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Magnetic Alignment of Polymer Macro-Nanodiscs Enables Residual-Dipolar-Coupling-Based High-Resolution Structural Studies by NMR Spectroscopy

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Abstract: *Experimentally measured residual dipolar couplings (RDCs) are highly valuable for atomic-resolution structural and dynamic studies of molecular systems ranging from small molecules to large proteins by solution NMR spectroscopy. Here we demonstrate the first use of magnetic-alignment behavior of lyotropic liquid-crystalline polymer macro-nanodiscs (>20 nm in diameter) as a novel alignment medium for the measurement of RDCs using high-resolution NMR. The easy preparation of macro-nanodiscs, their high stability against pH changes and the presence of divalent metal ions, and their high homogeneity make them an efficient tool to investigate a wide range of molecular systems including natural products, proteins, and RNA.*

Polymer nanodiscs are successfully used in structural and functional studies of membrane proteins.[1–6] A polymer nanodisc is composed of a planar lipid bilayer surrounded by an amphiphilic polymer belt.[7] A variety of polymers have been used to form nanodiscs using synthetic lipids or by directly extracting membrane proteins in their native lipid environment.[2,8,9] Starting with styrene maleic acid,[10] there have been several different polymers[11–13] and their derivatives[14–19] that were used to overcome limitations such as size control as well as metal-ion and low-pH instability.[14,15,20] Furthermore, the size control enables the use of macro-nanodiscs (>20 nm) in solid-state NMR due to their magnetic-alignment properties in the presence of an external magnetic field.[14,15,21] Herein we demonstrate for the first time the use of the magnetic-alignment property of macro-nanodiscs for high-resolution NMR studies of water-soluble biomolecules using residual dipolar couplings (RDCs).

The use of RDCs is a powerful technique in the structural characterization of biomolecules. RDCs have been shown to provide valuable global orientation constraints to determine and refine high-resolution structures of biomolecules.[22–24] RDCs are typically measured using an anisotropic environment created by magnetically oriented liquid-crystalline molecules or mechanically compressed/stretched gels.[23,25] Previous studies have shown that the degeneracy of bond orientations obtained from RDC values can be minimized using orthogonal tensors by measuring RDCs in different alignment media or modulating the interaction between the alignment medium and protein (or other molecular system under investigation).[26] Bicelles have been used to achieve the measurements of orthogonal alignment tensors by changing the net charge via doping with negatively charged lipids[26] or surfactants.[27] However, bicelles are not usable for many biomolecular systems due to the presence of a denaturing detergent. In this study we show that polymer nanodiscs can be used as an alignment medium for measuring RDCs. Our results demonstrate that polymer nanodiscs exhibit a highly ordered homogeneous alignment in the presence of a magnetic field, and the degree of alignment can be scaled by the concentration as well as temperature of the sample. Using cytochrome c (cyt c) as a model system, we show that the experimentally measured RDCs are in good agreement with calculated values from reported structures.

Styrene maleic acid quaternary ammonium (SMA-QA) polymer was synthesized as described previously.[15] Polymer macro-nanodiscs were prepared using a 1:0.5 w/w ratio of DMPC and SMA-QA (Figure 1 A). The resulting nanodiscs were purified using size-exclusion chromatography (SEC), and characterized by dynamic light scattering (Figures S1 and S2 in the Supporting Information) and transmission electron microscopy (TEM). The TEM images showed macro-nanodiscs that were $\approx 25 \pm 4$ nm in diameter (Figures 1 B and S3).

Macro-nanodiscs with a varying lipid concentration (10–100 mg mL^{-1}) were prepared in a 10 % D_2O solution containing a phosphate buffer. ^{2}H NMR was used to examine the anisotropic properties of the macro-nanodiscs. An isotropic peak was observed at a low lipid concentration (10 mg mL^{-1}) at 32 °C, whereas by increasing the concentration to 20 mg mL^{-1}, a doublet was observed with a ^{2}H quadrupolar splitting ($\Delta\nu_Q$) of $\approx$3.7 Hz from HOD (Figure 1 C). Further increase in the lipid concentration to 100 mg mL^{-1} increased the observed ^{2}H quadrupole splitting to $\approx$21.7 Hz. These observations suggest that the degree of magnetic alignment of the macro-nanodiscs is scalable by altering the lipid concentration; and the observed symmetric, narrow peaks (line width <2 Hz) indicate a highly ordered homogeneous alignment of the nanodiscs. The effect of temperature on the alignment was examined using 40 mg mL^{-1} lipid concentration of the nanodiscs. An isotropic peak was observed at 20 °C (which is below the gel-to-liquid crystalline-phase transition temperature of DMPC), while a doublet with a quadrupole splitting of $\approx$1.6 Hz appeared as the sample temperature increased to 30 °C. Further increase in temperature increased the observed quadrupole splitting as shown in Figure 1 C,

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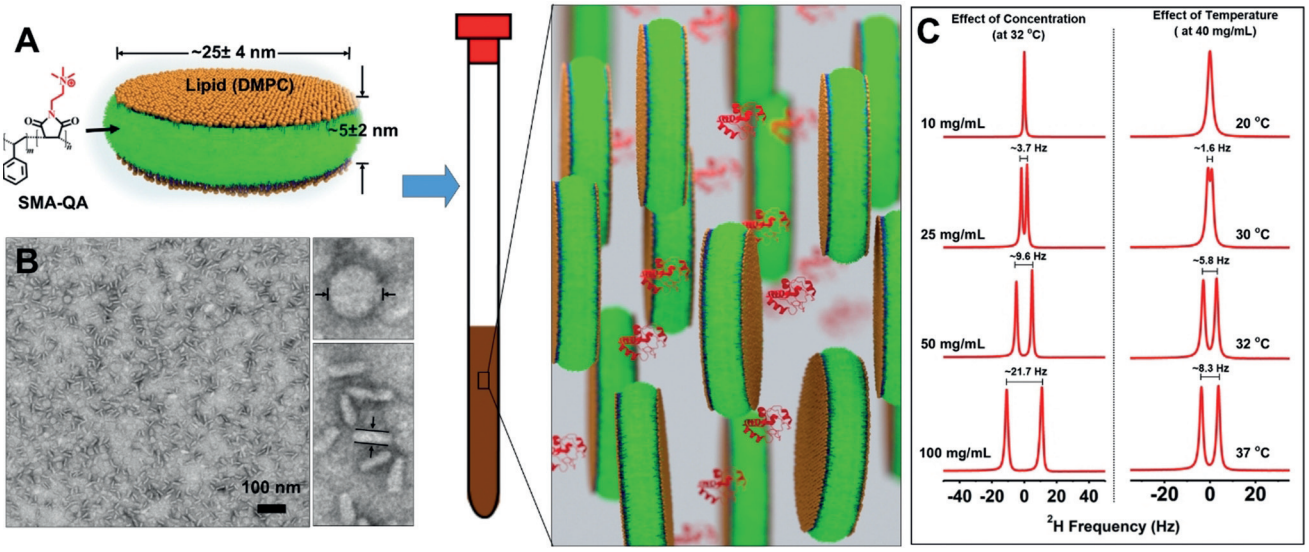

Figure 1. A) Schematic depiction of a polymer-lipid-bilayer nanodisc and its use as an alignment medium. B) Transmission electron microscopy image of macro-nanodiscs prepared using a 1:0.5 w/w ratio of DMPC:SMA-QA. C) Deuterium NMR spectra of the macro-nanodiscs with a varying lipid concentration (at 32 °C) and at different temperatures (at 40 mg mL^{-1}). All spectra were acquired using a 500 MHz NMR spectrometer.

suggesting that the macro-nanodiscs exhibit a lyotropic liquid-crystal behavior in which the liquid-crystalline phase depends on the temperature and concentration, and are suitable for RDC measurements.[22]

To demonstrate the feasibility of using nanodiscs as an alignment medium for NMR structural studies, uniformly ^{15}N-labeled cyt c was used as a model protein in this study. Cyt c is a positively charged, water-soluble protein with a pI value of 9.6.[28] We chose positively charged SMA-QA macro-nanodiscs to avoid any charge–charge interaction with cyt c.[29] ^{15}N-labeled cyt c was added to a solution of macro-nanodiscs (40 mg mL^{-1}) and used in ^{15}N–^{1}H in-phase anti-phase heteronuclear single-quantum coherence (IPAP-HSQC)[30] experiments to measure RDCs. 2D IPAP-HSQC spectra acquired at 20 °C showed that $^{1}J_{NH}$ couplings measured in the presence and absence of nanodiscs are comparable (Figure S4), suggesting that the nanodisc medium is isotropic at 20 °C. This is also confirmed from the appearance of an isotropic ^{2}H peak for the same sample (Figure S5). An anisotropic nanodisc medium was obtained by increasing the temperature to 32 °C, for which the alignment was confirmed from the appearance of a doublet in the deuterium NMR spectrum (Figure 2 A). A selected region of the 2D IPAP-HSQC spectrum is shown in Figure 2 C. RDCs were obtained by subtracting coupling values ($^{1}J_{NH}$ or $^{1}J_{NH}$ + D_{NH}) measured from the 2D IPAP-HSQC spectra acquired at isotropic (20 °C) and anisotropic (32 °C) conditions. Our experimental results show that the RDC values can be scaled by simply increasing the nanodisc concentration or the temperature, as evident from the increased RDCs obtained at higher concentrations: ±6 Hz for 40 mg mL^{-1} and ±40 Hz for 100 mg mL^{-1} (see Figures 2 C,D, 3 A,B and S6).

To validate the accuracy of the experimentally measured RDC values, RDCs were calculated using reported crystal structures of cyt c. Figure 3 C,D shows the correlation between experimental and calculated RDCs with R values of 0.958 for 40 mg mL^{-1} and 0.979 for 100 mg mL^{-1}. We further compared the experimental RDCs with values calculated from different high-resolution structures reported

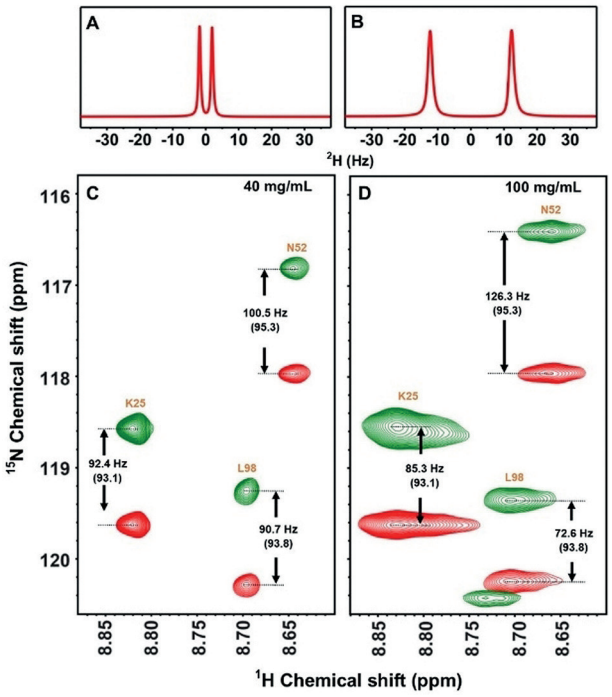

Figure 2. A), B) Deuterium NMR spectra of SMA-QA:DMPC macro-nanodiscs containing 200 μM ^{15}N-cyt c in 10% D_2O and a phosphate buffer at 32 °C with a lipid concentration of 40 mg mL^{-1} (A) and 100 mg mL^{-1} (B). C), D) Selected region of 2D ^{1}H-^{15}N IPAP-HSQC spectra obtained for a lipid concentration of 40 mg mL^{-1} (C) and 100 mg mL^{-1} (D) at 32 °C in a 800 MHz NMR spectrometer. Isotropic $^{1}J_{NH}$ values obtained at 20 °C are given in parentheses (see Figure S5). NMR spectra (Figures S10–14) and SEC profiles (Figure S15) are included in the Supporting Information.

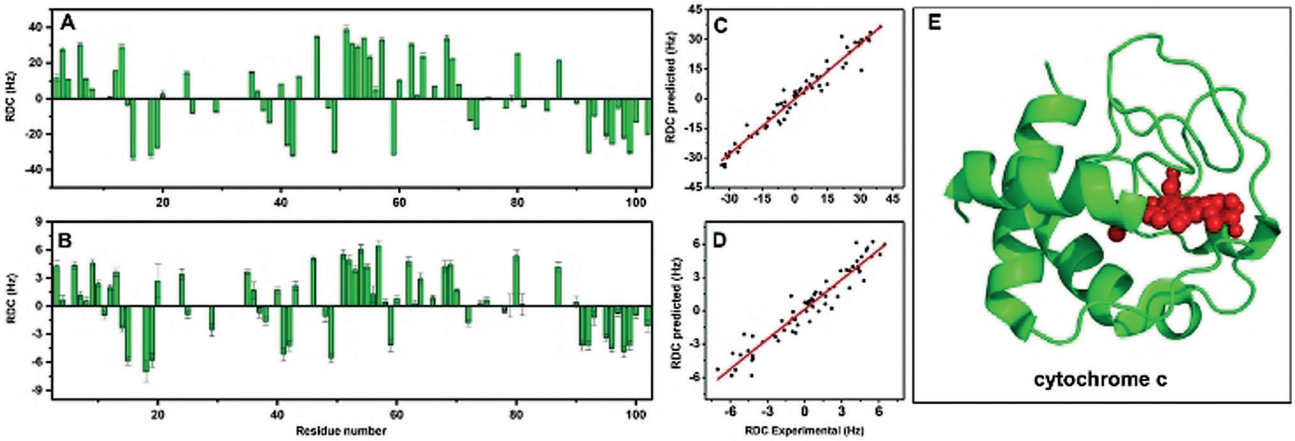

Figure 3. A), B) Experimentally measured residual dipolar couplings of ^{15}N-labeled cytochrome c for a lipid concentration of 100 mg mL^{-1} (A) and 40 mg mL^{-1} (B). C), D) Correlation of experimental and calculated RDCs at 100 mg mL^{-1} (C) and 40 mg mL^{-1} (D). E) X-ray crystal structure of cyt c (PDB:6FF5).

for cyt c (Figures S7–S9). These comparisons can be used to confirm the correct structure of the protein used in the NMR sample.

In summary, we have successfully demonstrated the use of polymer macro-nanodiscs as an effective alignment medium for the measurement of RDC values in structural studies using NMR experiments. Our results show that the degree of alignment can be scaled by varying either the lipid-nanodisc concentration or temperature. Using cyt c as a model system, we showed that the experimentally measured RDCs are in good agreement with the calculated values from known structures of the protein. It is remarkable that the pH-resistant and divalent-metal-ion-tolerant SMA-QA nanodiscs can be further exploited to study a variety of proteins and other biomolecules for RDC-based structural NMR studies under various conditions. The alignment tensor of the macro-nanodisc-alignment medium can be modulated by altering the interaction between the protein and the alignment medium via changing the lipid composition, and thereby enabling the measurement of RDCs with orthogonal tensors to restrict the determined bond orientations. Another advantage of polymer nanodiscs is that the protein (or any biomolecule) under study can be recovered from the sample using SEC after the NMR experiments. Due to these unique properties, we believe that the use of polymer nanodiscs as an alignment medium will be valuable for high-resolution structural studies on water-soluble biomolecules as well as to probe the interaction of molecules (such as peptides, proteins, ligands, or RNA) with lipid membranes.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: cytochrome c · magnetic alignment · NMR spectroscopy · polymer nanodiscs · residual dipolar couplings

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