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On-cell nuclear magnetic resonance spectroscopy to probe cell surface interactions

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Draft

Abstract

Nuclear magnetic resonance (NMR) spectroscopy allows determination of atomic-level information about intermolecular interactions, molecular structure, and molecular dynamics in the cellular environment. This may be broadly divided into studies focused on obtaining detailed molecular information in the intracellular context (“in-cell”) or those focused on characterizing molecules or events at the cell surface (“on-cell”). In this review, we outline some key NMR techniques applied for on-cell NMR studies through both solution-state and solid-state NMR and survey studies that have used these techniques to uncover key information. We particularly focus on application of on-cell NMR spectroscopy to characterize ligand interactions with cell surface membrane proteins such as G-protein coupled receptors (GPCRs), receptor tyrosine kinases, etc. These techniques allow for quantification of binding affinities, competitive binding assays, delineation of portions of ligands involved in binding, ligand bound-state conformational determination, evaluation of receptor structuring and dynamics, and inference of distance constraints characteristic of the ligand-receptor bound state. Excitingly, it is possible to avoid the barriers of production and purification of membrane proteins while obtaining directly physiologically-relevant information through on-cell NMR. We also provide a briefer survey of the applicability of on-cell NMR approaches to other classes of cell surface molecule.

Keywords: NMR spectroscopy; ligand-receptor interactions; intact cells; atomic-level characterization; biophysics

Introduction

Membrane proteins account for ~26% of the human proteome (Fagerberg et al. 2010). This class of proteins includes a wide variety of cell surface receptors that mediate communication and signalling, such as the large family of G-protein coupled receptors (GPCRs). Beyond the inherent challenges in the study of isolated membrane proteins (e.g., Pandey et al. 2016), the characterization of ligand-receptor interactions at an atomic or even sub-molecular level in their natural membrane environment is even more difficult. Recent advances in sample preparation for NMR spectroscopy studies of membrane proteins have come from the use of both protein- and polymer-scaffolded nanodiscs (e.g., Bayburt and Sligar 2010; Di Mauro et al. 2020; Hagn et al. 2013; Hardin et al. 2019; Huang et al. 2021; Knowles et al. 2009; Orwick-Rydmark et al. 2012; Ravula et al. 2019). Of note, polymeric nanodiscs allow for membrane protein reconstitution in native membrane while avoiding issues that may arise from protocols requiring detergents (Bada Juarez et al. 2020; Dorr et al. 2016; Lee et al. 2016). As an alternative, NMR spectroscopy studies are feasible in the context of intact cells through both “in-cell” and “on-cell” approaches, depending upon the specific localization of the target molecule of interest (Freedberg and Selenko 2014). Here, we specifically review on-cell nuclear magnetic resonance (NMR) spectroscopy techniques and studies as one potentially highly valuable experimental approach to characterize ligand-receptor interactions.

Solution-state on-cell NMR studies

Solution-state NMR spectroscopy techniques typically enable observation of relatively rapidly tumbling species in an isotropic environment. For cell-based studies, solution-state NMR has been widely applied to a range of sample types to determine many different classes of

experimental information (extensively reviewed in (Freedberg and Selenko 2014)). In on-cell solution-state NMR studies to date, two primary classes of experiment have been applied: saturation transfer difference (STD) and transferred nuclear Overhauser enhancement (trNOE) including WaterLOGSY experiments. ^{19}F NMR spectroscopy also has clear potential for on-cell NMR, although it has typically thus far been applied for in-cell NMR. Each of these well-established NMR techniques – as summarized in Figure 1 – will first be introduced, followed by a review of studies in which these have successfully been applied to understand ligand-receptor interactions in a cellular setting.

Primary solution-state on-cell NMR experimental techniques

STD NMR spectroscopy

STD NMR is a technique which allows the monitoring ligand binding (e.g., as reflected by dissociation constant, K_d) by magnetization transfer from a relatively slowly tumbling species, such as a large protein receptor, to a smaller ligand (Assadi-Porter et al. 2008; Mayer and Meyer 1999, 2001; Wang et al. 2004). For solution state on-cell NMR studies of membrane proteins, this technique is typically initiated by saturation of the ^1H spins on the receptor (Figure 1A, process 1) by selecting an irradiation frequency that is distinct to the receptor (Venkitakrishnan et al. 2012). The magnetization developed in this manner in the receptor is then transferred from the receptor to bound ligands (Figure 1A, process 2), leading to so-called saturation of NMR-active spins in the bound population. Upon dissociation, a saturated ligand molecule becomes part of the typically much larger pool of free ligands (Figure 1A, process 3). Following a period of saturation, the signal observed for the pool of free ligand molecules will be attenuated as a direct function of the proportion of saturated vs. unaffected ligand. A reference

experiment, without receptor saturation, is subtracted from the experiment under receptor-saturation conditions providing the STD spectrum, where only NMR signals that are affected by the saturation will be apparent.

Two primary advantages of the STD experiment are that it does not require expensive NMR-active isotope labelling and that it allows for the detection of K_d over a broad range, from ~ 10 nM – 1 mM. Practically speaking, the energy transfer in the STD phenomenon occurs relatively fast (e.g., ~ 100 ms) – the experiment is, thus, most sensitive for binding processes with a fast off rate (e.g., faster than ~ 0.1 Hz), with slower off rates leading to poor efficiency of the STD process (Meyer and Peters 2003). This experiment also requires a relatively low receptor concentration in the presence of a 20-1000 $\times$ stoichiometric excess of ligand. From both an economic standpoint and from an experimental feasibility standpoint, this is beneficial as it does not require overexpression of the receptor of interest on the cell surface (Assadi-Porter et al. 2008; Assadi-Porter et al. 2010). Because this technique monitors only the ligands, receptor molecular weight, with NMR behaviour accentuated by its even slower tumbling as a cell-surface species, is not problematic in terms of line broadening and slow longitudinal relaxation for solution-state NMR. Spin diffusion, which is beneficial for the saturation phenomenon, is also more efficient for a membrane-localized receptor protein than for a smaller molecule. STD experiments may also be applied to simultaneously monitor and distinguish between the binding - or lack thereof - of multiple potential ligands, provided that each ligand has NMR signals that may be distinguished (Fehér et al. 2008; Fruth et al. 2014). As an expansion upon the original STD technique, the saturation transfer double difference (STDD) experiment is highly effective in studying drug molecules bound to the surface of a living cell and/or to membrane proteins (Claasen et al. 2005; Cox et al. 2015; Pereira et al. 2009). STDD takes the double difference

between the STD spectrum of the cell/ligand mixture and that of the cell in the absence of ligand, providing a final double-difference spectrum free of any background residual receptor signal.

The trNOE and WaterLOGSY experiments

The nuclear Overhauser enhancement (NOE) is a phenomenon arising from dipolar cross-relaxation between nuclei that are within $\sim 6 \text{ \AA}$ of each other (Overhauser 1953a, b). In the context of ligand-receptor binding, the slowly-tumbling bound state of the ligand will generally have a much higher rate of cross-relaxation than the fast-tumbling free ligand state (Clore and Gronenborn 1982; Neuhaus and Williamson 2000; Wakamatsu et al. 1986; Wang et al. 1993). This, in turn, means that the NOEs may readily develop in the bound ligand (Figure 1B, process 1); following unbinding of the ligand (in the fast-exchange regime, on the NMR timescale), these appear in the fast-tumbling and spectroscopically observable free-state (Figure 1B, process 2). In other words, the NOEs that are observed are “transferred” from the bound-state conformation of the ligand to the free-state, providing insight into the structuring of the ligand in the bound-state although it is observed in the free-state. Alternatively, under the regime of slower exchange the trNOE may be applied in an intermolecular manner (i.e., transfer from ligand to receptor and vice versa; Figure 1C, process 1) to provide resonance-specific delineation of site(s) of the ligand that are directly binding to and interacting with the much larger and slower tumbling receptor (Figure 1C, process 2) (Campbell and Sykes 1991). Such intermolecular trNOE experiments, however, are complex to analyze due to the presence of several possible competing cross-relaxation pathways.

The trNOE phenomenon can be applied to monitor oligomerization and, similar to STD, to measure the K_d for complex formation. As with STD, it requires only a low receptor

concentration with excess ligand and does not rely upon over-expression of the protein of interest on the cell surface. To be effective, the ligand should bind with relatively low affinity (K_d from ~ 10 nM to 1 mM) with a dissociation rate in the millisecond range or faster. From an experimental perspective, a relatively short mixing time during which the NOE cross-relaxation is allowed to occur is required for quantitative analysis of the trNOE phenomenon vs. signal enhancement arising from spin diffusion (i.e., additional dipole-dipole transfer beyond the initial spin pair).

The conceptually-related WaterLOGSY experiment (Dalvit et al. 2000) monitors the NOE that develops between water (irradiated, as in Figure 1D, process 1) and a ligand either directly (Figure 1D, process 2) or via the protein binding partner (Figure 1D, process 3). Differences in NOE effects for ligands that are associated with bulk water vs. those bound to the protein are quite distinctive. Namely, ligands directly interacting with bulk water experience a positive NOE from the water resulting in signal enhancement (Figure 1D, process 2). Conversely, ligands that interact with the protein will experience a negative NOE mediated through the protein where this NOE has been initially transferred from water to protein and subsequently back to ligand (Figure 1D, process 3). In the latter case, upon dissociation the ligand will be observable (Figure 1D, process 4) and will experience a decrease in signal intensity at sites experiencing the protein-mediated NOE due to the negative enhancement factor. This difference in enhancement allows for distinguishing of molecules that bind to a given target macromolecule vs. those that do not provided that dissociation constants are relatively weak ($\sim \mu\text{M}$ to mM) provided that on rates are not too small (i.e., corresponding to a long residence time on the molecule) (Dalvit 2008). Recent optimization to the WaterLOGSY experiment also

allows for accurate K_d determination through this technique (Huang et al. 2017), further expanding its versatility.

^{19}F NMR spectroscopy

The above-mentioned techniques usually rely on ^1H -based NMR experiments in biological settings, with heteronuclear experiments applying a combination of ^1H with ^{13}C and/or ^{15}N also being feasible, particularly with isotopic-enrichment of at least one of the molecular species being studied. ^{19}F is a spin- $\frac{1}{2}$ nucleus with properties that makes it a highly effective probe for NMR studies. Namely, ^{19}F has sensitivity approaching that of ^1H , 100% natural abundance, a high gyromagnetic ratio and a wide chemical shift range (Kitevski-LeBlanc and Prosser 2012; Marsh and Suzuki 2014). Importantly, ^{19}F is also absent from natural biological systems, meaning that there are practically no background signals which must be distinguished. Cross-correlation experiments between relaxation arising from ^{19}F chemical shift anisotropy and ^{19}F - ^1H dipole-dipole contributions can be exploited to elucidate K_d values for target ligand-receptor interactions (Peng 2001). Adding to the versatility of ^{19}F NMR, effective variations of STD and WaterLOGSY experiments have been developed where ^1H nuclei are irradiated and magnetization is transferred to ^{19}F for detection (Diercks et al. 2009; Furihata et al. 2016; Sakuma et al. 2015). The large ^{19}F chemical shift anisotropy can also be taken advantage of in direct ^{19}F NMR-observed experiments for ligand screening, since this accentuates the linewidth difference between the bound and free states of a ligand allowing for detection of a smaller fraction of bound state molecules than in ^1H NMR-based experiments and reducing the required amount of binding partner protein while also expanding the range of affinities that can be detected (Dalvit et al. 2003; Dalvit et al. 2002). Membrane-interaction and binding by disparate

species can also be readily monitored by ^{19}F NMR spectroscopy (e.g., Marsh and Suzuki 2014), including otherwise challenging-to-study processes such as amyloid peptide oligomerization (Suzuki et al. 2013).

From a ligand-receptor interaction perspective, ^{19}F NMR spectroscopy has been applied to simultaneously monitor both ligand and receptor species in membrane-mimetic environments (Simmons et al. 2019), taking advantage of biosynthetic incorporation of fluorotryptophan into the receptor (Crowley et al. 2012; Kenward et al. 2018) giving distinct ^{19}F NMR signals from a trifluorophenylalanine in a synthetic peptide ligand (Murza et al. 2012). Moving into the natural membrane environment, fluorotryptophan labelling has been applied to characterize cytochrome b_5 reconstituted into nanodiscs, with influences on ^{19}F signals observed both as a function of the lipid bilayer composition and of binding to full-length cytochrome P450 2B4 (Bai et al. 2020). As a whole, although not yet realized in the on-cell context, ^{19}F NMR has great potential applicability for future on-cell NMR studies as seen in previous in-cell NMR studies (Bao et al. 2017; Shishmarev et al. 2018; Takaoka et al. 2013; Ye et al. 2018). ^{19}F NMR spectroscopy is also highly applicable and beneficial in the solid-state regime, with studies of this nature discussed below.

Probing peptide-receptor and protein-receptor interactions by solution-state on-cell NMR

In 2005, the Meyer group investigated the interaction of a pentapeptide cyclo(RGDfV; where f is D-Phe) and the surface glycoprotein integrin $\alpha_{\text{IIb}}\beta_3$ to characterize the binding interactions of cell surface proteins and the binding epitopes of ligands in native platelets (Claassen et al. 2005). Using STD NMR, a significantly higher affinity of cyclo(RGDfV) to integrin $\alpha_{\text{IIb}}\beta_3$ in native platelets was detected relative to in liposomes, reflecting a distinct

binding configuration in the natural environment. This difference in binding configuration was directly related to a different binding mode for the RG region in cyclo(RGDfV) for the native integrin receptor.

Potenza and co-workers looked at the interaction of 2 cyclic RGD- mimetic peptides with human platelets in 2008 (Potenza and Belvisi 2008) and subsequently, in 2011, with human endothelial ECV304 cells (Potenza et al. 2011) using STD and trNOE experiments to study the epitopes of these ligands. The RGD motif is known to interact with integrin $\alpha_v\beta_3$, found on the surface of both of these cell types. Epitopes of the ligands in close proximity to cell surface integrin $\alpha_v\beta_5$ were pinpointed using STD and preferred conformations of the bound ligands were evaluated by trNOE. Computational docking studies were carried out independently from the NMR experiments, allowing for direct confirmation of the NMR-derived binding features. Interestingly, although the two compounds tested only differed by configuration changes at two carbons, distinct modes of binding to the integrin were clear. In one instance, the interaction of the ligand benzyl ring with Tyr122 of subunit β_3 favoured the formation of a hydrogen bond network involving ligand Asp functionalities. In the other, the change in ligand configuration did not allow for this π - π interaction, instead favouring contact of the benzyl group with the Tyr178 side chain of subunit α_v and consequently preventing the interaction of the ligand with the β_3 subunit.

Along a similar vein, Mari et al. explored interactions of a series of cyclopeptides with two human cancer cell lines using trNOE experiments (Mari et al. 2010). Non-small cell lung carcinoma (MR300) and melanoma (MSR3) cell lines were chosen, both of which express integrin $\alpha_v\beta_3$. These cell types differ in expression of the CD13 antigen, with distinct phenotypes in terms of tumour growth and metastatic invasiveness. Through the trNOE, binding selectivity

of RGD-based (integrin $\alpha_v\beta_3$ -targeting) and NGR-based (CD13-targeting) peptide families were clearly demonstrated. Ligand competition experiments also allowed for direct comparison of binding affinities for ligands from the same family.

In an approach which was augmented by ^{15}N -enrichment, Assadi-Porter et al. examined both peptide and protein ligand binding to the heterodimeric human sweet receptor, a family 3 GPCR (Assadi-Porter et al. 2008). For the sweet-tasting protein brazzein, ^{15}N -enrichment was employed, enabling ^{15}N -selected ^1H -observed STD experiments to be carried out. Specifically, ^1H - ^{15}N STDD was used to characterize brazzein binding to the GPCR in membrane extracts of human embryonic kidney (HEK293) cells with clear differences in binding observed for a lower-affinity mutant of brazzein as reflected by a dramatic reduction in the ^{15}N -selected STDD intensity. This study also clearly showed the ability to selectively saturate a membrane-embedded receptor employing saturation at -1 ppm ^1H frequency, even if the species being observed in the ligand pool is another protein (molecular weight ~ 6.5 kDa in the case of brazzein). Standard ^1H STD experiments were also applied to characterize binding of a mixture of the dipeptide alitame and the artificial sweetener cyclamate the sweet receptor on intact cells, demonstrating on-cell NMR of peptide-receptor interactions.

A 2018 study by Brancaccio et al. investigated the interaction of peptide heptamer derivatives of the chemokine CXCL12 and the GPCR CXCR4 on the membranes of intact human T-Leukemia cells (CCRF-CEM cells) using STD, WaterLOGSY, and trNOE methods (Brancaccio et al. 2018). Through both STD and WaterLOGSY, a peptidic triad - the Arg4-Phe5-Phe6 motif – was found to be primarily involved in receptor interaction. Over and above this motif, a correctly-oriented positively charged residue at the 1-position was shown to be required to achieve high CXCR4 binding potency. Augmenting this by the use of trNOE experiments, the

presence of a well-defined side chain cluster involved in binding to CXCR4 on the cell surface was observed. Notably, computational docking studies augmented with on-cell NMR-derived restraints were shown to improve convergence over unrestrained simulations, with a great deal of ambiguity in the docking results without the experimental restraints. This is an exciting demonstration of the utility of on-cell NMR studies for direct characterization of peptide-GPCR interactions, with an experimental strategy that may be broadly applied for dissection of many other classes of cell surface interactions.

Through a distinct type of experimental design, the Pazos group used 1D and 2D ^1H NMR experiments to enable peptide ligand-specific chemical shift assignment under various on-cell conditions (Martin-Pastor et al. 2010). Specifically, interactions of the acylated ghrelin peptide, the endogenous ligand for the GH secretagogue receptor (GHS-R1a) receptor, and its des-acylated form by on-cell NMR using stably GHS-R1a transfected Chinese hamster ovary (CHO) and HEK293 cell lines. Comparison was made to both buffer and non-transfected control cells. In the presence of GHS-R1a-transfected cells, ghrelin showed a higher number of residues that underwent chemical shift perturbation or chemical exchange than des-acyl ghrelin. Using chemical shift-based secondary structural inferences (Wishart et al. 1992), structural calculations were carried out leading to the suggestion that the observed NMR data were most consistent with two sets of preferred peptide bond isomerization states at four prolines isomers in ghrelin that allowed a left-handed α -helix to form from Glu8 to Lys20. Binding of ghrelin to the GHS-R1a receptor thus appears to favour specific *cis/trans* isomerization states – whether the binding event promotes or follows these isomerization events taking place is uncertain. It should be explicitly noted that in this and the subsequent studies that focus on effects on chemical shift and nuclear spin relaxation, the observed perturbations are most likely attributable to ligands

undergoing exchange at an appropriate timescale to allow for solution-state NMR observation with a corresponding scaling of the observable perturbation on the basis of the stoichiometry (typically, large excess of ligand relative to receptor.) This makes interpretation challenging but does not preclude experimental observation or analysis of the underlying intermolecular interaction provided that sufficient control experiments are also carried out.

Expanding on this type of strategy of specific ligand chemical shift evaluation, Diana et al. applied on-cell NMR spectroscopy to investigate the functional binding surface of a β -hairpin Vascular Endothelial Growth Factor Receptor 2 (VEGF-2) to ^{15}N -enriched HPLW peptides (Diana et al. 2015). Chemical shift perturbation and variations in intensity and relaxation time constants as a function of position in the HPLW peptide were specifically made possible through use of ^{15}N -edited NMR spectroscopy experiments. This allowed precise characterization of the epitope of the HPLW peptide that is recognized by VEGF-2 in stably-transfected porcine aortic endothelial cells (PAEC). Further collaborative studies by the same research groups examined the VEGFR binding interaction with the VEGF-mimetic QK peptide under both *in vitro* and *in vivo* (PAEC-VEGFR1 or PAEC-VEGFR2) conditions (Di Stasi et al. 2018). This included both ^{15}N -based examination of the QK peptide alongside STD to test for competition between the QK peptide and the natural ligand VEGF165. Using both STD and chemical shift perturbation results, a molecular model of the QK/VEGFR1D2 complex was produced and demonstrated to be in agreement with the residues of QK identified as being involved in binding. These studies have elucidated the functional mechanisms underlying different modes of ligand binding to VEGFR, directly informing medicinal chemistry efforts.

Most recently, an on-cell saturation transfer difference (STD) NMR study of bombesin (BN) binding to gastrin-releasing peptide receptor (GRP-R) (Palmioli et al. 2020). To develop

new strategies for the selectively-targeted delivery of anticancer drugs to GRP-R and diagnostics directly applicable to tumour cells, detailed understanding of the GRP-R interaction with its natural ligand BN is needed. This study utilized on-cell STD NMR techniques for the screening and epitope mapping of GRP-R ligands in the physiological environment with GRP-R-overexpressing tumour cells. Excitingly, the key residues (Trp8, His12, Leu13 and Met 14) that make up BN binding epitope were specifically elucidated in this manner. Looking to the future, the groundwork provided in this study will allow for a move to rapid screening of new potential GRP-R ligands.

Other solution-state on-cell NMR studies

The first study demonstrating the potential application of STD under on-cell NMR conditions investigated polysaccharide-based ligand-receptor interaction. Namely, Mari et al. probed molecular recognition between a carbohydrate (*Saccharomyces cerevisiae* mannan) and a C-type lectin receptor (dendritic-cell-specific ICAM-3-grabbing non-integrin, DC-SIGN), a process which initiates phagocytosis as part of the immune response to pathogens (Mari et al. 2005). This study demonstrated a direct interaction between DC-SIGN and *S. cerevisiae* mannan, with the proposal that STD NMR would be valuable as a potential drug-screening technique for non-peptide ligands through direct observation of perturbation in this binding.

An alternative application of on-cell NMR to polysaccharides is the characterization of bacterial capsular polysaccharides. A study in 2006 applied on-cell NMR to compare *Neisseria meningitidis* serogroup B and *Escherichia coli* K1 cell capsular polysaccharides (Azurmendi et al. 2007). This study specifically focused on polysialic acid, a capsular polysaccharide with the distinguishing feature that it generates little or no immunogenic response in humans. In this

study, a variety of heteronuclear NMR experiments were applied (see, e.g., Cavanagh et al. 2007), including ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) and ^1H - ^{13}C HSQC experiments alongside 2D versions of the HNCA and HNCO experiments. Real-time monitoring of the NMR signal intensity increase upon cleavage of polysialic acid by *exo*-neuraminidase, an enzyme that can only access cell surface saccharides, was performed used to confirm that the observed polysialic acid was indeed located on the cell exterior. No substantial difference was observed between free and cell-bound polysialic acid, indicating that the poor immunogenicity of this saccharide does not arise from major structural differences between the free and cell-surface states. Although not explicitly discussed by the authors, it seems likely that the relatively high degree of intramolecular mobility and dynamics experienced by cell surface glycans (vs., e.g., a membrane-embedded receptor) would be responsible for providing sufficiently narrow lines to allow the observation of these resonances through solution-state methods.

A 2009 study utilized STDD to screen a marine natural product extract library for binding to two human GPRCs, the cannabinoid receptors CB1 and CB2 (Pereira et al. 2009). Using engineered Sf21 insect cells, STDD was applied to compare interactions of extracts between CB1-expressing, CB2-expressing and two control cell Sf21 lines. Through this approach, the compound haplosamate A was identified as a selective binder to CB1 and CB2.

In a recent study, the Potenza group investigated interactions between a trisaccharide derivative of sialic acid and two hemagglutinins from human and avian strains of influenza virus expressed on the surface of stably HA-transfected HEK293T human cells (Vasile et al. 2018a). In this context, the HAs were shown to retain their native trimeric conformation, demonstrated through reactivity with monoclonal antibodies, and host-cell binding properties through microscopy. Using STD NMR, different sugar epitopes were demonstrated to be recognized by

each HA species in this HEK293T cell surface setting. The trNOE experiment was also applied but did not provide diagnostic NOE data. In a follow-up study, both STD and trNOE experiments were applied to compare binding of two trisaccharide to this pair of HAs on the surface of HEK293T cells (Vasile et al. 2018b). Applying the binding epitope information determined through solution-state on-cell NMR, molecular dynamics simulations were carried out to determine trisaccharide conformations consistent with the bound-state. The conformations thus determined were noted to be consistent with prior co-crystal structures, nicely demonstrating the applicability of on-cell NMR as a means to provide experimentally-based restraints and inferences to directly inform structural calculations and screen for conformations that are consistent with the physiological setting.

Solid-state on-cell NMR studies

Solution-state NMR techniques have traditionally focused on molecular species and assemblies of ~50 kDa and less, although recent experimental advances have enabled studies of species of up to ~1 MDa in molecular weight (Rosenzweig and Kay 2014). In general, as molecular weight (or, more correctly, “apparent molecular weight”) increases, tumbling rates decrease leading to rapid transverse relaxation and broader NMR lines (Cavanagh et al. 2007). Similar issues occur with increased viscosity. The relative influences of different terms in the NMR spin Hamiltonian upon also change as tumbling slows or under conditions of anisotropy in comparison to an isotropic solution-state setting. Solid-state NMR techniques allow for characterization of nuclei under conditions where line-broadening make samples poorly amenable to solution-state techniques – this need not be strictly a “solid” sample, with a wide variety of different classes of biological sample being amenable to solid-state NMR study (e.g.,

various classes of protein (Demers et al. 2018; Goldberga et al. 2018; Mandala et al. 2018; Opella and Marassi 2017; Siemer 2020), RNA (Marchanka and Carlomagno 2019), and bacterial cells (Booth et al. 2017; Freedberg and Selenko 2014; Warnet et al. 2015). On-cell NMR using solid-state NMR has been more limited in application than solution-state to date, with four primary experimental techniques applied: magic angle spinning (MAS), rotational echo double resonance (REDOR), cross-polarization (CP), and dynamic nuclear polarization (DNP). These methods have the advantage over solution-state methods, which typically rely on inferences of the bound state through perturbation to the observable solution-state NMR properties (Figure 1A-D), of enabling study of both the ligand and the receptor in the physiological membrane environment (Figure 1E).

Primary solid-state NMR experimental techniques

In MAS (Andrew et al. 1958; Lowe 1959), the sample is packed into a rotor that is physically oriented at the so-called magic angle (54.74°) relative to the static magnetic field of the NMR spectrometer and rotated rapidly about this axis. The fundamental requirement is that the spinning rate must exceed the linewidth, which may be many kHz. In some instances, typically with soft solids or gels, a relatively slow spinning rate (~ 3000 - 5000 Hz) is applied under high-resolution MAS (HR-MAS) conditions, requiring a specialized probe to achieve liquid-state like resolution (Fitch et al. 1994). In samples with broader lines and/or slower motion that are not amenable to HR-MAS, the required spinning rates tend to be higher and distinct solids-oriented classes of experiment are applied. These may be subdivided into experiments observing nuclei other than ^1H or those observing ^1H . For the latter class, ^1H - ^1H dipolar couplings may be on the order of ~ 100 kHz or higher with “fast” or “ultra-fast MAS” required at

rates of 70-110 kHz using small rotors and correspondingly small sample mass (Zhou et al. 2007). Through MAS, orientationally-dependent broadening may be eliminated, providing narrow lines at the isotropic chemical shift at the expense of orientational information.

REDOR (Gullion and Schaefer 1989) is a specific MAS-based solid-state NMR technique whereby the effects of distance-dependent spin-spin dipolar interactions are reintroduced. Linewidths are narrow through the MAS phenomenon, with chemical shifts observed at the isotropic values. Through direct comparison of signal intensity attenuated by reintroduction of the dipole-dipole interaction relative to a reference experiment, distances between NMR-active nuclei may be determined. Variations on this experiment allow measurement of both homonuclear and heteronuclear dipolar interactions, giving a great deal of versatility in the systems that may be probed.

In the solids regime, short transverse relaxation times (T_2 's) leading to broad signals and long longitudinal relaxation times (T_1 's) requiring long delays before experimental repetition are characteristic of so-called dilute spins (e.g., Apperley et al. 2012; Duer 2004). To counteract the issue of long T_1 and to improve signal-to-noise for relatively insensitive nuclei (e.g., most non- ^1H spin- $1/2$ nuclei), cross-polarization (CP) methods are frequently employed. In CP, rather than directly exciting the insensitive/dilute nucleus, magnetization is transferred from a high-abundance and high-sensitivity nucleus such as ^1H (Hartmann and Hahn 1962). These high-abundance nuclei typically have shorter T_1 , allowing for much more rapid experimental acquisition for the nucleus undergoing enhancement.

The DNP phenomenon provides dramatic sensitivity enhancement over and above that of the CP method (Ni et al. 2013). A stable radical-containing polarizing agent must be doped into the sample. Its unpaired electron is polarized, a species which typically has a short T_1 . This

polarization is subsequently transferred to the NMR-active nuclei being studied through microwave irradiation, in turn increasing the polarization of the nuclear species by factors of up to 10,000. One caveat is that the process must be carried out at cryogenic temperatures, leading to some limitations in its suitability for application. Notwithstanding this limitation, DNP-enhanced MAS is becoming more and more frequently applied with several notable examples of its use for cell-based NMR studies.

The benefits of ^{19}F as a site-specific and background-free NMR-active label (*vide supra*) can also be directly applied to proteins in environments approaching those of intact cells using solid-state NMR. As an example, Polenova, Gronenborn and co-workers have applied MAS-based solid-state NMR to study human immunodeficiency virus 1 (HIV-1) capsid proteins in tubular assemblies (Lu et al. 2019; Wang et al. 2018b). In both of these studies, biosynthetic methods (Crowley et al. 2012) were applied to incorporate 5-fluorotryptophan into uniformly (with the exception of the tryptophan indole rings) $^{15}\text{N}/^{13}\text{C}$ -enriched capsid proteins. Through MAS-based methods alone, applying a fast-MAS spinning frequency of up to 60 kHz, ^{19}F - ^{19}F distances of up to 20 Å within HIV-1 capsid protein tubes could be measured (Wang et al. 2018b). With the addition of DNP-enhancement, ^{13}C - ^{19}F heteronuclear correlation (HETCOR) experiments were possible under MAS (Lu et al. 2019). This, in turn, allowed for development of a series of distance-dependent ^{13}C - ^{19}F correlations up to ~12 Å in length, providing insight into the HIV-1 capsid protein assembly and architecture in the native tubular environment. Both of these classes of ^{19}F -based distance determination techniques have exciting potential to provide structural constraints in challenging environments, including the on-cell perspective.

Studies enabled by solid-state on-cell NMR spectroscopy

From the end of the 1990's until now, HR-MAS on-cell NMR techniques have been developed and successfully used but have not focused on understanding ligand-receptor interactions (Gudlavalleti et al. 2006; Jachymek et al. 1999; Niedziela et al. 2006; Szymanski et al. 2003; Zandomeneghi et al. 2012). In a pioneering 1999 study, Jachymek et al. applied HR-MAS to compare and contrast characterize polysaccharides on the surface of *Yokenella regensburgei* strains (Jachymek et al. 1999). Strain-specific differences in acetylation of O-specific polysaccharides on the cell surface lipopolysaccharide layer were identified, with HR-MAS allowing this to be demonstrated both for isolated lipopolysaccharides and for living bacterial cells. This seminal study demonstrated applicability of NMR spectroscopy in obtaining atomic-level insight into cell surface molecules.

Following this work, a variety of other studies have characterized both molecular structures and interactions on bacterial surfaces. A series of studies from the Schaefer group probed antibiotics on the surface of intact bacteria using both REDOR (Kim and Schaefer 2008; Kim et al. 2009) and REDOR coupled with CP-MAS (Patti et al. 2009). Positioning and conformational plasticity of antibiotics relative to cell wall components was achieved through ^{19}F -labelling of antibiotics, allowing for specific REDOR-based distance determination to both peptidoglycan (^{15}N - and ^{13}C -enriched (Kim and Schaefer 2008; Kim et al. 2009; Patti et al. 2009)) and outer membrane lipids (^{31}P (Kim et al. 2009)). Mechanistic insight was also derived through direct evaluation of effects of antibiotics on cell wall components through $^{13}\text{C}\{^{15}\text{N}\}$ -REDOR experiments (Patti et al. 2009). Subsequent studies of lipopolysaccharides have employed HR-MAS (Zandomeneghi et al. 2012), while bacterial cell wall peptidoglycan in intact cells has been probed using ^{31}P MAS NMR (Kern et al. 2010) as well as CP-MAS and REDOR

(Zhou and Cegelski 2012) techniques. DNP enhancement has also been demonstrated for peptidoglycan in intact bacterial cells (Takahashi et al. 2013). It should also be noted that the structural features and interactions of polysaccharides have also been studied in intact plant (Dick-Perez et al. 2011) and fungal (Kang et al. 2018) cell walls using MAS-based techniques, with DNP enhancement employed in the latter case, implying that these classes of cell may also be amenable to future on-cell NMR studies.

From a somewhat distinct standpoint, solid-state NMR has been demonstrated to be extremely amenable for studies of antimicrobial peptide interactions with bacterial cell surfaces, as has been recently reviewed by Booth et al. (Booth et al. 2017). Studies of this nature have tended to focus upon the manner in which bacterial membrane dynamics are modulated by antimicrobial peptides. This is facilitated by ^2H -labelling of lipids, allowing determination of order parameters representative of lipid mobility and state as a function of position in the lipid acyl chain through ^2H NMR – usually under MAS conditions, and ^{31}P NMR, which is reflective of the headgroup state.

It should also be noted that HR-MAS provides additional versatility in terms of characterization of molecular profiles and behaviour in biologically-relevant environments. Tissue-specific metabolic profiling is achievable (Somashekar et al. 2011), for example, allowing for comparison of carcinomas collected from different anatomical locations and under different tumour grades. Amyloid- β oligomers have also been studied using ^1H - ^1H radiofrequency driven dipolar coupling (RFDR) experiments enabled by HR-MAS, with a key being that purification was not required allowing for direct and high-resolution observation of supramolecular assembly in a messy and challenging environment (Kotler et al. 2015). This HR-MAS-based methodology was subsequently employed to monitor aggregation of amyloid A β peptides in real time, with

differences observable both in terms of the applied spinning rate and the peptide concentration (Wang et al. 2018a). These classes of study can be readily envisioned as being extendable to the on-cell context, where supramolecular assembly processes or metabolite behaviour may be monitored in the cell-surface context.

On-cell solid-state NMR also has strong potential for characterization of interactions from the perspective of cell surface proteins. The integral membrane protein PagL has been characterized by MAS in both whole cell and isolated cell envelopes of *E. coli* (Renault et al. 2012b), with ^{15}N and ^{13}C resonances assigned for 13 of 150 residues in diverse regions of the protein demonstrating excellent agreement with the X-ray crystallography-based structure. In this study, Renault et al. also characterized the 56-residue lipoprotein Lpp-56 – which forms a three-stranded coiled-coil that can be peptidoglycan-anchored or free – in *E. coli* in both whole cell and cell envelope settings. The observed ^{15}N - ^{13}C and ^{13}C - ^{13}C chemical shift correlation patterns agreed extremely well with those predicted on the basis of the high resolution X-ray crystal structure of the Lpp-56 homotrimer over the core of the coiled-coil, with deviations observed only at the N- and C-termini consistent with the potential for changes in conformation and/or dynamics in the cell or cell envelope context relative to the crystal. Clear evidence was also observed for Lys residues corresponding to both peptidoglycan-bound and free states of Lpp-56. In a follow-up study, DNP-enhancement was demonstrated to be achievable for both PagL and Lpp-56 in both *E. coli* cell envelope and whole samples (Renault et al. 2012a).

Cytochrome- b_5 , which has a single transmembrane helix and larger soluble domain, has also been demonstrated to be suitable for on-cell NMR using DNP/MAS techniques in intact *E. coli* (Yamamoto et al. 2015). Reasonably well-resolved ^{13}C - ^{13}C correlation MAS spectra were observed for cytochrome- b_5 with a reported correlation range of up to ~ 5.5 Å in the

transmembrane region, allowing for inter-residue ^{13}C - ^{13}C correlations to be observed. This proof-of-principle study demonstrates the feasibility and applicability of DNP-MAS for atomic-level study of structure and dynamics in membrane-bound proteins with large soluble domains in intact cells. Follow-up work from Yamamoto et al. demonstrated the effectiveness of DNP-MAS-based methodologies for studying the full-length cytochrome P450-cytochrome-b₅ in the bilayer context (Yamamoto et al. 2017), indicating that these much larger protein complexes are also amenable to these techniques.

The demonstrated potential to probe integral membrane proteins in intact cells by solid-state NMR (Renault et al. 2012a; Renault et al. 2012b; Yamamoto et al. 2015) logically couples with the suitability of DNP-enhancement to allow evaluation of protein-receptor interactions (Kaplan et al. 2016; Linden et al. 2011) and intact integral membrane proteins (Jacso et al. 2012) or complexes (Kaplan et al. 2015; Yamamoto et al. 2017) in native membrane extracts. Namely, progression from membrane extracts to whole cells is likely to become increasingly feasible and commonly applied, especially given the demonstration of experimental feasibility and comparability of results between membrane/cell envelope and intact cell environments demonstrated both by Renault et al. (Renault et al. 2012a; Renault et al. 2012b) and by Yamamoto et al. (Yamamoto et al. 2015). Looking forward, this approach as a whole holds great potential for even more physiologically-relevant on-cell NMR studies probing both molecular structure and dynamics as well as intermolecular interactions.

Summary and outlook

On-cell NMR provides an exciting and relatively newly-developed strategy for probing interactions between ligands and receptors (summarized in Table 1). From the ligand

perspective, NMR techniques optimized for observation of a ligand can be applied to map binding epitopes and single out active/binding ligands in a mixture to enhance understanding of a given ligand-receptor interaction at the atomic-level on a cell surface. Techniques targeted at cell surface receptors themselves, or at other classes of cell surface molecules, typically take advantage of the ability to study molecules in a wide variety of physical contexts through solid-state NMR methodologies. It should also be emphasized that many of the techniques discussed and reviewed herein are useful for other classes of system that have been traditionally difficult to characterize, including amyloid fibre self-assembly processes and architecture at mimetics of cell surfaces (e.g., Brender et al. 2012; Kotler et al. 2014; Okada et al. 2019; Sahoo et al. 2020) or protein liquid-liquid phase separation (e.g., Murthy and Fawzi 2020; Reichheld et al. 2017), among others. As a whole, the studies reviewed here highlight the value of continued advancement of on-cell NMR techniques to avoid the necessity to produce and purify challenging species such as membrane proteins while enabling study of truly multi-component systems in physiologically-relevant settings. Given the potentially wide applicability of on-cell NMR spectroscopy, it seems likely that these techniques will become key in both the elaboration of fundamental aspects of processes on cell surfaces and in applied roles such as therapeutic screening, drug development and diagnostics.

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Table

Table 1 – Overview of potential classes of information about cell surface ligand-receptor interactions obtainable via solution-state or solid-state on-cell NMR spectroscopy methodologies.

Information Method	K _d	Ligand competition or discrimination	Binding site on ligand	Binding site on receptor	Ligand conformation and/or dynamics	Receptor conformation and/or dynamics	Ligand- receptor distance constraints
STD ^{a,b}	●	●	●				
trNOE ^a	●	●	●		●		●
WaterLOGSY ^{a,b}	●	●	●				
Solution-state NMR-based chemical shift and/or spin relaxation ^c		●	●		●		
Solid-state NMR-based chemical shift and/or spin relaxation ^{c,d}			●	●	●	●	
Solid-state NMR-based dipolar interactions ^{c,d,e}			●	●	●	●	●

^a Solution-state NMR experiments that typically observe ¹H, with potential to take advantage of a heteronucleus (¹⁵N, ¹³C, ¹⁹F) in selection or editing of the experimental result

^b Also effective ¹H-saturated, ¹⁹F-observed variations developed

^c May take advantage of versatile nuclear species including ¹H (typically limited to solution-state NMR, except under fast or ultra-fast MAS), ²H (solid-state NMR), ¹³C, ¹⁵N, ¹⁹F, ³¹P, etc.

^d MAS-based methods, usually employing CP and with potential for DNP-based enhancement, discussed herein

^e Including, but not limited to, REDOR or the ¹⁹F-based correlations discussed herein

Figure Caption

Figure 1 – Schematic illustration of the primary NMR spectroscopy techniques reviewed herein.

The techniques depicted in (A-D) are solution-state NMR methodologies. (A) Saturation transfer difference (STD) experiment, whereby (1.) the receptor is saturated by radiofrequency (RF) excitation, (2.) this is transferred to bound ligand, and (3.) the bound, saturated ligand dissociates and joins a larger pool of unsaturated, free ligands; resonances involved in binding will be attenuated in the STD-derived difference spectrum in direct relation to the binding. (B) Intramolecular and (C) intermolecular transfer nuclear Overhauser enhancement (trNOE), where (1.) the NOE process occurs more effectively in the ligand-receptor complex than for free ligand, with (2.) resulting intramolecular (B) and/or intermolecular (C) NOE's being observed in released and excited free ligand following dissociation. (D) WaterLOGSY experiment, where (1.) water molecules are excited and produce an NOE either (2.) for free ligands, typically with a positive enhancement, or (3.) via bound waters in the ligand-receptor complex that leads to a negative enhancement (4.) observed in ligands following dissociation. (E) Solid-state NMR strategies allow for direct observation of ligand and/or receptor, as well as their interactions, through modulation of chemical shift (δ), spin relaxation, and/or through measurable energy transfers dependent on (e.g.) internuclear distance.

Figure 1: