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Review Article

Observation of nucleic acids inside living human cells by in-cell NMR spectroscopy

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The intracellular environment is highly crowded with biomacromolecules such as proteins and nucleic acids. Under such conditions, the structural and biophysical features of nucleic acids have been thought to be different from those *in vitro*. To obtain high-resolution structural information on nucleic acids in living cells, the in-cell NMR method is a unique tool. Following the first in-cell NMR measurement of nucleic acids in 2009, several interesting insights were obtained using *Xenopus laevis* oocytes. However, the in-cell NMR spectrum of nucleic acids in living human cells was not reported until two years ago due to the technical challenges of delivering exogenous nucleic acids. We reported the first in-cell NMR spectra of nucleic acids in living human cells in 2018, where we applied a pore-forming toxic protein, streptolysin O. The in-cell NMR measurements demonstrated that the hairpin structures of nucleic acids can be detected in living human cells. In this review article, we summarize our recent work and discuss the future prospects of the in-cell NMR technique for nucleic acids.

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The interior of living cell is highly crowded with biomacromolecules. The intracellular concentrations of proteins and nucleic acids are estimated to be 50–250 g/L and 20–50 g/L, respectively, in mammalian cells [1]. Under such crowded conditions, biophysical properties such as specific/non-specific interactions, the excluded volume effect, water activity, and viscosity are different from those under *in vitro* dilute conditions. These differences are thought to affect the biophysical properties such as the structure, dynamics, and interaction of nucleic acids. Indeed, theoretical and experimental studies of the crowding effects on nucleic acids have been reported. To create molecular crowded conditions, and to simulate the intracellular behavior of proteins and nucleic acids *in vitro*, artificial crowding agents have been utilized [2]. For example, in the presence of polyethylene glycol, the thermal stability of a duplex structure was affected depending on the length of the duplex [3]. In addition, non-canonical structures of nucleic acids, such as the G-quadruplex and i-motif, that are formed by guanine- and cytosine-rich sequences, respectively, are also influenced by the molecular crowding. The G-quadruplex structure is considered to be a regulatory element for various biological events such as DNA replication, RNA transcription, protein translation,

◄ *Significance* ►

Inside a living cell, where nucleic acids exist, is highly crowded with biological macromolecules. The nucleic acids inside the cells have been thought to possess different structural features from those *in vitro*. To investigate those features of nucleic acids in living cells, the in-cell NMR method is a powerful tool. We have successfully expanded the in-cell NMR method for nucleic acids used for *Xenopus laevis* oocytes to human cells. We performed the first measurements of in-cell NMR spectra of nucleic acids forming hairpin structures in living human cells.

and telomere elongation [4]. The i-motif structure is also currently considered to be involved in regulation of gene transcription [5–7]. The G-quadruplex and i-motif structures are reportedly more stable in the presence of polyethylene glycol than they are under dilute solution conditions [8,9]. Furthermore, on addition of polyethylene glycol, the antiparallel-type and (3+1)-type G-quadruplex structures were converted to a parallel-type G-quadruplex [10]. The accumulated results indicated that the structural and biophysical properties of nucleic acids are affected by molecular crowding and those properties in crowded intracellular environments should be different from those under *in vitro* dilute conditions. However, the effects of such crowding agents on the features of nucleic acids depend on the kind of crowding agent [2,11]. For example, some guanine-rich DNA forms parallel type G-quadruplex structure in the presence of polyethylene glycol, while the same DNA forms the other type of G-quadruplex structure in the presence of the other crowding agent, Ficoll [11]. Therefore, investigation of the structural and biophysical properties of nucleic acids in living cells is essential to understand how nucleic acids behave in nature.

To analyze nucleic acids in living cells, in-cell NMR is one of the powerful methods. In-cell NMR is an application of NMR spectroscopy and allows observation of the NMR signals of proteins and nucleic acids of interest in living cells without modifications such as fluorescence labeling. For in-cell NMR experiments, a suspension of living cells including proteins or nucleic acids of interest is used as a sample. The proteins or nucleic acids of interest are delivered into living cells or expressed in the living cells. The in-cell NMR method for proteins was applied to structure determination [12–14], observation of protein–protein [15,16] and protein–drug [17] interactions, detection of structural disorder of a protein [18], and monitoring of chemical reactions [19–21] in living cells. In-cell NMR studies on proteins have been performed using various kinds of cells such as *E. coli* [12,15,22], yeast [23], *Xenopus laevis* oocytes [24], insect cells [14], and human cells [17–20].

The first in-cell NMR study on nucleic acids was reported in 2009 [25]. Those authors reported in-cell NMR spectra of DNAs and RNAs that form hairpin and G-quadruplex structures. Following the first report [25], the same group detected the in-cell NMR signals of specifically ^{15}N-labeled DNA that revealed the intracellular conformation of telomeric DNA. They detected two G-quadruplex conformations that were different from those formed in *in vitro* water-depleted conditions [26]. This indicated that the structure of nucleic acids is affected by the intracellular crowding environment. This also indicated that mimicking the intracellular crowding environment *in vitro* is difficult. Salgado and coworkers observed the DNA–ligand interaction inside cells [27]. Additionally, several nucleoside analog probes involving a ^{19}F-label were developed and

were applied to in-cell NMR to investigate the structure of the G-quadruplex of DNA and RNA [28–30]. Interestingly, Bao and coworkers demonstrated that the telomeric RNA (12-nt) forms a stacked tetrameric G-quadruplex structure in living cells, while it forms a non-stacked dimeric G-quadruplex *in vitro* [28]. This indicated that the mode of the assembly of nucleic acids is affected by the intracellular crowding environment.

In contrast to proteins, these in-cell NMR studies on nucleic acids reported only involved *Xenopus laevis* oocytes until two years ago [25–31]. This was due to the fact that a method for the introduction of a sufficient amount of nucleic acids into a large number of living human cells had not been established. Nonetheless, it is preferable to perform the in-cell NMR experiments of nucleic acids in human cells. Structural and biophysical features of nucleic acids such as genomic DNAs, micro RNAs (miRNAs), non-coding RNAs (ncRNAs) are affected by interactions with molecules such as proteins, ions and metabolites. Therefore, the investigation of nucleic acids in the presence of physiologically meaningful binding counterparts is highly desired. For example, screening of the drug candidates targeting DNAs and RNAs by in-cell NMR should be conducted in living human cells. Thus, the in-cell NMR experiments of nucleic acids in human cells have been waited.

We have applied the method using a bacterial toxin, streptolysin O (SLO), to introduce nucleic acids into living human cells. The whole living cells into which nucleic acids were introduced were subjected to in-cell NMR experiments. The NMR signals of nucleic acids inside the living human cells were observed for the first time [32,33]. In this review article, we summarize our recent works and discuss the future prospects of the in-cell NMR technique for nucleic acids.

Delivery of nucleic acids into living human cells

A large number of living cells that include a sufficient amount of the biomacromolecules of interest were required for in-cell NMR experiments. In the case of in-cell NMR studies on proteins, the protein of interest is overexpressed in or introduced into living cells by several methods. For in-cells NMR of nucleic acids, exogenous nucleic acids are introduced into *Xenopus laevis* oocytes by only the microinjection method [34,35]. The nucleic acids of interest are delivered into *Xenopus laevis* oocytes by microinjection, one by one, and finally, about 200 oocytes that include nucleic acids of interest are prepared. Because most human cells are small (in the case of a HeLa cell, 50 μm (Fig. 1a)) and a large number of the cells (at least 2×10^7 cells (Fig. 1b)) are required, the application of the microinjection method for human cells is not feasible.
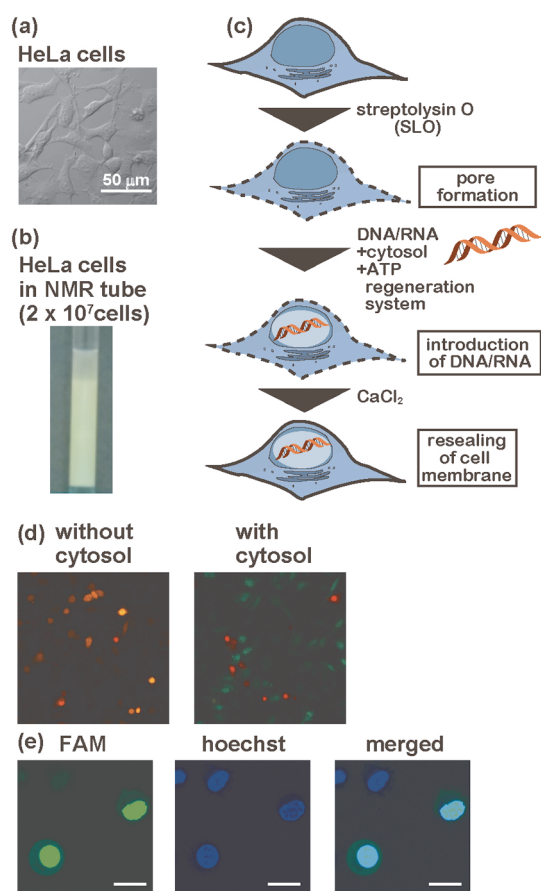
To overcome this difficulty, pore-formation on the cell

Figure 1 Sample preparation for in-cell NMR of nucleic acids using human cells. (a, b) Hela cells on culture dish (a) and in NMR tube (b). (c) Scheme of SLO treatment. (d) Fluorescence images of HeLa cells after resealing without (left) and with (right) cytosolic extract. Green indicates fluorescence of FITC dye that was introduced by SLO. Red indicates fluorescence of propidium iodide that can only enter dead cells. (e) Fluorescence images of HeLa cells with FAM-labeled RNA delivered by SLO. Green indicates fluorescence of FAM that was conjugated with RNA. Blue indicates fluorescence of hoechst 33342 dye that stained cell nuclei. ((a, b) Adapted from reference [36], (c) reproduced from reference [33], and (e) adapted from reference [32] with permission from the PCCP Owner Societies).

membrane and resealing of cells utilizing a bacterial toxin, SLO, have been applied. SLO, a toxic protein derived from Streptococcus, binds to the cell membrane depending on cholesterol and forms a homo-oligomer. The SLO oligomer generates a transmembrane channel of 25 to 30 nm internal diameter [37]. The nucleic acids of interest were incubated with the membrane-permeabilized cells and the nucleic acids entered the cells through the pores formed by SLO. The pores can be resealed by the addition of $CaCl_2$ (Fig. 1c). This technique has been applied to deliver various kinds of membrane-impermeable molecules into living cells [38–40]. For an in-cell NMR study, firstly, this technique has been applied to proteins [16,19,21]. We applied the pore-formation and resealing cells utilizing SLO to an in-cell NMR study on nucleic acids with some modifications [32] to the previous in-cell NMR studies [16,19].

When the cells were permeabilized by SLO, endogenous molecules leaked out to some extent. As modifications, a cytosolic extract and an ATP-regenerating system (ATP, creatine kinase, and creatine phosphate) were supplied to the SLO-treated cells. These additives supported the recovery of the resealed cells and improved their survival rate (Fig. 1d). This modification is essential for the efficient introduction of nucleic acids into cells permeabilized by SLO.

Intracellular localization of the delivered nucleic acids was assessed by confocal fluorescence microscopy. The fluorescence-labeled nucleic acids that were introduced into HeLa cells by the SLO method were mainly present in cell nuclei and uniformly dispersed throughout the cell nuclei (Fig. 1e) [32]. The localization at the cell nuclei is suitable for *in situ* observation of DNAs (and some RNAs) that work in cell nuclei.

In-cell NMR measurement of nucleic acids using human cells

For in-cell NMR studies, nucleic acids that form hairpin structures were introduced into living human cells (Fig. 2a). The hairpin structures of nucleic acids are an intramolecular structure composed of a base-paired region (stem region) and an un-base-paired region (loop region) (Fig. 2a, b). DNA hairpin structures are formed through trinucleotide repeat expansion associated with neurological disorders. RNA hairpin structures are usually present in rRNAs, mRNAs, and tRNAs, and may also occur in non-coding RNAs.

Firstly, NMR spectra of HeLa cells without SLO treatment were recorded. The imino proton signals of genomic DNAs, rRNAs, and mRNAs are possibly observed in the imino proton region (10 to 16 ppm) of NMR spectra but there was no signal in that region (Fig. 2c). This is supposed to be due to either line broadening caused by the large molecular weights or low intracellular concentrations of these DNAs and RNAs. In the NMR spectra of HeLa cells that were treated with SLO without exogenous nucleic acids, no imino proton signal was observed either (Fig. 2d). This means that almost no background NMR signals are present in the imino proton region. Next, the NMR spectra of HeLa cells with exogenous DNA and RNA introduced by SLO were recorded. Some signals of imino protons involved in Watson–Crick base pairs of these exogenous DNA and RNA were observed in the NMR spectra of cell suspensions (Fig. 2e). After the measurement, each NMR sample was centrifuged and the supernatant of the HeLa cell suspension was collected. In the NMR spectrum of the supernatant, there were weak imino proton signals (Fig. 2f). These signals were derived from the nucleic acids that had leaked out from dead cells during the in-cell NMR measurement. Therefore, the net in-cell NMR spectrum of
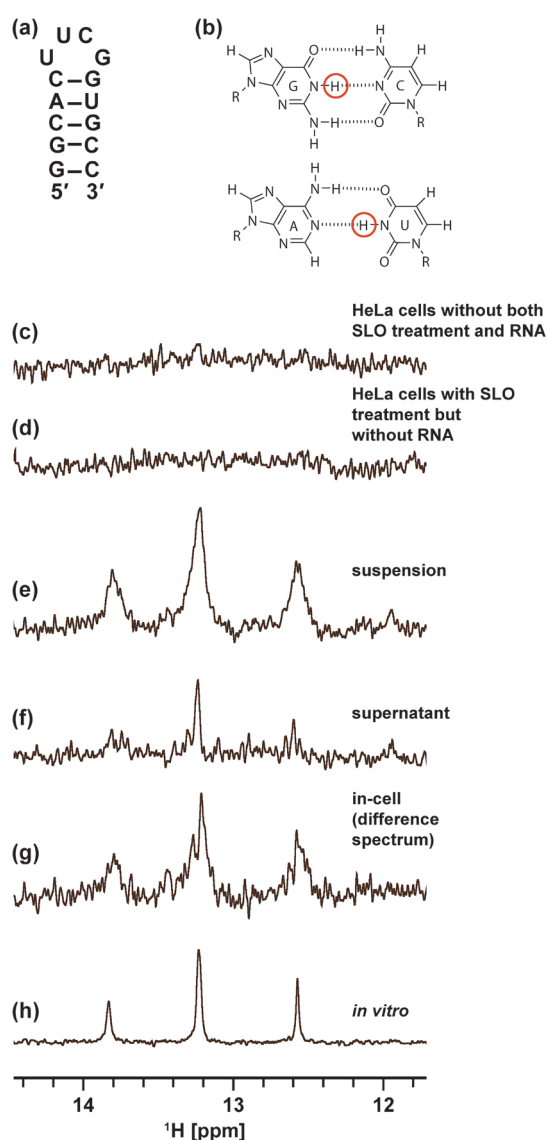
Figure 2 Series of NMR spectra of hairpin RNA. (a) The secondary structure of hairpin RNA. (b) Scheme of Watson–Crick base pairs. (c, d) NMR spectra of HeLa cells without SLO treatment and introduction of RNA (c), and treated with SLO without introduction of RNA (d). (e) NMR spectrum of HeLa cell suspension with RNA introduced by SLO. (f) NMR spectrum of supernatant collected after in-cell NMR measurement. (g) In-cell NMR spectrum obtained on subtraction of spectrum of supernatant subtracted by the spectrum of suspension. (h) NMR spectrum of RNA recorded *in vitro*. ((e)–(h) Adapted from reference [32] with permission from the PCCP Owner Societies and reproduced from reference [33]).

nucleic acid was obtained on subtraction of spectrum of supernatant from the spectrum of suspension (Fig. 2g). This is the first reported in-cell NMR spectrum of nucleic acid in living human cells [32].

The NMR signals of imino protons can be observed only when the imino protons are involved in hydrogen bonds or protected from exchange with the bulk water (Fig. 2b). Therefore, the observation of imino proton signals in the difference spectrum (Fig. 2g) indicated the formation of

base pairing of oligo RNA in living human cells. Furthermore, the difference spectrum is very similar to the NMR spectrum of hairpin RNA recorded under *in vitro* conditions (Fig. 2h). The chemical shifts of imino proton signals depend on the mode of base-pairs such as Watson–Crick and non-Watson–Crick ones, and also the formation of the tertiary structure. Therefore, comparison of in-cell and *in vitro* NMR spectra indicated that the oligo RNA formed the hairpin structure in living human cells as well as under *in vitro* conditions [32].

Based on the same principle, Dzatko and coworkers revealed the formation of the i-motif structure of oligo DNA in living human cells [41]. The i-motif structure is composed of non-Watson–Crick base pairs, $C–C^+$, that have imino protons involved in hydrogen bonding. They detected the imino proton signals of the $C–C^+$ base pairs and the results indicated that the i-motif structure can exist in living human cells.

Conclusions and perspectives

Now, in-cell NMR of nucleic acids using living human cells has been applied to the detection of the hairpin [32], i-motif [41], and G-quadruplex [42] structures. Bao and coworkers applied a nucleoside analog probe including a ^{19}F-label and detected the structural polymorphism of telomeric DNA. The telomeric DNA forms two hybrid-type and two-tetrad antiparallel-type G-quadruplex structures in living human cells. Additionally, the interactions between an mRNA–antisense drug [43] and DNA–small compounds [44] have also been investigated by the in-cell NMR method. Krafcikova and coworkers demonstrated that three kinds of compounds bound to a T–T mismatched DNA with similar affinities *in vitro*, while only two of them could bind to this DNA in living human cells. The other one could not bind to this DNA in living human cells due to its low binding specificity [44]. These studies demonstrated that investigation of nucleic acids in the native intracellular environment is valuable for understanding the structural and interactive basis of nucleic acid biology and developing drugs targeting nucleic acids. The in-cell NMR of nucleic acid is a powerful tool to achieve this.

For further investigations, the short lifetime of the human cells including nucleic acids of interest in the NMR tube can be a problem. Because inside of the NMR tube is highly dense, nutrients and oxygen rapidly run out. To extend the lifetime of the cells, a bioreactor system that continuously supplies fresh culture medium to the cells in the NMR tube is applicable as well as protein in-cell NMR [16,45–47]. Additionally, improvement of the sensitivity of in-cell NMR measurement is also an essential issue. Recently, dynamic nuclear polarization (DNP)–assisted solid-state NMR for in-cell NMR of proteins in human cells was demonstrated [48]. Notably, Schlagnitweit and

coworkers applied DNP–assisted solid-state NMR for in-cell NMR of nucleic acids in living human cells and improved the sensitivity [43]. These methods may expand the potential of in-cell NMR for nucleic acids in living human cells. The in-cell NMR is a powerful tool to understand the true nature of nucleic acids in living human cells.

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Conflicts of Interest

All the authors declare that there are no conflicts of interest.

Authors Contributions

Y. Y., T. N., T. S., and M. K. wrote the manuscript.

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