



Magic-Angle-Spinning Solid-State NMR of Membrane Proteins

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Abstract

Solid-state NMR spectroscopy (ssNMR) provides increasing possibilities to examine membrane proteins in different molecular settings, ranging from synthetic bilayers to whole cells. This flexibility often enables ssNMR experiments to be directly correlated with membrane protein function. In this contribution, we discuss experimental aspects of such studies starting with protein expression and labeling, leading to membrane protein isolation or to membrane proteins in a cellular environment. We show that optimized procedures can depend on aspects such as the achieved levels of expression, the stability of the protein during purification or proper refolding. Dealing with native membrane samples, such as isolated cellular membranes, can alleviate or entirely remove such biochemical challenges. Subsequently, we outline ssNMR experiments that involve the use of magic-angle-spinning and can be used to study membrane protein structure and their functional aspects. We pay specific attention to spectroscopic

issues such as sensitivity and spectral resolution. The latter aspect can be controlled using a combination of tailored preparation procedures with solid-state NMR experiments that simplify the spectral analysis using specific filtering and correlation methods. Such approaches have already provided access to obtain structural views of membrane proteins and study their function in lipid bilayers. Ongoing developments in sample preparation and NMR methodology, in particular in using hyperpolarization or proton-detection schemes, offer additional opportunities to study membrane proteins close to their cellular function. These considerations suggest a further increase in the potential of using solid-state NMR in the context of prokaryotic or eukaryotic membrane protein systems in the near future.



1. INTRODUCTION

Membrane proteins (MPs) are involved in a diverse range of biological functions but pose unique challenges for structural biologists. Their amphipathic, heterogeneous native environment is challenging to mimic *in vitro*, complicating not only isolation of these proteins but also the interpretation of data obtained in nonnative settings. The choice of environmental mimetic, such as detergents or synthetic bilayers, can have a significant impact on the structure, function, and stability of MPs. Solid-state NMR spectroscopy (ssNMR) provides a method by which to examine MPs in different membrane systems, ranging from synthetic bilayers to whole cells (Baker & Baldus, 2014). This flexibility enables ssNMR experiments to be directly correlated with complementary approaches probing structure on different length scales or function via functional assays or methods such as electrophysiology.

As with other spectroscopic methods, sensitivity is a critical factor for NMR studies on MPs. Unless specific experimental conditions are established (such as low temperature; Rocchigiani, Ciancaleoni, Zuccaccia, & Macchioni, 2011) and/or hyperpolarization methods are used, it is not uncommon for a single ssNMR sample to contain 5–10 mg of the MP of interest as well as environmental molecules such as water and lipids. At the same time, “NMR-active” nuclei such as (^{13}C or ^{15}N , respectively) must be incorporated into the molecule, usually by adding isotopically labeled molecules to the growth medium. Separate from the issue of sensitivity, spectral resolution in ssNMR studies on MPs is most easily established by conducting experiments under magic-angle-spinning (MAS) ssNMR. Under MAS, a randomly packed sample is rotated about an axis at an angle of 54.7° relative to the magnetic field (the “magic angle”), at speeds ranging

from 1 to 100 kHz, to average out some of the interactions and mimic the effects of molecular tumbling. The rest of this chapter will assume the use of MAS ssNMR, as it is applicable to a wide range of specimens.

Figure 1 summarizes the overall workflow for MAS ssNMR experiments on MPs. Although each step will be discussed in detail below, the choices made at each step have repercussions throughout, so it is worthwhile to

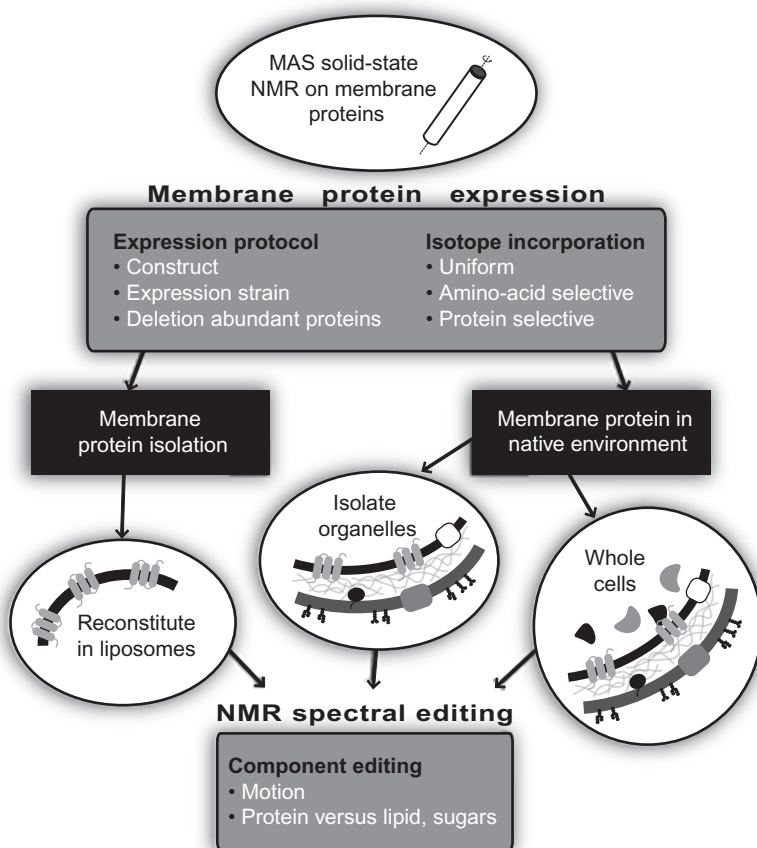
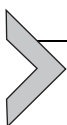


Figure 1 Experimental pathways for magic-angle-spinning solid-state NMR of membrane proteins. In general, the first step is to establish expression conditions for the protein of choice. Since NMR requires significant quantities of isotope-labeled protein, this step will determine the outcome of subsequent steps. After suitable expression has been obtained, a variety of samples can be prepared, using synthetic or native membranes. Specific NMR experiments can be used to target specific types of molecules in heterogeneous membrane samples.

consider the experimental path as a whole. In particular, the choice of membrane system for the final sample will depend on the achieved levels of expression, the stability of the protein during purification, and the need for refolding (such as for the purification of overexpressed β -barrels from inclusion bodies discussed below). These considerations underline the fact that there is no single general approach that works best for every MP.

With sufficient expression levels of folded protein, correctly inserted into the membrane, native membrane samples, such as isolated cellular membranes, can remove many of the biochemical challenges associated with purifying large quantities of MPs. This approach also has the potential to reduce the volume of cell culture required to produce samples (Baker, Daniëls, van der Cruijssen, Folkers, & Baldus, in preparation), significantly reducing costs. In addition, the choice of membrane system will influence the type of NMR experiments that are best suited to the system—for example, spectroscopic editing of lipid signals from fully isotopically labeled native membrane samples.



2. PRODUCTION OF RECOMBINANT PROTEINS IN *ESCHERICHIA COLI*

Production of recombinant proteins in *E. coli* is a well-established (Studier, Rosenberg, Dunn, & Dubendorff, 1990) and frequently summarized method for preparation of protein samples for structural and functional studies (Acton et al., 2011; Kubicek, Block, Maertens, Priestersbach, & Labahn, 2014). We will therefore focus on techniques specifically useful for the preparation of MP samples for ssNMR.

2.1 Optimization of expression conditions using small-scale cultures

The choice of expression conditions can have a significant impact on the yield of MPs, either correctly folded in the native membrane environment or in inclusion bodies. In a benchmark study for a set of soluble proteins, it was observed that relatively subtle changes in expression protocols can easily change expression levels by an order of magnitude (Berrow et al., 2006). Since expression parameters are not independent, multidimensional optimization of expression conditions is required, and small-scale cultures serve as a convenient way to test many dependent conditions. In our experience, small-scale expression screening experiments serve as a good predictor for

Table 1 Optimization parameters for expression of MPs in *E. coli*

	Starting condition	Possible variations
Bacterial strain	BL21 Rosetta2	BL21, Lemo21, C41/C43, Origami, KRS
Culture medium	M9	LB, SB/TB, autoinduction media
Induction (OD ₆₀₀)	0.6–0.8	0.25–2.0
Temperature (°C)	18	16, 20, 28, 37
Induction time (h)	12	1–24

large-scale sample production, provided that the conditions used for screening closely mimic the production process (Folkers, van Buuren, & Kaptein, 2004). In Table 1 and below, we summarize some of the culture conditions that may have a large effect on recombinant protein expression. For a more detailed protocol for isotope labeling and bacterial growth, see (Studier, 2005, 2014).

2.1.1 Bacterial strain

The choice of bacterial strain can critically affect both expression and solubility. For the expression of eukaryotic proteins in *E. coli*, it is crucial to use strains that overexpress tRNAs that are frequently used in eukaryotes but not in *E. coli* (e.g., Rosetta, EMD Millipore). The so-called Walker strains C41 (DE3) and C43(DE3), derived from BL21 cells, often permit expression of MPs in high amounts with lower toxicity (Miroux & Walker, 1996). A study by Wagner et al. (2008) suggested that mutations in these cells affect the expression of the T7 polymerase, leading the authors to engineer a BL21-derived strain called Lemo21 (New England Biolabs), which allows for regulation of T7 polymerase activity via controlled expression of T7 lysozyme, its natural inhibitor. However, the gene for T7 lysozyme is under the control of a rhamnose-inducible promotor that is inhibited by glucose, complicating its use when growing in M9 media. We have seen improved expression in Lemo21 cells in M9 cultures for a MP, but only under specific culture conditions.

For cellular preparations, a BL21 strain that is deficient in the genes for the highly abundant outer MPs OmpA and OmpF can be used to reduce background signals (Renault, Tommassen-van Boxtel, et al., 2012). However, the use of this strain is not needed when specific labeling is achieved by treatment with rifampicin (as described in Section 2.2).

2.1.2 Preculture conditions

Since MPs are often toxic for the host strain, it is important to prevent expression of the protein prior to induction (see also [Studier, 2014](#)). In most cases, both T7 polymerase and recombinant protein expression are suppressed by the lac repressor. To keep the lac repressor in its active state, 0.5–1% (w/v) glucose can be added during transformation and culturing. Similarly, we found that for toxic proteins, bacteria kept in the exponential growth phase at low densities throughout all precultures exhibit better expression levels during induction ([Romanuka, van den Bulke, Kaptein, Boelens, & Folkers, 2009](#)).

2.1.3 Culture medium

As described above, isotope labeling is required for sample preparation for NMR and expression is normally done in a defined minimal medium. As such, we generally perform expression screening in minimal medium. Although the overall yield per liter in richer media (Luria Broth (LB), Super Broth, Terrific Broth) is often better, the amount of correctly folded protein per cell is not necessarily so. It is possible that the slower growth rate in minimal media enables more effective protein folding, increasing the expression yield.

For MPs, uniformly labeled samples often are too complex and crowded for resonance assignment. More sophisticated labeling schemes have been described previously ([Filipp, Sinha, Jairam, Bradley, & Opella, 2009](#); [Verardi, Traaseth, Masterson, Vostrikov, & Veglia, 2012](#)), but general strategies include amino acid specific labels and specific ^{13}C incorporation via metabolic precursors. When adding labeled amino acids to the growth media, it is important to consider the metabolic pathways associated with each amino acid. For example, threonine can be converted to isoleucine via α -ketobutyrate; to label threonine but not isoleucine, $^{12}\text{C}^{14}\text{N}$ isoleucine may be added to the media in addition to $^{13}\text{C}^{15}\text{N}$ threonine. For a summary of the metabolic interconversion of amino acids, please see (e.g., [fig. S5, Sinnige et al., 2014](#)). In our experience, addition of amino acids to M9 cultures can change growth patterns and expression levels, and expression conditions might need to be reoptimized. Amino acid labeling schemes also may use combinations of singly labeled amino acids (i.e., only ^{13}C or ^{15}N labeled) for assignment of specific sequential correlations (i.e., between ^{13}C in residue (i) and ^{15}N in residue ($i+1$)). Spectra can also be simplified using

specifically labeled metabolic precursors as a carbon source, such as glycerol labeled at either the C2 or the C1/3 positions. These strategies results in each amino acid ^{13}C labeled at specific positions (Filipp et al., 2009).

Lastly, introducing deuterons via D_2O and/or appropriate precursors provides additional routes in ssNMR spectroscopy, from the level of spectral editing to ^1H detection schemes to the analysis of water molecules (see Section 5). In general, the doubling time of *E. coli* in D_2O is significantly slower than in H_2O , and an additional preculture in D_2O should be included to give the bacteria time to adapt before induction of protein expression. D_2O can be introduced between the LB and M9 cultures as deuterated LB or subsequent to the M9 H_2O culture (see below).

2.1.4 Expression conditions

Our experience with both soluble (Berrow et al., 2006) and MPs (Fig. 2) reveals that both expression levels and amount of folded protein can be optimized by changing the timing of induction according to the growth phase of the bacteria. Although induction at an OD_{600} of 0.6–0.8 serves

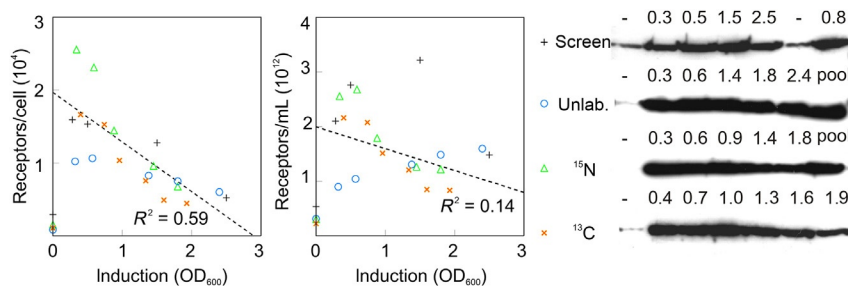


Figure 2 Effect of growth phase on the expression of membrane protein expression. The GPCR NTR1 (UniProt ID: [P20789](#)) was expressed in *E. coli* DH5 α and induced at various densities (OD_{600}) and various media: small-scale expression screening in unlabeled M9 medium (+), or in 2-L flasks containing 500 mL unlabeled M9 medium (○). ^{15}N -enriched medium (Δ) or ^{13}C 15N-enriched medium (×). The left two panels show the number of receptors as quantified by a radioligand-binding assay. The left panel shows the number of receptors per cell, while the middle panel shows the number of receptors per mL of culture. Both the total amount of protein/mL cell culture as well as the amount of protein per cell is negatively affected by the OD_{600} of induction. The right panel shows a Western blot of the total expression of an equivalent volume of cell culture for each condition, as a function of cell density at the time of induction (OD_{600}) as labeled above each row. The label “pool” represents a combined “average” sample from all expression conditions.

as a good starting point, induction earlier or later in the exponential phase, and occasionally in the stationary growth phase, can result in better expression and folding. Optimization of this condition is particularly important for NMR of native membranes, since the amount of folded protein per cell, not the total amount of protein in a culture, is critical. The length of time between induction and harvesting also needs to be optimized, especially for toxic proteins where cultures can stop growing almost immediately after induction. Lastly, growth temperature at induction, as well as the concentration of the induction agent (such as IPTG), should be optimized. Slower growth due to lower temperature can be beneficial for MP folding.

2.1.5 Evaluation of results

To evaluate small-scale expression screening, SDS-PAGE can be used to compare the total and membrane fractions of a lysed cell culture in a detergent-containing lysis buffer. If expression levels are low, or the MP of interest stains poorly, Western blotting can be used. Ideally, a functional assay or ligand-binding assay would be used to quantify the amount of properly folded MP produced, providing a quantitative optimization target.

2.1.6 Protocol: Generalized small-scale expression tests

- (1) Transformation: plate on LB + antibiotics + glucose—overnight
- (2) Preculture: a few colonies, each in 2 mL LB + antibiotics + glucose—4–6 h
- (3) Media change: once cells have reached OD₆₀₀ of 0.5–1.0, centrifuge for 3 min at 6000 rpm in a benchtop minicentrifuge and remove supernatant.
- (4) Preculture: resuspend cells in M9 + antibiotics, and split into 3 × 25 mL cultures. Grow one culture at 20, 30, and 37 °C overnight.
- (5) For subsequent experiments, the precultures with an OD₆₀₀ of ~1.0 can be used to inoculate 50 mL freshly prepared M9 at OD₆₀₀ 0.05–0.1.
- (6) This preculture is then split evenly into 10 × 5.0 mL in 50-mL Greiner tubes, each with a different expression condition.
- (7) Approximately 30 min prior to induction, cultures are transferred to the temperature used for induction, and when the required OD₆₀₀ is reached, expression is started by adding inducers such as IPTG.
- (8) Harvest cells at different time points after induction by centrifuging at 4000 × *g* for 15 min.

- (9) Resuspend 100 μL of cell culture in 100- μL SDS sample buffer per OD_{600} 1.0 culture density, for SDS-PAGE analysis. The remainder of the culture can be used for small-scale cell envelope preparation, lysis in a suitable buffer or functional characterization.

2.2 Selective isotope incorporation

For preparation of NMR samples of MPs in their native cellular environments, it is desirable that only the protein of interest be isotopically labeled, and the cellular background be NMR silent. Rifampicin, an antibiotic that targets the native *E. coli* RNA polymerase (Di Mauro et al., 1969; Wehrli, Knüsel, Schmid, & Staehelin, 1968), and the single-protein production (SPP) system (Suzuki, Zhang, Liu, Woychik, & Inouye, 2005), which makes use of the highly specific endoribonuclease MazF, both inhibit background protein production prior to translation and have been used for NMR in both the solution (Almeida et al., 2001; Mao et al., 2009) and solid states (Baker et al., in preparation; Mao et al., 2011). The SPP method requires replacement of any ACA sequences in the mRNA of the target protein to avoid degradation, while the rifampicin method requires use of a nonbacterial RNA polymerase (such as the T7 system) for expression. A detailed protocol for the use of the SPP system exists (Suzuki, Mao, & Inouye, 2007). Since the rifampicin procedure is applicable for the generally used T7 expression system, we will focus here on this method. This protocol has been used to produce cellular membrane samples of YidC from *E. coli* (UniProt ID: P25714) and KcsA from *S. lividans* (UniProt ID: P0A334) for ssNMR (Baker et al., in preparation).

2.2.1 Protocol: Selective isotope incorporation for native membrane samples

- (1) Preculture: 5-mL LB + antibiotics—8 h at 37 °C
- (2) Preculture: 50-mL M9 + antibiotics—overnight at 37 °C
- (3) (If deuteration desired, 50–100 mL preculture in D_2O M9 + antibiotics) *optional*
- (4) Culture: 50–500 mL M9 + antibiotics— ^{12}C , ^{14}N , $^{1/2}\text{H}$
 - a. Inoculate at $\sim\text{OD}$ 0.1 and grow to $\text{OD} \sim 1.8$
 - b. Pellet cells ($4000 \times g$, 10–15 m, 20 °C)
 - c. Replace media with appropriately isotopically labeled M9 + antibiotics

- d. Add IPTG to 1 mM final concentration and grow 15–30 min at 25–28 °C
- e. Add rifampicin to 100 µg/mL final concentration and grow at 25–28 °C overnight, shielded from light¹

For solid-state NMR of MPs, an additional aspect that needs to be taken into account arises for native membrane samples prepared with either rifampicin or the SPP method. Labeling with ¹³C–glucose results in labeled lipids in the cellular membranes, resulting in high background levels in experiments not involving ¹⁵N. The high background levels can be overcome spectroscopically (using magnetization transfer via ¹⁵N—see [Section 5](#)), or by labeling via amino acids instead of glucose and ammonium chloride, or by the use of cerulenin, which inhibits phospholipid biosynthesis ([Mao et al., 2011](#)).



3. ISOLATION OF CELLS AND CELLULAR MEMBRANES

3.1 From cells to ssNMR samples of MPs

All ssNMR samples begin with a similar isolation protocol. The differences for whole cell, native membrane, purified membranes, and purified and reconstituted samples are indicated in the general protocol below.

3.1.1 Protocol: Isolation of cells and membranes

- (1) Harvest cells by centrifugation at $4000 \times g$ for 15 min at 4 °C.
- (2) Resuspend cells in ~5–10 mL (depending on cell volume) of an ice-cold buffer of choice (e.g., 50 mM Tris pH 7.4, 100 mM NaCl, or phosphate-buffered saline (PBS)). Cells may be frozen at –80 °C at this point for several months.

Note: Whole cells may be washed with PBS by repeating the centrifugation (step 1) and packed into a ssNMR rotor.

- (3) Lyse cells with a chilled (4 °C minimum 1 h prior to lysis) French press (8000 psi)—it normally takes about four cycles for complete lysis (can be monitored by measuring OD₆₀₀)
- (4) Pellet cell debris at $8000 \times g$ for 15 min.
 - a. Optional step: Isolation of inclusion bodies for further purification by centrifugation at $25,000\text{--}100,000 \times g$ for 15 min.
- (5) Pellet membranes at $\sim 100,000 \times g$ for 1 h

¹ *Note:* rifampicin is light sensitive—keep stock solutions and bacterial cultures covered in aluminum foil.

Note: At this stage, membranes are ready for protein purification, if desired (see [Section 4](#) for a discussion of next steps for purified protein samples).

- (6) Wash membranes in ~ 20 -mL 10 mM phosphate buffer (e.g., pH 6.8)
- (7) Pellet membranes at $\sim 100,000 \times g$ for 1 h.

Note: At this stage, membranes are ready for separation of inner and outer membranes, if desired (see below for a discussion of next steps for membrane separation).

- (8) Resuspend in 1-mL 10 mM phosphate buffer (e.g., pH 6.8).
- (9) Pellet membranes at $\sim 125,000 \times g$ for 2–3 h.
- (10) Remove supernatant and pack into a ssNMR MAS rotor for native membrane experiments.

3.2 Purification of specific membranes

If expression levels in native membrane samples are limiting, further purification of the membranes containing the MP of interest can improve the sensitivity of ssNMR experiments. Inner and outer *E. coli* membranes can be separated by the following protocol.

3.2.1 Protocol: Separation of inner and outer bacterial membranes

- (1) Harvest membranes as described above (steps 1–7).
- (2) Prepare a sucrose gradient in 50 mM Tris pH 8.0 buffer. For a 27-mL gradient, layer:
 - a. 2 mL of 55% (w/v) sucrose
 - b. 8 mL of 51% (w/v) sucrose
 - c. 8 mL of 45% (w/v) sucrose
 - d. 5 mL of 36% (w/v) sucrose
 - e. Membranes resuspended in 4 mL of 20% (w/v) sucrose
- (3) Centrifuge overnight at $100,000 \times g$, preferably in a swinging bucket rotor (e.g., SW32-Ti (Beckman)). A fixed angle rotor will result in angled layers that will need to be kept flat.
- (4) Harvest with a syringe:
 - a. Outer membranes at the interface of the 55% and 51% sucrose steps.
 - b. Inner membranes at the interface of the 45% and 36% sucrose steps.
- (5) Wash membranes in ~ 20 -mL 10 mM phosphate buffer (pH 7).
- (6) Pellet membranes at $\sim 100,000 \times g$ for 1 h and discard supernatant.
- (7) Repeat steps 5 and 6 to remove any sucrose and Tris from the samples.

- (8) Resuspend in 1-mL 10 mM phosphate buffer (pH 6.8)
- (9) Pellet membranes at $\sim 125,000 \times g$ for 2–3 h
- (10) Remove supernatant and pack into a ssNMR MAS rotor for native membrane experiments.

In our experience, after running sucrose gradients with membrane preparations from *E. coli* BL21-derived cell lines grown in M9, an additional band is observed at the interface between the 51% and 45% sucrose steps. This band seems to be composed of a mixture of inner and outer membranes and reduces the amount of purified components that can be obtained. This band is not observed from membrane preparations of these cell lines grown in LB.



4. PURIFICATION AND RECONSTITUTION OF MPs FOR ssNMR

Although unnecessary for native membrane samples, purification and reconstitution of MPs can provide valuable insight into the stability and environmental sensitivity of the MP of interest. As each protein requires a different treatment during purification, we only provide guidelines for parts of the process that are consistent for two broad classes of MPs: those that are produced folded in membranes, and those produced in inclusion bodies. Detailed discussions of chromatography of MPs, for example, can be found elsewhere (Asenjo & Andrews, 2009; Crowe et al., 1994; Saraswat et al., 2013).

4.1 Detergent solubilization of folded MPs

Once membranes have been harvested (see [Section 3.1.1](#)), they are solubilized with detergent to produce soluble protein-detergent complexes that can then be purified by traditional chromatography. In general, solubilization is done with high detergent concentrations, which can be an important cost factor, and it is worth optimizing solubilization conditions to improve protein yield and efficiency. [Table 2](#) below summarizes conditions that can be varied during solubilization and gives starting conditions to try for new target MPs. As for optimization of expression conditions, the best conditions are most easily determined by comparison of yields by SDS-PAGE or functional assays. Many of the parameters that affect solubilization will also affect the stability of the protein during purification; however, the same conditions might not be best for both steps. If protein stability is an issue, consider a second optimization for the subsequent purification process.

Table 2 Common parameters for optimization of detergent solubilization of folded MPs

	Starting condition	Possible variations
Type of detergent	Dodecyl maltoside	CHAPS, Triton, Digitonin, decyl maltoside
Detergent concentration	10 × critical micelle concentration (CMC)	5–20 × CMC
Solubilization time	2 h	30 min—overnight
Solubilization temperature	4 °C	4–25 °C
Salt concentrations	250 mM NaCl	0–500 mM NaCl, KCl, MgSO ₄
Glycerol concentration	10% (v/v)	0–30% (v/v)

4.2 β -Barrel purification and refolding

Overexpression as cytoplasmic inclusion bodies in *E. coli* can lead to high-recombinant protein yields. Commonly, β -barrel proteins can be refolded from a denatured state into detergent micelles or preformed lipid bilayers. In general, inclusion bodies are solubilized in a denaturing agent and diluted into a refolding buffer-containing detergent. However, buffer conditions and refolding protocols need to be established to obtain a sufficiently good yield of refolded protein. Table 3 shows common parameters to optimize during denaturation and refolding. In the choice of detergent, the critical micelle concentration (CMC) should be taken into account—the CMC should be sufficiently high to enable efficient removal of the detergent during the subsequent reconstitution in proteoliposomes (see Section 4.3). In addition, the amount of detergent required for optimal refolding should be cost efficient. Varying the concentration of detergent in the refolding buffer as well as the dilution factor of the unfolded protein into this buffer can help mitigate potential expenses.

β -Barrel proteins have the additional advantage that folded and unfolded protein can be discriminated on (regular) SDS-PAGE or in some cases on semisensitive SDS-PAGE, a native gel that is run on ice in the presence of 0.2% SDS in the sample buffer and 0.075% SDS in the running buffer. The folded protein migrates faster than heat-denatured species (see Dekker, Merck, Tommassen, & Verheij, 1995; Heller, 1978; Nakamura & Mizushima, 1976; Robert et al., 2006), quantification of the band intensities leads to estimates of the refolding yield (Fig. 3A).

Table 3 Common parameters for optimization of β -barrel refolding

	Starting condition	Possible variations
Denaturant for inclusion body solubilization	8 M urea	6 M guanidine hydrochloride
Denatured protein concentration	100 μ M	20–500 μ M
Type of detergent	<i>N</i> -dodecyl- <i>N,N</i> -dimethylamine- <i>N</i> -oxide (LDAO)	Octyl glucoside, tetraethylene glycol monoethyl ether (C8E4), diheptanoyl phosphocholine (DHPC), dodecylphosphocholine (DPC), sulfobetaine 12 (SB12)
Detergent concentration	10 \times critical micelle concentration (CMC)	5–20 \times CMC
Dilution speed	Fast (rapid addition with stirring)	Slow (drop-wise addition with stirring)
Dilution factor (protein solution:refolding buffer)	1:10	1:2–1:100

4.3 Reconstitution of detergent solubilized MPs into lipid bilayers

Protein can be reconstituted in proteoliposomes by addition of lipids and removal of the detergent. For the latter, biobeads that absorb the detergent or dialysis are most commonly used. In some cases, biobeads do not result in the appearance of proteoliposomes that are sufficiently large for ssNMR sample preparation, possibly due to the quick removal of the detergent. Dialysis has the disadvantage that it takes longer to remove the detergent compared to biobeads, but depending on the MP this may be the preferential method.

4.3.1 Protocol: Reconstitution of detergent-solubilized MPs into lipid bilayers

- (1) Remove any aggregates by centrifugation for 20 min at $4000 \times g$ and 4 °C.
- (2) If necessary, concentrate the protein with a centrifuge concentrator (Millipore) and remove aggregates by centrifugation for 20 min at $4000 \times g$ and 4 °C.

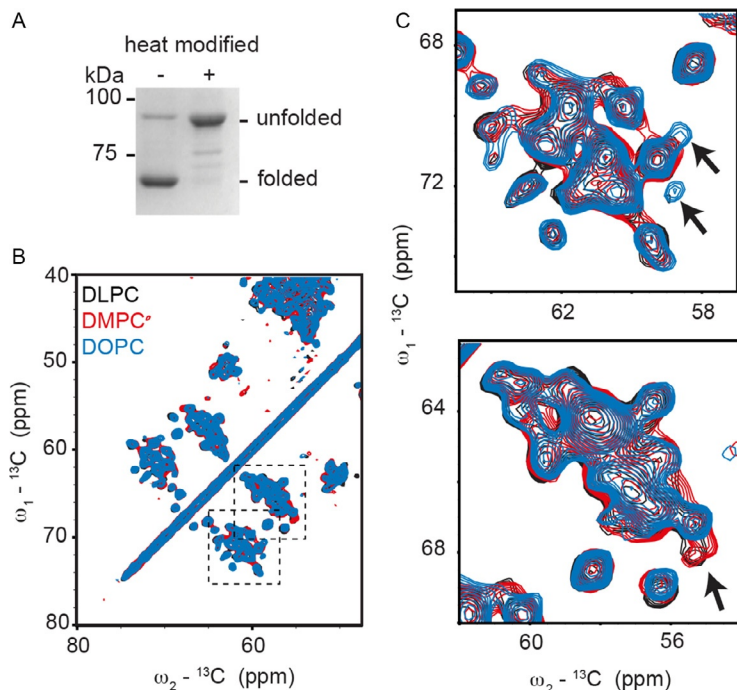


Figure 3 Proteins with β -barrel folds such as BamA (β -barrel assembly machinery A, UniProt ID: [P0A940](#)) from *E. coli* can be studied by ssNMR. (A) Folding of BamA analyzed on semisensitive SDS-PAGE. BamA reconstituted in DOPC vesicles at a molar lipid: protein ratio of 25:1 is shown. Native, folded BamA shows a characteristic shift of the electrophoretic mobility from 88 to 70 kDa, compared to heat-denatured protein. (B) and (C) Comparison of BamA reconstituted in different lipid bilayers. Shown in (B) is a section of a 2D ^{13}C , ^{13}C correlation experiment with 30 ms PARIS mixing of BamA in DLPC (black), DMPC (red), and DOPC (blue) at a molar lipid: protein ratios of 25:1. (C) Close-ups of the boxed regions in (B) reveal small differences in certain regions of the spectra.

- (3) Dry the lipids (such as *E. coli* lipids) in chloroform under a stream of nitrogen, followed by >1 h vacuum drying.
- (4) Take up the lipid film in 1-mL reconstitution buffer and incubate the lipids for 5 min at 37 °C (necessary for certain lipids to reach the liquid crystalline phase).
- (5) Resuspend the lipids thoroughly by vortexing to form multilamellar vesicles.
- (6) Add the lipids to the detergent-solubilized protein (the suspension should become clear as the detergent micelles solubilize the lipids).

- (7) (a) If removing detergent with dialysis, add reconstitution buffer to dilute below the CMC of the detergent and incubate 30 min at 37 °C.
- (b) If using biobeads, wash the correct amount (according to manufacturer's recommendations for the amount of detergent in the sample) of beads 3× with water and then 3× with reconstitution buffer and add the beads to the protein and lipid solution.
- (8) (a) Dialyze against reconstitution buffer (optional if using biobeads) at room temperature or 4 °C for 1–7 days, changing the dialysis buffer once or twice a day, until no more detergent (bubbles or foam) is observed when replacing the buffer. The protein–lipid solution has become turbid.
- (b) If using biobeads, separate the biobeads from the protein–lipid suspension on a gravity flow column.
- (9) Harvest the proteoliposomes by ultracentrifugation for 1–2 h at 100,000–125,000 × *g* and 4 °C and pack into a MAS rotor.

For reconstitution, it is useful to test a variety of pure lipids and lipid mixtures (see Fig. 3B and C, such as *E. coli* polar lipid extract). The lipid-to-protein ratio, as well as the composition of the reconstitution buffer (pH, addition of salts) should also be screened to give optimal results. Reconstitution yields can be checked on SDS-PAGE by comparing the amount of protein in the proteoliposome pellet and that in the supernatant. For β-barrels, the amounts of folded protein can be estimated on (semimative) SDS-PAGE as described above. Ultimately, however, ssNMR experiments such as a 2D ¹³C, ¹³C correlation experiment are most suited (see Section 5) to judge the success of sample preparation. It is not unusual for the best samples to yield ¹³C line widths of 0.7–1 ppm. An example is shown in Fig. 4 for the bacterial KcsA channel that was reconstituted in Asolectin. The (¹³C, ¹³C) correlation spectrum was obtained using proton-driven spin diffusion with a mixing time of 30 ms (Fig. 4A and 4B) shows results of an NCA experiment (see, e.g., Ref. Weingarth et al. (2014) and references therein).



5. DEDICATED ssNMR EXPERIMENTS

In the last decade, a series of multidimensional ssNMR correlation experiments have been developed to obtain resonance assignments and structurally characterize solid-phase proteins. In principle, all these

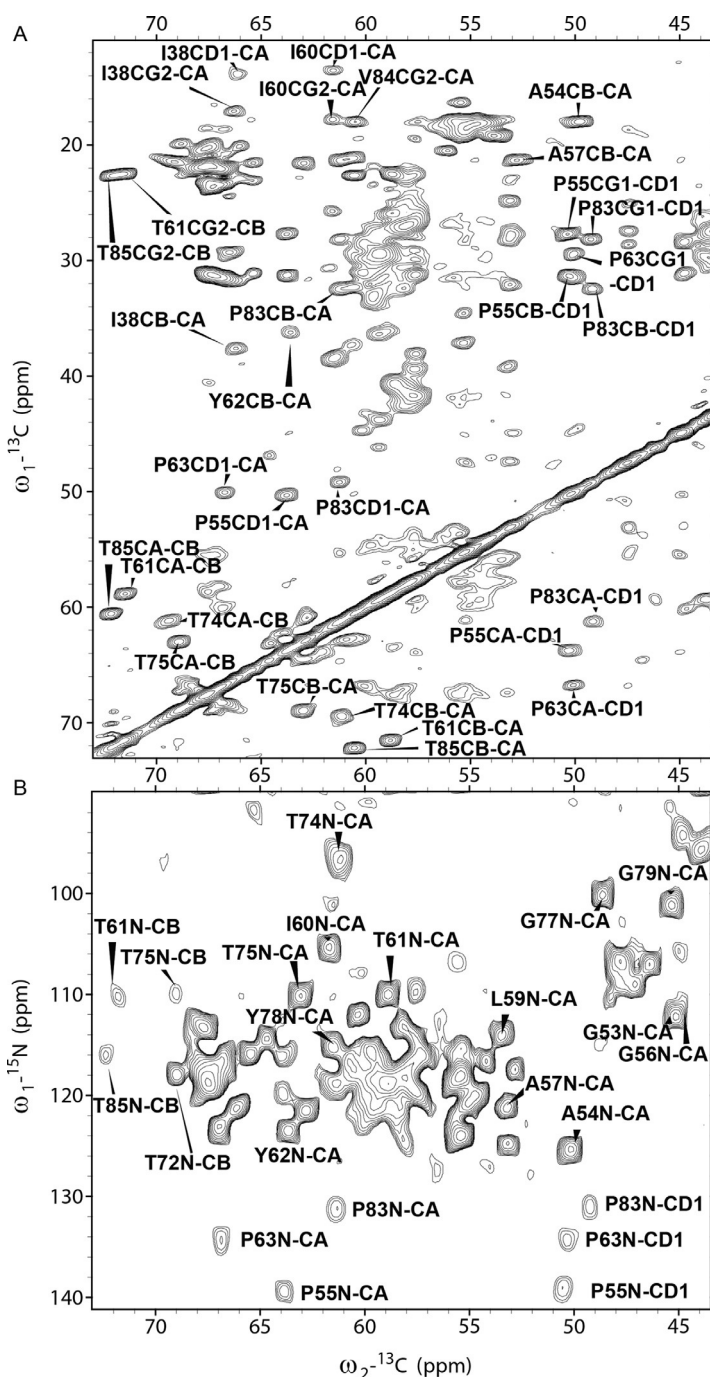


Figure 4 High resolution spectra of U[¹³C, ¹⁵N] labeled KcsA reconstituted in asolectin lipids. (A) 2D ¹³C, ¹³C correlation experiment recorded with 600 μ s CP time, 30 ms proton-driven spin diffusion, and 10.921 KHz MAS. (B) 2D NCA spectrum recorded with 600 μ s CP time, 3.2 ms SPECIFIC CP time. Both spectra were recorded at 700 MHz at 273 K.

experiments are applicable to MPs embedded in synthetic membranes or when associated with cellular membranes/organelles or cells as described above.

To this end, most ssNMR-studies have used experiments that correlate N–C moieties or C–C spin networks in two, three, or even four spectral dimensions. With such techniques several 3D structures have been obtained and functional aspects of MPs have been elucidated (see, e.g., (Baker & Baldus, 2014) for a recent review). More recently, proton detected experiments have been used for MPs embedded in synthetic bilayers (Linser et al., 2011; Weingarth et al., 2014), and already helped to understand structural aspects related to their mode of action.

With increasing protein size or the presence of endogenous proteinaceous or nonproteinaceous components, dedicated signal filtering and spectral simplification methods become an important aspect in ssNMR. As discussed in Section 2, signal overlap or unwanted molecular contributions may be suppressed by dedicated preparation procedures. Alternatively, the use of ^{15}N -edited dimensions greatly reduces the spectral components in nonproteinaceous cellular samples, for example, leaving ^{13}C spectra largely containing protein signals or, depending on the experimental conditions and labeling procedures, containing labeled lipids (Cukkemane & Baldus, 2013) or nucleotides (Renault, Pawsey, et al., 2012). Another powerful means to deduce structural information in large integral MPs or to zoom on specific molecular components in native preparations relates to solid-state NMR filtering approaches. Different motional scales often characterize signals from water-exposed and transmembrane protein segments. Accordingly, mobile protein loops and rigid transmembrane segments might be detected using through-bond and through-space transfer pathways, respectively (Andronesi et al., 2005). Finally, spectral complexity can also be reduced by “divide-and-conquer” approaches. Here, NMR data are obtained on reference samples such as in synthetic bilayers (Renault, Tommassen-van Boxtel, et al., 2012) or in solution to aid the analysis of membrane-embedded MPs by ssNMR. We have used such methods in the case of membrane-embedded histidine kinases (Etzkorn et al., 2008), retinal complexes (Etzkorn et al., 2010) or, more recently for the outer MP BamA (Sinnige et al., 2015).

On the other hand, correlating signals for the MP of interest to molecular interaction partners, or the surrounding cellular setting offers a unique opportunity to study the supramolecular structure of MPs or the functional roles. As we have described recently, there are a variety of experimental

approaches that have been successfully applied to, mostly, synthetic bilayer preparations. Combining such experimental data with information from computational resources such as docking or molecular dynamics simulations further enhances the possibility to study MPs and their functional aspects (Weingarth & Baldus, 2013).



6. CONCLUSIONS

The protocols and tips provided in this chapter are only a starting point for studying MPs by ssNMR. One of the strongest advantages of solid-state NMR is its flexibility, and experiments can be designed to investigate highly specific questions unique to each biological system. In addition to this experimental flexibility, there is also the potential to compare a reconstituted system, where tight control over the molecular constituents is possible, to a native system such as a cellular membrane, where biological relevance is ensured but heterogeneity must be addressed. Although we restricted our discussion here to bacterial MP preparations, reports in the literature (Goncalves et al., 2013) as well as ongoing work in our own laboratory suggest that future ssNMR studies of MP will also be in many cases possible for eukaryotic systems.

As with other spectroscopic methods, sensitivity and spectral resolution are a critical factor. However, the development of hyperpolarization methods that were already demonstrated in the context of cellular ssNMR studies (Renault, Pawsey, et al., 2012) and the further development of ultra-high field NMR systems are likely to further improve the prospects of studying complex MP systems by ssNMR under *in situ* conditions.

ACKNOWLEDGMENTS

Work done in the authors' laboratories and described in this review was funded in part by the Max-Planck society, the DFG, NIH, and NWO (grant numbers 700.11.344 and 700.58.102). L. B. thanks the International Human Frontier Science Program for a Long Term Fellowship. We thank Mark Daniëls for his expertise in the wetlab.

REFERENCES

- Acton, T. B., Xiao, R., Anderson, S., Aramini, J., Buchwald, W. A., Ciccocanti, C., et al. (2011). Preparation of protein samples for NMR structure, function, and small-molecule screening studies. *Methods in Enzymology*, 493, 21–60. <http://dx.doi.org/10.1016/B978-0-12-381274-2.00002-9>.
- Almeida, F. C., Amorim, G. C., Moreau, V. H., Sousa, V. O., Creazola, A. T., Américo, T. A., et al. (2001). Selectively labeling the heterologous protein in *Escherichia*

- coli for NMR studies: A strategy to speed up NMR spectroscopy. *Journal of Magnetic Resonance*, 148(1), 142–146.
- Andronesi, O. C., Becker, S., Seidel, K., Heise, H., Young, H. S., & Baldus, M. (2005). Determination of membrane protein structure and dynamics by magic-angle-spinning solid-state NMR spectroscopy. *Journal of the American Chemical Society*, 127, 12965–12974. <http://dx.doi.org/10.1021/ja0530164>.
- Asenjo, J. A., & Andrews, B. A. (2009). Protein purification using chromatography: Selection of type, modelling and optimization of operating conditions. *Journal of Molecular Recognition*, 22(2), 65–76. <http://dx.doi.org/10.1002/jmr.898>.
- Baker, L. A., & Baldus, M. (2014). Characterization of membrane protein function by solid-state NMR spectroscopy. *Current Opinion in Structural Biology*, 27, 48–55.
- Baker, L. A.; Daniëls, M.; van der Cruijssen, E. A. W.; Folkers, G. E.; & Baldus, M. (in preparation). Improved cellular solid-state NMR of membrane proteins with specific target labeling.
- Berrow, N. S., Büsow, K., Coutard, B., Diprose, J., Ekberg, M., Folkers, G. E., et al. (2006). Recombinant protein expression and solubility screening in *Escherichia coli*: A comparative study. *Acta Crystallographica. Section D Biological Crystallography*, 62(Pt 10), 1218–1226. <http://dx.doi.org/10.1107/S0907444906031337>.
- Crowe, J., Döbeli, H., Gentz, R., Hochuli, E., Stüber, D., & Henco, K. (1994). 6xHis-Ni-NTA chromatography as a superior technique in recombinant protein expression/purification. *Methods in Molecular Biology (Clifton, N.J.)*, 31, 371–387. <http://dx.doi.org/10.1385/0-89603-258-2:371>.
- Cukkemane, A., & Baldus, M. (2013). Characterization of a cyclic nucleotide-activated K(+) channel and its lipid environment by using solid-state NMR spectroscopy. *Chembiochem: A European Journal of Chemical Biology*, 14(14), 1789–1798. <http://dx.doi.org/10.1002/cbic.201300182>.
- Dekker, N., Merck, K., Tommassen, J., & Verheij, H. M. (1995). In vitro folding of *Escherichia coli* outer-membrane phospholipase A. *European Journal of Biochemistry/FEBS*, 232(1), 214–219.
- Di Mauro, E., Synder, L., Marino, P., Lamberti, A., Coppo, A., & Tocchini-Valentini, G. P. (1969). Rifampicin sensitivity of the components of DNA-dependent RNA polymerase. *Nature*, 222(5193), 533–537.
- Etzkorn, M., Kneuper, H., Dünnwald, P., Vijayan, V., Krämer, J., Griesinger, C., et al. (2008). Plasticity of the PAS domain and a potential role for signal transduction in the histidine kinase DcuS. *Nature Structural & Molecular Biology*, 15(10), 1031–1039. <http://dx.doi.org/10.1038/nsmb.1493>.
- Etzkorn, M., Seidel, K., Li, L., Martell, S., Geyer, M., Engelhard, M., et al. (2010). Complex formation and light activation in membrane-embedded sensory rhodopsin II as seen by solid-state NMR spectroscopy. *Structure (London, England: 1993)*, 18(3), 293–300. <http://dx.doi.org/10.1016/j.str.2010.01.011>.
- Filipp, F. V., Sinha, N., Jairam, L., Bradley, J., & Opella, S. J. (2009). Labeling strategies for ¹³C-detected aligned-sample solid-state NMR of proteins. *Journal of Magnetic Resonance (San Diego, Calif: 1997)*, 201(2), 121–130. <http://dx.doi.org/10.1016/j.jmr.2009.08.012>.
- Folkers, G. E., van Buuren, B. N. M., & Kaptein, R. (2004). Expression screening, protein purification and NMR analysis of human protein domains for structural genomics. *Journal of Structural and Functional Genomics*, 5(1–2), 119–131. <http://dx.doi.org/10.1023/B:JSFG.0000029200.66197.0c>.
- Goncalves, J., Eilers, M., South, K., Opefi, C. A., Laissue, P., Reeves, P. J., et al. (2013). Magic angle spinning nuclear magnetic resonance spectroscopy of G protein-coupled receptors. *Methods in Enzymology*, 522, 365–389. <http://dx.doi.org/10.1016/B978-0-12-407865-9.00017-0>.

- Heller, K. B. (1978). Apparent molecular weights of a heat-modifiable protein from the outer membrane of *Escherichia coli* in gels with different acrylamide concentrations. *Journal of Bacteriology*, 134(3), 1181–1183.
- Kubicek, J., Block, H., Maertens, B., Spriestersbach, A., & Labahn, J. (2014). Expression and purification of membrane proteins. *Methods in Enzymology*, 541, 117–140. <http://dx.doi.org/10.1016/B978-0-12-420119-4.00010-0>.
- Linser, R., Dasari, M., Hiller, M., Higman, V., Fink, U., Lopez del Amo, J.-M., et al. (2011). Proton-detected solid-state NMR spectroscopy of fibrillar and membrane proteins. *Angewandte Chemie (International Ed. in English)*, 50(19), 4508–4512. <http://dx.doi.org/10.1002/anie.201008244>.
- Mao, L., Inoue, K., Tao, Y., Montelione, G. T., McDermott, A. E., & Inouye, M. (2011). Suppression of phospholipid biosynthesis by cerulenin in the condensed Single-Protein-Production (cSPP) system. *Journal of Biomolecular NMR*, 49(2), 131–137.
- Mao, L., Tang, Y., Vaiphei, S. T., Shimazu, T., Kim, S.-G., Mani, R., et al. (2009). Production of membrane proteins for NMR studies using the condensed single protein (cSPP) production system. *Journal of Structural and Functional Genomics*, 10(4), 281–289. <http://dx.doi.org/10.1007/s10969-009-9072-0>.
- Miroux, B., & Walker, J. E. (1996). Over-production of proteins in *Escherichia coli*: Mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *Journal of Molecular Biology*, 260(3), 289–298. <http://dx.doi.org/10.1006/jmbi.1996.0399>.
- Nakamura, K., & Mizushima, S. (1976). Effects of heating in dodecyl sulfate solution on the conformation and electrophoretic mobility of isolated major outer membrane proteins from *Escherichia coli* K-12. *Journal of Biochemistry*, 80(6), 1411–1422.
- Renault, M., Pawsey, S., Bos, M. P., Koers, E. J., Nand, D., Tommassen-van Boxtel, R., et al. (2012). Solid-state NMR spectroscopy on cellular preparations enhanced by dynamic nuclear polarization. *Angewandte Chemie (International Ed. in English)*, 51(12), 2998–3001.
- Renault, M., Tommassen-van Boxtel, R., Bos, M. P., Post, J. A., Tommassen, J., & Baldus, M. (2012). Cellular solid-state nuclear magnetic resonance spectroscopy. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 4863–4868.
- Robert, V., Volokhina, E. B., Senf, F., Bos, M. P., Van Gelder, P., & Tommassen, J. (2006). Assembly factor Omp85 recognizes its outer membrane protein substrates by a species-specific C-terminal motif. *PLoS Biology*, 4(11), e377. <http://dx.doi.org/10.1371/journal.pbio.0040377>.
- Rocchigiani, L., Ciancaleoni, G., Zuccaccia, C., & Macchioni, A. (2011). Low-temperature kinetic NMR studies on the insertion of a single olefin molecule into a Zr-C bond: Assessing the counterion-solvent interplay. *Angewandte Chemie, International Edition*, 50(49), 11752–11755. <http://dx.doi.org/10.1002/anie.201105122>.
- Romanuka, J., van den Bulke, H., Kaptein, R., Boelens, R., & Folkers, G. E. (2009). Novel strategies to overcome expression problems encountered with toxic proteins: Application to the production of Lac repressor proteins for NMR studies. *Protein Expression and Purification*, 67(2), 104–112. <http://dx.doi.org/10.1016/j.pep.2009.05.008>.
- Saraswat, M., Musante, L., Ravidá, A., Shortt, B., Byrne, B., & Holthofer, H. (2013). Preparative purification of recombinant proteins: Current status and future trends. *BioMed Research International*, 2013, 312709. <http://dx.doi.org/10.1155/2013/312709>.
- Sinnige, T., Weingarth, M., Renault, M., Baker, L., Tommassen, J., & Baldus, M. (2014). Solid-state NMR studies of full-length BamA in lipid bilayers suggest limited overall POTRA mobility. *Journal of Molecular Biology*, 426(9), 2009–2021. <http://dx.doi.org/10.1016/j.jmb.2014.02.007>.
- Sinnige, T., Houben, K., Pritisanac, I., Renault, M., Boelens, R., & Baldus, M. (2015). Insight into the conformational stability of membrane-embedded BamA using a

- combined solution and solid-state approach. *Journal of Biomolecular NMR*. <http://dx.doi.org/10.1007/s10858-014-9891-6>, in press.
- Studier, F. W. (2005). Protein production by auto-induction in high density shaking cultures. *Protein Expression and Purification*, 41(1), 207–234.
- Studier, F. W. (2014). Stable expression clones and auto-induction for protein production in *E. coli*. *Methods in Molecular Biology (Clifton, N.J.)*, 1091, 17–32. http://dx.doi.org/10.1007/978-1-62703-691-7_2.
- Studier, F., Rosenberg, A., Dunn, J., & Dubendorff, J. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods in Enzymology*, 185(1986), 60–89.
- Suzuki, M., Mao, L., & Inouye, M. (2007). Single protein production (SPP) system in *Escherichia coli*. *Nature Protocols*, 2(7), 1802–1810. <http://dx.doi.org/10.1038/nprot.2007.252>.
- Suzuki, M., Zhang, J., Liu, M., Woychik, N. A., & Inouye, M. (2005). Single protein production in living cells facilitated by an mRNA interferase. *Molecular Cell*, 18(2), 253–261. <http://dx.doi.org/10.1016/j.molcel.2005.03.011>.
- Verardi, R., Traaseth, N. J., Masterson, L. R., Vostrikov, V. V., & Veglia, G. (2012). Isotope labeling for solution and solid-state NMR spectroscopy of membrane proteins. *Advances in Experimental Medicine and Biology*, 992, 35–62. http://dx.doi.org/10.1007/978-94-007-4954-2_3.
- Wagner, S., Klepsch, M. M., Schlegel, S., Appel, A., Draheim, R., Tarry, M., et al. (2008). Tuning *Escherichia coli* for membrane protein overexpression. *Proceedings of the National Academy of Sciences of the United States of America*, 105(38), 14371–14376. <http://dx.doi.org/10.1073/pnas.0804090105>.
- Wehrli, W., Knüsel, F., Schmid, K., & Staehelin, M. (1968). Interaction of rifamycin with bacterial RNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America*, 61(2), 667–673.
- Weingarth, M., & Baldus, M. (2013). Solid-state NMR-based approaches for supramolecular structure elucidation. *Accounts of Chemical Research*, 46, 2037–2046. <http://dx.doi.org/10.1021/ar300316e>.
- Weingarth, M., Van der Cruysen, E. A., Ostmeier, J., Lievestro, S., Roux, B., et al. (2014). Quantitative analysis of the water occupancy around the selectivity filter of a K⁺ channel in different gating modes. *Journal of the American Chemical Society*, 136, 2000–2007. <http://dx.doi.org/10.1021/ja411450y>.