Real-time In-cell ^{19}F NMR Study on Uptake of Fluorescent and Nonfluorescent ^{19}F-Octaarginines into Human Jurkat Cells

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Real-time in-cell ^{19}F NMR spectroscopy is developed to determine the time scale of cellular uptake of fluorescent and nonfluorescent ^{19}F-octaarginines. The peptide concentration as low as 10-μM order is successfully detected with a time resolution of 1–2 min. The concentration analysis shows that fluorescein isothiocyanate (FITC) induces different kinetics for the peptide uptake into natural human cells. The ability of ^{19}F NMR to detect the real-time uptake of bioactive molecules is shown with minimal perturbation.

Fluorescent probing has been most widely used to visualize vital functions in cell biology.[1] Its sensitivity is high enough to monitor intracellular deliveries of bioactive peptides and proteins under physiological conditions. Fluorophores are, however, artificially introduced modifiers and may disturb the system under investigation. Here we show how differently fluorescein isothiocyanate (FITC)-labeled and nonlabeled ^{19}F-octaarginines[2] are delivered into human Jurkat cells by applying real-time in-cell ^{19}F NMR. NMR is advantageous because it allows us to make a quantitative (concentration) analysis in relation to the molecular mechanism of biological interest. The sensitivity has been improved dramatically by the highly computerized instrumentation, the high-quality probe, and the stable and homogeneous generation of the high magnetic field.[3]

Fluorine-19 is powerful in in vivo NMR studies[4] because of no backgrounds in natural living cells. Here a low concentration of 10-μM order can be detected with a time resolution of 1–2 min. The sensitivity is notably high as compared to previous in-cell NMR studies.[5] For the reliable monitoring of such a dilute ^{19}F NMR signals, we have introduced 4-trifluoromethyl-D-phenylalanine (TFMe-D-Phe)[6] to both N- and C-termini of D-octaarginine (D-Arg)$_8$.[7] Fluorescent ^{19}F-octaarginine was obtained by the introduction of FITC to the N-terminus of ^{19}F-octaarginine using γ-aminobutyric acid (GABA) as a spacer. The design is summarized in Table 1. The system ***I*** contains a fluorescent probe and ***II*** does not.

Table 1. Fluorescent and nonfluorescent ^{19}F-octaarginines designed, together with the cell binding affinity and cell viability

	Peptide	Concentration / μM[a]			Viability
		Initial	Unbound	Bound	%
I	FITC-GABA-(TFMe-D-Phe)-(D-Arg)$_8$-(TFMe-D-Phe)-NH$_2$	15	≈0[b]	≈15	80[c]
		40	≈0[b]	≈40	39[c]
II	(TFMe-D-Phe)-(D-Arg)$_8$-(TFMe-D-Phe)-NH$_2$	30	27 ± 2	3 ± 2	82[c]
		80	57 ± 3	23 ± 3	74[c]

[a]Determined by the ^{19}F signal intensity relative to a reference of known concentration. [b]^{19}F signal intensity within the experimental error. [c]The control value without the peptide was 85%.

Real-time in-cell ^{19}F NMR measurements were performed at a frequency of 564.7 MHz, using a high-resolution, NMR spectrometer (JEOL JNM-ECA600) at 37 °C. A newly developed high-power probe, JEOL T10A, for the 10-mm-diameter tube was used. Jurkat (final concentration, 2×10^7 cells/mL) from human T-cell leukemia was suspended in phosphate-buffered saline (PBS, pH 7.4) and put into a NMR tube. To minimize the cellular toxicity, D_2O amount used for the signal lock was suppressed to 10%. The sample was rotated. The field-gradient shimming was applied to quickly attain the spectral resolution. The measurement started immediately after the peptide was added and mixed within the NMR tube.[8] Cell viability was assessed by the trypan blue staining after the NMR measurement; see Table 1.

To scrutinize whether the fluorophore influences the intracellular delivery of octaarginine, we have observed the real-time in-cell NMR spectra of FITC-labeled ***I*** at low concentrations. As illustrated in Figure 1a, two signals are observed; a broad peak at −61.547 ppm and a sharp one at −61.650 ppm, assigned to TFMe-D-Phe at the N- and C-termini of (D-Arg)$_8$, respectively. The assignment is established by introducing TFMe-D-Phe only to the N-terminus of (D-Arg)$_8$. When the FITC-peptide is added

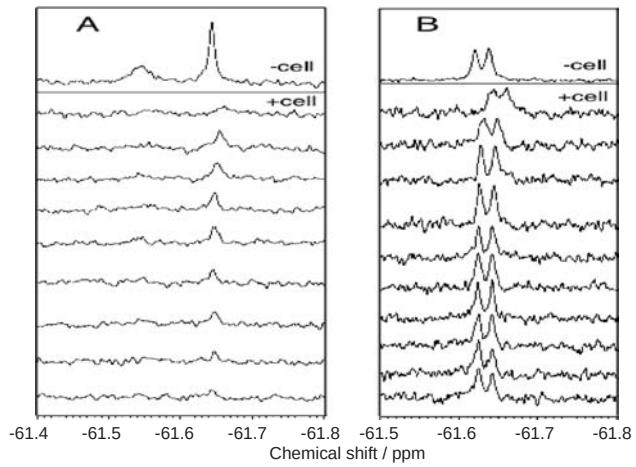

Figure 1. Real-time in-cell ^{19}F NMR spectra of (A) 40 μM FITC-^{19}F-octaarginine ***I*** without cells (upper column), and 3, 5, 7, 9, 11, 13, 15, 17, and 19 min after the addition to Jurkat cells (lower column from top to bottom) and (B) 30 μM nonfluorescent ***II*** without cells (upper column), and 3, 4.5, 6, 7.5, 9, 10.5, 15, 30, 45, and 60 min after the addition to cell (lower column from top to bottom). Since the samples are not in thermal equilibrium, the initial signals are somewhat broadened and show an upfield shift.

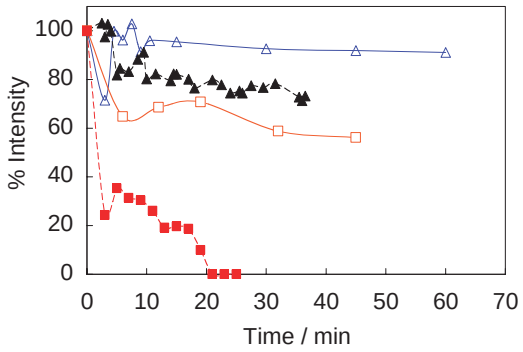

Figure 2. Real-time change in the signal intensities of 15 (open squares) and 40 μM (filled squares) fluorescent ^{19}F-octaarginines ***I*** and 30 (open triangles) and 80 μM (filled triangles) nonfluorescent ^{19}F-octaarginines ***II*** after mixing with Jurkat cells. The intensities are obtained from the integration of the two TFMe-D-Phe peaks and relative to the initial values before the mixing.

to the cell, the intensity of the signals is immediately decreased. Almost no signals can be observed 20 min after the addition of ***I*** to the cell.

In-cell NMR spectroscopy allows us to make a concentration analysis according to the signal intensity. In Figure 2 is shown the real-time variation of the signal intensity or concentration of ^{19}F-octaarginines in the presence of the cell. The significant decrease in intensity is observed for both 15 and 40 μM FITC-^{19}F-octaarginine within several minutes. It is surprising that the signal finally vanishes at 40 μM.

To elucidate whether the fluorophore influences the real-time uptake of octaarginine, we have examined nonfluorescent ^{19}F-octaarginine ***II*** for comparison. The spectra are shown in Figure 1B at the peptide concentration of 30 μM.[9] The signal intensity of the nonfluorescent ***II*** is not so much decreased. As shown in Figure 2, almost 90% is retained after 30 μM ^{19}F-octaarginine is added to the cell. The intensity decrease is relatively large when the concentration is increased to 80 μM. More than 70% is not lost, however, in contrast to the fluorescent ***I***.

The NMR signal intensity shows the concentration decrease after the addition of ^{19}F-octaarginines to the cell. The decrease corresponds to the peptide insertion into the cell membrane; the intensity loss is due to the dramatically restricted motion of the peptide in viscous membrane environments.[3b] The addition of the peptide to lipid vesicles does not change the signal intensity, different from the cellular uptake.

The hydrophobic nature of the fluorophore stabilizes the peptide binding to the cell membrane and accelerates the peptide uptake. In fact, no signals of FITC-^{19}F-octaarginine ***I*** can be observed in the supernatant after the cell is centrifuged; almost all peptides are bound to the cell (Table 1). The intensity loss means the immobilization of the peptide in the cell because the peptide signal reappears after the cell is solubilized with acetic acid, identical to the reappearance of the hydrophobic anesthetic signal in the cell solubilized with chloroform (in preparation). The strong affinity of the fluorescent ***I*** for the cell is evident, as compared to the nonfluorescent ***II*** where less than 30% is bound; see Table 1. The fluorophore is found to serve as a stabilizing anchor to the cell membrane, immobilize octaarginine at the cellular surface, and facilitate the peptide entry into the cell. Not only electrostatic but also hydrophobic interactions play a significant role in the efficient uptake.[10] In contrast, neither N- nor C-terminus of the nonfluorescent ***II*** anchors to the cell membrane; the intensities of the two signals are always equal (Figure 1b).

Consider how much of the initial intensities is finally lost by the cellular uptake. The signal of 40 μM FITC-^{19}F-octaarginine finally vanishes (red line in Figure 2). This is in marked contrast to nonfluorescent ^{19}F-octaarginine of 80 μM (in black) where more than 70% of the intensity is retained. To confirm the influence of fluorophores at lower concentrations, we apply 15 μM FITC-^{19}F-octaarginine to the cell. The intensity loss by 40% (in orange) is larger than that of the nonfluorescent ^{19}F-octaarginine at 30 μM (in blue) where 90% is retained. The intracellular deliveries of the fluorescent and nonfluorescent peptides are different even at such a low concentration.

In conclusion, the real-time in-cell ^{19}F NMR is realized for the first time to show that the fluorophore facilitates the cellular uptake of octaarginine. The influence is unavoidable even at low fluorophore concentrations. It is confirmed by monitoring 10-μM-order concentrations with a time resolution of 1–2 min. Although the high sensitivity makes the fluorescence technique less invasive,[11] the strong affinity of fluorophore for cell membranes may disrupt the membrane interior and enhance the cellular toxicity. The fluorescent probing is influenced by the hydrophobic nature and needs to be treated with care.[1] The present work demonstrates the ability of ^{19}F NMR to detect real-time processes of cellular uptake of bioactive molecules in the noninvasive manner.

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6 Nonnatural TFMe-D-Phe has minimal influence on the cellular uptake, as compared to Phe-free ^{19}F-octaarginine (in preparation).
7 The D-isomers are selected to avoid peptide fragmentation by the intracellular peptidase. All the peptides were prepared by Fmoc-solid-phase synthesis; see Supporting Information.
8 The digital resolution of the spectra was 0.2 Hz, corresponding to the uncertainty of the chemical shifts of ±0.0005 ppm.
9 Signals at −61.625 and −61.643 ppm are assigned to TFMe-D-Phe at the N- and C-termini of $(D\text{-Arg})_8$, respectively; notice that the N-terminus is not so broad as that of FITC-^{19}F-octaarginine ***I***.
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