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On-Cell NMR Contributions to Membrane Receptor Binding Characterization

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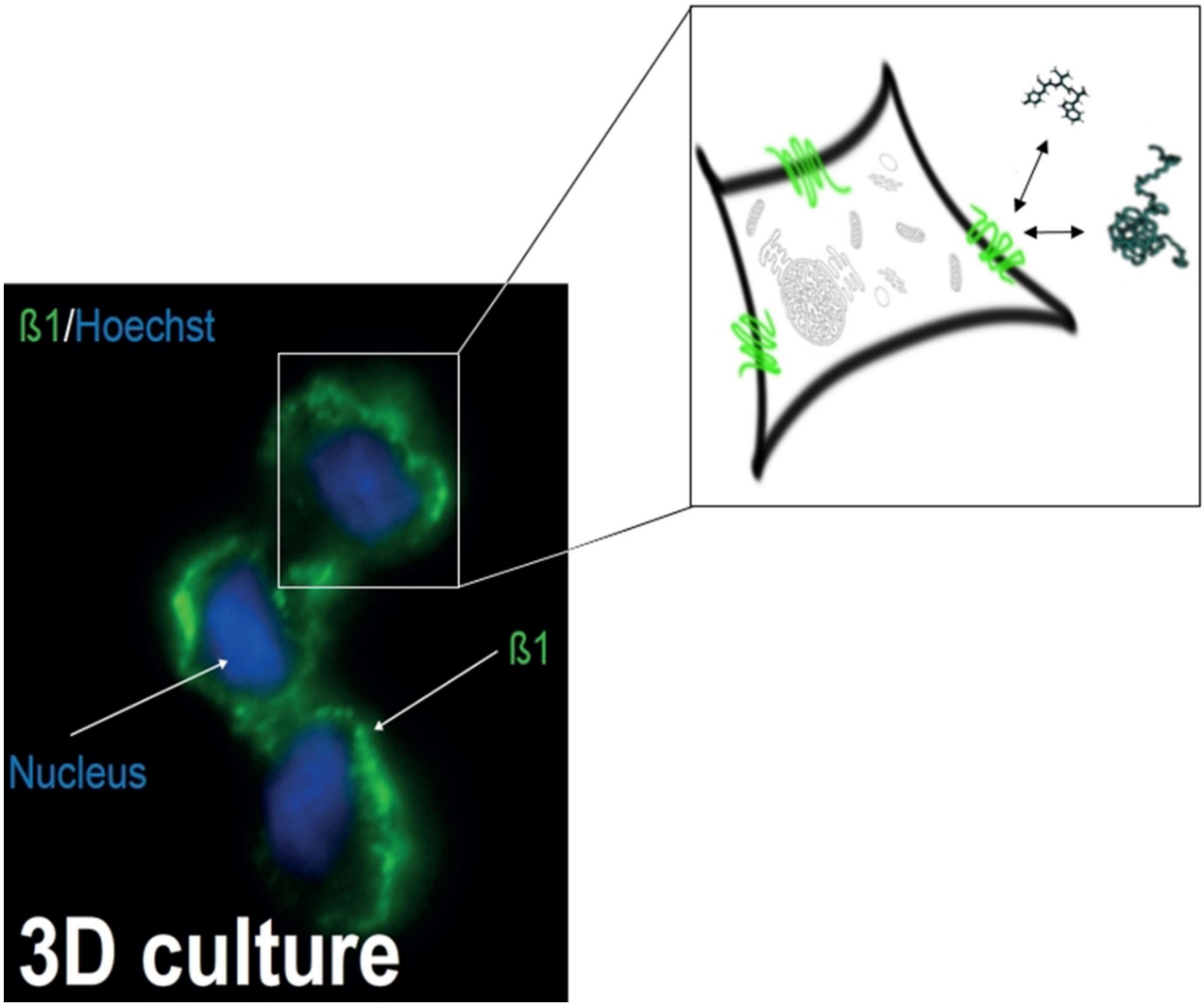

NMR spectroscopy has matured into a powerful tool to characterize interactions between biological molecules at atomic resolution, most importantly even under near to native (physiological) conditions. The field of in-cell NMR aims to study proteins and nucleic acids inside living cells. However, cells interrogate their environment and are continuously modulated by external stimuli. Cell signaling processes are often initialized by membrane receptors on the cell surface; therefore, characterizing their interactions at atomic resolution by NMR, hereafter referred as on-cell NMR, can provide valuable mechanistic information. This review aims to summarize recent on-cell NMR tools that give information about the binding site and the affinity of membrane receptors to their ligands together with potential applications to in vivo drug screening systems.

1. Introduction

One of the most important features of living organisms is the bidirectional communication capability to respond to their environment by reacting to stimuli from the extracellular space and respond by producing and secreting signaling molecules to shape their surroundings. The essential response system comprises different membrane proteins embedded in the plasma membranes of cells. The extracellular parts of membrane receptors typically interact with a variety of molecules, such as proteins, neurotransmitters, hormones, metabolites, ligands, and ions. Upon binding, the membrane receptor activates different physiological processes by triggering signaling cascades. Given their eminent biological functionalities, they are attractive targets for many drug discovery projects. For example, approximately 30% of all protein-coding genes in humans account for integral membrane proteins and 40% of all FDA-approved drugs target membrane proteins[1] [2].

The structural and biochemical characterization of membrane proteins and their binding partners is still challenging, although various improvements in fields of sample preparation, hardware and data processing have been accomplished in recent years.[3] Specifically, X-ray crystallography and cryo-EM have been established as the methods of choice for large macromolecules, as they provide structural information about macromolecules with high-resolution.

A unique method to probe molecular interactions and dynamics at atomic resolution, however, is nuclear magnetic resonance (NMR), be it in the solid-state (SS-NMR) or liquid-state (LS-NMR). Therefore, it has emerged as an indispensable tool in drug discovery, as NMR can also detect changes of the conformation and dynamics upon ligand binding, providing unique opportunities to target allosteric binding sites.[4,5] Most importantly, the results of *in vitro* assays might be misleading or inconclusive as the functionally relevant molecular interactions occur either in the cell's cytoplasm, at the external surface of living cells or in the extracellular matrix (ECM). *Ex vivo* NMR involves interaction studies with cells, tissues or organs and therefore, provides unique possibilities to study biomolecules and their interactions in the near to native environment. It should not be confused with *in vivo* NMR, which refers to interactions studies with living organisms, e.g. MRI. Additionally, with *ex vivo* NMR, important information about how the drug is affected under physiological conditions can be achieved, as a drug might not be able to cross the cellular membrane, might be pumped out of the cell, is metabolized or interacts with other cellular components instead of the therapeutic target.[6] *Ex vivo* NMR divides into investigations inside (*in-cell* NMR), at the membrane (*on-cell* NMR) and outside (*ex-cell* NMR) of the living cell. In this review, we focus on the studies of membrane receptors and their interactions by *on-cell* NMR. We will address important considerations covering the expression of membrane receptors with living mammalian cells, quantification of ligand binding, binding site mapping, and ultimately determination of binding poses. The aim of this mini-review is to guide the reader through the opportunities and difficulties of on-cell NMR and eventually simplify their entry to the new field.

2. NMR in Biological Environments

2.1. The cellular environment

Typical structural biology projects are carried out in solutions containing few salts in a buffer that adjusts the pH. The cellular environment, however, is way more complex and contains a plethora of other molecules such as metabolites, osmolytes, other proteins, nucleic acids, lipids and polymeric assemblies, depending on the specifics of the cellular (sub)-system.[7] The cell's interior, for example, is highly crowded, which influences not only the translational and rotational diffusion of the molecules in the cell but also the function of biomolecules.[9,10] Several NMR studies provided insightful information on the protein stability and function in crowded cellular conditions.[9,11–13] Additionally, subcellular compartments, like cytoplasm, nucleus, mitochondria and lysosomes, confine the cell's volume. Crowding and confinement have important consequences for protein stability and reactivity, as proteins can interact with cellular milieu molecules, either by excluded volume of preferential interactions,[14] and are altered by post-translational modifications (PTMs).[15] For more details on in-cell NMR, we recommend other reviews that focus on this topic.[7,16–18]

When investigating membrane receptor interactions on the cell surface the details of the extracellular matrix has also to be considered, as it contains a significant number of polysaccharides (both in free form or as a part of protein glycosylation

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sites), important for several molecular recognition events, and secondary metabolites that are relevant for intercellular communication.[19] Therefore, the ECM not only supports the mechanical stability of the cells but also is essential for outside-in or inside-out signalling mechanism.[20]

2.2. Receptor characterization

When investigating membrane receptors in mammalian cells, it is important to know precisely the identity and quantity of the protein. Both can be achieved by using mass spectrometry, commercial available microarrays and RNA sequencing.[21] Additionally, as a negative control, a cell line not expressing the membrane receptor should be established. To this end, CRISPR/Cas9 technology has emerged as an excellent tool to manipulate the genome of cells.[22] With that methodology, tailored cell lines can be designed to address a specific question involving the membrane receptor of interest. This method has a great potential in mimicking disease-related conditions by introducing point mutations, gene deletion or insertions.[23] Furthermore, CRISPR/Cas can be used in designing and developing new drugs, especially for GPRCs, as they have a highly selective pattern of expression and activity levels in healthy and disease tissues.[24] Using CRISPR/Cas, it is also possible to introduce a variety of point mutations in the binding region of the receptor. With that, the binding site can be mapped from the receptor's perspective and investigate how ligand binding is altered in disease-related conditions. Additionally, this genome-editing tool can be modified to target different physiological processes, for example, targeted transcriptional regulation. For this purpose, deactivated Cas9 was fused to transcriptional activators or repressors, which can, therefore, activate or repress a certain genomic region with high specificity compared to other transcriptional regulators such as interference RNA.[25] Control experiments have to be carried out, to make sure that the right membrane receptor is expressed in the desired amounts. Fluorescence activated cell sorting (FACS) is a widespread method of choice, because it is a versatile platform to monitor both the presence of reporter molecules of cellular fate and the expression of specific receptor types using monoclonal antibodies. For example, it is possible to detect the presence of pre-apoptotic cells with the annexin V assay as Annexin V binds to phosphatidylserines that migrates to the outer plasma membrane in apoptosis. In parallel, cell viability can be checked by propidium iodide or trypan blue staining. Thus, in a single assay, several reporters of viability, membrane receptor status and cellular phenotype can be monitored.

An essential prerequisite for the recording of NMR experiments with living cells is to maintain cell viability. To this end, the usage of bioreactors that enable the continuous exchange of media in the NMR instruments have been proposed,[26–30] or alternatively cells can be encapsulated within agar or alginate hydrogels.[27] The problem, however, associated with these approaches is the fact that highly viscous gels are not suitable for LS-NMR as they prevent the free tumbling of the protein under study in solution. To circumvent this problem our laboratory has recently developed an alternative experimental strategy involving methylcellulose hydrogels to prevent cells from sedimentation. This approach ensures proper tumbling of extracellular components, while retaining the cells attached to a (hydrogel) scaffold. Depending on the molecular weight and the concentration of the methylcellulose, the size of the pores can be modulated. It allows water-soluble compounds to tumble freely in solution though large cells are encapsulated within the void space. This easy-to-handle technique, which is already well-established in 3D cell cultures, can maintain cell integrity and viability in the NMR tube. Furthermore, it was demonstrated that this approach reduces cell sedimentation and avoids membrane receptor internalization, without disturbing the actual binding event.[8] It was also successfully applied to monitor the metabolic changes by real-time NMR.[31]

Finally, one of the most important aspects of *in vivo* NMR is to maintain the stability of the cell in the NMR tube within the required experimental time. The conditions are drastically different from cell cultures, as there are limited amounts of

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nutrients and oxygen. In addition, the elimination of metabolic by-products, acidification of the media, sedimentation of the cells itself and overexpressed protein of interest (POI) can induce significant changes in the cell viability and the metabolism of the cell. The lack of cell attachment sites leads to the internalization of membrane receptors, the trigger of apoptosis and degradation of cellular components. Depending on the concentration of the overexpressed POI and the complexity of NMR experiments, the duration can range from minutes to hours, where changes in the cell viability can already occur. Embedding cells in a methylcellulose hydrogel (*see above*) solved the problem for LS-NMR, and similar techniques exist for SS-NMR, where high-resolution magic-angle spinning (HR-MAS) techniques have been explored to maintain cell stability for the experimental duration and to prevent cell sedimentation.[32,33]

2.3. Protein isotope labelling

In order to distinguish the POI or its binding partner from the background of the cell, the macromolecule has to be labelled with stable isotopes. The most commonly used isotopes are ^{15}N and ^{13}C, followed by ^{19}F. ^{19}F is especially interesting, as it is highly sensitive and due to its missing in biological molecules, the spectra is background free.[34,35] Depending on the origin of the interaction partner, the expression of labelled proteins and peptides is very straightforward and accomplished in *E.coli*. To increase the sample yield, smaller proteins and peptides are fused to an expression tag, like Maltose-binding protein (MBP), glutathione S-transferase (GST), Histidine or a Streptomycin tag.[36] Furthermore, *in-organelle* NMR approaches were also possible by fusion of specific targeting sequences.[37] In SS-NMR, the membrane receptor and the interaction partner can be labelled with stable isotopes, as in this approach the molecular rotational tumbling rate of the labelled protein does not influence the line broadening. These facts make the *on-cell* NMR approach more feasible and easier to handle in comparison to *in-cell* NMR, of course, depending on the specific question at stake. However, the expression and purification of membrane receptors, especially human membrane receptors, is still a challenging task. The most applicable system for expressing human membrane receptors is the usage of mammalian cell lines. The most used cell lines are human embryo kidney (HEK) and Chinese hamster ovary (CHO), as these cell types have the highest rates of transfection via polyethyleneimine (PEI), calcium phosphate or electroporation.[38] With that, one can ensure that the membrane receptor is expressed in the correct fold and has all the required PTMs to be functional. Moreover, two or more proteins can be expressed in human cells, whereas only one is selectively labelled by controlling the timing of expression by using a silencing vector for one of the proteins.[39] The major drawback of expressing the POI in mammalian cells, labelled or unlabelled, are its relative high cost and the low yields compared to bacterial expression.[40]

3. Membrane Receptor Interactions by On-Cell NMR

NMR spectroscopic parameters are extremely sensitive probes of molecular binding events as even subtle changes of the chemical environment lead to pronounced changes of chemical shifts, relaxation parameters, diffusion coefficients, transfer Nuclear Overhauser Effect (trNOEs) and saturation transfer differences (STDs).[36,41] In general, *on-cell* NMR studies of membrane receptor interactions can either exploit ligand- or receptor-based detection schemes, depending on the exchange kinetics between free and receptor-bound state. Figure 1A illustrates the different NMR observation strategies. The two approaches (receptor *vs* ligand detection) complement each other and have their individual benefits. Clearly, looking at the receptor directly provides direct (atomistic) information about binding site residues and potential structural changes accompanying ligand binding. Fortunately, signal intensities in SS-NMR are insensitive to the molecular rotational tumbling rate and thus allows to study non-soluble or large cell surface macromolecules.[6,42–44] Therefore, SS-NMR employing heteronuclear $^{13}C/^{15}N$-detected HR-MAS (high-resolution magic angle spinning) NMR experiments is a very promising approach.[45–47] Using this technique, detailed information about large structures on living cells, for example, keratin, collagen and other components of the ECM, were achieved.[48–52] Another way of increasing the sensitivity in SS-NMR is by exploiting the enhancement of dynamic nuclear polarization (DNP) by the usage of paramagnetic labels. With that method, it was even possible to detect naturally abundant proteins and nucleotides in living bacteria.[53] More insightful reviews on this strategy can be found in the recent literature.[54,55] As represented in Figure 1A, direct observation by SS-NMR allows to resolve the signals of the bound state (Figure 1A, bottom left), by comparison with the free form obtained by, for example, conventional LS-NMR (i.e. 2D ^{15}N-^{1}H HSQC, Figure 1A, bottom right) allows the mapping of receptor-binding sites. Additionally, measurement of relaxation parameters and dipolar couplings allows the probing of structural dynamics in the bound state. It is important to note, that with the same technique (provided sensitivity allows) observation of the membrane receptor is feasible, as has been reported recently.[49,56,57]

Upon binding to a membrane receptor, the ligand behaves as a part of a huge macromolecule (i.e., slow molecular tumbling), where LS-NMR is not capable to directly detect any signal from the bound state due to broad signals and decreased signal-to-noise ratios, and one has to resort to indirect observables. Ligands that display weaker binding affinities and fast exchange rates are easily accessible to LS-NMR methods such as trNOEs, water LOGSY and STD NMR spectroscopy, which, although observing the free ligand, provide valuable information about the receptor-bound state. The trNOESY is a useful method to determine the conformations of bound ligands, provided the exchange of the ligand is fast enough ($K_D > 10^{-6}$ M) and a sufficient amount of free ligand is observable.[58–60] For a detailed explanation of trNOE and water-

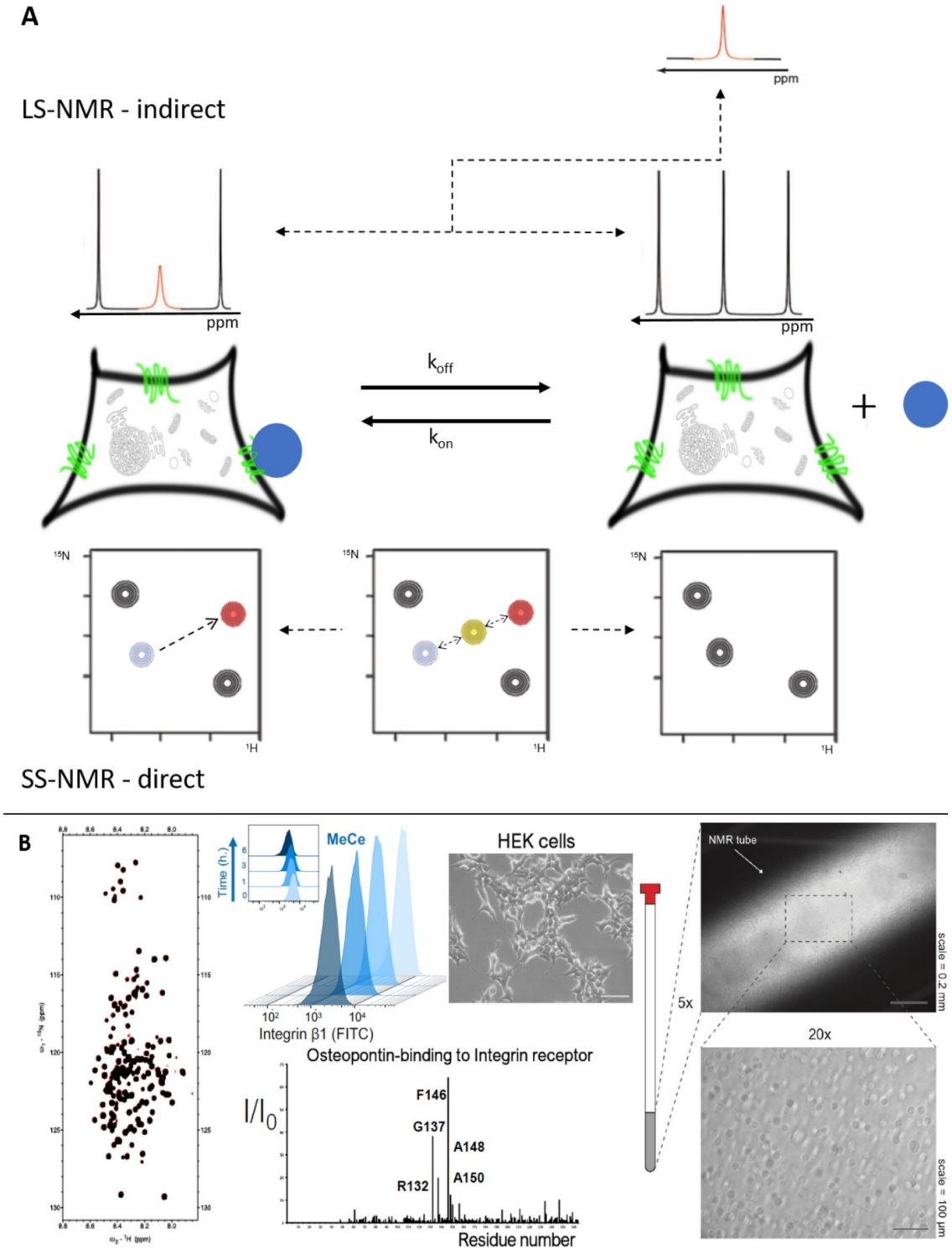

Figure 1. (A) Scheme of membrane receptor interactions probing by NMR. Interaction of extracellular ligand (blue, protein or small molecule) with membrane-bound receptor (green) can be probed indirectly (top) via solution saturation-transfer difference techniques (LS-NMR, STD) or directly (bottom) by observing chemical shifts in the solid state (SS-NMR, HR-MAS). Provided the exchange between free (apo) and receptor-bound state is slow on the NMR time scale separate NMR signals can be (directly, bottom) observed and resolved depending on the relative populations (concentrations and dissociation constant, K_D). In case of fast exchange, a population-averaged chemical shift will be observed. In contrast, solution NMR techniques (STD, top) probe the bound state indirectly via relaxation effects (NOE, spin diffusion, see text) exploiting the intense signal of the free (apo) state. Reversible exchange between free and bound form leads to an averaging of the relaxation effects. Due to the huge molecular weight difference of free and receptor-bound form the observed STD effect is entirely dominated by the bound state and therefore reports on the structural dynamics of the ligand when it is bound to cellular surface. (B) NMR studies of the interaction between the IDP Osteopontin (15 N-1H-HSQC, intensity ratio of apo and bound form, left and middle), a cytokine located in the extracellular matrix and $\alpha_V\beta_3$ integrin receptors in living HEK cells embedded in Methylcellulose hydrogel (light microscope images, right). Via FACS, membrane receptor internalization of $\alpha_V\beta_1$ was monitored (top, middle), adapted from.[8]

LOGSY concepts and applications, we address the reader elsewhere.[61] One of the most powerful experiments to identify the binding site from the ligand's perspective is STD.[62] The STD experiment relies on spin diffusion, which transfers saturation from the protein, in our case the receptor, to the bound ligand. Due to the long correlation times of high-molecular weight system (i.e. membrane-bound receptors), saturation of resonances by the applied (protein on-resonance) radio-frequency (RF) fields is highly efficient. After dissociation of the complex and release of the ligand from the membrane, saturation still prevails and produces the disappearance of the signal. As reference, the same RF pulse is applied off-resonance. The resulting STD spectrum is obtained by subtracting the saturated from the reference spectra. To exclude STD signals arising from non-specific interactions between the ligand and the cell membranes, additional STD experiments in the absence of the ligand and the membrane receptor, by using a cell line that does not express the protein, should be carried out.[63–65] STD-NMR experiments were applied to probe the interaction of membrane receptors to small peptides, sugars, lipopolysaccharide, proteins and peptide-mimetic drugs.[8,60,66–68] STD experiments reveal important information about the protein-ligand interaction. First, the binding site of the ligand is described at a detailed atomic level. Second, as the degree of saturation is also dependent on the ratio of the protein and ligand concentration, one gets additional information about the affinity of the complex as it displays the k_{on} and k_{off} rates. The dissociation constant K_D can also be derived by using the initial growth rate approach of the STD amplification factor (STD-AF). It is defined as the product of the STD intensity and the excess of the ligand concentration, whereas the K_D is obtained from the fit of the STD-AF values as a function of the ligand concentrations.[69] Third, one needs very small amounts of the receptor, as the preferred concentration for STD experiments lies for the binding partner in the nM-μM range.[65]

Most importantly, ligands in this context are not necessarily small molecular weight compounds. In our recent publication we demonstrated that the STD methodology can be adapted to study interactions between proteins (IDPs) from the extracellular matrix and membrane receptors in living cells.[8] Figure 1B shows experimental results for the interaction between the ECM IDP Osteopontin and the $\alpha_V\beta_3$ integrin receptor embedded in cell membranes of HEK cells. This important extension offers exciting possibilities particularly in drug design programs targeting hitherto unexplored ("undruggable") protein targets.

Compared to other screening assays, one major advantage of NMR spectroscopy is to provide detailed information at different stages of pre-clinical drug discovery processes. One of the earliest techniques that accomplished this is SAR by NMR (Structure-Activity Relationships). SAR by NMR is a target-based method where NMR parameters are monitored while adding a small molecule or a mixture of them.[70,71] Applied in an iterative way, it is an important tool to optimize lead compounds in drug design programs and has contributed to a better understanding of protein-ligand interactions, as it can describe the target's half-life, residence time, the equilibrium state and the transition states of the interactions in the cell's environment.[72,73] The possibility to quantitatively study the interactions between extracellular proteins and membrane-receptor in living cells offers exciting possibilities in future drug design programs (i.e. competition binding assays). Particularly, by introducing site-specific mutations in the receptor and/or the ligand protein, it is possible to (i) resolve key residues and (ii) detect changes in the binding affinity,[55] and thereby providing unique information about receptor-target specificity.

In addition to chemical shifts and NOE-based methods (such as the STD experiment), paramagnetic NMR spectroscopy can give unique information about membrane receptor interactions. With the introduction of a paramagnetic group, for example TEMPO or MTSL, on the ligand or membrane receptor, one gets specific information about the dynamical behaviour of the complex. Depending on the paramagnetic label, proton signals within a distance of 10—20 Å from the paramagnetic centre are weakened or quenched. In addition to established applications in structural biology, PREs also have been introduced to screening applications in drug design, for example SLAPSTIC (spin labels attached to protein side chains as a tool to identify interacting compounds) from Jahnke et al.[74] It will be interesting to explore possible SLAPSTIC applications when investigating ligand interactions with naturally abundant membrane receptors,[75] for example, by monitoring NMR spin relaxation changes due to the presence of the paramagnetic spin label.

4. Summary and Outlook

Membrane receptors interact with numerous endogenous and external ligands, and upon binding, different physiological processes inside or outside the cell are stimulated. As a result, investigations of membrane receptors and their ligands in living cells are of high interest for biological research. NMR spectroscopy is a very versatile technique to map binding sites, the application of *on-cell* NMR can help to close the knowledge gap of membrane receptor interactions *in vivo*. In addition, HR-MAS SS NMR has proven to be a powerful method to characterize not only the structure of large insoluble or membrane-surrounded protein complexes, but also their binding affinities to their ligands. LS-NMR had the problem of cell sedimentation and viability when investigating whole cells. Using a bioreactor specifically made for a typical cell type one can bypass this problem. The use of hydrogels can be adapted to mimic specific disease-relevant ECM compositions.[27] One limiting point is the correct and sufficient expression of the membrane receptor, where the newly arising field of CRISPR/Cas genome-editing tool can facilitate cell handling for *in situ* structural biology. Additionally, altered cell lines, produced by the CRISPR/Cas system, can help to identify the key residues in the receptor binding site through site-specific mutations and therefore establish structure-affinity-relationship (SAR).[55] These cell lines are reflecting the native conditions of expression and activity level more accurately. With all these possible manipulations of the investigating system, *on-cell* NMR achieves highly detailed information about the binding site, kinetics, dynamics and

structures in various different environments of the cell. It can thus be safely anticipated that the described techniques will find widespread applications both in structural biology as well as in rational drug design programs.

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Conflict of Interest

The authors declare no conflict of interest.

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[1] J. A. Lyons, A. Shahsavar, P. A. Paulsen, B. P. Pedersen, P. Nissen, *Curr. Opin. Struct. Biol.* **2016**, *38*, 137–144.
[2] W. A. Hendrickson, *Nat. Struct. Mol. Biol.* **2017**, *23*, 464–467.
[3] C. C. Valley, A. K. Lewis, J. N. Sachs, *BBA-Biomembranes* **2017**, *1859*, 1398–1416.
[4] B. Meyer, T. Peters, *Angew. Chem. Int. Ed. Engl.* **2003**, *42*, 864–90.
[5] A. D. Gossert, W. Jahnke, *Prog. Nucl. Magn. Reson. Spectrosc.* **2016**, *97*, 82–125.
[6] C. Li, J. Zhao, K. Cheng, Y. Ge, Q. Wu, Y. Ye, G. Xu, Z. Zhang, W. Zheng, X. Zhang, X. Zhou, G. Pielak, M. Liu, *Annu. Rev. Anal. Chem.* **2017**, *10*, 157–182.
[7] A. Pastore, P. A. Temussi, *Arch. Biochem. Biophys.* **2017**, *628*, 114–122.
[8] B. Mateos, M. Sealey-Cardona, K. Balazs, J. Konrat, G. Staffler, R. Konrat, *Angew. Chem. Int. Ed.* **2020**, *59*, 3886–3890; *Angew. Chem.* **2020**, *132*, 3914–3918.
[9] W. B. Monteitha, G. J. Pielak, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 11335–11340.
[10] F.-X. Theillet, A. Binolfi, T. Frembgen-Kesner, K. Hingorani, M. Sarkar, C. Kyne, C. Li, P. B. Crowley, L. Gierasch, G. J. Pielak, A. H. Elcock, A. Gershenson, P. Selenko, *Chem. Rev.* **2014**, *114*, 6661–6714.
[11] M. M. Dedmon, C. N. Patel, G. B. Young, G. J. Pielak, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 12681–12684.
[12] J. Danielsson, X. Mu, L. Lang, H. Wang, A. Binolfi, F. X. Theillet, B. Bekei, D. T. Logan, P. Selenko, H. Wennerström, M. Oliveberg, W. A. Eaton, *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 12402–12407.
[13] A. P. Schlesinger, Y. Wang, X. Tadeo, O. Millet, G. J. Pielak, *J. Am. Chem. Soc.* **2011**, *133*, 8082–8085.
[14] G. Rivas, A. P. Minton, *Trends Biochem. Sci.* **2016**, *41*, 970–981.
[15] I. M. Kuznetsova, K. K. Turoverov, V. N. Uversky, *Int. J. Mol. Sci.* **2014**, *15*, 23090–23140.
[16] B. Mateos, M. Maffei, M. Pons, *eLS* **2018**, 1–9.
[17] P. Selenko, G. Wagner, *J. Struct. Biol.* **2007**, *158*, 244–253.
[18] E. Luchinat, L. Banci, *IUCrJ* **2017**, *4*, 108–118.
[19] L. Unione, M. P. Lenza, A. Ardá, P. Urquiza, A. Laín, J. M. Falcón-Pérez, J. Jiménez-Barbero, O. Millet, *ACS Cent. Sci.* **2019**, *5*, 1554–1561.
[20] R. P. Mecham, *Curr. Protoc. cell Biol.* **2012**, *Chapter 10*, Unit 10.1.
[21] P. A. Insel, A. Wilderman, A. C. Zambon, A. N. Snead, F. Murray, N. Aroonsakool, D. S. McDonald, S. Zhou, T. McCann, L. Zhang, K. Sriram, A. M. Chinn, A. V. Michkov, R. M. Lynch, A. C. Overland, R. Corriden, *Mol. Pharmacol.* **2015**, *88*, 181–187.
[22] R. M. Gupta, K. Musunuru, *J. Clin. Invest.* **2014**, *124*, 4154–4161.
[23] G. Yang, X. Huang, *Cell Regen.* **2019**, *8*, 33–41.
[24] P. A. Insel, K. Sriram, M. W. Gorr, S. Z. Wiley, A. Michkov, C. Salmerón, A. M. Chinn, *Trends Pharmacol. Sci.* **2019**, *40*, 378–387.
[25] H. Manghwar, B. Li, X. Ding, A. Hussain, K. Lindsey, X. Zhang, S. Jin, *Adv. Sci.* **2020**, *7*.
[26] E. Luchinat, L. Barbieri, T. F. Campbell, L. Banci, *Anal. Chem.* **2020**, *92*, 9997–10006.
[27] 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.
[28] N. G. Sharaf, C. O. Barnes, L. M. Charlton, G. B. Young, G. J. Pielak, *J. Magn. Reson.* **2010**, *202*, 140–146.
[29] K. Inomata, A. Ohno, H. Tochio, S. Isogai, T. Tenno, I. Nakase, T. Takeuchi, S. Futaki, Y. Ito, H. Hiroaki, M. Shirakawa, *Nature* **2009**, *458*, 106–109.
[30] L. Cerofolini, S. Giuntini, L. Barbieri, M. Pennestri, A. Codina, M. Fragai, L. Banci, E. Luchinat, E. Ravera, *Biophys. J.* **2019**, *116*, 239–247.
[31] I. Alshamleh, N. Krause, C. Richter, N. Kurrle, H. Serve, U. L. Günther, H. Schwalbe, *Angew. Chem. Int. Ed.* **2020**, *59*, 2304–2308; *Angew. Chem.* **2020**, *132*, 2324–2328.
[32] W. Jachymek, T. Niedziela, C. Petersson, C. Lugowski, J. Czaja, L. Kenne, *Biochemistry* **1999**, *38*, 11788–11795.
[33] S. Narasimhan, S. Scherpe, A. Lucini Paioni, J. van der Zwan, G. E. Folkers, H. Ovaa, M. Baldus, *Angew. Chem. Int. Ed.* **2019**, *58*, 12969–12973; *Angew. Chem.* **2019**, *131*, 13103–13107.
[34] C. Li, G.-F. Wang, Y. Wang, R. Creager-Allen, E. A. Lutz, H. Scronce, K. M. Slade, R. A. S. Ruf, R. A. Mehl, G. J. Pielak, *J. Am. Chem. Soc.* **2010**, *132*, 321–327.
[35] Z. Serber, A. T. Keatinge-Clay, R. Ledwidge, A. E. Kelly, S. M. Miller, V. Dötsch, *J. Am. Chem. Soc.* **2001**, *123*, 2446–2447.
[36] S. Tapaneeyakorn, A. D. Goddard, J. Oates, C. L. Willis, A. Watts, *Biochim. Biophys. Acta Biomembr.* **2011**, *1808*, 1462–1475.
[37] L. Barbieri, E. Luchinat, L. Banci, *Biochim. Biophys. Acta Mol. Cell Res.* **2014**, *1843*, 2492–2496.
[38] K. H. Khan, *Adv. Pharm. Bull.* **2013**, *3*, 257–263.
[39] E. Luchinat, E. Secci, F. Cencetti, P. Bruni, *Biochim. Biophys. Acta* **2016**, *1860*, 527–533.
[40] L. Barbieri, E. Luchinat, L. Banci, *Nat. Protoc.* **2016**, *11*, 1101–1111.
[41] D. Potenza, F. Vasile, L. Belvisi, M. Civera, E. M. V. Araldi, *ChemBioChem* **2011**, *12*, 695–699.
[42] V. Wai, C. Wong, D. G. Reid, W. Ying, R. Rajan, M. Green, R. A. Brooks, M. J. Duer, *J. Biomol. NMR* **2015**, *63*, 119–123.
[43] M. P. Bokoch, Y. Zou, S. G. F. Rasmussen, C. W. Liu, R. Nygaard, D. M. Rosenbaum, J. J. Fung, H.-J. Choi, F. S. Thian, T. S. Kobilka, J. D. Puglisi, W. I. Weis, L. Pardo, R. S. Prosser, L. Mueller, B. K. Kobilka, *Nature* **2010**, *463*, 108–112.
[44] A. McDermott, *Annu. Rev. Biophys.* **2009**, *38*, 385–403.
[45] I. C. Felli, L. Gonnelli, R. Pierattelli, *Nat. Protoc.* **2014**, *9*, 2005–2016.
[46] A. Alik, C. Bouguechtouli, M. Julien, W. Bermel, R. Ghouil, S. Zinn-Justin, F. Theillet, *Angew. Chem. Int. Ed.* **2020**, *59*, 10411–10415; *Angew. Chem.* **2020**, *132*, 10497–10501.
[47] 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.
[48] I. Goldberga, R. Li, M. J. Duer, *Acc. Chem. Res.* **2018**, *51*, 1621–1629.
[49] D. Bolton, R. A. Munro, L. S. Brown, V. Ladizhansky, *Nat. Commun.* **2019**, 1–11.
[50] L. Krabben, B. J. Van Rossum, F. Castellani, E. Bocharov, A. A. Schulga, A. S. Arseniev, C. Weise, F. Hucho, H. Oschkinat, *FEBS Lett.* **2004**, *564*, 319–324.
[51] L. Krabben, B. J. van Rossum, S. Jehle, E. Bocharov, E. N. Lyukmanova, A. A. Schulga, A. Arseniev, F. Hucho, H. Oschkinat, *J. Mol. Biol.* **2009**, *390*, 662–671.
[52] P. T. F. Williamson, B. H. Meier, A. Watts, *Eur. Biophys. J.* **2004**, *33*, 247–254.
[53] M. Renault, S. Pawsey, M. P. Bos, E. J. Koers, D. Nand, R. Tommassen-, V. Boxtel, M. Rosay, J. Tommassen, W. E. Maas, M. Baldus, *Angew. Chem. Int. Ed.* **2012**, *51*, 2998–3001; *Angew. Chem.* **2012**, *124*, 3053–3056.
[54] S. Narasimhan, G. E. Folkers, M. Baldus, *ChemPlusChem* **2020**, *85*, 760–768.
[55] A. Kaiser, I. Coin, *Molecules* **2020**, *25*, 1–41.
[56] M. Teucher, H. Zhang, V. Bader, K. F. Winklhofer, A. J. García-Sáez, A. Rajca, S. Bleicken, E. Bordignon, *Sci. Rep.* **2019**, *9*, 1–15.
[57] C. A. P. Whittaker, S. G. Patching, M. Esmann, D. A. Middleton, *Org. Biomol. Chem.* **2015**, *13*, 2664–2668.
[58] C. B. Post, *Curr. Opin. Struct. Biol.* **2003**, *13*, 581–588.

[59] T. Haselhorst, J.-F. Espinosa, J. Jiménez-Barbero, T. Sokolowski, P. Kosma, H. Brade, L. Brade, T. Peters, *Biochemistry* **1999**, *38*, 6449–6459.
[60] S. Mari, C. Invernizzi, A. Spitaleri, L. Alberici, M. Ghitti, C. Bordignon, C. Traversari, G. Rizzardi, G. Musco, *Angew. Chem. Int. Ed.* **2010**, *49*, 1071–1074; *Angew. Chem.* **2010**, *122*, 1089–1092.
[61] B. Meyer, T. Peters, *Angew. Chem. Int. Ed.* **2003**, *42*, 864–890; *Angew. Chem.* **2003**, *115*, 890–918.
[62] M. Mayer, B. Meyer, *J. Am. Chem. Soc.* **2001**, *123*, 6108–6117.
[63] B. Claasen, M. Axmann, R. Meinecke, B. Meyer, *J. Am. Chem. Soc.* **2005**, *127*, 916–919.
[64] F. M. Assadi-porter, M. Tonelli, E. Maillet, K. Hallenga, M. Max, J. L. Markley, *J. Am. Chem. Soc.* **2009**, *130*, 7212–7213.
[65] F. M. Assadi-Porter, M. Tonelli, E. L. Maillet, J. L. Markley, M. Max, *Biochim. Biophys. Acta Biomembr.* **2010**, *1798*, 82–86.
[66] A. Bhunia, S. Bhattacharjya, S. Chatterjee, *Drug Discovery Today* **2012**, *17*, 505–513.
[67] R. P. Venkitakrishnan, O. Bernard, M. Max, J. L. Markley, *Methods Mol. Biol.* **2013**, 47–63.
[68] S. Mari, D. Serrano-Gómez, F. J. Cañada, A. L. Corbí, J. Jiménez-Barbero, *Angew. Chem. Int. Ed.* **2004**, *44*, 296–298.
[69] G. Künze, J. P. Gehrcke, M. T. Pisabarro, D. Huster, *Glycobiology* **2014**, *24*, 1036–1049.
[70] S. L. Swann, D. Song, C. Sun, P. J. Hajduk, A. M. Petros, *ACS Med. Chem. Lett.* **2010**, *1*, 295–299.
[71] S. B. Shuker, P. J. Hajduk, R. P. Meadows, S. W. Fesik, *Science* **1996**, *274*, 1531–1534.
[72] L. Xia, H. De Vries, E. B. Lenselink, J. Louvel, M. J. Waring, L. Cheng, S. Pahle, M. J. Petersson, P. Schell, R. I. Olsson, L. H. Heitman, R. J. Sheppard, A. P. Ijzerman, *J. Med. Chem.* **2017**, *60*, 9545–9564.
[73] D. Guo, L. H. Heitman, A. P. Ijzerman, *ChemMedChem* **2015**, *10*, 1793–1796.
[74] W. Jahnke, S. Rüdisser, M. Zurini, *J. Am. Chem. Soc.* **2001**, *123*, 3149–3150.
[75] L. F. Pinto, J. Correa, L. Zhao, R. Riguera, E. Fernandez-Megia, *ACS Omega* **2018**, *3*, 2974–2983.

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