NMR Spectroscopy

International Edition: DOI: 10.1002/anie.201913585
German Edition: DOI: 10.1002/ange.201913585

NMR Characterization of Surface Receptor Protein Interactions in Live Cells Using Methylcellulose Hydrogels

*Borja Mateos[+], Marco Sealey-Cardona[+], Katja Balazs, Judith Konrat, Guenther Staffler, and Robert Konrat**

Abstract: *Interactions of transmembrane receptors with their extracellular ligands are essential for cellular communication and signaling and are therefore a major focus in drug discovery programs. The transition from in vitro to live cell interaction studies, however, is typically a bottleneck in many drug discovery projects due to the challenge of obtaining atomic-resolution information under near-physiological conditions. Although NMR spectroscopy is ideally suited to overcome this limitation, several experimental impairments are still present. Herein, we propose the use of methylcellulose hydrogels to study extracellular proteins and their interactions with plasma membrane receptors. This approach reduces cell sedimentation, prevents the internalization of membrane receptors, and increases cell survival, while retaining the free tumbling of extracellular proteins.*

The extracellular matrix (ECM) is an intricate network of fibrous proteins, glycoproteins, and non-protein components that provides physical support for cells and determines a wide range of biological functions.[1] Apart from its structural properties, the ECM is the niche for many adaptative cellular communication processes established through tightly controlled protein–protein interactions (PPIs).[2–4] Dysregulation of these PPIs leads to impaired cellular functions and/or disease phenotypes and can, for example, promote cell mobility and tumour metastasis.[5] Interfering with protein interactions occurring on cell membranes and thereby altering cellular responses from outside the cells has become an interesting intervention strategy and a major focus of drug discovery projects.[6]

[*] B. Mateos,[+] M. Sealey-Cardona,[+] J. Konrat, Prof. R. Konrat
Department of Structural and Computational Biology,
Max Perutz Labs, Vienna Biocenter Campus 5, 1030 Vienna (Austria)
E-mail: robert.konrat@univie.ac.at
K. Balazs, G. Staffler
AFFiRiS AG, Karl-Farkas-Gasse 22, 1030 Vienna (Austria)
M. Sealey-Cardona[+]
Present address: Calyxha Biotechnologies GmbH
Karl-Farkas-Gasse 22, 1030 Vienna (Austria)
[+] These authors contributed equally to this work.
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201913585.

The ultimate goal of macromolecular interaction studies is to obtain atomic-resolution information to identify binding epitopes or to decipher the mechanism of action of a particular ligand. Solution nuclear magnetic resonance (NMR) spectroscopy is a robust and well-established method to characterize molecular interactions in solution.[7,8] The saturation transfer difference (STD) experiment is one of the most widely used experiments for epitope mapping of ligand binding to receptors.[9–11] Therefore, STD experiments are a promising approach to characterize membrane protein interactions with living cells and have already been successfully used to characterize the binding of small molecules, carbohydrates, and peptides to membrane proteins.[12–17]

It is important to note that for the above-mentioned STD applications, the required experimental time is relatively short (a few seconds to minutes) due to the use of monodimensional (1D) NMR experiments, and in consequence, the cell viability in the NMR tube is not a major issue. However, to characterize protein–protein interactions, multidimensional experiments are necessary to resolve the signals in an isotopically labelled protein sample. Since these NMR experiments require a considerable amount of time (from tens of minutes to hours) cell sedimentation, poor oxygen diffusion, protein leakage, and nutrient depletion are major drawbacks for the application of these highly informative experiments.[18,19] Kubo et al. developed an elegant bioreactor system to maintain living cells inside an NMR tube and successfully characterized the binding interface of an externally introduced protein using STD.[20] Other strategies, involving agar or alginate gels have been used to encapsulate cells and increase their viability.[21,22] However, when studying extracellular components, these highly viscous gels are not suitable because the embedded proteins no longer tumble freely in solution. In this study, we introduce methylcellulose (MeCe) hydrogels for NMR studies of extracellular proteins interacting with receptors exposed on the membranes of living cells (ex cell NMR). Although here we focus on protein interactions between cellular receptors and proteins from the extracellular space our findings are also highly relevant for ongoing in-cell NMR experiments[23–26] under physiological conditions,[27,28] for which cell integrity and viability are essential, as well as metabolomic studies.[45]

MeCe is particularly favorable for these studies as it is a hydrophilic polymer derived from cellulose and has a wide range of cell biology applications, such as 3D cell cultures.[29] Although it forms a viscous hydrogel, depending on the molecular weight and concentration used, it consists of sizeable pores (exhibiting large porosity) and therefore allows water-soluble compounds to tumble freely in solution.

Because of its colloidal properties, it is easy to handle and pipette. In contrast to agarose, a widely used hydrogel for the design of NMR bioreactors, MeCe allows the attachment of cells and, therefore, mimics the physical support of the ECM. The potential advantage for NMR studies of extracellular proteins with living cells is thus obvious, large cells are encapsulated within the void space of the hydrogel (Figure 1 A), thereby preventing cell sedimentation, (Figure 1 B), while proteins are sufficiently small to tumble freely within the pores.

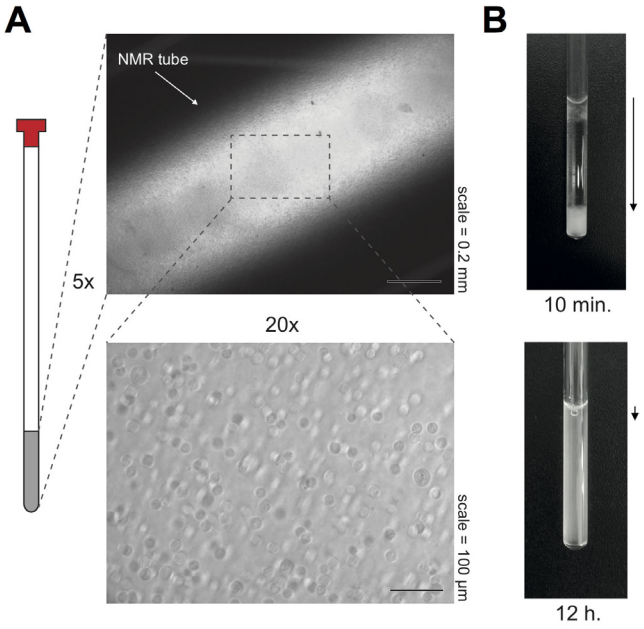

Figure 1. A) Phase contrast bright-field images of HEK293T cells embedded in the MeCe hydrogel. Cells are homogenously distributed along the 5 mm NMR tube. B) Sedimentation velocities are drastically different in buffer (upper image) and in the hydrogel (lower image).

In order to demonstrate the beneficial impact of MeCe hydrogels on living cells we decided to monitor cell sedimentation, viability, and survival using HEK293T cells. Viability was followed by propidium iodide staining (Supporting Information, Figure S1). Under PBS buffer conditions, the highly crowded cellular environment generated by sedimented cells showed an elevated cell death.

In contrast, the more even distribution of the cells inside the NMR tube and the possibility of adhering to the three-dimensional mesh of MeCe significantly increased cell viability at all tested temperatures (Supporting Information, Figure S1). It also allowed the growth of cells in 3D clusters, also called 3D cultures, provided that sufficient amounts of nutrients were available (namely, nutrient-rich cell media instead of PBS buffer) (Supporting Information, Figure S2).

After successfully demonstrating the favorable cell viability within MeCe scaffolds, an important next consideration was to verify the presence of receptors on the cell surface for a prolonged period of time. Immunostaining of HEK293T cells showed that cells keep integrin receptor β1 at the cellular membrane despite the apparently different cell morphology in the 3D MeCe matrix (Figure 2 A). We checked the presence of integrins and CD44 on the plasma membrane of cells used for the NMR assays (Supporting Information, Figure S5). Furthermore, flow cytometry assays showed that the pool of integrin β1 positive cells do not decay within MeCe while sedimented cells in PBS experience a decrease of 50–60 % after 6 hours (Figure 2 B). The decreased rate of receptor turnover and the correct exposure of integrin receptors for a prolonged period of time is crucial for the intended NMR measurements probing the interaction between extracellular proteins and membrane receptors. It should be noted that cell viability and membrane-receptor internalization occur on different time scales. This has to be considered not only in the ex cell NMR experiments described here but also in in cell NMR studies. In conclusion, our cell biology experiments convincingly demonstrated the

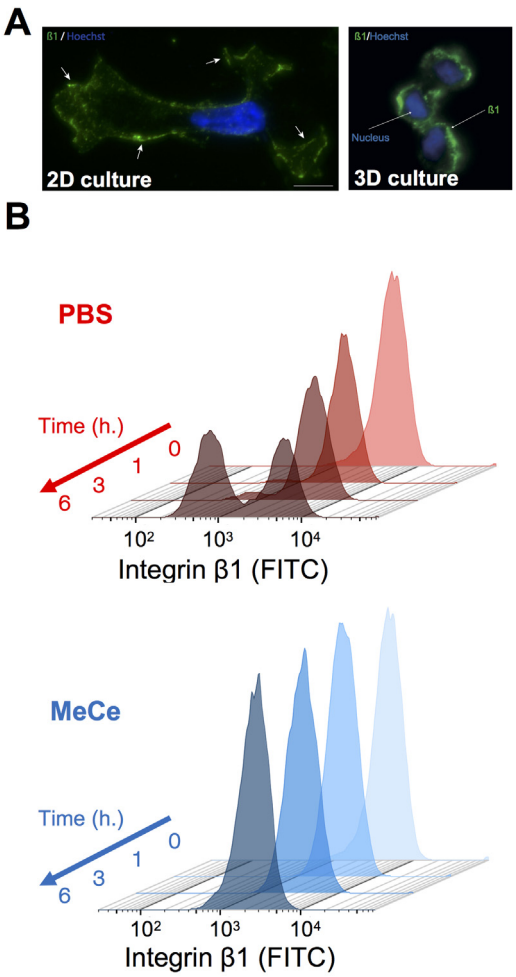

Figure 2. A) Immunostaining of HEK 293T cells attached to a surface in a 2D culture (left) or in a 3D MeCe 3D mesh (right). Nuclei are stained with Hoechst in blue and integrin β1 is shown in green. Scale bar = 5 µm. B) Flow cytometry populations of integrin-β1-stained cells (FITC filter channel). Sedimented cells in buffer (top) show a decreasing number of stained cells over time while cells in the hydrogel (bottom) maintain the integrin-β1-positive population. The integrin-β1-positive and unstained cells appear at fluorescence intensity values of 10^4 and 10^3, respectively.

integrity of cells and the correct exposure of membrane receptors.

Finally, we investigated the potential impact of MeCe hydrogels on the NMR properties of dissolved proteins. Osteopontin (OPN), an intrinsically disordered protein (IDP), was used as a test case. OPN is an extracellular IDP that exerts its function by binding to numerous components of the ECM, as well as two types of membrane receptors: integrins (αvβ3, αvβ5, αvβ8, α8β1, α5β1, and αIIbβ3) and CD44. Dysregulation of OPN receptor binding has been related to metastasis and abnormal cell function.[30,31] Interestingly, OPN is not entirely unstructured but contains regions with significant compaction that comprise the integrin receptor arginine-glycine-aspartic acid (RGD) interaction motif.[32] 2D ^{1}H-^{15}N HSQC spectra obtained for OPN in isotropic solution or dissolved in MeCe were virtually identical, both in terms of peak positions and signal intensities (Supporting Information, Figures S3 and S4 A).

^{15}N relaxation times also showed that molecular tumbling is unchanged. Relaxation data were analyzed as proposed by Li and Pielak[33] based on previous work of Kneller and Bracken.[34] The product of longitudinal and transverse relaxation (R_1·and R_2, respectively) is independent from the overall protein correlation time (τ_c) and anisotropic motion, while the ratio (R_2/R_1) retains these dependencies. Using this elegant NMR method, we observed no changes between the protein in PBS buffer and in presence of 1% of MeCe (viscosity around 3000 MPa; Supporting Information, Figure S4B).

Encouraged by the beneficial properties of the MeCe hydrogel matrix and as a proof-of-concept for protein receptor interaction studies on living cells using NMR, we studied the binding of OPN to integrin receptors on HEK293T cells.

Integrin binding to OPN was probed via two-dimensional STD ^{1}H-^{15}N HSQC experiments (schematically represented in Figure 3 A) with ^{15}N-labeled OPN samples in buffer, MeCe, and MeCe-encapsulated HEK293T cells. STD experiments were performed as described[9,35] (see the Supporting Information). Since receptor binding leads to a huge decrease of ^{15}N transverse relaxation time, T_2, and extreme line-broadening irradiation far from the proton aliphatic region

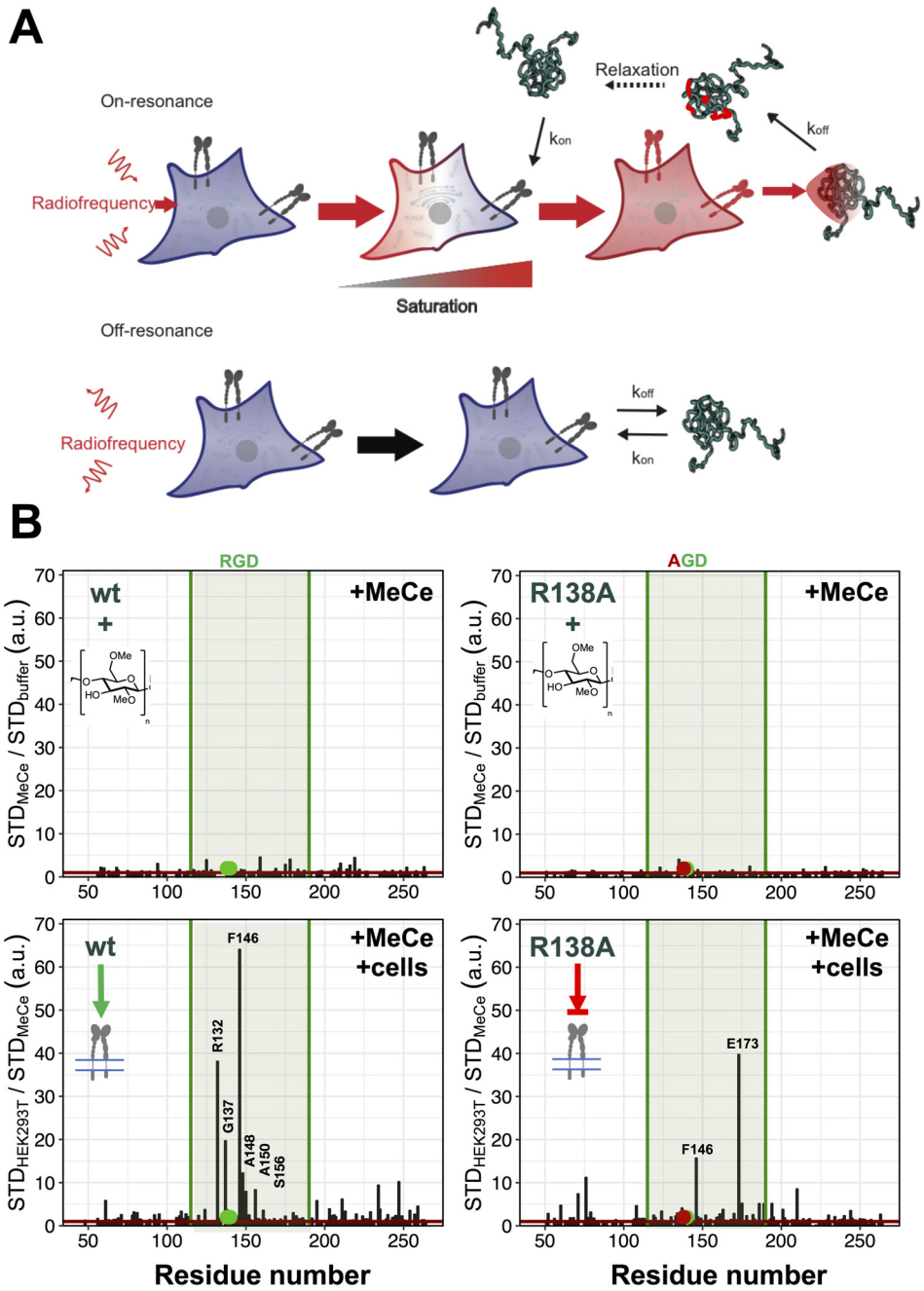

Figure 3. A) Schematic representation of the STD experiments. Figure adapted with permission from ref. [26], Copyright John Wiley & Sons, Ltd., 2018. B) Normalized STD amplifications from the MeCe (compared with a condition in buffer alone) (top) or the HEK293T cells (compared with a condition in MeCe alone) (bottom) of the wild-type (left) and the R138A mutant (right). The region encompassing the central compact state is indicated in green. The red line corresponds to a ΔSTD value of 1, which indicates no change between the compared conditions.

(0–4 ppm) exclusively saturates the receptor-bound protein species. Intermolecular (across the interaction interface between receptor and protein) as well as intramolecular (within the rigid, structurally stabilized binding epitope) dipolar cross-relaxation mechanisms lead to a change in signal intensities for different OPN residues. Detection of these differential signal intensities (or STD effects) reveals the affected residue positions and can easily be monitored in the free form of OPN and exchanging with the bound species. In order to validate our findings several control experiments were performed. First, potential contributions from the MeCe hydrogel were ruled out by performing STD experiments without cells (Figure 3B, top). Transfer of saturation was not observed from the matrix while in the presence of cells some signals around the RGD motif showed significant STD amplification (Figure 3B, bottom left). This experiment was repeated three times and showed that qualitatively the same signals experienced the transfer of saturation (Supporting Information, Figure S6A). However, when the RGD motif in OPN was mutated (R138A), this transfer of saturation was drastically decreased, indicating specific OPN binding to integrins via the canonical RGD-motif (Figure 3B, right). Interestingly, even in the mutated form, some signals showed a STD amplification, presumably due to RGD-independent binding to other membrane components with lower affinity. In order to support and prove these data an independent experiment was performed using a complementary cell adhesion assay (see the Supporting Information). It showed that HEK293T cells adhered to significantly lower concentrations of coated wild-type OPN compared to the R138A mutant protein. Already 10 nM of wild-type OPN resulted in an almost maximal adhesion, while the R138A mutant protein showed significantly lower (yet detectable) adhesion (Figure 4). The independent cell adhesion assay data corroborate our NMR findings and convincingly demonstrate the reliability of the new ex cell NMR methodology.

Finally, potential experimental saturation transfer artifacts were discarded. STD experiments rely on extensive line broadening of NMR signals in the bound state (in contrast to the free state which is observed), thus all other contributions that lead to extreme signal broadening have to be ruled out. Firstly, one of the most important line-broadening effects is the binding of paramagnetic ions, such as Mn^{2+} to OPN. Mn^{2+} is necessary for the activation of integrin receptors at the extracellular site,[36] and in consequence, proximal to the integrin-binding interface. Binding of Mn^{2+} to OPN leads to extensive line-broadening in some distinct regions (Supporting Information, Figure S7A) and subsequently increased transfer of saturation (Supporting Information, Figure S7B,C). Most importantly, however, these regions did not overlap with the integrin-binding RGD motif. Secondly, intermolecular NOEs due to spin diffusion may lead to undesired magnetization transfers from low-affinity binding to MeCe hydrogels. A fully deuterated OPN sample was prepared and the STD amplifications were drastically reduced in the presence of MeCe but still present when cells were added (Supporting Information, Figure S6B,C). Thus, OPN binding to living cells is mainly through binding to authentic integrin receptors and not affected by other line-broadening or spin diffusion effects. In principle, this methodology can be employed to any kind of protein, both folded and disordered, provided sensitivity allows.

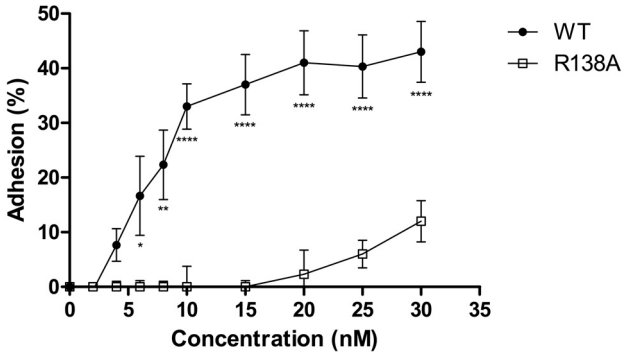

Figure 4. HEK293T cell adhesion assays. Cellular adhesion in percent of HEK 293T cells from 0 to 30 nM of recombinant wild-type (wt) or R138A OPN. Depicted are the means ± SEM of three independent experiments. Each experiment was set up in quadruplet. Asterisks indicate significance of cellular adhesion of R138A OPN in comparison to wt OPN (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.001$).

In summary, we have shown that MeCe hydrogels are a suitable scaffold to i) homogenously distribute living cells inside an NMR tube, ii) sustain cell viability and receptor externalization, and iii) maintain solution properties of dissolved proteins. As a proof of concept, we have studied the binding of OPN to integrin receptors in living cells by STD experiments. Given the ease of implementation valuable applications can be envisaged, such as screening of potential ligands for membrane-attached receptors. Interestingly, MeCe hydrogels could be combined with the flowNMR devices[37] equipped with membranes with low molecular weight cut-off to facilitate the exchange of nutrients, as proposed by Cerofolini et al.[38] Furthermore, further implementations by water hyperpolarization could improve the signal-to-noise[39–41] and NMR ^{13}C-detected tailored experiments would allow measurements at elevated temperatures and higher pH values.[42–44] The application of inert hydrogels demonstrated herein provide an exciting framework for NMR applications with living cells and helps to bridge the gap between in vitro and in vivo environments in membrane receptors drug discovery.

Acknowledgements

Thomas Sauer and Johanna Straner are acknowledged for the training and use of FACS equipment at the Max Perutz Labs, Vienna. B.M. acknowledges the DK Integrative Structural Biology funded by the Austrian Science Fund (FWF, project number AW0125821). R.K. acknowledges the grant of the Austrian Federal Ministry of Science, Research and Economy and the National Foundation for Research, Technology and Development.

Conflict of interest

The authors declare no conflict of interest.

Keywords: live-cell NMR · molecular recognition · NMR spectroscopy · protein–protein interactions · saturation transfer difference (STD)

How to cite: *Angew. Chem. Int. Ed.* **2020**, *59*, 3886–3890
Angew. Chem. **2020**, *132*, 3914–3918

[1] J. K. Mouw, G. Ou, V. M. Weaver, *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 771–785.
[2] M. J. Bissell, H. G. Hall, G. Parry, *J. Theor. Biol.* **1982**, *99*, 31–68.
[3] N. Boudreau, M. J. Bissell, *Curr. Opin. Cell Biol.* **1998**, *10*, 640–646.
[4] V. Vogel, A. G. M. Paschalia, M. Mountziaris, S. N. Tzouanas, L. Luca, A. L. Rougemont, B. H. Walpoth, L. Boure, A. Tami, J. M. Anderson, O. Jordan, R. Gurny, et al., *Int. J. Mol. Sci.* **2018**, *1840*, 353–387.
[5] K. J. Lenos, D. M. Miedema, S. C. Lodestijn, L. E. Nijman, T. van den Bosch, X. Romero Ros, F. C. Lourenço, M. C. Lecca, M. van der Heijden, S. M. van Neerven, et al., *Nat. Cell Biol.* **2018**, *20*, 1193–1202.
[6] A. L. Hopkins, C. R. Groom, *Nat. Rev. Drug Discovery* **2002**, *1*, 727–730.
[7] W. Becker, K. C. Bhattiprolu, N. Gubensäk, K. Zangger, *ChemPhysChem* **2018**, *19*, 895–906.
[8] M. Pellecchia, I. Bertini, D. Cowburn, C. Dalvit, E. Giralt, W. Jahnke, T. L. James, S. W. Homans, H. Kessler, C. Luchinat, et al., *Nat. Rev. Drug Discovery* **2008**, *7*, 738–745.
[9] M. Mayer, B. Meyer, *J. Am. Chem. Soc.* **2001**, *123*, 6108–6117.
[10] A. Bhunia, S. Bhattacharjya, S. Chatterjee, *Drug Discovery Today* **2012**, *17*, 505–513.
[11] S. Monaco, L. E. Tailford, N. Juge, J. Angulo, *Angew. Chem. Int. Ed.* **2017**, *56*, 15289–15293; *Angew. Chem.* **2017**, *129*, 15491–15495.
[12] S. Mari, D. Serrano-Gómez, F. J. Cañada, A. L. Corbí, J. Jiménez-Barbero, *Angew. Chem. Int. Ed.* **2005**, *44*, 296–298; *Angew. Chem.* **2005**, *117*, 300–302.
[13] B. Claasen, M. Axmann, R. Meinecke, B. Meyer, *J. Am. Chem. Soc.* **2005**, *127*, 916–919.
[14] F. M. Assadi-Porter, M. Tonelli, E. Maillet, K. Hallenga, O. Benard, M. Max, J. L. Markley, *J. Am. Chem. Soc.* **2008**, *130*, 7212–7213.
[15] D. Potenza, L. Belvisi, *Org. Biomol. Chem.* **2008**, *6*, 258–262.
[16] D. Potenza, F. Vasile, L. Belvisi, M. Civera, E. M. Araldi, *ChemBioChem* **2011**, *12*, 695–699.
[17] S. Mari, C. Invernizzi, A. Spitaleri, L. Alberici, M. Ghitti, C. Bordignon, C. Traversari, G. P. Rizzardi, G. Musco, *Angew. Chem. Int. Ed.* **2010**, *49*, 1071–1074; *Angew. Chem.* **2010**, *122*, 1089–1092.
[18] C. O. Barnes, G. J. Pielak, *Proteins Struct. Funct. Bioinf.* **2011**, *79*, 347–351.
[19] E. Luchinat, L. Banci, *IUCrJ* **2017**, *4*, 108–118.
[20] S. Kubo, N. Nishida, Y. Udagawa, O. Takarada, S. Ogino, I. Shimada, *Angew. Chem. Int. Ed.* **2013**, *52*, 1208–1211; *Angew. Chem.* **2013**, *125*, 1246–1249.
[21] N. G. Sharaf, C. O. Barnes, L. M. Charlton, G. B. Young, G. J. Pielak, *J. Magn. Reson.* **2010**, *202*, 140–146.
[22] K. Inomata, H. Kamoshida, M. Ikari, Y. Ito, T. Kigawa, *Chem. Commun.* **2017**, *53*, 11245–11248.
[23] E. Luchinat, L. Banci, *J. Biol. Chem.* **2016**, *291*, 3776–3784.
[24] F. Theillet, A. Binolfi, T. Frembgen-Kesner, K. Hingorani, M. Sarkar, C. Kyne, C. Li, P. B. Crowley, L. Gierasch, G. J. Pielak, et al., *Chem. Rev.* **2014**, *114*, 6661–6714.
[25] L. Barbieri, E. Luchinat, L. Banci, *Nat. Protoc.* **2016**, *11*, 1101–1111.
[26] B. Mateos, M. Maffei, M. Pons, *eLS* **2018**, DOI: 10.1002/9780470015902.a0027154.
[27] D. S. Burz, K. Dutta, D. Cowburn, A. Shekhtman, *Nat. Protoc.* **2006**, *1*, 146–152.
[28] J. Xie, R. Thapa, S. Reverdatto, D. S. Burz, A. Shekhtman, *J. Med. Chem.* **2009**, *52*, 3516–3522.
[29] J. E. Hotchin, *Nature* **1955**, *175*, 352.
[30] C. C. Kazanecki, D. J. Uzwiak, D. T. Denhardt, *J. Cell. Biochem.* **2007**, *102*, 912–924.
[31] P. H. Anborgh, J. C. Mutrie, A. B. Tuck, A. F. Chambers, *J. Cell. Mol. Med.* **2010**, *14*, 2037–2044.
[32] D. Kurzbach, T. C. Schwarz, G. Platzer, S. Höfler, D. Hinderberger, R. Konrat, *Angew. Chem. Int. Ed.* **2014**, *53*, 3840–3843; *Angew. Chem.* **2014**, *126*, 3919–3922.
[33] C. Li, G. J. Pielak, *J. Am. Chem. Soc.* **2009**, *131*, 1368–1369.
[34] J. M. Kneller, M. Lu, C. Bracken, *J. Am. Chem. Soc.* **2002**, *124*, 1852–1853.
[35] R. P. Venkitakrishnan, O. Benard, M. Max, J. L. Markley, F. M. Assadi-Porter, *Membrane Protein Structure and Dynamics*, Humana Press, Totowa, **2012**, pp. 47–63.
[36] J. Takagi, B. M. Petre, T. Walz, T. A. Springer, *Cell* **2002**, *110*, 599–611.
[37] D. A. Foley, E. Bez, A. Codina, K. L. Colson, M. Fey, R. Krull, D. Piroli, M. T. Zell, B. L. Marquez, *Anal. Chem.* **2014**, *86*, 12008–12013.
[38] L. Cerofolini, S. Giuntini, L. Barbieri, M. Pennestri, A. Codina, M. Fragai, L. Banci, E. Luchinat, E. Ravera, *Biophys. J.* **2019**, *116*, 239–247.
[39] A. Sadet, C. Stavarache, M. Bacalum, M. Radu, G. Bodenhausen, D. Kurzbach, P. R. Vasos, *J. Am. Chem. Soc.* **2019**, *141*, 12448–12452.
[40] T. Harris, O. Szekely, L. Frydman, *J. Phys. Chem. B* **2014**, *118*, 3281–3290.
[41] K. R. Keshari, J. Kurhanewicz, R. E. Jeffries, D. M. Wilson, B. J. Dewar, M. Van Criekinge, M. Zierhut, D. B. Vigneron, J. M. Macdonald, *Magn. Reson. Med.* **2010**, *63*, 322–329.
[42] I. Bertini, I. C. Felli, L. Gonnelli, M. V. Vasantha Kumar, R. Pierattelli, *Angew. Chem. Int. Ed.* **2011**, *50*, 2339–2341; *Angew. Chem.* **2011**, *123*, 2387–2389.
[43] I. C. Felli, L. Gonnelli, R. Pierattelli, *Nat. Protoc.* **2014**, *9*, 2005–2016.
[44] S. Gil, T. Hošek, Z. Solyom, R. Kümmerle, B. Brutscher, R. Pierattelli, I. C. Felli, *Angew. Chem. Int. Ed.* **2013**, *52*, 11808–11812; *Angew. Chem.* **2013**, *125*, 12024–12028.
[45] I. Alshamleh, N. Krause, C. Richter, N. Kurrle, H. Serve, U. L Günther, H. Schwalbe, *Angew. Chem. Int. Ed.* **2019**, https://doi.org/10.1002/anie.201912919; *Angew. Chem.* **2019**, https://doi.org/10.1002/ange.201912919.

Manuscript received: October 24, 2019
Revised manuscript received: November 11, 2019
Accepted manuscript online: November 13, 2019
Version of record online: January 29, 2020

