared with either TEMPO or spin-labeled stearic acid at a molar ratio of 1:100, and resuspended in 50 mM NaH_iPO_i at pH 7.4. The DMPC dispersions were used to form DMPC-Lipodisq particles by solubilizing with SMA-polymer in 50 mM NaH_iPO_i at pH 7.4. Both the DMPC dispersion and Lipodisq solution were prepared at a final lipid concentration of 25 mg mL^{-1} (37 mM). Cw EPR measurements were carried out on a Bruker BioSpin GmbH EMX spectrometer at X-band fitted with a nitrogen cryostat.

DSC experiments were carried out on a VP-DSC microcalorimeter (MicroCal, LLC, Northampton, MA, USA) with a heating rate of 2° per hour on 6.5 mM DMPC and Lipodisq solutions prepared as above using 50 mM NaH_iPO_i at pH 7.4 as the buffer. The data were analyzed using Origin 5.0 software (Microcal, Inc.) employing a two-state model.

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