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In-cell nuclear magnetic resonance spectroscopy for studying intermolecular interactions

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Abstract Studies on the interactions of proteins with partner molecules at the atomic resolution are essential for understanding the biological function of proteins in cells and for developing drug molecules. Solution NMR spectroscopy has shown remarkably useful capability for investigating properties on the weak to strong intermolecular interactions in both diluted and crowded solution such as cell lysates. Of note, the state-of-the-art in-cell NMR method has made it possible to obtain atomistic information on natures of intermolecular interactions between target proteins with partner molecules in living cells. In this mini-review, we comprehensively describe the several technological advances and developments in the in-cell NMR spectroscopy.

Keywords in-cell NMR, protein-protein interaction, drug discovery

Introduction

Proteins are main players for numerous biological functions mainly through intermolecular interactions. Practical application using proteins to biotechnology and nanotechnology has been actively performed. Studies on intermolecular interactions of proteins with natural and artificial partner molecules are also important for drug discovery. Interprotein interactions show a range of affinity, weak, intermediate, and strong intermolecular interactions, and have been essential criteria to characterize properties of interplays among molecules. In general, biophysical and biochemical approaches detect the intermolecular affinity ranging several sub-nanomolar to several hundred molar dissociation constant (K_d). However, too weak and strong affinity is still in the limit of detection. Solution NMR spectroscopy has been one of the powerful approaches to investigate intermolecular interactions in solution with a high resolution, i.e., atomic and residue, for a long time. Furthermore, it reveals even weak interactions which cannot be examined using other approaches.

The elucidation of structural and physicochemical properties of proteins in physiologically-relevant conditions has offered the direct and fundamental insights into the molecular mechanisms of numerous biological processes of the target protein and the development of new drugs. However, broadening and overlapping of NMR signals of target molecules, coming from the decrease in the rotational correlation time due to the increase in the molecular weight and the non-specific intermolecular interaction, are major obstacles to limit the in-depth study and application of solution NMR, especially, inherently low resolution technique of in-cell NMR spectroscopy.

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In-cell NMR spectroscopy is a challenging method since there are a lot of things to be overcome. It has revealed three dimensional structures and intermolecular interactions in living cells, which opened a new era of the study on proteins inside cells not just in test tubes. Efforts have been made for the improvement of the in-cell NMR technique. One of drawbacks of in-cell NMR is in the severe background noises. However, the significant improvement of the sensitivity and resolution of solution NMR based on the recent development of NMR hardwares (e.g. probe, magnet, and spectrometer), softwares (pulse sequences and data processing programs), and sample preparation techniques has expanded the application of solution NMR to allow intermolecular interactions of proteins at the atomic resolution even in the inside of living cells.[1-3] The in-cell NMR methodology offers chances of structural, physicochemical, thermodynamic, kinetic, and functional studies on biomacromolecules including proteins in cells without considering artificial and non-physiological conditions *in vitro*. We herein describe briefly various methods of sample preparations as well as how the in-cell NMR method can be used for the study of intermolecular interactions.

In-cell NMR using various host cells

Various host cells for in-cell NMR experiments have been reported such as *Escherichia coli* (*E. coli*), yeast (e.g. *Pichia pastoris*), insect cells (e.g. sf9), *Xenopus*

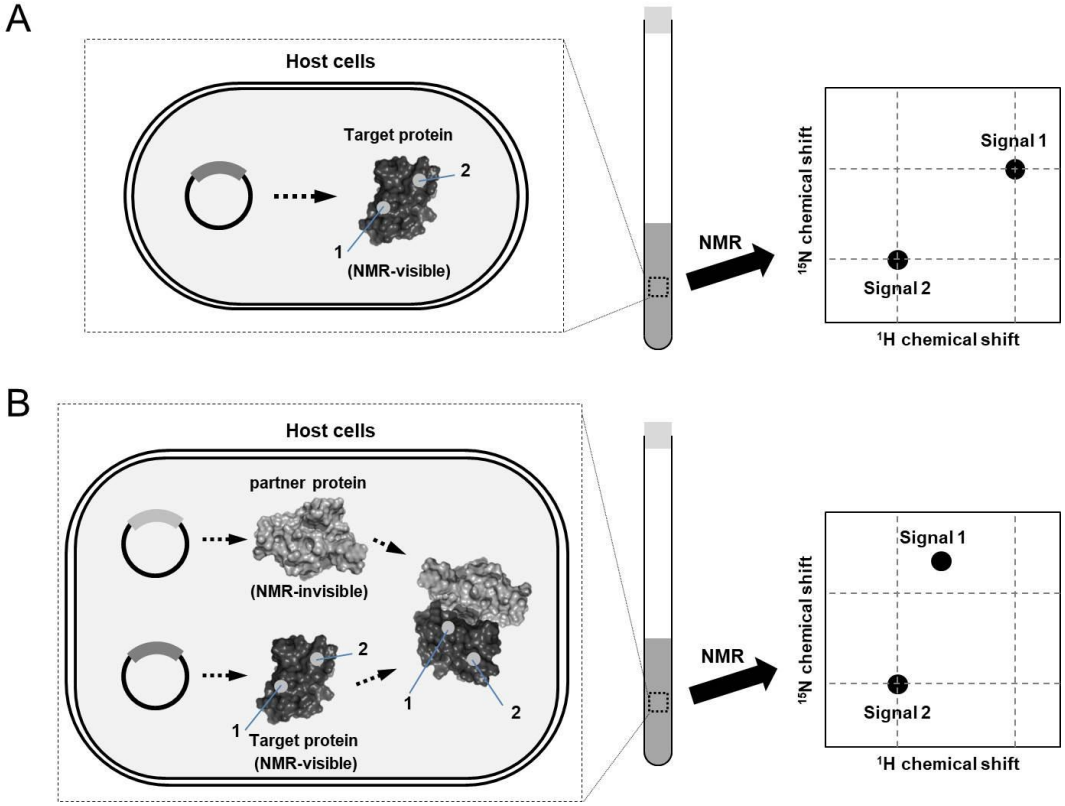

Figure 1. Illustration of sample preparation for in-cell NMR experiments. (A) Isotopically (e.g. ^{13}C, ^{15}N, or ^{19}F)-labeled target proteins are overexpressed in host cells (e.g. *Escherichia coli*) and NMR signals of the target protein (e.g. two-dimenstional ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) are observed in a NMR spectrum. (B) Isotopically-labeled target protein (the molecule colored by dark gray) and unlabeled partner protein (the molecule with light gray) are concurrently overexpressed in host cells. By comparing the intensity and the chemical shift of NMR signals in spectra with and without over-expression of partner molecules, interaction sites on the target proteins for partners in living cells can be revealed. The numbers of "1" and "2" in the host cell are correspond to the "Signal 1" and "Signal 2" denoted on the schematic illustration of in NMR spectra on right, respectively. In this case, a region around the residue of "Signal 1" should be a binding site as the change in the chemical shift of only "Signal 1" is observed.

oocyte, and mammalian cells (e.g. HeLa and HEK293T).[4,5]

In many cases of *E. coli*, yeast, or insect cells, isotopically (e.g. ^{13}C, ^{15}N, and ^{19}F)-labeled target proteins are directly over-expressed in the host cells,[5] and the cell suspension is transferred to a NMR tube (Fig. 1A). Thus, NMR signals of the target protein in the host cells are directly observed with no purification steps. In addition, this simple and convenient experimental procedure has encouraged methodologies for examining of protein-protein interaction (PPI) in living cells using *E. coli*-based in-cell NMR techniques (Fig. 1B) (refer to a section below).

On the other hand, for *Xenopus* oocyte and mammalian cells, isotopically-labeled proteins, which were recombinantly expressed and purified in advance by *E. coli* expression system and several column chromatography, were physically injected into living host cells using microinjection, cell-penetrating peptides (CPPs), pore-forming toxin, or electroporation (Fig. 2).[4,5] Then, suspended cells

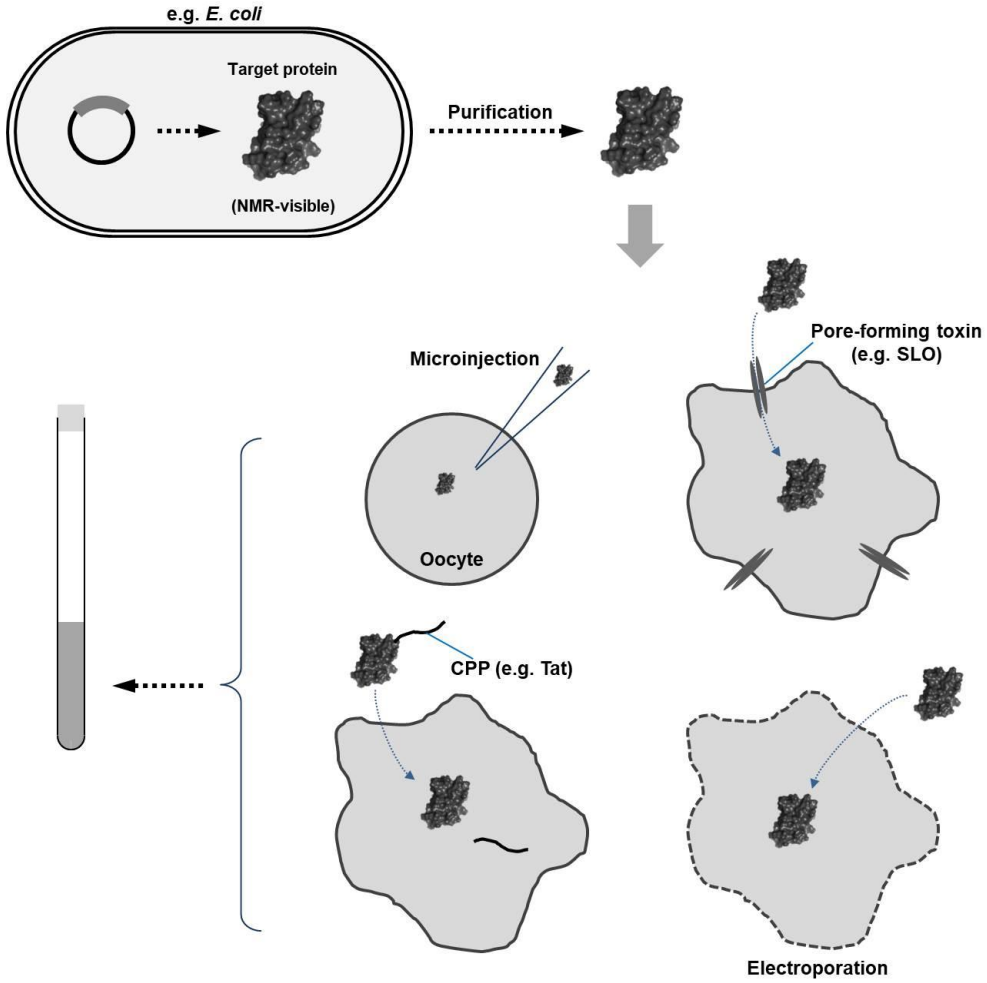

Figure 2. Valiation of sample preparation and strategies for in-cell NMR experiments. (A) Isotopically-labeled target proteins are first overexpressed by using an appropriate protein expression system (e.g. *Escherichia coli* (*E. coli*)), and purified target proteins are introduced into host cells for in-cell NMR experiments. (Upper left) In the case of oocyte as a host cell, the target proteins are introduced into cells by microinjection. (Upper right) In many cases of mammalian cells, the target proteins are penetrated into cells by using pore-forming toxin (e.g. Streptlysin O (SLO)), (lower right) by performing electroporation, or (lower left) aby fusing a cell-penetrating peptide (CPP) (e.g. Tat peptide) to the target protein. CPP can be eliminated in the host cells when CPP-fused target proteins eneter successfully cells.

containing isotope-labeled target proteins are transferred to a NMR tube. Important processes are to wash cells as thoroughly as possible and re-suspend them using fresh media prior to transfer of the cells into a NMR tube in an effort to keep out detection of target proteins that are leaked or exist outside of host cells.

NMR signals derived from the target proteins outside of cells show much stronger intensity and narrower line-width than those obtained using in-cell NMR spectroscopy. Just small amounts of proteins outside cells produce large NMR signals, which, therefore, significantly impedes the obtaining true in-cell NMR signals and spectra.

In-cell NMR using prokaryotic cells (bacteria and yeast cells) – *E. coli* is the most widely used host cell among prokaryotic cells for preparation of recombinant proteins and in-cell NMR experiments due to several advantages as follows. *E. coli* systems are easy to handle and cost performance is more effective than other expression systems. In addition, the cytosol of *E. coli* cells provides more physiological environments for target proteins compared to solution in test tubes, for instance the presence of non-specific intermolecular interactions and slow molecular tumbling due to molecular crowding effects.[6] Thus, in-cell NMR technology using *E. coli* has been the basis of the modern in-cell NMR spectroscopy.

A couple of NMR scientists, Volker Dötsch, Gary Pielak and Congang Li, and Alexander Shekhtman, have contributed to the development of *E. coli*-based in-cell NMR. Dötsch and Pielak groups individually reported how to prepare samples for successful in-cell NMR data correction in *E. coli*.[2,7,8] Furthermore, there have been a lot of studies which demonstrate that *E. coli*-based in-cell NMR is a useful and powerful approach for the structures and dynamics of intrinsically disordered proteins such as α–synuclein at the atomic resolution.[9-12]

Similarly to *E. coli*, yeast cells such as *Pichia pastoris* are suitable for over-expression of recombinant target proteins and also applicable as a host cell for in-cell NMR spectroscopy.[13] Yeast cells have been used for a model organism of eukaryotic cells as they have intracellular compartments and organelles such as the endoplasmic reticulum and golgi apparatus. Therefore, yeast-based in-cell NMR allows us to investigate the structure and dynamics of eukaryotic target proteins under more physiologically relevant environments.

In-cell NMR using eukaryotic cells (insect and mammalian cells) – Insect cells have been used for in-cell NMR spectroscopy. Hamatsu and his co-workers reported that the isotopically-labeled target proteins, *Streptococcus* protein GB1 domain (GB1), *T. Thermophilus* HB8 (TTHA1718), rat calmodulin, and human HAH1, were successfully over-expressed in sf9 cells. They could observe in-cell NMR signals of GB1 and TTHA1718 with high quality.[14] On the other hand, over-expression of isotopically-labeled target proteins in mammalian cells with sufficient concentrations for detecting NMR signals is not easy due mainly to the high costs. Therefore, in order to assure the enough concentration of target proteins, methods of incorporation of isotopically-labeled recombinant proteins prepared in advance to mammalian host cells have been widely utilized. Several approaches have been developed based on CPPs, pore-forming toxins, and electroporation.[4,15-20]

CPPs such as Tat peptide (YGRKKRRQRRR) are highly positively-charged with lysine and arginine, and thus effectively enter cells. In general, CPPs are fused to N and/or C terminal parts of the isotopically-labeled target proteins and cleaved from target proteins in cells by being reduced as two cysteins in the terminal part of CPP and a target protein is covalently bonded through a disulfide bond.[15,16]

A method using pore-forming toxin, such as Streptolysine O (SLO), uses the formation of pores on the lipid membrane of the host cells. Pore formation gives chances for isotopically-labeled target proteins to diffuse (i.e., enter) to cells in a passive manner. Pores which may cause the cell death can be recovered by supplying Ca^{2+} ions to cell media.[17] Shimada and his co-workers developed a

unique and innovative bioreactor system for the in-cell NMR experiment, which continuously supplies fresh media to the host cells in a NMR tube during NMR measurements.[21] The bioreactor system remarkably improved cell viability, which opened the door for the long-time in-cell NMR experiments more than several days as general time limits of in-cell NMR spectroscopy had been a few hours.

A new methodology using electroporation has been suggested.[19,20,22,23] This method reversibly permeabilizes the plasma membrane of the host cells by applying the electric pulse to the mixture suspensions of cells and isotope-labeled proteins. During the electric treatment, target proteins diffuse to cells like the method using pore-forming toxin. NMR signals of α-synuclein and SOD1 in living cells were detected.

PPI investigation using in-cell NMR

In-cell NMR experiments for PPI analyses have been technically developed with *E. coli*.[24-26] Furthermore, Shekhtman and his co-workers established experimental systems for coexpression of several proteins with the selective labeling to investigate PPIs using *E. coli*-based in-cell NMR spectroscopy, so-called the STINT-NMR method (Fig. 2B).[27-29] They showed over-expression of isotopically-labeled target proteins and unlabeled partner proteins to examine the changes in the peak intensity and chemical shift of target proteins.[29] Of note, a markedly useful analysis of the chemical shift perturbation was introduced. The principal component analysis (PCA) based on the single value decomposition (SVD) method was successfully applied to investigate PPI in cells even for broadened in-cell NMR signals which, in general, are not best situations for the accurate and precise data analyses.[30] Although a lot of factors such as biological relevance of results obtained in *E. coli* regarding eukaryotic PPI should be clear, *E. coli*-based in-cell NMR spectroscopy allow us to study PPI in living cells and to challenge the development of in-cell NMR spectroscopy.[31]

Drug discovery using in-cell NMR spectroscopy

Elucidation of the binding affinity between target proteins and druggable compounds and interacting sites on target proteins is key to the field of drug discovery. However, in many cases, weak intermolecular interactions between target molecules and (potent) drug molecules in physiological conditions are difficult to be demonstrated using conventional biophysical tools. Therefore, by adjusting experimental conditions where PPI becomes strong, one can obtain a chance to examine information of PPI at the level of test tubes. However, PPI in non-physiological conditions is obviously different from that in cells, which may be one factor making clinical trials unsuccessful.

One of the merits of solution NMR spectroscopy is in the detecting capability for weak intermolecular interactions in physiological conditions at the level of atoms (K_d = ~1 mM), and thereby elucidating the binding site and affinity. Therefore, solution NMR is a powerful tool to discover druggable molecules, and, furthermore, in living cell using in-cell NMR spectroscopy.

Recently, a unique in-cell NMR-based method to screen novel fragment compounds which can disrupt deleterious PPI using in-cell NMR, SMILI-NMR, has been suggested.[32-34] It should be noted that fluorine 19(^{19}F)-NMR spectroscopy is useful for studying PPI and developing drug discovery in cells as ^{19}F nuclei shows the relatively narrow signal width and the chemical shift of ^{19}F is sensitive to chemical environment changes caused by intermolecular cross-talk including the PPI and protein-ligand interaction.[35] In addition, ^{19}F in-cell NMR provides inherently low background NMR signals as fluorine atoms even less exist compared to other NMR-active nuclei in the biological systems such as carbon.

Perspectives

Although a lot of limits still remain to be clear, in-cell NMR-based techniques are innovative and deserve to be developed. Advances in the direct preparation of isotope-labeled samples in cells and in

the efficient incorporation of target proteins to cells will make in-cell NMR methods more general and popular. Improvements of hardwares and pulse sequenced tailored for in-cell NMR spectroscopy will greatly contribute to wide application of this technology to multiple fields. At the same time, the tool and algorithms for analyses such as PCA are expected to allow ones to interpret results in more details based on biological and physiological relevances. Gradual and systematic changes in experimental conditions of test tubes including macromolecular crowding effects, redox states, ionic strengths, and membranes toward conditions best reflecting physiological conditions where target molecules exist will provide rational semi-in-cell systems which bridge gaps between in vitro and *in vivo* data.

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