HHS Public Access

Author manuscript

Chem Commun (Camb). Author manuscript; available in PMC 2021 June 18.

Published in final edited form as:

Chem Commun (Camb). 2020 June 18; 56(48): 6511–6514. doi:10.1039/d0cc01737j.

Detergent-free extraction, reconstitution and characterization of membrane-anchored cytochrome-b5 in native lipids

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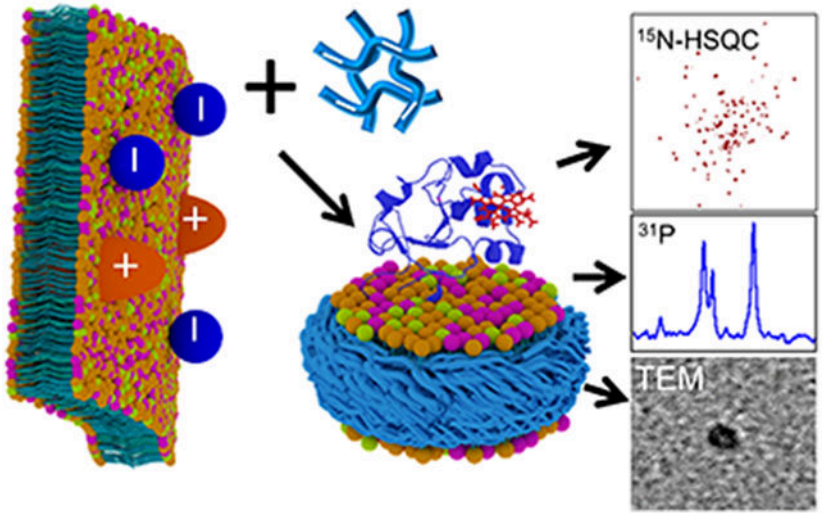
Abstract

Despite their denaturing properties, detergents are used to purify and study membrane proteins. Herein, we demonstrated a polymer-based detergent-free extraction of the membrane protein cytochrome-b5 along with *E. coli* lipids. Nuclear magnetic resonance experiments revealed the suitability of using nanodiscs for high-resolution studies and revealed the type of native lipids associated with the protein.

Graphical Abstract



Despite the need for high-resolution structures of membrane proteins to better understand their numerous functions, only <3% of the structures reported in the Protein Data Bank are of membrane proteins. Although a combination of a variety of biophysical techniques is used to overcome the challenges to investigate high-resolution structures and dynamics of membrane proteins,[1, 2] the key issue is related to the preparation of samples exhibiting stable

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

Conflicts of interest

There are no conflicts to declare.

and natively-folded structures that retain the function of the membrane proteins.[3, 4] Although detergents, and micelles as membrane mimetics, are continued to be used in the structural studies, mainly the X-ray crystallography studies, it is known that detergents destabilize protein structures and disrupt their functions.[5] Bicelles, although considered to be better membrane mimetic systems, have also been shown to distort protein structures and functions due to the diffusion of the detergent molecules from the bicelle rim to the planar lipid bilayer region.[6] These difficulties have been well demonstrated for the cytochrome proteins containing large soluble domains.[6] To overcome these difficulties, recent reported works have focused on the feasibility of directly extracting membrane proteins from cell lysates by using synthetic polymers[7-10] without the use of any detergents and reconstituting the proteins in polymer-based nanodiscs.[11-13] In this study, we demonstrated the feasibility of a direct extraction of a ~16-kDa cytochrome-b5 from *E. coli* using a synthetic styrene-*co*-maleic acid-ethanolamine (SMA-EA) polymer and reconstituting the cytochrome-b5 protein in polymer nanodiscs along with native lipids from *E. coli* (Fig. 1).

Recent studies have reported low-molecular-weight styrene-maleic acid (SMA)[14] polymer derivatives such as anionic SMA-EA[15] and cationic SMA-QA[16] that have been successfully used in the formation of nanodiscs with synthetic lipids and the ability to vary the size of the lipid nanodisc by simply changing the lipid-to-polymer ratio. The large nanodiscs (>20 nm, called macro-nanodiscs) have been shown to align in the presence of an external magnetic field, and therefore can be used in solid-state nuclear magnetic resonance (NMR)-based structural studies under static conditions.[15-17] In the current study, we showed that these polymer variants can be used for extracting cytochrome-b5 directly from *E. coli* without the use of any detergent and also showed the feasibility of carrying out structural studies of this protein using well-established solution NMR and other biophysical techniques. Since the anionic transmembrane protein cytochrome-b5 contains a large soluble domain with a large number of negatively charged residues, we used this protein to further demonstrate the need to avoid any non-specific interaction between the membrane protein to be extracted and the polymer used in order to successfully stabilize the native structure, folding, and function of a membrane protein in nanodiscs. The feasibility of identifying and quantifying the lipids, directly extracted along with cytochrome-b5 from *E. coli*, by performing simple 1D ^{31}P NMR experiments was also demonstrated.

Cytochrome-b5 is a single transmembrane protein containing heme as a prosthetic group.[18] This protein functions by donating the second electron to the membrane-bound cytochrome-P450 enzyme, which requires two electrons to perform the metabolism of drugs and xenobiotics. Cytochrome-b5 also delivers electrons to various other proteins that regulate fatty acid metabolism.[19, 20] The 16-kDa cytochrome-b5 consists of an N-terminal heme-binding domain (residues 1-89), a 15-residue flexible linker region (residues 90-104), and a hydrophobic C-terminal domain (residues 105-134).[18]

Cytochrome-b5 is a negatively charged protein with a net charge of −8.8 at pH 7.4;[21] and, therefore, a negatively charged synthetic SMA-EA polymer (Figs. 1 and S1) was used for the direct extraction to avoid non-specific interactions between the protein and polymer. The 7.4 pH was maintained throughout the purification protocol. Cytochrome-b5 was expressed in *E. coli* C41 cells as reported previously[22] and briefly explained in Supporting

Information. The membranes containing cytochrome-b5 were collected by subjecting cell lysate to centrifugation, and were resuspended in potassium phosphate buffer (pH 7.4); and the concentration of SMA-EA polymer was adjusted to have a 1:1 membrane-to-polymer ratio (w/w). The cytochrome-b5 in SMA-EA nanodiscs containing native *E. coli* lipids was purified using Ni-NTA affinity chromatography and then size-exclusion chromatography (SEC).

The directly extracted cytochrome-b5 in SMA-EA polymer nanodiscs containing native *E. coli* lipids was experimentally examined using SEC, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), dynamic light scattering (DLS), transmission electron microscopy (TEM) and NMR spectroscopy as shown in Figures 2, 3 and 4. The SEC elution volume for cytochrome-b5 was between 21 and 26 mL, much lower than the expected elution volume for cytochrome-b5 alone (MW ~16 kDa), indicating the presence of a complex larger than cytochrome-b5 alone (Fig. 2A). The protein showed a single band (MW ~16 kDa) on the SDS-PAGE gel, indicating the high purity of the detergent-free extracted cytochrome-b5 (Fig. 2B). The final protein yields were about 2.5 to 3.0 mg per L culture. UV-visible absorption spectra were recorded in the absence and presence of sodium dithionite. Shifts from 409 nm (oxidized form) to 424, 526 and 556 nm were observed upon the addition of sodium dithionite (Fig. S2). These characteristics are typical of the oxidized state of cytochrome-b5.[23] A ^{1}H NMR spectrum showed peaks corresponding to lipids (0.84 and 3.14 ppm), SMA-EA polymer (6.5 to 7.4 ppm from the styrene group) and protein (amide-proton resonances in the 7-9 ppm region), which further confirmed the presence of cytochrome-b5 in the SMA-EA native *E. coli* lipid nanodiscs (Fig. 2C). The nanodiscs were further characterized using DLS, which showed a hydrodynamic radius of ~5 nm (Fig. 3A), and by acquiring TEM images of them (Fig. 3B), which revealed the presence of nanodiscs having dimensions of ~10 nm.

Native *E. coli* lipids present in the nanodiscs were analyzed using ^{31}P NMR. The ^{31}P NMR spectrum exhibited a very low signal-to-noise ratio with broad spectral lines (with a line width of ~250 Hz) for the extracted native lipids in the SMA-EA nanodiscs. The observed line broadening can be attributed to the unaveraged ^{1}H-^{1}H dipolar couplings, due to the slow tumbling of the ~10-nm-sized nanodiscs, contributing to ^{31}P spin-spin relaxation (Fig. S3). In addition, the applied weak radio-frequency irradiation for proton decoupling during ^{31}P signal acquisition may not have been sufficient to completely suppress ^{1}H-^{31}P dipolar couplings. In order to resolve the overlapped NMR peaks and hence identify the lipids directly extracted by the polymer from *E. coli*, the nanodiscs were dissolved by adding the non-denaturing detergent sodium cholate at a concentration of 100 mM, and the resultant solution was used to record the ^{31}P NMR spectrum. A reference ^{31}P NMR spectrum was also recorded from commercially available synthetic lipids in the same buffer conditions (Fig. S3). Three well-resolved resonances were observed in the ^{31}P NMR spectrum for the extracted native lipids, as shown in Figure 4A. Based on the spectrum of the reference sample (Fig. 4A), the major peaks observed in the ^{31}P NMR spectrum for the extracted lipids were assigned to phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL). The unassigned low-intensity peak (denoted with an asterisk) appearing in the downfield region of the spectrum was likely due to uncharacterized lipids of the *E. coli* membrane. The observation of all types of *E. coli* native lipids in the polymer nanodiscs

indicated that cytochrome-b5 does not have a preference to interact with any *E. coli* lipids. This observation was found to be in agreement with our recent study that utilized the lipid exchange process in peptide-based nanodiscs to report a non-specific preference of cytochrome-b5 for lipids.[24]

The polymer nanodiscs consisting of the directly extracted lipids and cytochrome-b5 are small (~10 nm); therefore, the isotropic nature of the nanodiscs should enable the application of solution NMR experiments to study the structure and dynamics of cytochrome-b5. To demonstrate this feasibility, uniformly ^{15}N-labelled cytochrome-b5 was expressed in *E. coli* and directly extracted in native *E.coli* lipids using the polymer as described above, and the resultant nanodiscs were characterized using the above-mentioned biophysical and NMR experiments. A 2D ^{1}H/^{15}N TROSY-HSQC NMR spectrum of cytochrome-b5 reconstituted in native *E. coli* lipids was then acquired, and is shown in Fig. 4B. This spectrum exhibited well-dispersed resonances both in the ^{1}H and ^{15}N dimensions, indicating the correctly folded conformation of the protein, and in good agreement with previous studies.[18] The spectrum also showed narrow spectral lines, which were attributed to the highly dynamic nature of the large soluble domain of cytochrome-b5, as reported in the previous studies. On the other hand, broad spectral lines invisible in this 2D ^{1}H/^{15}N TROSY-HSQC solution NMR spectrum have been observed in an ^{19}F NMR spectrum[25] and also according to solid-state NMR experiments,[26] and were attributed to the residues in the transmembrane domain undergoing a slow motion, on the milliseconds or slower time scale. The narrow spectral lines observed for the soluble domain residues in the 2D ^{1}H/^{15}N TROSY-HSQC NMR spectrum confirmed the native-like mobility of residues in the soluble domain of cytochrome-b5 reconstituted in the nanodiscs and also proved the absence of any interaction between the polymer belt and the residues in the reconstituted protein. Taken together, these results suggested that SMA-EA polymer-lipid nanodiscs with cytochrome-b5 were intact under the purification conditions employed in this study.

A direct extraction of cytochrome-b5 was also attempted using a positively charged SMA-QA polymer (Fig. S1) in order to determine the effect of both polymer and protein charges on the solubilization and purification of membrane proteins. The *E. coli* membranes were solubilized using the SMA-QA polymer under conditions otherwise identical to those described above for the negatively charged SMA-EA polymer. The SMA-QA polymer-protein fraction from the Ni-NTA chromatography column showed, on an SDS-PAGE gel, a protein band with an intensity much lower than that obtained for the SMA-EA protein-fraction (Fig. S4). Unlike the case for SMA-EA, when the membranes were solubilized with SMA-QA, most of the cytochrome-b5 was in the insoluble portion of the membranes and buffer flow-through, indicating the role played by the interaction of the positively charged SMA-QA polymer with the negatively charged cytochrome-b5. Therefore, the charge of the polymer must be the same as that of the membrane protein under investigation to avoid any non-specific interaction between the polymer and the membrane protein.[27, 28]

Despite the recent advances in the direct extraction of membrane proteins using synthetic polymers, the results reported in this study demonstrated the need to consider a few limitations for achieving a successful membrane protein extraction and reconstitution. As demonstrated for cytochrome-b5, the charge of the polymer used needs to be carefully

chosen for a successful reconstitution/extraction of a membrane protein from the cell membrane — in agreement with a recent study on the effect of polymer charge on the stability and functional folding of the reconstituted protein.[27, 28] As demonstrated in the current study, to avoid any charge-charge interaction between the polymer and cytochrome-b5, we chose the negatively charged SMA-EA polymer to purify the negatively charged cytochrome-b5. Note that due to the presence of carboxylic groups in the SMA-EA polymer, the non-specific binding of the polymer to Ni-NTA beads required extensive washing steps, which reduced the final yield of the cytochrome-b5 (Fig. S5). Therefore, the use of other purification tags[29] in place of the 6His-tag would be helpful to overcome such non-specific interactions.

In conclusion, the membrane-anchored heme-containing cytochrome-b5 was successfully extracted from *E. coli* and reconstituted in native *E. coli* lipids by using a negatively charged synthetic SMA-EA polymer. The type of native *E. coli* lipids present in the nanodiscs was identified using ^{31}P NMR. These results demonstrated that ^{31}P NMR can be used to identify the type of native lipids, and their ratio, associated with the directly extracted membrane protein. The information obtained on the lipids from ^{31}P NMR experiments can also be useful to optimize the conditions/procedure used to successfully reconstitute the membrane proteins extracted using detergents when following the well-established protocols. Our results revealed that the directly extracted ^{15}N-labelled cytochrome-b5 was of high purity as indicated by the 2D ^{1}H/^{15}N TROSY-HSQC NMR spectrum, which also demonstrated the correctly folded state of the protein and the feasibility to carry out structural and dynamical measurements using the well-established multidimensional solution NMR techniques. Although both SMA-QA and SMA-EA polymers were observed to behave similarly in solubilizing membranes, the polymer charge was found to have a significant effect on the protein purification. The attempts to purify the cytochrome-b5 using the positively charged polymer SMA-QA failed due to the charge-charge interactions between the protein and the polymer. Therefore, our results suggested that the polymer charge should be seriously considered based on the net charge of the desired protein that is to be purified at a given pH. We expect the electrostatic interactions between the polymer and protein to be highly significant for most membrane proteins containing a large soluble domain, such as single-pass and double-pass transmembrane proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This study was supported by NIH (GM084018 to A.R.).

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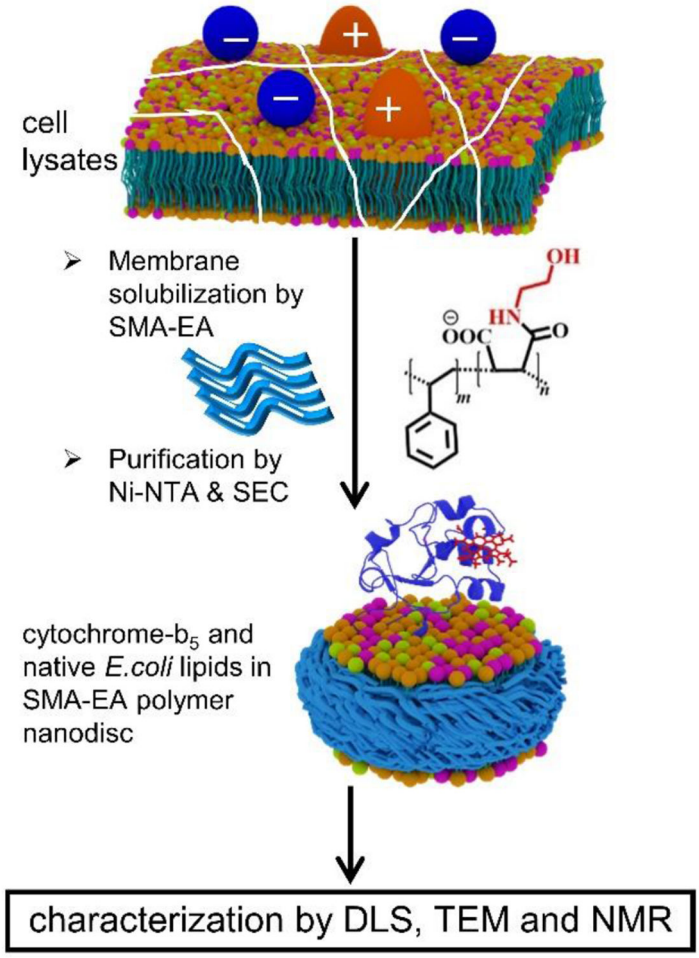

Figure 1.

A schematic representation of the direct extraction of a 16-kDa rabbit cytochrome-b5 in native *E. coli* lipids using a negatively charged SMA-EA polymer. + and − indicate the net charge of a membrane protein. SEC: size-exclusion chromatography.

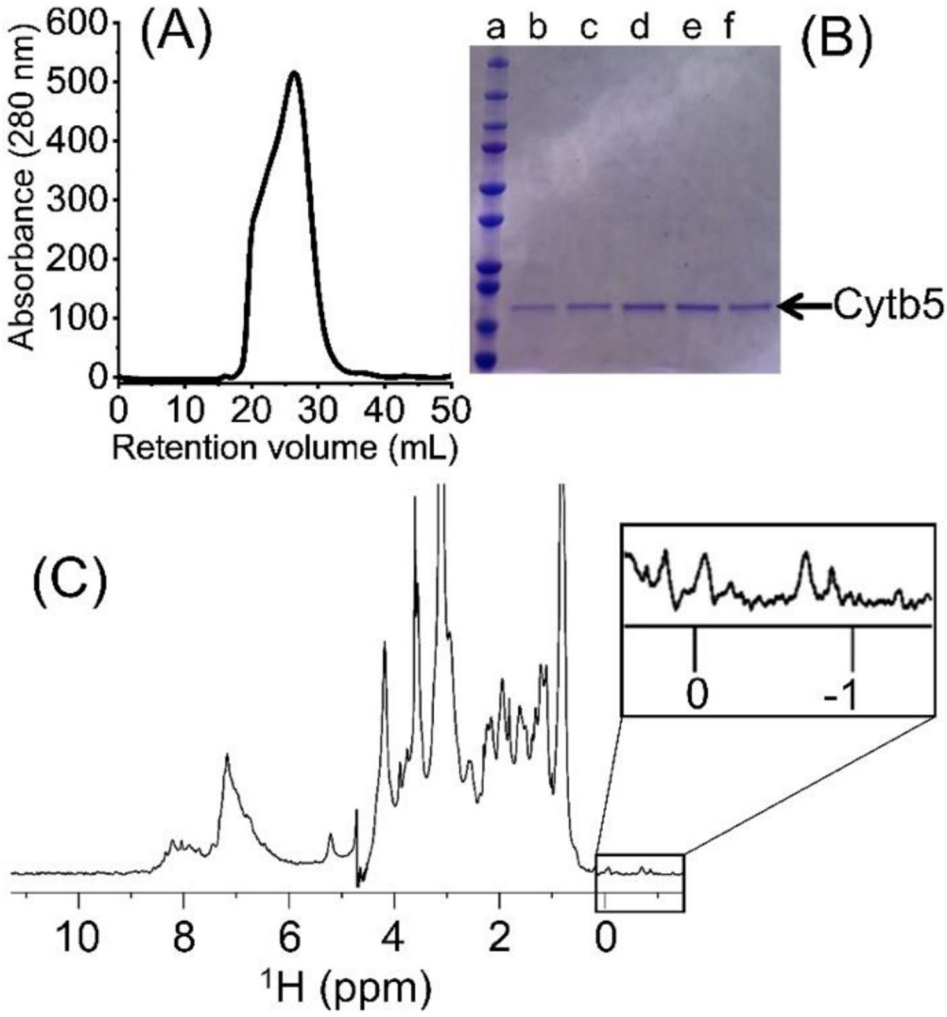

Figure 2.

(**A**) Size-exclusion chromatogram (SEC) of the extracted cytochrome-b5. (**B**) SDS-PAGE analysis of the directly extracted cytochrome-b5. Lane a includes the protein markers and lanes b-f the SEC protein fractions. (**C**) ^{1}H NMR spectrum of the directly extracted cytochrome-b5. Methyl resonances in the upfield region (boxed) indicating the folded conformation of the protein. The protein sample was prepared in 20 mM potassium phosphate buffer pH 7.4, 50 mM NaCl. The NMR spectrum data were collected at 25 °C using a Bruker 500 MHz NMR spectrometer equipped with a TXI probe.

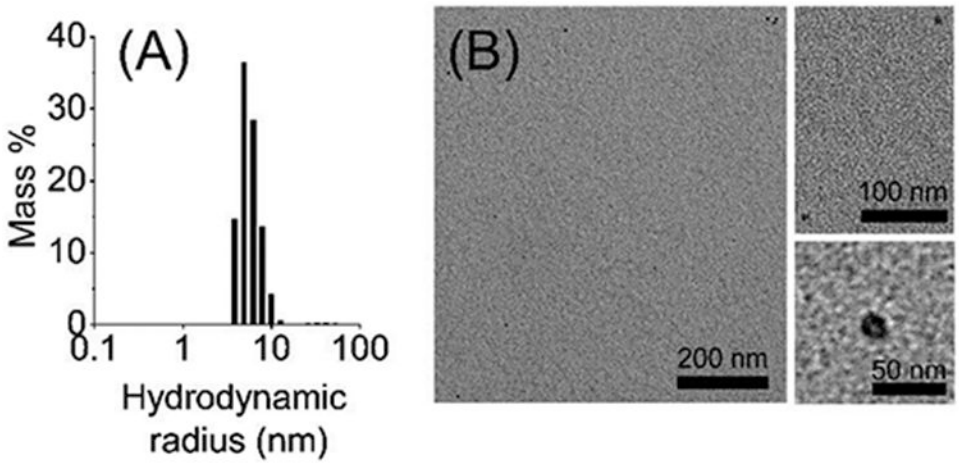

Figure 3.

(**A**) Dynamic light scattering profile of the nanodiscs showing the presence of a large complex with a hydrodynamic radius of ~5 nm. (**B**) Transmission electron microscopy images showing SMA-EA-based nanodiscs containing directly extracted cytochrome-b5 and *E. coli* lipids; enlarged images are shown on the right side of the panel for clarity.

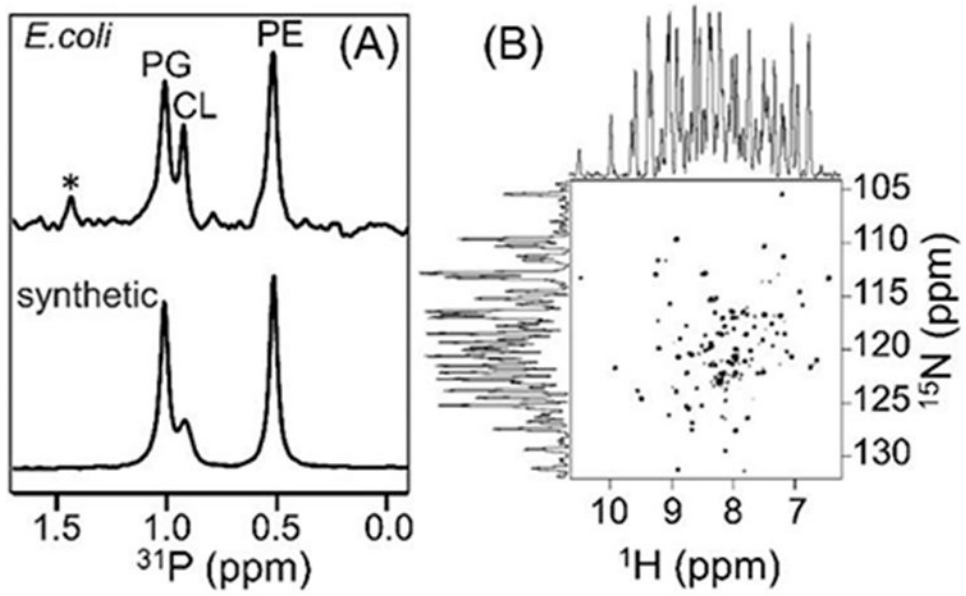

Figure 4.

(**A**) ^{31}P NMR spectra of native *E. coli* lipids present in the polymer nanodiscs and of the artificially reconstituted synthetic lipids (a reference sample) acquired under by decoupling protons. The protein sample was prepared in 20 mM HEPES buffer pH 7.4 containing 50 mM NaCl and 100 mM sodium cholate. The spectra were recorded using a 500 MHz Bruker NMR spectrometer equipped with a room-temperature ^{1}H/^{31}P/^{2}H BB reverse probe. The synthetic lipids (PE: phosphatidylethanolamine, CL: cardiolipin, and PG: phosphatidylglycerol) were prepared in a PE:CL:PG molar ratio of 7:1.2:4.3. * indicate the uncharacterized *E. coli* lipids. (**B**) 2D ^{1}H/^{15}N TROSY-HSQC NMR spectrum of 65 μM ^{15}N-labelled cytochrome-b5 in native nanodiscs. The ^{1}H and ^{15}N line widths were measured to be 27-35 Hz and ~18 Hz, respectively. The protein sample was prepared in 20 mM potassium phosphate buffer pH 7.4, 50 mM NaCl. The 2D NMR spectrum was recorded at 25 °C using a Bruker Avance III 800-MHz spectrometer equipped with a cryogenically cooled triple-resonance probe.