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Macrodiscs Comprising SMALPs for Oriented Sample Solid-State NMR Spectroscopy of Membrane Proteins

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ABSTRACT Macrodiscs, which are magnetically alignable lipid bilayer discs with diameters of >30 nm, were obtained by solubilizing protein-containing liposomes with styrene-maleic acid copolymers. Macrodiscs provide a detergent-free phospholipid bilayer environment for biophysical and functional studies of membrane proteins under physiological conditions. The narrow resonance linewidths observed from membrane proteins in styrene-maleic acid macrodiscs advance structure determination by oriented sample solid-state NMR spectroscopy.

Styrene-maleic acid (SMA) macrodiscs advance structure determination of membrane proteins by providing a detergent-free bilayer environment that affords superior alignment in the magnetic field of an NMR spectrometer. Membrane proteins are high-profile targets for structure determination. They account for ~30% of all expressed genes, and their locations within the membrane barriers of cells, viruses, and organelles endow them with unique biological functions, including as receptors, ion channels, and transporters. The need for new techniques for structure determination of membrane proteins is evident in the limited number of structures that have been determined. Moreover, with few exceptions, the accuracy of the available structures is compromised by the experimental requirements of current methods, e.g., truncated or modified protein sequences, detergent-containing samples, or non-native conditions. Notably, NMR spectroscopy has the potential to be a general method for determining the structures of membrane proteins under near-native conditions. Although earlier studies employed a variety of micelle, bicelle, and amphipol samples, current NMR approaches reflect the availability of protein-containing phospholipid bilayer samples, such as unoriented liposomes studied by magic angle spinning solid-state NMR (1,2), rapidly reorienting nanodiscs studied by solution NMR (3,4), and aligned bilayers studied by oriented sample (OS) solid-state NMR (5–8).

The modern era of bilayer samples for membrane proteins started with the development of nanodiscs by Sligar and coworkers (9). They introduced a class of amphipathic helical proteins to the role of membrane scaffold proteins, which self-assemble as a circular "belt" around phospholipids in a bilayer to form nanodiscs with ~10 nm diameter and are suitable for chemical, physical, and functional studies of membrane proteins (10). Moreover, because protein-containing nanodiscs are a chemically defined system, many parameters can be manipulated, such as lipid composition, diameter, and lipid-to-protein ratio.

SMA is an amphipathic copolymer with alternating styrene (hydrophobic) and maleic acid (hydrophilic) moieties that spontaneously solubilizes biological membranes by forming circular boundaries of defined diameter around lipid bilayers (11–13) in a manner similar to that of a 14-residue amphipathic polypeptide (14A) (14). As a result, SMA polymers offer a detergent-free route to the isolation and purification of membrane proteins. They also enable the preparation of samples from purified proteins in liposomes with specified lipid compositions. Following the initial incorporation of PagP and bacteriorhodopsin into SMA nanodiscs (11), a variety of membrane proteins have been studied using similar preparations (12,15,16). Further development has led to similar polymers with greater pH stability and decreased susceptibility to divalent metal cations, among other favorable properties (17–22).

Previously, we described the use of 14A to form both small (nano, ~10 nm diameter) and large (macro, ~30 nm diameter) discs by varying the lipid-to-peptide ratio (14). In contrast to nanodiscs, macrodiscs are large enough to "immobilize" the proteins on NMR timescales and are

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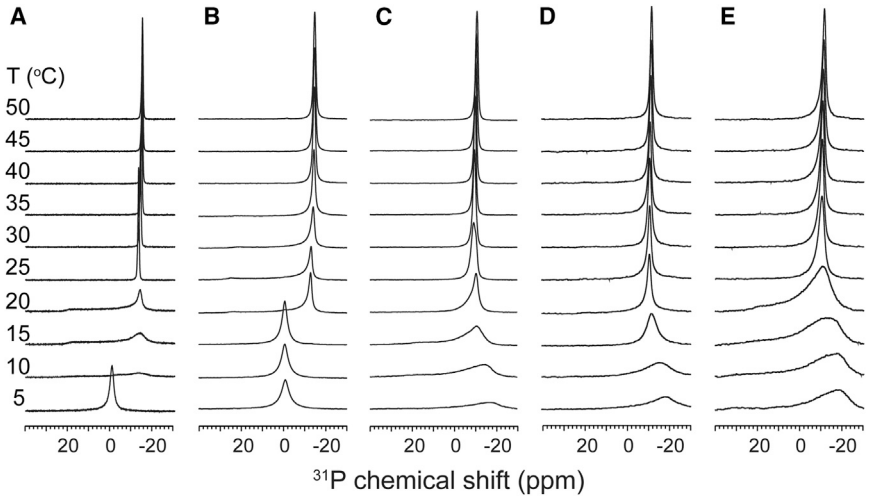

FIGURE 1 **^{31}P chemical shift NMR spectra of DMPC bilayers as a function of temperature at a resonance frequency of 283 MHz with ^{1}H decoupling. (*A*) Bicelles consist of DMPC and Triton X-100 with $q = 5$. (*B*) Macrodiscs consist of DMPC and 14A at a molar ratio of 13.3. (*C–E*) Macrodiscs consist of DMPC and three different SMA polymers. (*C*) SMA(1.4:1) are shown with $q_d = 7.4$. (*D*) SMA(2:1) are shown with $q_d = 27.7$. (*E*) SMA(3:1) are shown with $q_d = 49.1$. The lipid concentration in all samples is 10% (w/v).**

magnetically alignable. Higher lipid-to-SMA polymer ratios result in the formation of larger diameter discs (23–26). Chemically modified SMA macrodiscs have been shown to align in a magnetic field, and a solid-state NMR spectrum of an associated protein has been obtained (17).

Here, we describe the formation of macrodiscs from three different unmodified SMA polymers and phospholipids. These discs have favorable properties for OS solid-state NMR spectroscopy, which we demonstrate with one- and two-dimensional spectra that display the narrowest linewidths observed to date from an aligned protein sample. Highly homogeneous and translucent macrodiscs are obtained by solubilizing liposomes with polymers having average styrene: maleic acid monomer ratios of 1.4:1, 2:1, and 3:1, with molar ratios of lipid to SMA polymer (q_d) of 7.4, 27.7, and 49.1, respectively. Each q_d was optimized by titration of the polymers to the liposomes. The longer the length of the polymer (SMA(3:1) > SMA(2:1) > SMA(1.4:1)), the smaller the amount needed to form macrodiscs. Nanodiscs typically form immediately upon addition of amphipathic peptides or SMA polymers to liposomes, whereas SMA macrodiscs require many hours to form (Fig. S3).

In Fig. 1, the alignment and phase behavior of three SMA macrodisc samples are compared to those of samples of high q dimyristoylphosphatidylcholine (DMPC)/Triton X-100 bicelles (27) and DMPC/14A macrodiscs (14). The ^{31}P NMR spectra of bicelles (Fig. 1 *A*), 14A macrodiscs (Fig. 1 *B*), and three different SMA macrodiscs (Fig. 1, *C–E*) demonstrate that the DMPC bilayers are well aligned between 30 and 50°C with their normals perpendicular to the direction of the applied magnetic field because they display a single resonance with a chemical shift of -13 ± 2 ppm. However, differences are observed among the samples at temperatures below the gel to liquid crystal phase transition of DMPC. The ^{31}P chemical shift of DMPC in SMA macrodiscs does not change between 20 and 50°C for SMA(2:1) macrodiscs or between 25 and 50°C for SMA(1.4:1) and SMA(3:1) macrodiscs. This indicates that their magnetic alignments are stable over a wide range of temperatures. Triton X-100 bicelles exist in an isotropic phase below 10°C. The 14A macrodiscs exist in an isotropic phase between 5 and 15°C. Notably, SMA macrodiscs display no evidence of an isotropic phase above 5°C and exist as aligned discs above 25°C.

The protein-containing SMA macrodiscs were prepared by solubilizing the proteoliposomes with SMA polymer (see Supporting Materials and Methods for further details). The ^{15}N amide backbone resonances of Pf1 coat protein in SMA macrodiscs (Fig. 2 *A*; Fig. S1 *A*) have linewidths as

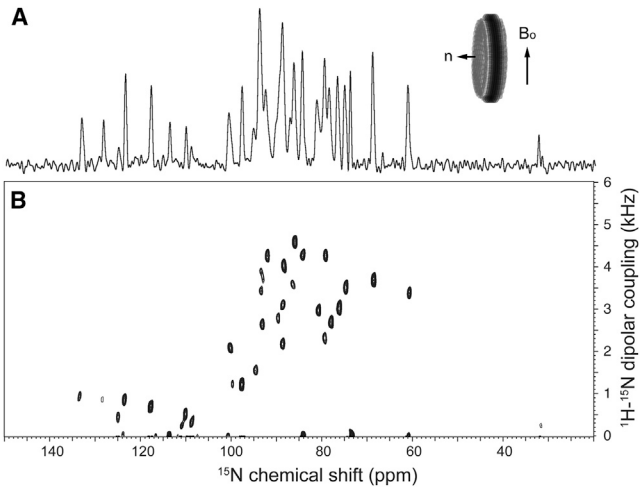

FIGURE 2 **Solid-state NMR spectra of the membrane-bound form of uniformly ^{15}N-labeled Pf1 coat protein in macrodiscs consisting of DMPC/dimyristoylphosphatidylglycerol (1:1) and SMA(3:1) with $q_d = 49.1$. The samples are aligned with their bilayer normals (n) perpendicular to the direction of the 21.1 T magnetic field, as illustrated in the cartoon. (*A*) One-dimensional ^{15}N chemical shift spectrum was obtained by cross-polarization with a 25 ms acquisition time. (*B*) Two-dimensional ^{1}H-^{15}N dipolar coupling/^{15}N chemical shift spectrum was obtained using polarization inversion spin exchange at the magic angle with 80 t_1 increments. Both spectra were obtained at 40°C with 45.5 kHz ^{1}H irradiations and 1 ms cross-polarization mix times.**

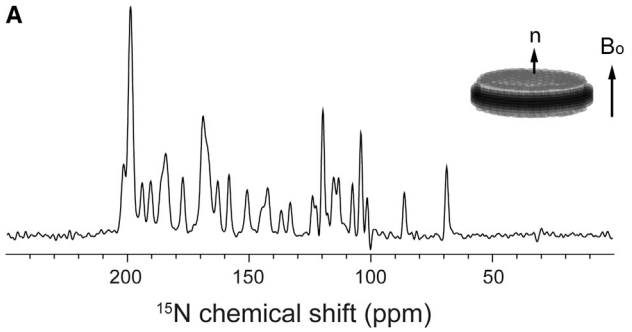

FIGURE 3 (*A*) ^{15}N chemical shift NMR spectrum of "flipped" Pf1 coat protein in SMA(3:1) macrodiscs was obtained by cross-polarization after addition of 5 mM $TmCl_3$ to the sample used in Fig. 2. The membrane normal is parallel to the field, as illustrated in the cartoon. (*B*) ^{31}P chemical shift NMR spectrum of DMPC:SMA(3:1) macrodiscs in the presence of 4 mM $YbCl_3$. (Also shown in Fig. S4)

narrow as 0.3 ppm, which provide better spectral resolution than previously observed in Triton X-100 bicelles (27) (Fig. S1 *B*) and 14A macrodiscs (14). The chemical shift differences between the spectra of Pf1 coat protein in SMA macrodiscs and in Triton X-100 bicelles are due mainly to the larger order parameter of the SMA macrodiscs (Figs. S1 and S2). Resonances from residues in the transmembrane helix (60–100 ppm) as well as those from residues in the adjacent loop region (110–140 ppm) have similar, uniform line shapes, suggesting that the protein adopts a single conformation in SMA macrodiscs.

The two-dimensional ^{1}H/^{15}N polarization inversion spin exchange at the magic angle (28) spectrum shown in Fig. 2 *B* is fully resolved with narrow linewidths in both the ^{15}N chemical shift and ^{1}H-^{15}N dipolar coupling frequency dimensions. The orientationally dependent frequencies of the resonances provide the angular constraints used in protein structure calculations. We note that two sets of signals (G23/I26 and G24/G28) that overlap in the equivalent spectrum of the protein in Triton X-100 bicelles (14) (Fig. S2 *B*) are fully resolved in the spectra in Fig. 2 *B* and Fig. S2 *A* because of the narrower linewidths observed in SMA macrodiscs.

The lipid bilayer normal of magnetically aligned bicelles and 14A macrodiscs is generally perpendicular to the direction of the magnetic field, as illustrated by the cartoon in Fig. 2. However, it is possible to "flip" the normal to the parallel direction with the addition of lanthanides (29). This is also the case for SMA macrodiscs, as shown by the spectra of the protein and lipids in Fig. 3, which were obtained after the addition of $TmCl_3$ or $YbCl_3$. The total span of the ^{15}N chemical shift frequencies is increased to ~170 ppm (Fig. 3 *A*), and that of the ^{31}P chemical shift frequencies is increased to 18.5 ppm (Figs. 3 *B* and S4), which is consistent with a 90° change of the direction of alignment of the SMA macrodiscs. Quantitative titration of $YbCl_3$ to SMA macrodiscs did not yield spectra with any ^{31}P chemical shift values other than those observed in Fig. 1 *E* and Fig. 3 *B*; thus, we find no evidence of intermediate orientations of the bilayer normal in SMA macrodiscs (Fig. S4). This is in contrast to the continuous range of ^{31}P chemical shift frequencies observed for macrodiscs prepared from chemically modified SMA polymers (17).

SMA macrodiscs provide a stable lipid bilayer environment that is well suited for biophysical and functional studies of membrane proteins. They can be prepared using a variety of phospholipids and a number of different SMA polymers. They are well aligned over a broad range of temperatures, and their orientation can be shifted from perpendicular to parallel with the addition of lanthanides. Moreover, they are applicable to membrane proteins and binding partners that are sensitive to detergents. SMA macrodiscs are particularly well suited for immobilizing and aligning membrane proteins for OS solid-state NMR structure determination. Notably, in these samples the proteins are in a near-native environment under physiological conditions, as required to ensure that their structures represent the functional conformations of the proteins.

SUPPORTING MATERIAL

Supporting Materials and Methods and four figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(18)30627-1.

AUTHOR CONTRIBUTIONS

J.R., S.H.P., and S.J.O. designed the research. J.R. and S.H.P. performed the experiments. J.R., S.H.P., and S.J.O. analyzed the data and wrote the manuscript.

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SUPPORTING CITATIONS

References (30–32) appear in the Supporting Material.

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