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A Gel-Encapsulated Bioreactor System for NMR Studies of Protein–Protein Interactions in Living Mammalian Cells**

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In-cell NMR spectroscopy is a method used to observe isotopically labeled molecules within living cells.[1] The first in-cell NMR experiment was performed with an *E. coli* overexpressing a ^{15}N-labeled protein.[2] For the first application of the in-cell NMR method with eukaryotic cells, isotopically labeled target proteins were introduced, by microinjection, into *Xenopus laevis* oocytes.[3,4] Recently, Inomata et al. reported a novel in-cell NMR method utilizing a cell-penetrating tag,[5] which opens the way for the application of in-cell NMR spectroscopy to mammalian cells. Our group also reported an in-cell NMR method for mammalian cells; we used a pore-forming toxin, streptolysin O (SLO), to introduce target proteins by diffusion.[6] By using these methods, protein–drug interactions and intracellular post-translational modifications, such as phosphorylation and acetylation, were successfully detected in vivo.[5–7]

However, the major limitation of the in-cell NMR experiments is the occurrence of cell death during the NMR measurement. As the suspension contains a high density of cells ($>1\times10^7$ mL), nutrient depletion occurs rapidly in the anaerobic environment within the NMR tube, thus causing the deterioration of conditions and resulting in cell death during NMR measurements.[8,9] Therefore, the observation of the NMR signals from proteins in living cells is hampered by the sharp NMR signals derived from the isotopically labeled proteins leaked from the cells.[10] Therefore, in-cell NMR experiments for eukaryotic cells currently have limited applications, such as for obtaining a single NMR spectrum measured within a very short time. Although sparse sampling methods have been utilized to shorten the time required to acquire multidimensional NMR spectra,[11] many existing in vitro NMR experiments that are used to provide information regarding dynamics and protein interactions take several hours to perform.

To suppress the cell death during NMR measurements over a longer period of time, we utilized a bioreactor to perfuse the cells in the NMR sample tube.[18] In the early in vivo NMR studies, the perfused cells were entrapped in an agarose gel in a 10 mm NMR tube to sequester them in the signal detection coil, and the ^{31}P signals of intracellular ATP metabolites in yeast and mammalian cells were observed for a period of several days.[12,13] In this study, we developed a bioreactor for in-cell NMR spectroscopy (Figure 1); in this bioreactor the cells are encapsulated within Mebiol gel, which thermoreversibly forms the gel state at temperatures above 25 °C, and fresh Dulbecco's Modified Essential Medium (DMEM) is supplied continuously to the bottom of a 5 mm NMR tube through a glass capillary. By using a Pasteur pipette we prepared the Mebiol gel as a coil-shaped string, so that the exchange of the medium and the removal of the leaked protein would be efficient. The excess medium was aspirated by a tube inserted into the middle of the NMR sample tube.

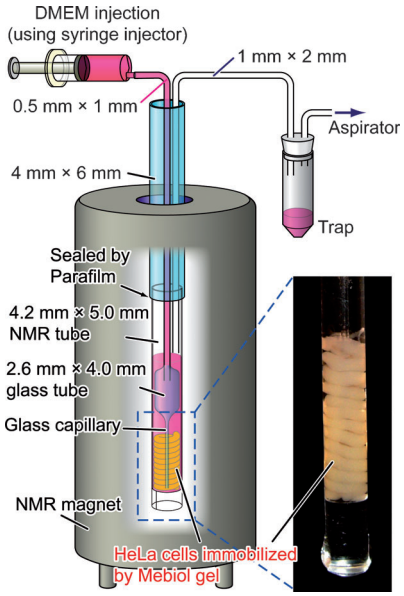

Figure 1. A schematic illustration of the gel-encapsulated bioreactor system.

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To evaluate the effectiveness of the bioreactor, we examined the time-dependent changes of the ATP concentration inside the HeLa S3 cells by ^{31}P NMR spectroscopy. In the absence of the bioreactor, the signals derived from the three phosphate groups of ATP completely disappeared after a 30 min incubation period (Figure 2), thus suggesting that the

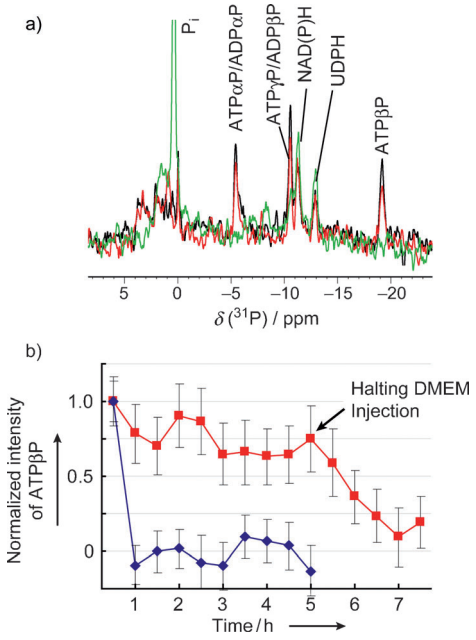

Figure 2. a) ^{31}P NMR spectra of freshly harvested HeLa cells were acquired immediately (black), after 30 min incubation in DMEM (green), and after 5 h incubation in the bioreactor (red). b) Time course of the signal intensity originating from the β P atom of intracellular ATP, with (red) and without (blue) the bioreactor. The injection was stopped at the time point indicated by the arrow. The error bar is the signal-to-noise ratio for each spectrum.

intracellular ATP was entirely depleted within 30 min. After a 15 h incubation, the amount of dead cells had increased by more than 80 % of the total cell population (Figure S1 a in the Supporting information). In contrast, for the ^{31}P NMR spectra acquired in the presence of the bioreactor, the signal intensity of the ATP remained almost constant during the 5 h of measurement and rapidly declined when the injection of DMEM was stopped (Figure 2). In the presence of the bioreactor the amount of dead cells after a 15 h incubation period was 21 %, which is dramatically lower than the amount of dead cells observed in the absence of the bioreactor (Figure S1 b in the Supporting Information). Therefore, we demonstrated the gel-encapsulated bioreactor system can maintain the cells in a metabolically active state during in-cell NMR measurements taken over a long period of time.

We used the new bioreactor to obtain an in-cell NMR spectrum of the CAP-Gly1 domain (CG1), which is the 9 kDa microtubule (MT) binding domain of CLIP-170. To introduce the CG1 protein into HeLa S3 cells, HeLa S3 cells were treated with a range of concentrations of SLO (Figure S2 a and b in the Supporting Information). After protein influx and pore resealing, dead cells were removed by centrifugation in the presence of 25 % Percoll. The highest population of CG1-treated cells (85 %) was obtained when cells were treated with 10 ng mL^{-1} SLO (Figure S2 a and c in the Supporting Information). Confocal microscopy confirmed that the prepared cells contain CG1 within their cytoplasm (Figure S3 in the Supporting Information). When cells were treated with 1 mM of the CG1 solution, the average CG1 concentration within the cell was estimated to be 180 μM, based on the NMR signals of the cell lysate (Figure S4 in the Supporting Information).

NMR signals of the intracellular CG1 were observed by a methyl-TROSY technique.[14] For the methyl-TROSY experiments, ILV-CG1, in which the methyl groups from Ile δ1, Leu δ1/2, and Val γ1/2 are selectively labeled with ^{1}H and ^{13}C in a highly deuterated background, was introduced into HeLa cells, and a ^{1}H–^{13}C band-selective optimized-flip-angle short-transient heteronuclear multiple quantum coherence (SOFAST-HMQC) spectrum was acquired.[15] Without the bioreactor, the NMR signals derived from the CG1 leaked from the cells were observed in the spectrum of the supernatant after a 1 h incubation period (Figure 3 a). Although we

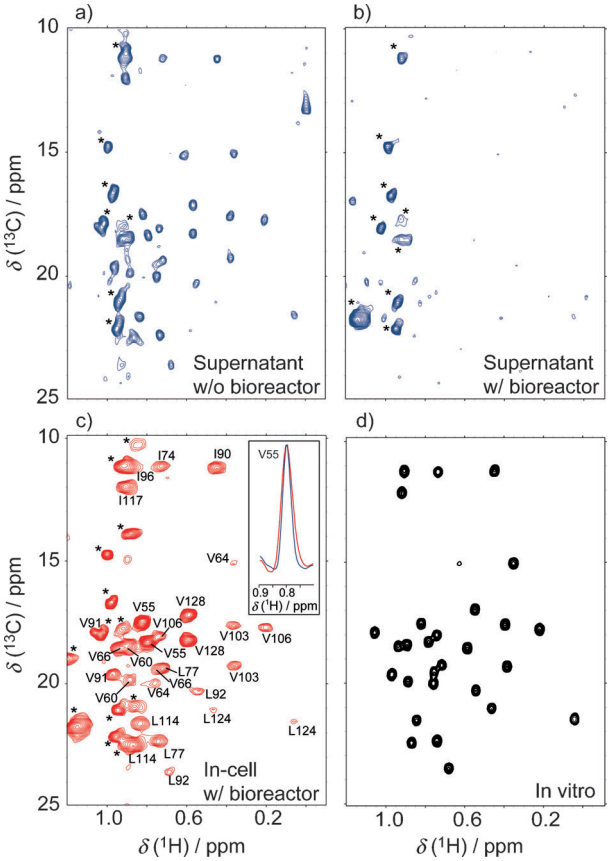

Figure 3. ^{1}H–^{13}C methyl-TROSY spectra of supernatant collected after in-cell NMR experiments after 1 h incubation in the absence of the bioreactor (a) and after 4.5 h incubation in the bioreactor (b). c) ^{1}H-^{13}C methyl-TROSY spectrum of the CG1-treated cells acquired after 5 h incubation in the bioreactor. The in-cell NMR spectrum in the absence of the bioreactor was measured 15 min before signals of the leaked protein were detected in the supernatant. Asterisks indicate signals from the components of the cell and the medium (Figure S10 in Supporting Information). For comparison of the linewidths, the inset shows the signal corresponding to V55 in the in-cell 1D NMR spectra, with (red) and without the bioreactor (blue). d) ^{1}H-^{13}C methyl-TROSY spectrum of purified CG1.

estimated the amount of leaked CG1 to be at most 10–20% of the total amount of CG1 introduced inside the cells (Figure S5 in the Supporting Information), this extracellular CG1 gave much stronger NMR signals than the CG1 remaining in the cell because of the faster tumbling motion. On the other hand, when the bioreactor was used, no signals derived from leaked CG1 were detected in the methyl-TROSY spectrum of the supernatant after an incubation period of 5 h (Figure 3b). The in-cell methyl-TROSY spectrum acquired with the bioreactor exhibited all of the methyl signals similar to those observed in the in vitro experiment, except for the signals originating from the medium and small cellular compounds (Figure 3c and d). These results clearly demonstrated that the cellular conditions in the NMR sample tube can be maintained during the experiment, and that the issue of contamination by a small amount of the leaked protein was effectively removed. A comparison of the signal linewidth of the spectra obtained with and without the bioreactor showed that the spectral resolution was not significantly affected by the immobilization in the Mebiol gel and the insertion of the glass capillary (Figure 3c, inset). Therefore, we concluded that it was possible to obtain NMR measurements over long periods of time by utilizing the gel-encapsulated bioreactor system.

Having established the ability to obtain NMR measurements over long periods of time, we utilized the transferred cross-saturation (TCS) method to identify the binding interface of externally introduced CG1 for endogenous MTs[16] that are present in the cytoplasm in concentrations of more than 20 μM. In the TCS method, the radio frequency (RF) saturation applied to unlabeled endogenous molecules is transferred to the protons of the ^{13}C-labeled methyl groups of CG1 at the binding site to the MTs. Therefore, the CG1 residues that are interacting with MTs can be identified on the basis of the decrease in the NMR signal intensities in the methyl-TROSY spectrum of CG1 in the unbound state. In this experiment, ILV-CG1 was introduced in a buffer containing 50% D_2O to suppress spin diffusion. We confirmed that a D_2O concentration below 60% does not significantly affect the viability of the HeLa cells for several hours (Figure S6 in the Supporting Information). After in-cell TCS measurements for 5.5 h in the presence of the bioreactor, a comparison of the ratios of intensity reduction of the signals in the presence and absence of RF irradiation revealed that L77, I90, L92, V103, and I117 were significantly affected, as their ratios of intensity reduction were greater than 0.50 (Figure 4a). These residues are localized on the same side of the CG1 structure (Figure 4b), and are consistent with the binding region of the CG domains of CLIP-170 for MTs that was identified in the in vitro study (Figure 4d).[17]

To determine whether the significant intensity reductions in the in-cell TCS experiment were attributable to the interaction with endogenous MTs in the cell, we performed the following control experiments. First, we examined the chemical shift changes for CG1 that were caused by the interactions with MTs. A comparison of the HMQC spectra of ILV-CG1 obtained under the in-cell and in vitro (without MTs) conditions revealed that the signals corresponding to V103 and V128, which are proximal to the MT-binding surface, exhibited the largest chemical shift changes. Similar chemical shift changes for V103 and V128 were observed for the in vitro spectrum in the presence of purified MTs, thus suggesting that the chemical shift changes observed in the in-cell spectrum reflect the interactions with endogenous MTs, and that the MTs are the major binding partner of the CG1 introduced in the cell (Figure S7 in the Supporting Information). Secondly, we assessed the contribution of unspecific interactions with endogenous molecules by competitive inhibition TCS experiments, in which an excess amount of

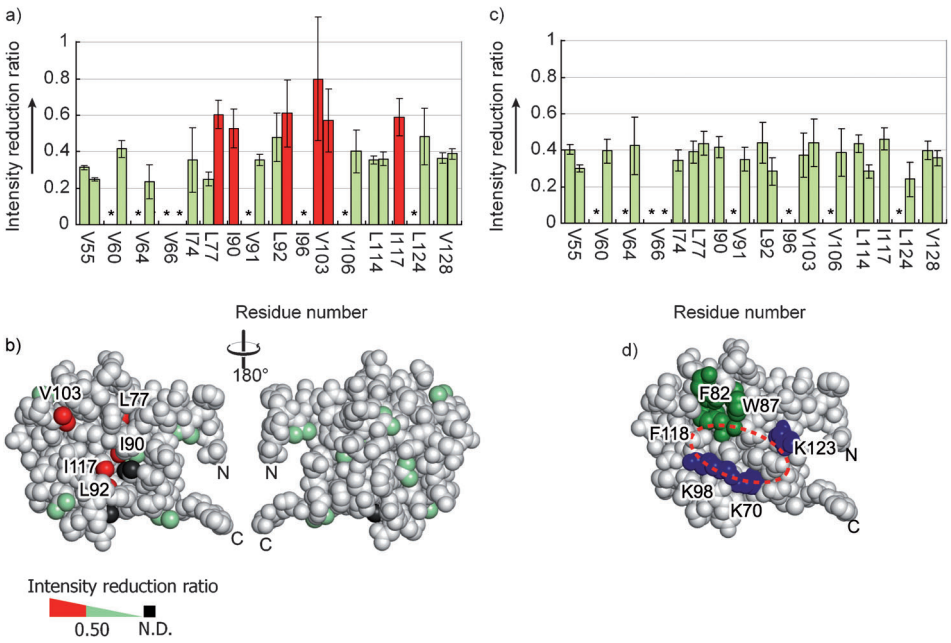

Figure 4. a) An in-cell TCS experiment performed in the presence of the bioreactor. The ratios of reduction of the signal intensities with or without RF irradiation are shown as a bar graph. Signals with asterisks were excluded from the analyses because of overlapping signals or line broadening. The methyl groups with ratios of intensity reduction greater than or less than 0.5 are colored red and light green, respectively. b) The result of the TCS experiments in a) was mapped on a sphere representation of the CG1 structure (PDB code, 2CP5). The methyl groups with ratios of intensity reduction greater than or less than 0.5 are colored red and light green, respectively, and black spheres represent methyl groups for which TCS data was unavailable. c) The competitive inhibition TCS experiments, performed in the presence of an excess amount of nonlabeled CG1. d) Hydrophobic (green) and basic (blue) residues that are important for interactions with MTs. The putative MT-binding region of CG1 is indicated by the red dashed circle.

unlabeled CG1 was introduced into the cell during the protein-influx step of the SLO treatment (Figure S8 in the Supporting Information). In the presence of unlabeled CG1, the chemical shift change for V103 of ILV-CG1 was decreased, presumably because the binding sites for CG1 on the MTs were saturated by the excess amount of unlabeled CG1 (Figure S9 in the Supporting Information). Therefore, we performed the competitive in-cell TCS experiment, by introducing the ILV-CG1 into the cell at the same time as unlabeled CG1 at a ratio of 1:10, thus only the effect of nonspecific interactions with the endogenous molecules should be observed. As a result, the ratios of intensity reduction for all residues were suppressed to below 0.5 (Figure 4c). These control experiments indicated that the binding surface identified by the in-cell TCS experiment reflected the specific interaction of CG1 with endogenous MTs in HeLa cells. Thus, we concluded that the binding surface of an exogenously introduced protein for an endogenous macromolecular complex can be identified by the in-cell TCS method.

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