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A Split-Intein-Based Method for the Efficient Production of Circularized Nanodisks for Structural Studies of Membrane Proteins

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Phospholipid nanodisks are a native-like membrane mimetic that is suitable for structural studies of membrane proteins. Although nanodisks of different sizes exist for various structural applications, their thermal and long-term stability can vary considerably. Covalently circularized nanodisks are a perfect tool to overcome these limitations. Existing methods for the production of circularized nanodisks can be time-consuming and technically demanding. Therefore, an easy in vivo approach, in which circularized membrane scaffold proteins (MSPs) can be directly obtained from *Escherichia coli* culture, is reported herein. *Nostoc punctiforme* DnaE split-intein fusions with MSPs of various lengths are used and consistently provide circularized nanodisks in high yields. With this approach, a large variety of circularized nanodisks, ranging from 7 to 26 nm in diameter, that are suitable for NMR spectroscopy and electron microscopy (EM) applications can be prepared. These nanodisks are superior to those of the corresponding linear versions in terms of stability and size homogeneity, which affects the quality of NMR spectroscopy data and EM experiments. Due to their long-term stability and homogeneity, the presented small circular nanodisks are suited for high-resolution NMR spectroscopy studies, as demonstrated with two membrane proteins of 17 or 32 kDa in size. The presented method will provide easy access to circularized nanodisks for structural studies of membrane proteins and for applications in which a defined and stable nanodisk size is required.

Phospholipid nanodisks have gained increasing popularity in structural biology in recent years. This detergent-free membrane mimetic is based on a real lipid bilayer membrane in which two copies of an engineered apolipoprotein A-1 (apoA-1) or membrane scaffold protein (MSP) are wrapped around a patch of lipid bilayer membrane.[1] Due to this straightforward assembly mechanism, the length of the MSP directly defines the diameter of the nanodisk particle. Nanodisks are suitable for cryoelectron microscopy[2] and NMR spectroscopy.[3] Furthermore, the introduction of smaller nanodisks, ranging from 6 to 10 nm in diameter, permit the adaptation of the nanodisk size to a particular membrane protein of interest, which is essential for solution-state NMR spectroscopy, in which smaller particles give rise to sharper NMR signals.[3a,4] In addition, nanodisks are suited to the study of membrane-attached proteins and complexes with membrane proteins in a native environment.[5] Furthermore, the long-term stability of nanodisks is also essential for biochemical assays, for example, for probing amyloid binding to lipid surfaces.[6] To improve the homogeneity of nanodisks for structural applications, circular MSP (cMSP) proteins have been produced by using sortase A mediated protein ligation[7] and optimized by the addition of detergents.[8] However, this time-consuming enzymatic reaction often needs to be optimized and ligation yields can vary considerably, which may limit the wider use of circular nanodisks in structural biology or biochemical assay development.

Herein, we present a versatile and easy method for the production of cMSP nanodisks by using in vivo split intein ligation in *Escherichia coli*. By fusing the two parts of a *Nostoc punctiforme* (*Npu*) DnaE split intein[9] to various MSPs, we were able to obtain high amounts of pure cMSP directly from *E. coli* culture. These cMSPs can be purified within 1 day and the obtained nanodisks show superior stability and size homogeneity. With the circularization procedure, stable nanodisks ranging from 7 to 26 nm can be obtained within 2 days. Due to stability issues in the linear form, nanodisks below 8 nm in diameter are of limited use for structural studies. Circularization of the corresponding MSPs is an elegant solution to this problem. We show herein that small circular nanodisks are suitable for long-term NMR spectroscopy experiments at elevated temperatures, resulting in enhanced spectral quality, compared with regular linear nanodisks. Furthermore, any other biochemical and structural biology application that relies on stability and size homogeneity will benefit from the presented method. Thus, we believe that, by enabling easy access to circular nanodisks, the presented split-intein-based method will be useful for a large variety of applications in structural biology and membrane protein biochemistry without the need for an elaborate optimization of ligation conditions.

To achieve efficient intein splicing in living *E. coli* cells, we designed a suitable construct by using the *Npu* DnaE split intein.[9,10] This well-characterized, natural, split intein is highly active,[9,11] leading to quantitative splicing of the precursor protein in vivo and the subsequent formation of a cMSP.

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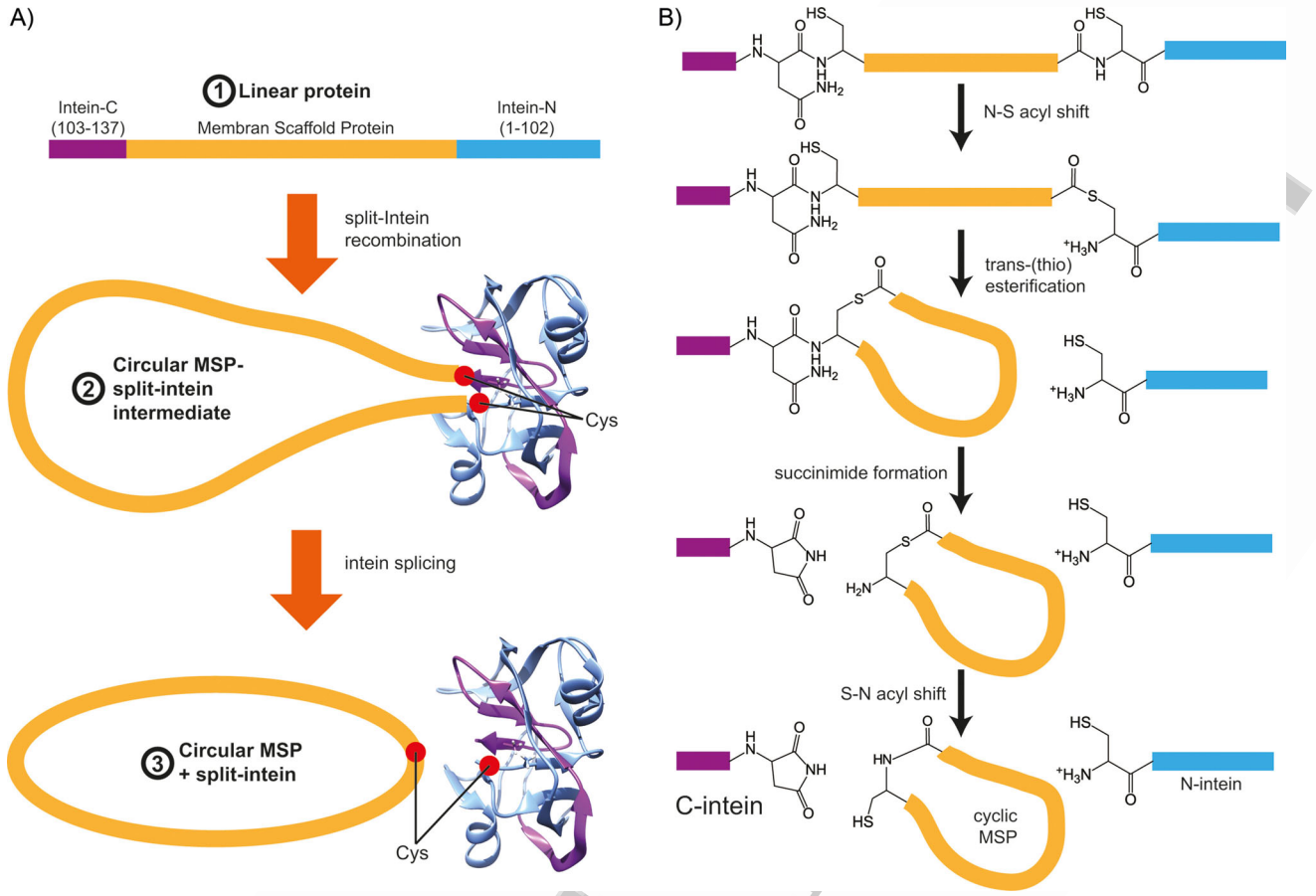

Figure 1. Split intein-based strategy for the production of cMSPs in vivo. A) Design of the *Npu* DnaE split-intein MSP fusion construct and mechanism of intein splicing in vivo, leading to the generation of cMSP. N-Intein (aa 1–102) and C-intein (aa 103–137) are shown in blue and magenta, respectively. Intein structural coordinates were taken from ref. [13] (PDB ID: 2KEQ). The essential cysteine residues are marked by red dots. B) Mechanism of intein splicing with the MSP and two split intein fragments colored according to the scheme in A).

For circularized MSP$\Delta H5$ and MSP1D1, we designed a construct (Figure 1 A) that contained an N-terminal His_6 tag, followed by the C-intein (residues 103–137, IKA…ASNCFN), a single glycine spacer, the particular MSP variant, a single glycine residue and the N-intein (residues 1–102, CLS…LPN), and a His_6 tag (Figure 1 A) at the C-terminal end. With this domain organization, the precursor protein can be efficiently spliced in vivo in the cytoplasm (Figure 1) by a cysteine- and thioester-based mechanism, as reported previously for the expressed protein ligation (EPL) method (Figure 1 B).[12]

After protein synthesis, the two intein fragments recombine and form a functional intein domain that is competent to perform an efficient splicing reaction (Figure 1 B). We arranged the two intein fragments in a reverse manner that led to the generation of a circular protein, as previously demonstrated with cyclic peptides[14] by using the *Ssp* DnaE intein. We used the *Npu* DnaE split intein,[9,10] which has been demonstrated to be more efficient than that of *Ssp* DnaE. The mechanism of intein-mediated ligation has been described by Kent and co-workers for the semisynthetic preparation of protein–peptide adducts by using proteins containing an N-terminal cysteine and peptides harboring C-terminal thioesters; this method was named expressed protein ligation (EPL).[12] A major advantage of the intein ligation method is the direct production of circular proteins in vivo without the need for a downstream enzymatic reaction for MSP circularization, as required with sortase A mediated ligation;[7,8] this reduces the time for the recombinant production and purification of cMSPs from >4 to 2 days. Furthermore, we obtain consistently high yields of cMSPs (Table 1). Our method leads to the incorporation of a single cysteine residue in the cMSP that can be utilized for downstream chemical modifications or surface immobilization for biochemical assays. The high yield of cMSP protein production can readily be observed in whole-cell extracts by using SDS-PAGE, through which the two intein fragments (6 and 12 kDa for I_C and I_N, respectively) and the cMSP (cM, 19 kDa) can already be identified as pronounced bands (Figure 2 A).

Table 1. Average yields of cMSPs of various diameters per liter of *E. coli* culture obtained by in vivo intein splicing.

Construct	Yield [mg L^{-1}]	Construct	Yield [mg L^{-1}]
cMSP$\Delta H4$-6	10	cMSP1D1	20
cMSP$\Delta H45$	8	cMSP26	8
cMSP$\Delta H5$	25		

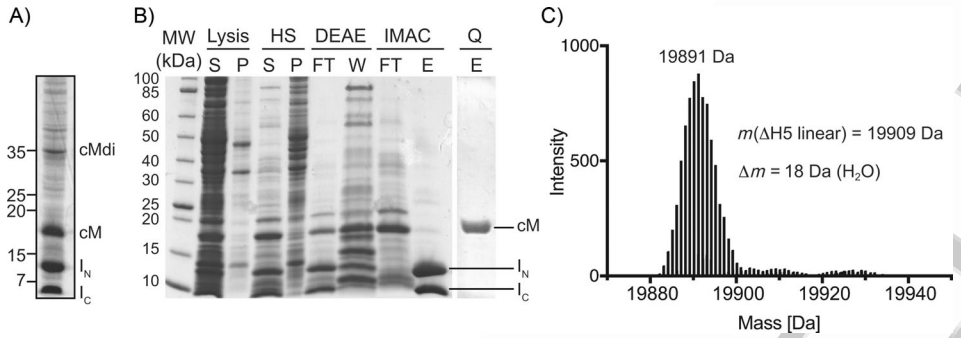

Figure 2. In vivo intein splicing in *E. coli* cells. A) SDS-PAGE of whole-cell extracts show three abundant protein fragments that correspond to the two split inteins (I_C and I_N) and the circular MSPD1Δ*H5*, as well as a small portion of dimeric cMSPD1Δ*H5*. B) Purification of cMSP from *E. coli* culture is achieved by heat shock followed by ion exchange and Ni-NTA chromatography. C) ESI-MS analysis of purified cMSP confirms the reduction in mass by 18 Da, which corresponds to one water molecule that is released upon amide-bond formation upon circularization. cM: circular MSP1D1Δ*H5*, cMdi: dimeric MSP1D1Δ*H5*, I_N: N-intein, I_C: C-intein, HS: heat shock, DEAE: DEAE cellulose column (weak anion exchange resin to remove DNA), IMAC: Ni-NTA affinity, Q: Q-sepharose column (strong anion exchange), E: elution fraction, FT: flow-through, S: soluble fraction, P: pellet, W: wash fraction.

A small portion of the cMSP tends to covalently dimerize in vivo to give rise to a band at about 35 kDa (cMdi). By using sortase A, oligomerization of the MSP is a prominent side reaction that can be reduced by lowering the MSP concentration[7] and/or the use of detergents.[8]

However, optimization of the reaction conditions has to be done in each case and previous work has mostly focused on larger nanodisks. Small circular nanodisks, which are relevant for solution-state NMR spectroscopy studies, have not been described in these previous studies. We developed a fast and easy purification strategy for our cMSPs (Figure 2B). We take advantage of the higher thermal stability of cMSPs, compared with that of most other proteins in *E. coli*. Thus, we apply a 70 °C heat shock, which leads to the precipitation of a wide range of proteins, whereas the cMSP and intein fragments can be found in the soluble fraction. We then use a weak anion-exchange resin (DEAE) to remove DNA and pull out the His-tag containing intein fragments by Ni-NTA chromatography. Finally, we use a strong anion exchange column (Q) in 8 M urea to obtain, after dialysis to buffer without urea, highly pure cMSP for further use. This procedure was necessary to obtain highly pure cMSPs for further use.

To confirm the existence of a peptide bond between the termini of the cMSP, we performed ESI-MS analysis, which indicated a mass for the cMSP that was 18 Da lower than that of the calculated mass of the linear protein, corresponding to one H_2O molecule that was released upon peptide-bond formation (Figure 2C).

Using this strategy, we inserted a large variety of MSP constructs into our split-intein plasmid. For the very small MSP variants MSPΔ*H*45 and MSPΔ*H*4-6[4a] or the large MSP26 variant shown to form 15 nm nanodisks because it most likely adopts a double ring around a lipid patch,[7,8] we removed the His_6-tags flanking the split inteins and added the His_6-tag to the final cMSP. Thus, Ni-NTA and Q-sepharose chromatography in 8 M urea could be used as a main purification strategy (see the Methods section in the Supporting Information). With this protocol, we were able to obtain 8–25 mg of cMSP per liter cell culture (Table 1).

Next, we investigated the use of these cMSPs for nanodisk assembly. Employing standard assembly conditions, as reported herein (see the Methods section in the Supporting Information) or elsewhere,[1b,3a,d,4a,7] we managed to obtain homogenous nanodisks, as probed by size exclusion chromatography (SEC; Figure 3A) or negative-stain electron microscopy (EM; Figure 3B).

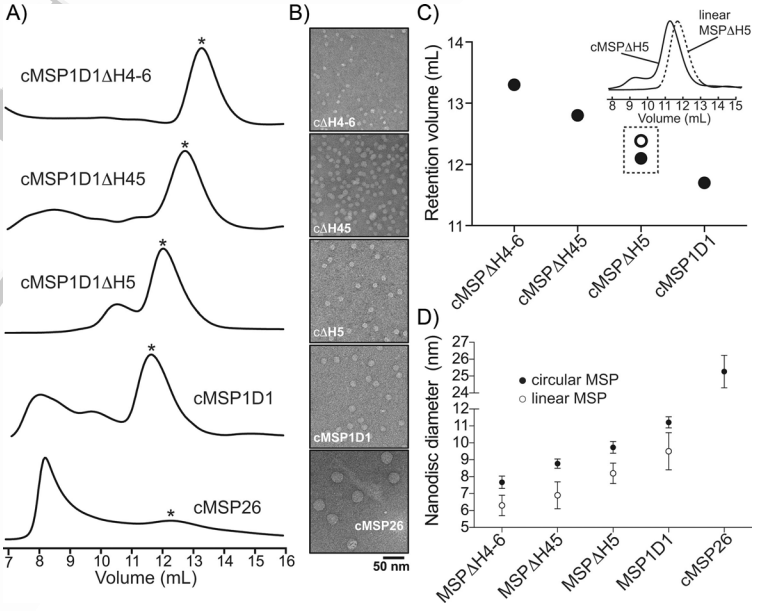

Figure 3. Size homogeneity of cMSP nanodisks. A) S200 SEC with nanodisks assembled with cMSP constructs and DMPC lipids (nanodisk peak indicated by an asterisk). Due to its larger size, for MSP26, a Superose-6 column is used. Nanodisks elute at around 12.5 mL, whereas the main peak contains excess lipids. B) Electron micrographs of these nanodisks. C) Elution volumes of the nanodisk preparations decrease gradually with nanodisk size. For comparison, the elution volume and the SEC chromatogram of linear MSP1D1Δ*H5* nanodisks is shown. D) Average diameters and standard deviations of the circular nanodisks shown in (B) relative to previous data[4a] on nanodisks formed with the corresponding linear MSP constructs.

As expected, the size of the assembled nanodisks decreases with subsequently shorter cMSPs, as monitored by an increase in the retention volume in SEC and by direct visualization through negative-stain EM. The variation in diameter is, in all cases, lower than that observed for linear MSP nanodisks by using the same methods[4a] (Figure 3D). Furthermore, we managed to obtain circular MSP26, which was shown to form nanodisks of 15 nm in diameter,[7] and were able to assemble 26 nm disks after screening the lipid-to-MSP ratio used for nanodisk assembly.

At lower lipid-to-MSP ratios, this MSP presumably forms a double ring around the lipid patch, giving rise to a smaller nanodisk. In general, cMSP nanodisks are around 1 nm larger in diameter than that of the linear versions, possibly due to the participation of the entire MSP in the formation of the covalent protein ring. In linear MSPs, the terminal amino acid stretches are not in direct contact with lipids, and thus, do not contribute to the nanodisk size, as shown by subsequent N-terminal truncations[15] and a recent NMR structure of empty MSP1D1DH5 nanodisks.[16]

So far, the use of very small nanodisks assembled with linear MSPs (ΔH45 and ΔH4-6) was restricted to experiments of short duration due to their limited long-term stability.[4a] A likely reason for this behavior is increasing strain within the MSP that is generated upon wrapping around a small patch of lipid bilayer. For the previously characterized medium-sized, smaller nanodisks MSP1D1ΔH4 or ΔH5 variants,[4a] this strain is apparently lower than the energy gained by hydrophobic interactions between the lipids and the MSP, whereas in the shorter MSPs the strain is higher than these stabilizing interactions. Thus, we anticipated that circularization would remedy this situation and render the smaller disks more stable, that is, suitable for use in the structural analysis of small membrane proteins. To probe the stability of dimyristoylphosphatidylcholine (DMPC) nanodisks assembled with linear and cMSPs, we performed circular dichroism (CD)-detected thermal melting experiments by monitoring the CD signal at $\lambda = 222$ nm, which is characteristic for the occurrence of an α-helical secondary structure of the MSP "belt" that encircles the lipid patch (Figure 4A). Nanodisk melting behavior and the influence on target protein melting has been characterized previously by means of differential scanning calorimetry and small-angle X-ray scattering (SAXS),[17] with which mainly lipid phase transitions have been investigated up to about 60 °C, as well as the properties and dimensions of the bilayer membrane. In one of these reports,[17a] an increase in size and decrease in membrane thickness of linear nanodisks at the phase transition temperature of the used lipids has been described, potentially leading to nanodisk rapture at higher temperatures. We used CD spectroscopy to selectively monitor the stability of the MSP belt up to higher temperatures.

The existence of intact nanodisks at ambient temperatures has been monitored by EM and SEC (Figure 3). We assume that MSP secondary-structure unfolding coincides with thermal disintegration of the respective nanodisk particles. We observe cooperative unfolding traces, that is, rapid CD signal change around the melting temperature of the MSP (Figure 4A), which

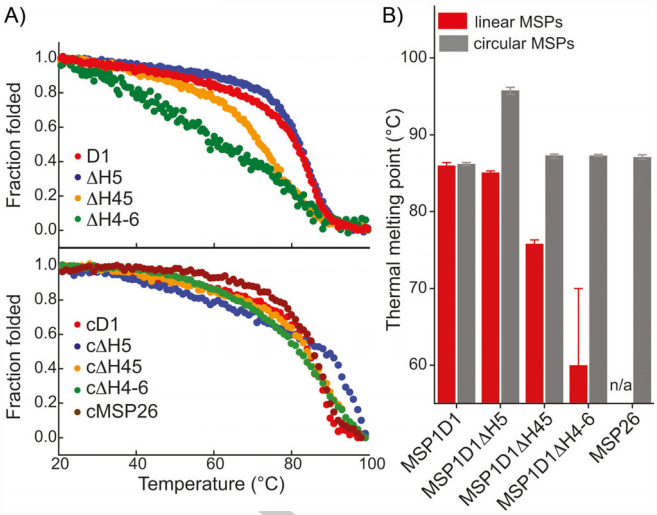

Figure 4. Thermal stability of linear versus circular nanodisks. A) CD-detected thermal melting curves detected at $\lambda = 222$ nm to monitor unfolding of the MSP α-helical secondary structure. Top panel: linear MSP; lower panel: cMSP nanodisks. B) Melting points for linear (red bars) and circular (gray bars) nanodisks extracted from the data in (A) show that circular nanodisks show higher stability, in particular, for the smaller disks that are not very stable in a linear form. The integrity of the nanodisk preparations was probed by SEC and EM (Figure 3).

is indicative of the occurrence of a compact particle. This behavior has first been demonstrated for soluble proteins in which tertiary- and secondary-structure unfolding occurs in a cooperative manner.[18]

The extracted thermal melting points in each case (Figure 4B) show that the thermal stability of the linear MSP nanodisks rapidly decreases in the case of the ΔH45 and ΔH4-6 variants, whereas their circular counterparts show equally high stability to that of the more relaxed, larger nanodisks. The cooperativity of MSP unfolding is lower for the shorter MSP variants, which suggests that these particles are not as tightly packed as the longer MSP nanodisks. cMSP1D1ΔH5 showed an increased thermal stability of 95 °C, relative to the corresponding linear MSP. Thus, the strategy of MSP circularization is highly beneficial for nanodisk stability and offers the possibility to use these nanodisks for structural investigations or for applications in which the nanodisk size needs to be tightly and stably controlled. As shown previously for linear nanodisks, the thermal melting behavior directly correlates with long-term nanodisk stability.[4a] Thus, the exceptionally high thermal stability of the presented circularized nanodisks confers long-term stability. In line with this assumption is a complete lack of sample precipitation over weeks at elevated temperatures (up to 50 °C), as well as the observation of identical NMR spectra of inserted membrane proteins.

To demonstrate the benefit of these cMSP nanodisks for solution NMR spectroscopy studies of membrane proteins, we inserted two membrane proteins of different sizes into linear and cMSP nanodisks and subsequently recorded 2D [^{15}N,^{1}H] TROSY experiments. Any other membrane protein can be inserted into these nanodisks with assembly conditions similar to those used for linear nanodisks. As for linear nanodisks,

the yields of membrane protein insertion into circularized nanodisks is highly dependent on the particular protein, its size, degree of oligomerization, and lipid composition, as recently summarized.[1b, 3a]

We used the bacterial outer-membrane protein X (OmpX, 17 kDa) and the human voltage-dependent anion channel subtype 1 (VDAC1, 32 kDa) as model systems, with which we typically observed nanodisk insertion yields of >50%. Assembly conditions and yields for a G protein-coupled receptor (GPCR) have been extensively described recently,[7] and can be directly transferred to our cMSP systems. Due to the relatively small size of OmpX, we inserted it into linear and circular MSP1D1Δ*H5* and the smallest circular MSP1D1Δ*H4*-6 nanodisks and recorded 2D NMR spectroscopy experiments. As shown in Figure 5A, the NMR spectral quality of OmpX increases from linear to circular Δ*H5* disks, for which more and sharper resonances appear in the circular nanodisk. Furthermore, in circular Δ*H4*-6 disks, OmpX shows an increased spectral quality to that of the cΔ*H5* variant. This visual judgement is corroborated by T2 relaxation experiments (TRACT[19]), which can be used to estimate the rotational correlation time of inserted isotope-labeled OmpX.

The apparent correlation time, τ_C, decreases from 35 to 21 and 18 ns in linear, circular Δ*H5*, and circular Δ*H4*-6 disks, respectively. This information indicates that NMR spectroscopy is facilitated in the circular nanodisk systems, which enables

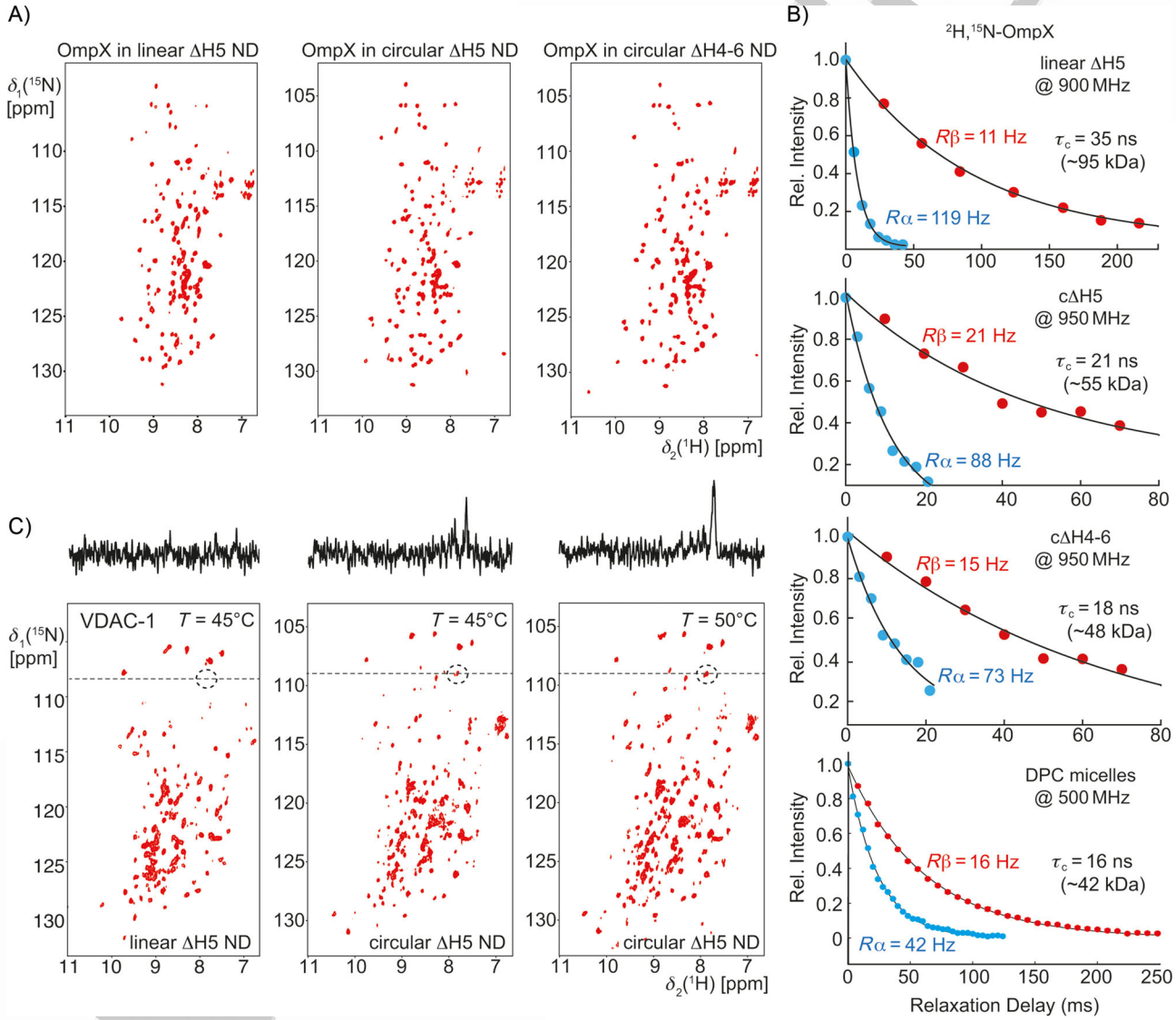

Figure 5. Circular nanodisks for high-resolution solution-state NMR spectroscopy. A) 2D [^{15}N,^{1}H] TROSY experiments of U-^{2}H,^{15}N-labeled OmpX incorporated into linear MSP1D1Δ*H5* (left) and circular MSP1D1Δ*H5* (middle) or MSP1D1Δ*H4*-6 (right) nanodisks at 45 °C show increased spectral quality for the circular nanodisks and suitability of the smallest circular nanodisk for long-term NMR spectroscopy experiments. B) Analysis of the NMR relaxation properties of OmpX in nanodisks, at 45 °C as shown in (A). [^{15}N,^{1}H] TRACT[19] experiments have been performed to estimate the rotational correlation time (τ_C) of OmpX in the indicated nanodisks. For comparison, a correlation time of OmpX in dodecylphosphocholine (DPC) micelles of 16 ns is observed. C) 2D [^{15}N,^{1}H] TROSY experiments of U-^{2}H,^{15}N-labeled VDAC-1 in the linear (left) and circular (middle) MSP1D1Δ*H5* nanodisks at 45 °C. The circular nanodisks strongly improve NMR spectral quality and provide enhanced long-term stability suitable for multidimensional NMR spectroscopy measurements at elevated temperatures of up to 50 °C (right). Top: Spectral slices along the ^{1}H axis (the broken lines in the 2D spectra) show that the signal intensity increases from linear to circular nanodisks and with increasing temperature.

high-resolution structure determination in a native membrane environment in solution. As shown in Figure 3, the size of circularized nanodisks is slightly larger than that for the linear versions. However, we assume that, due to the enhanced size homogeneity of circularized nanodisks, the NMR spectral properties are better, despite their slightly larger size. For our NMR spectroscopy experiments, we are well above the phase transition temperature of the DMPC/1,2-dimyristoyl-*sn*-glycero-3-phosphorylglycerol (DMPG) lipids. It has been shown through SAXS experiments that the diameter of linear MSP nanodisks increases above the phase transition temperature of the lipids used.[17a] Due to the restrictive nature of circular nanodisks, this behavior cannot occur in this case, leading to nanodisks of stable size over a wide range of temperatures, and thus, leading to improved NMR spectroscopy properties of these nanodisks.

We recently showed that structure determination of membrane proteins in 8 nm nanodisks was possible.[3a,4] Here, the spectral quality is even better that that reported in these previous studies, which will further facilitate the use of nanodisks for structural studies in a native lipid environment. The NMR spectral properties of the Δ*H*4-6 disks are very close to the smallest possible scenario, such as small detergent micelles. Thus, we measured TRACT experiments with OmpX in DPC micelles (micellar size ≈20 kDa) and obtained a correlation time of 16 ns at 45 °C, only slightly shorter than that in the best nanodisk case (Figure 5B). These results demonstrate that the Δ*H*4-6 disks are the smallest possible achievable nanodisk size that can be used for structural studies.

To demonstrate the use of circular nanodisks for a larger membrane protein, we inserted the VDAC1 anion channel into linear and circular MSP1D1Δ*H*5 nanodisks and recorded 2D [^{15}N,^{1}H] TROSY experiments (Figure 5C). The signal intensity and number of observed resonances in the spectra increases markedly if circular nanodisks are used instead of the linear version. Furthermore, the circular nanodisks are highly stable; thus enabling measurement at 50 °C for hours to days without sample precipitation. The quality of the TROSY spectrum of VDAC1 in circular MSP1D1Δ*H*5 nanodisks is markedly improving at higher temperature (45 vs. 50 °C), which gives rise to sharper NMR signals. The subsequent appearance of 2D NMR signals in the three cases is demonstrated in the upper half of Figure 5C by using a well-resolved signal at $\delta = 108$ ppm for the ^{15}N chemical shift, for which no signal can be observed in the linear nanodisk, whereas a signal of subsequently sharper line width can be seen in the circular nanodisk at increasing temperature. Furthermore, as mentioned above, we do not observe any precipitation of either protein in circular nanodisks, which demonstrates highly improved long-term sample stability, even at elevated temperatures.

In summary, we showed herein that cMSPs could be efficiently produced by intein splicing in living *E. coli* cells and rapidly purified within 2 days. The resulting cMSP nanodisks showed exceptional size homogeneity and thermal stability. As shown recently,[4a] the limited long-term stability of very small nanodisks (< 8 nm) restricts their use in structural biology applications. In contrast, circularization of the shortest MSP constructs yields highly stable nanodisks, as shown in thermal melting experiments. These nanodisks are thus suitable for EM and high-resolution NMR spectroscopy, and will be useful for a wide range of biophysical and biochemical applications, for which high thermal and long-term stability, as well as size restriction, is required. Apart from structural biology applications, as demonstrated herein, trapping of the monomeric versus the oligomeric state[7] of a membrane protein could be achieved with circular nanodisks. Furthermore, the herein introduced 26 nm disk will be of great value for the investigation of pore-forming proteins or large membrane proteins and their complexes by EM. In the case of heterogeneous membrane pores, circular nanodisks might be useful to restrict the size of protein oligomers and improve the homogeneity of such pores for subsequent structural analysis.

Experimental Section

Experimental details on protein production and purification, nanodisk assembly, analytical methods, and NMR spectroscopy experiments are provided in the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: electron microscopy · membrane proteins · nanostructures · NMR spectroscopy · structural biology

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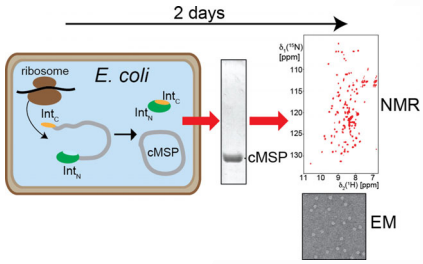
A Split-Intein-Based Method for the Efficient Production of Circularized Nanodisks for Structural Studies of Membrane Proteins



A split-intein saves nine! A versatile and easy method for the rapid and high-yield production of circularized nanodisks for membrane scaffold protein (cMSP) research based on intein splicing in living *E. coli* cells is introduced. These nanodisks offer exceptional size homogeneity and stability and are thus a robust tool for structural biology and biochemical applications.

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D.G. Conceptualization: Supporting; Investigation: Supporting; Resources: Supporting.

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