Structure-based drug design: NMR-based approach for ligand–protein interactions

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The realization of the powerfulness in analyzing ligand–protein interactions at the atomic resolution has made NMR techniques increasingly attractive in drug discovery and development. With some significant new method developments during the past few years, NMR-based approaches will undoubtedly be helpful in high throughput screening and in providing structural and interaction information beneficial for new drug developments. Here in this review, instead of providing an exhaustive account of applications of NMR, we will seek to highlight some key points related to the solution state NMR methods for extracting information from both ligands and proteins.

Introduction

As an important drug discovery tool, nuclear magnetic resonance (NMR) can provide valuable information on the molecular structure, dynamics and interactions between drugs and their targets. Ever since the well-known NMR method, so-called ‘SAR by NMR’, was introduced in 1996 [1] to reveal the structure-activity relationships (SAR) in discovering high-affinity ligands for proteins, many new NMR techniques for measuring protein–ligand interactions have been developed during the past few years [2–6], although such development will be predictably accelerated even further to achieve better understandings for the structure of ligand–protein complex and dynamics of the interaction. A number of reviews have already been published [4,7–10] on using NMR approach in drug discovery and screening. The aims of this review will seek to provide a description of the technical aspects of solution state NMR-based methods applicable to ligand–protein interactions and to address some of the key issues including the current NMR strategies aimed to extract interaction information from both proteins and ligands in free and bound states.

Protein-based experiments

The binding of ligands to a protein often results in changes in the protein’s structure and dynamics, in addition to the local environmental changes on the binding surface. Properties of the ligand are also affected by the interactions. These changes will be in turn manifested in the NMR properties of both ligands and the protein, such as chemical shifts, relaxation rates and line-shapes of NMR peaks. Therefore, these parameters can be used as indicators for the interactions, the relative orientation and motions of domains within proteins, which are key factors affecting the multivalent recognition.
Chemical shift perturbation

The strong binding of a ligand to a protein often results in changes of protein chemical shifts. This has made the chemical shift perturbation experiment one of the most widely used methods for probing ligand–protein interactions [11–13] by measuring the chemical-shift changes of the protein during titration using a ligand. The amino acid residues that experienced progressive chemical changes were generally located on the binding surface or in the domain of structural changes; the larger the chemical-shift change is, the closer the residues or spins are to the binding site. Using the fast exchanging model, the dissociation constant ($k_d$) can be derived from chemical-shift changes as function of the ligand concentration [14].

Protein dynamics

Dynamics are vital to protein functions and alterations in its three dimensional structure due to specific molecular interactions will lead to changes to its dynamics. The dynamics and their alteration can often be readily measured in auto-relaxation that is one of the most extensively explored methods for accessing the dynamics of the protein backbone. The spin properties of $^{15}$N, $^{13}$C or $^2$H sites in proteins, including both longitudinal and transverse relaxation rates ($R_1$ and $R_2$), are sensitive to the dynamics of the proteins in particular the local flexibility [15]. Upon binding, the changes will occur in the relaxation rates of the nuclear spins at the interactive interface. In addition, the transferred cross-correlated relaxation [16] and transferred residual dipolar coupling are also important parameters for probing the ligand–proteins interactions [17] in terms of the binding geometries. Furthermore, a number of novel experiments have been introduced to probe the side chain dynamics [18] during past few years, because the side chains are always related to the protein surface properties and their motions are essential for ligand binding. For example, methyl groups of the human intestinal fatty acid binding proteins (IFABP) are often found in the hydrophobic cores, the binding of oleic acid results in decreased mobility of most of its methyl groups in the binding region [19]. The protein-targeted experiment can provide information about interaction interface and structure. However, extra care has to be taken to distinguish the structural and dynamic changes in the binding surface and in more extended regions of the protein molecule. It is also worth noting that the chemical shift perturbation may also result from the variation of pH and/or salt concentration although it is an excellent indicator of allosteric processes [20].

Ligand-based experiments

The ligand-based methods are established on the basis of the fact that upon binding to a target protein, the molecular tumbling rates of ligands in solution are altered. Generally, most of the NMR screening libraries consist of small molecules that always exhibit slow relaxation ($T_1$, $T_2$) and fast diffusion. When binding to a large receptor, nuclear spin relaxation time and molecular self-diffusion coefficient of the ligand will be dramatically reduced. These changes enable the ligand–protein interaction to be accessed by directly observing the ligand NMR signals.

Relaxation-edited methods

Relaxation-based methods are more sensitive than diffusion-based methods because of increment of apparent molecular weight upon binding [21]. In particular, the paramagnetic induced relaxation approach, such as so-called SLAPSTIC (the spin labeled attached to protein side chains as a tool to identify interacting compounds), offers a sensitive method for interaction mapping [22]. This is because that the magnetic moment of the electron is thousands times larger than that of the proton, and the amount of the biological targets required will be reduced by 1 to 2 orders of magnitude, alleviating the necessity of high ligand solubility. In addition, because relaxation is a spin property, a spin or nucleus that closed to the target protein or binding site will experience larger relaxation-rate change (similar to chemical shift). Therefore, the relaxation rate measurement can also provide information of the ligand orientation at the binding site.

Diffusion-edited experiment

Measuring self-diffusion coefficient is a convenient way to study the ligand binding without prior separation of the components [23]. Because diffusivity is a principle property of the whole molecule, all NMR signals of a ligand are decayed identically in the experiment with pulsed-field-gradients (PFG). In solution containing a target protein and ligands, the bound ligands will have small diffusion coefficients and their NMR signal intensities will be less attenuated in the PFG-NMR diffusion experiment. By selecting a suitable diffusion-attenuation factor such as the gradient strength, the NMR signals from nonbinding ligands will be filtered out because of their fast diffusion, and thus, the bound ligands will be selectively detected from the mixture. The efficiency for PFG-NMR depends on the differences in the observed diffusion coefficients between binding and nonbinding ligands. Because free and bound ligands are generally in fast exchanging on the diffusion time scale, the measured diffusion coefficients are the weighted average of the free and bound forms. This will narrow the gap of diffusivities between binding and nonbinding ligands, leaving a small window to identify ‘active’ components in the mixture [24,25]. In using PFG-NMR, one often has to disentangle the transferred nuclear Overhauser effect (trNOE) from the diffusion. Nevertheless, the decay of trNOE can also be used as an effective way to gain insight into ligand–protein interaction during the diffusion time [24–27].
**NOE pumping and reverse NOE pumping experiment**

The nuclear Overhauser effect (NOE) results from dipole–dipole interactions of atomic spins (through space); its strength is inversely related to the interspin distances \( r^{-6} \). In the NOE and reverse NOE pumping (RNP) experiments, the details in protein–ligand interactions are obtained through the dipole–dipole interactions to transfer the magnetization from protein to bound ligands or from binding ligands to protein. Therefore, a state where only the ligand signals or the protein signals are preserved has to be prepared by the diffusion \([24]\) or relaxation \([25]\) weighted experiments. Because of fast exchange between free (slow relaxation) and bound (fast relaxation) ligands, the magnetization transferred from protein is accumulated in the free ligands, resulting in a pumping effect for the NOE. The method can give reliable results for an interacting pair without the necessity of chemical separation or isotope labeling.

However, the NOE/RNP is generally limited to the systems with large differences in transverse relaxation time or diffusion coefficients between the receptor and ligands, and requires the binding reaction to be in the fast exchange regime between the free and bound states. In the case of slow exchange systems, isotope filtering/editing technique can be used to prepare nonequivalent states without the \( T_2 \) relaxation limitation.

**Isotope filtering/editing NMR techniques**

Isotope filtering/editing NMR techniques make use of isotopic \((^{13}\text{C},^{15}\text{N},\text{etc})\) labeling to selectively detect the spectrum of the labeled protein or ligand \([28]\), and the spectral signals of the other components are filtered out. By combining filtering/editing approach with conventional two dimensional or three dimensional experiments, the structure of the bounded proteins or ligands can be directly obtained. To maintain high sensitivity, the target component has to be completely labeled with NMR active isotopes followed with higher performance of the isotope filtering technique to suppress the unlabeled component. This kind of experiment can also be carried with direct detection of the labeling nuclear spin, \(^{13}\text{C}\) or \(^{15}\text{N}\) NMR. Because of low gyromagnetic ratio \((\gamma)\), \(^{13}\text{C}\) and \(^{15}\text{N}\) NMR is less sensitive than proton \((^1\text{H})\) NMR.

**Saturation transfer difference (STD) experiment**

Saturation transfer difference usually starts from saturating one of the resonances (peaks) of protein \([29,30]\) and until a steady state of saturation is achieved. There are magnetization-transfer mechanisms, spin diffusion and cross-relaxation, happened during the saturation. Because of spin diffusion, all signals of the protein will be saturated. Most importantly, cross-relaxation carries the saturation effect to ligands that interacts with the target protein. By taking difference of two spectra obtained with and without saturation, respectively, one can get a spectrum contained the resonances of a protein and its binding ligands. NMR peaks of the nonbinding ligands in the sample are not affected by the saturation and, thus, canceled by the difference. Therefore, the STD experiment is useful in identifying all potential ligands that interact with protein in a mixture. For weak interactions, the STD experiment is more sensitive than the NOE-based approach and, therefore, low concentration of protein can be used \([31,32]\). WaterLOGSY experiment (Water–Ligand Observation via Gradient SpectroscopY) is another intermolecular magnetization-transfer difference experiment based on saturation of water resonance \([33]\) instead of protein resonance used in the STD approach. Because water plays an important role in protein surface, saturation of water signals definitely saturated the protein and the bound compounds. Because of the fast exchange between bound water and bulk water, the large quantity of bulk water magnetization increases the sensitivity of the WaterLOGSY experiment.

**NOE/trNOE experiment**

Nuclear Overhauser effect provides another mechanism for both inter and intramolecular magnetization transfers. Because magnitude of NOE enhancement between two nuclei spins are exponentially related to the internuclear distance \( r^{-6} \), NOE related experiments have been widely used for determining three dimensional structures of protein and ligand–protein complex, orientations of the binding domain in protein and ligand inside or toward the binding site, as well as for deriving dynamic information for the ligand–protein interactions. It is worth noting that NOE experiments work well for the high affinity interactions, where stable ligand–protein structure is formed. In the case of low affinity binding or weak interactions, transferred NOE (trNOE) spectroscopy \([34–36]\) can be the method of choices. Transferred NOE provides information about orientation of the ligand at the protein binding site. In practice, trNOE as detected in NOEY experiments generally reaches a maximum within a few hundred milliseconds after which differential trNOE for individual ligand functionalities is equalized by spin diffusion of the NOE throughout the ligand proton spin system. Therefore, when long mixing times are used, NOE may diffuse throughout a proton spin system, affecting remote nuclei not directly involved in dipolar coupling \([37]\).

**Future perspective**

It is obvious that study of ligand–protein interaction in biological systems at atomic resolution is an ideal way for understanding the true pharmacological mechanism happening in living systems. From a technical point of view, such a goal can only be achieved by a combination of methods that are used for characterizing the target protein or ligand, solving the spectral overlapping and the other problems associated with the complexity of the system.
One of the examples is the study on interactions between ibuprofen, a potential drug for inhibiting low-density lipoprotein oxidation and lipoproteins in intact blood plasma [38]. Using the diffusion-edited experiment, chemical shift up-field drifts were observed for certain resonances of the lipoproteins after ibuprofen was introduced into the blood plasma. From two-dimensional (2D) correlation spectroscopy (COSY) experiment, chemical shift and line-shape analysis, it was possible to assign the resonances that experienced the chemical shift perturbation to the protons of –N[CH3]3 moieties of phosphatidylcholine (PC) and sphingomyelin (SM), olefinic chains (–CH=CH−, –CH=CHCH2CH=CH−, –(CH2)nCH2CH=−), and (CH3)3 groups and CH3 groups from unsaturated lipids in lipoprotein particles. This revealed that the negatively charged carboxyl group of ibuprofen may interact with the positively charged trimethyl head group of PC and SM, whereas the phenyl and isobutyl groups of the ibuprofen may take part in the hydrophobic interaction with the olefinic chains, (CH3)3, and CH3 groups of the unsaturated lipids [38]. The results were further improved by observation of intermolecular NOE, relaxation rate and diffusion coefficient measurements. These results may elucidate the mechanism by which the drug protects against oxidative modification of lipoproteins.

The recent development of the in-cell NMR technique [39] makes it possible to study protein–protein interactions in physiological conditions [40] without the time-consuming purification procedures and the use of buffer. In solution NMR, buffering is critical in drug-protein interactions because the solubility and stability of the investigated macromolecules are seriously affected by the solvent conditions. It is conceivable that studies of ligand–protein interaction in intact biological system will attract more and more attention.

When the proteins are not stable in solution or have poor solubility, solid state NMR may be a method of choice. This is exemplified by the 1H-19F REDOR measurements [41] as the first example of the distance measurements in an RNA-peptide complex.

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