J|A|C|S COMMUNICATIONS

Published on Web 05/18/2009

# Membrane Proteins Solubilized Intact in Lipid Containing Nanoparticles Bounded by Styrene Maleic Acid Copolymer

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Approximately one-third of eukaryotic proteins are integrated within membranes, as are the targets of 40% of approved drugs. However, the lack of a general means to solubilize and characterize stable and active membrane proteins has frustrated efforts to define and exploit their mechanisms. Here we report that monodispersed lipid disks formed by styrene maleic acid (SMA) copolymer preserve the integrity of transmembrane (TM) proteins and form biocompatible, thermostable, and soluble nanoparticles for their biophysical analysis in a lipid environment.

Amphipathic polymers including SMA adsorb to and destabilize membranes *via* a pH-dependent conversion from extended chains to secondary structures.[1] In the presence of lipids they form polymer/lipid assemblies such as nanometer-sized disks. A proprietary polymer/lipid assembly termed "Lipodisq" has recently been developed by Malvern Cosmeceutics Ltd. as a delivery vehicle for hydrophobic pharmaceutical agents.[2]

Here we integrate an α-helical bundle and β-barrel TM protein within an SMA/lipid particle (SMALP). Bacteriorhodopsin (bR) contains 7 TM helices while PagP forms an 8-stranded β-barrel. The PagP and bR proteins were integrated in SMALPs by adding SMA to protein in dimyristoyl phosphatidylcholine (PC) containing liposomes. The resulting nanoparticles were purified by nickel affinity chromatography or optimized to minimize vacant nanoparticles. The sample homogeneity is evident by gel filtration chromatography (Figure 1), revealing stable, monodispersed species, and with bR retaining its chromophore retinal as shown by its absorbance at 550 nm.

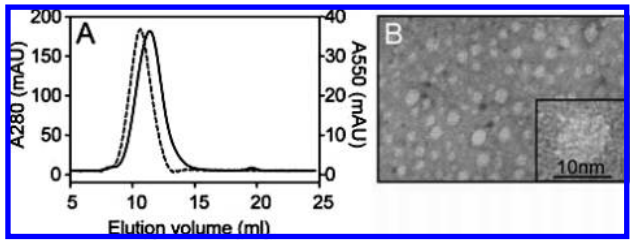


***Figure 1.*** (A) Size exclusion chromatography of PagP (solid line) and bR (dashed line) incorporated into SMALPs. Absorbance measured at 280 nm and 550 nm, respectively. (B) TEM micrograph of uranyl acetate stained SMALPs (×100 000), with the insert showing a single nanoparticle.

Previous studies have shown that discoidal bilayers composed of synthetic phospholipids and apolipoproteins form stacked structures.[3] However, electron microscopic analysis of negatively stained SMALPs shows dispersed disks with average diameters of 10.2 nm, consistent with the 9.0 ± 1.1 nm diameter estimated from dynamic light scattering (DLS), noting that negative staining can overestimate particle size.[3] The PagP-SMALPs are slightly larger, with DLS estimating their diameter as 11.2 ± 1.4 nm. The complex mass estimated from DLS is ∼200 kDa; however this is likely to be an overestimate due to the assumption of a globular particle shape.

The composition of PagP-containing SMALPs was determined. A ratio of 10.7 ± 0.7 molecules of PC per protein molecule was estimated by a phosphate assay and 280 nm absorbance,[4] respectively. Circular dichroism (CD) spectra were obtained to estimate the structural states of PagP and bR in the nanoparticles (Figure 2). Since no protein is required to form the disks they have negligible CD absorbance, unlike other systems.[5] Furthermore,

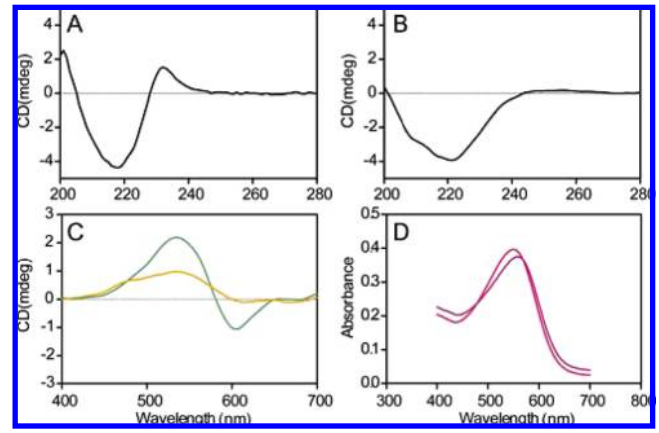


***Figure 2.*** Spectroscopy of SMALPs. Far UV CD of (A) PagP and (B) bR. (C) Visible CD of bR (orange) compared to native purple membrane (green). (D) UV absorption of dark (red) and light adapted (purple) bR.

conventional CD analysis of TM proteins requires either detergent solubilization, which can be denaturing, or integration within liposomes, which scatter light. The SMALPs are nondenaturing and exhibit minimal scattering, enabling unobscured analysis to below 200 nm, rivalling the quality of spectra afforded by synchrotron radiation sources of liposome-bound TM proteins.[6]

The PagP structure[4] is maintained in SMALPs. Far-UV CD spectra show a minimum at 218 nm and shoulders at 222 and 208 nm, indicating the expected β-barrel and N-terminal α-helical content (Figure 2). The peak observed at 232 nm has been attributed to native aromatic packing. The far UV CD spectrum of bR has a trough at 222 nm and shoulder at 208 nm, both being characteristic of its folded state.

Analysis of bR's chromophore reveals its association state within the nanoparticles. Trimeric bR in purple membrane gives a characteristic bilobed visible CD spectrum[7] (Figure 2C), while bR

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10.1021/ja810046q CCC: $40.75 

in SMALPs rather appears monomeric. Furthermore the absorbance maxima of bR in both detergent and SMALPs are blue-shifted relative to the crystalline form in purple membrane, consistent with its monomeric state, and exhibit a dark adapted maximum at 550 nm which shifts to a slightly higher wavelength upon light adaptation (Figure 2D), confirming its activity.

We investigated whether SMALPs offer improved stability over detergents. PagP solubilized in $\beta$-octylglucoside ($\beta$-OG) detergent denatured at 84 °C while neither PagP in SMALPs nor liposomes unfolded up to the maximum experimental temperature of 90 °C (Figure 3 and Supporting Information). Slight differences were apparent in the melts, particularly with a slight increase in $\beta$-strand

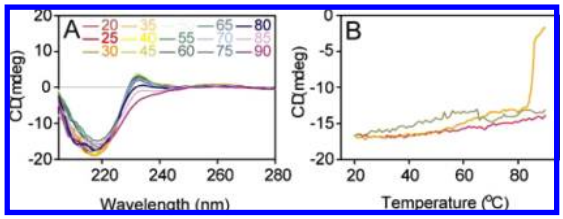


***Figure 3.*** CD thermal denaturation studies of PagP. CD thermal denaturation assay of PagP in (A) SMALPs, (B) 218 nm CD unfolding profiles for PagP in SMALPs (gray), liposomes (red), and $\beta$-OG (orange).

signal observed above 75 °C; however, the $\beta$-barrel fold is clearly maintained throughout. This suggests that SMALPs offer comparable stability to PC liposomes and a clear improvement over detergent solubilization.

The activity of PagP in SMALPs was demonstrated. Native PagP transfers a palmitate chain from phospholipid to the lipid A moiety of lipopolysaccharide,[8] thus contributing to bacterial virulence.[9] However PagP also catalyzes a phospholipase reaction[4] which can be monitored by $^{13}$C NMR.[10] Spectra of $^{13}$C- carbonyl labeled PC confirm that PagP in SMALPs acts as a phospholipase that forms the expected *sn*-1 lysophospholipid product[10] (Figure 4). The *sn*-2 product then accumulates due to the migration of the palmitate chain.[11] The assay requires solubilization of the $^{13}$C-labeled dipalmitoyl PC substrate in low levels of CYCLOFOS-7, as previously demonstrated.[10] The absence of protein signals in the $^{1}$H NMR spectrum of active PagP-SMALP samples indicates that PagP is not solubilized in micelles. Instead, it appears to be fully bound to SMALPs as the NH cross peaks are broadened beyond detection due to slow tumbling of the particles.

In conclusion, we have shown that PagP and bR can be assembled into nanoparticles using SMA copolymer. The disks are ~11 nm in diameter and contain ~11 PC lipids and a single protein molecule, consistent with the 10:1 ratio of bound lipids to crystallized bR.[12] The phospholipase activity of PagP shows that its central pore must be exposed and accessible. Together with a previous study showing a discoidal shape with lipid exposed on both surfaces, this suggests that the polymer forms an annulus around the phospholipid and protein core in a manner analogous to lipoprotein assemblies.[1] This advance provides a simple and effective method for solubilizing TM proteins from liposomal fractions and potentially from bilayers without requiring any detergent. SMALPs offer advantages over other solubilizing agents such as lipopeptides,[13] amphipols,[14] or PreserveX detergents (Tebu-Bio), as the lipid environment is maintained and detergents are not needed, consequently native structure and bound lipids can be retained. The absence of scattering and overlapping signals while maintaining the protein in a lipid environment enhances its spectral clarity. Many TM proteins and complexes are unstable in detergents, and SMALPs offer a new route for solubilization of intact states for structural and

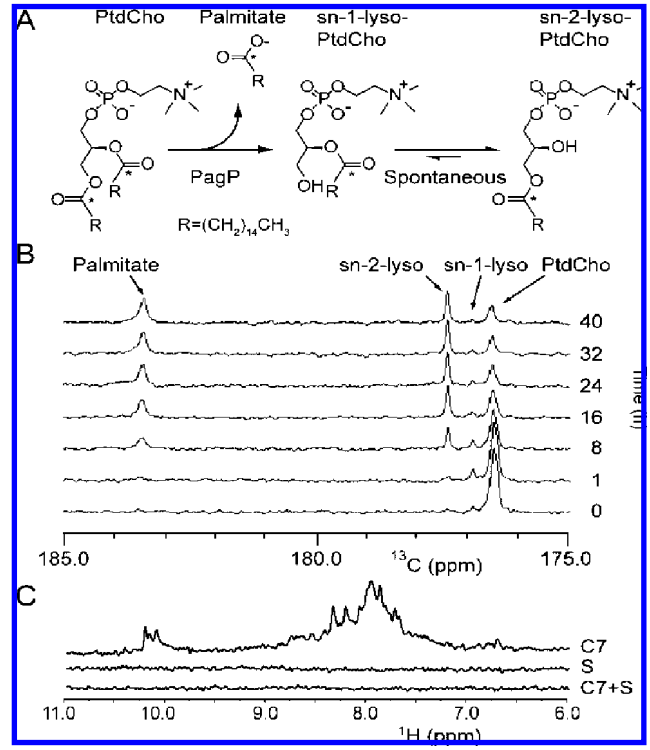


***Figure 4.*** PagP activity. (A) The phospholipase reaction with $^{13}$C-labels marked with asterisks and (B) monitored by $^{1}$H NMR of 100 $\mu$M PagP in SMALPs. (C) 1D TROSY spectra of PagP (100 $\mu$M) in CYCLOFOS-7 (C7), SMALPs (S), and SMALPs+CYCLOFOS-7 (C7+S).

functional analysis. Furthermore they have potential applications in drug discovery including screening of TM protein targets and characterization of ligand-induced changes within a lipid environment.

**Acknowledgment.** We thank S. Tonge and A. Harper for providing access to Lipodisq technology, C. Wharton for assistance with FTIR, A. Rodger for discussions, L. Kay for the PagP construct, and HWB-NMR. This work was funded by the BBSRC [T.D., T.J.K., and M.O.], EU PRISM, and Technology Transfer Fund (M.O.).

**Supporting Information Available:** Description of the experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA810046Q