Labeling Strategy and Signal Broadening Mechanism of Protein NMR Spectroscopy in *Xenopus laevis* Oocytes

Yansheng Ye, Xiaoli Liu, Yanhua Chen, Guohua Xu, Qiong Wu, Zeting Zhang, Chendie Yao, Mai Li, and Conggang Li*
Abstract: We used Xenopus laevis oocytes, a paradigm for a variety of biological studies, as a eukaryotic model system for in-cell protein NMR spectroscopy. The small globular protein GB1 was one of the first studied in Xenopus oocytes, but there have been few reports since then of high-resolution spectra in oocytes. The scarcity of data is at least partly due to the lack of good labeling strategies and the paucity of information on resonance broadening mechanisms. Here, we systematically evaluate isotope enrichment and labeling methods in oocytes injected with five different proteins with molecular masses of 6 to 54 kDa. $^{19}$F labeling is more promising than $^{15}$N, $^{13}$C, and $^2$H enrichment. We also used $^{19}$F NMR spectroscopy to quantify the contribution of viscosity, weak interactions, and sample inhomogeneity to resonance broadening in cells. We found that the viscosity in oocytes is only about 1.2 times that of water, and that inhomogeneous broadening is a major factor in determining line width in these cells.

NMR spectroscopy can provide conformational and dynamic information about proteins at the atomic level in living cells. Bacterial cells, almost exclusively Escherichia coli, are the most popular system for in-cell protein NMR spectroscopy because of the ease of isotope enrichment and labeling. Xenopus laevis oocytes, a model eukaryotic cell system, can be employed through micro-injection of proteins labeled or enriched with NMR-active nuclei. Unfortunately, few proteins have been studied in oocytes. One reason for the lack of data might be the insensitivity of $^{15}$N-1H HSQC spectra, the most common experiment for in-cell protein NMR spectroscopy. Here we explore labeling strategies and resonance broadening mechanisms in Xenopus oocytes with the goal of expanding the applicability of this cell type.

Four globular proteins, the B1 domain of protein G (GB1, 6.3 kDa), ubiquitin (UBQ, 8.5 kDa), calmodulin (CaM, 16.8 kDa), and protein disulfide isomerase (PDI, 54.4 kDa), and one disordered protein, α-synuclein (SYN, 14.4 kDa), were chosen to evaluate isotope enrichment and labeling techniques.

First, the HSQC spectra of the uniformly $^{15}$N-enriched proteins were acquired in simple buffered solution and in injected oocytes (Figure 1). GB1, UBQ, and CaM spectra have been reported and are shown in Figure S1 in the Supporting Information. Consistent with other reports, GB1 yielded a high-resolution spectrum in oocytes. The UBQ spectrum, however, is nearly undetectable because of the interactions between the hydrophobic surface patch and other cytoplasmic molecules. CaM displays broad cross-peaks. PDI, the largest protein examined in oocytes, shows a few cross-peaks, mainly from its disordered C terminus. The disordered protein SYN, which is studied in Xenopus oocytes by using NMR spectroscopy for the first time here, gave a spectrum similar to that obtained in dilute solution, except that its resonances were broadened. No protein cross-peaks were detected in solutions taken from above the oocytes (ca. 200 μL) after the experiment, indicating that protein leakage did not occur.

Deuterated reduces dipolar interactions, which should enhance the resolution of $^{15}$N-1H HSQC spectra. Injection of deuterated, $^{15}$N-enriched CaM gave improved sensitivity and resolution (Figure 2a and 2b), but some cross-peaks that were present in buffer, were absent in oocytes (Figure 2c). The residues with missing resonances are presumably located near the Ca$^{2+}$ binding sites, in which exchange between the Apo- and Mg$^{2+}$-bound states might make a large contribution to line-broadening. The cross-peaks from the $^1$H, $^{15}$N-enriched CaM in oocytes nearly overlay those from the Ca$^{2+}$-free (Apo) form in buffer (Figure 2C), indicating that the injected CaM mainly exists as Ca$^{2+}$-free form in oocytes.

Having shown that $^{13}$C-methyl enrichment of Ile, Leu, Val, and Ala (ILVA enrichment) is beneficial for detecting proteins in E. coli, we acquired 1D $^{13}$C spectra and 2D $^{13}$C-1H HMQC spectra of ILV-enriched CaM in oocytes (Figure 3). In contrast to E. coli, strong background signals in the methyl region were present. In both the 1D and 2D spectra, most of target protein resonances are below or overlapped with the background, but a few CaM resonances peaks can be distinguished in 1D spectrum from oocytes. The few resolved peaks (red arrows in Figure 3C) from injected Apo-CaM have almost the same chemical shifts as those from Apo-CaM in buffer (Figure 3a), indicating that the Ca$^{2+}$-free form of CaM exists in the oocytes, consistent with the $^{15}$N-1H HSQC results.

$^{19}$F NMR spectroscopy is promising because there are few background signals, and it can be used to study large proteins in E. coli. However, its applicability to Xenopus oocytes has
not been tested. $^{19}$F spectra of 5-fluorotryptophan (5FW)-labeled GB1 and PDI, and 3-fluorotyrosine (3FY)-labeled UBQ, CaM, and SYN in oocytes, E. coli cells, and buffer are shown in Figure 4. GB1 contains one tryptophan residue (W43), and only one $^{19}$F resonance was observed in oocytes. The line width is similar to that observed in E. coli. UBQ has a single tyrosine residue (Y59), and only one resonance was observed in oocytes.

The width is less than that observed in E. coli, suggesting the protein experiences weaker interactions in oocytes. CaM contains two tyrosine residues (Y99 and Y138), and two peaks were observed in oocytes. Their widths are similar to those observed in E. coli. The $^{19}$F NMR shifts in oocytes are almost the same as those from 3FY-labeled Apo-CaM in buffer, indicating that the injected CaM mainly remains in the Ca$^{2+}$-free form, consistent with the $^{19}$F NMR data, the 1D $^{13}$C data, and previous results. SYN contains four tyrosine residues (Y39, Y125, Y133, and Y136), but only one broad $^{19}$F envelope is observed in oocytes because of overlap, and the line width in oocytes is similar to that observed in E. coli. CaM binds five tryptophan residues (W35, W111, W347, W379, and W390). Four resolved resonances were observed in oocytes, and their widths were less than those observed in E. coli, in which two strong broad resonances were observed because of overlap with resonances from free 5FW and 5-fluoroindole (5FI). In summary, the $^{19}$F NMR resonances of all five proteins can be observed in injected oocytes, and their widths are similar to, or less than, those observed in E. coli cells. $^{19}$F NMR spectroscopy is more promising for studying larger proteins in Xenopus oocytes compared with isotope enrichment techniques.

Viscosity, weak interactions, and sample inhomogeneity are the main reasons that resonances from inside E. coli cells are broader than those obtained in simple buffers. Here, we quantify the source of the broadening in Xenopus oocytes by using 5FW-labeled GB1 and 3FY-labeled Y3FGB1 (tyrosine changed to phenylalanine at position 3), and our previously reported methods for assessing cytoplasmic viscosity. The intracellular viscosity is obtained from the $^{19}$F longitudinal relaxation time ($T_1$), based on the linear dependence of $T_1$ on solution viscosity, with the assumption that the internal dynamics of the labeled residue is the same in cells and buffer, which we have shown to be true for W43 and Y45. The viscosity of Xenopus oocyte cytosol is 1.2–1.3 times that of water (Figure S2A and B in the Supporting Information), less viscous than the E. coli cytoplasm, which is about twice that of water. The $T_1$ of Y33 is not linearly dependent on viscosity (Figure S2C in the Supporting Information) possibly because of the internal dynamics on the 20–100 ps time scale. This residue is not a suitable probe as we have discussed previously. The $^{19}$F transverse relaxation rate ($R_2$) is more sensitive to weak interactions than $T_1$, and the difference in viscosities inferred from $R_2$ and $T_1$ is indicative of these interactions. GB1 experienced an apparent viscosity of only about twice that of water in oocytes (Figure S2D and E in the Supporting Information), but the value was 6–11 times that of water in E. coli. These results suggest that GB1 experiences weaker interactions in Xenopus oocytes than it does in E. coli and that the oocyte cytosol is 1.2–1.3 times that of water (Figure S2A and B in the Supporting Information).
toplam is less viscous than that of E. coli. The conclusion seems to contradict our observation that GB1 resonances display similar widths in Xenopus oocytes and in E. coli cells. What are the reasons for this apparent contradiction?

Homogeneous broadening arises from cellular viscosity and weak interactions, whereas inhomogeneous broadening arises from subtle chemical shift differences involving sample inhomogeneity. Inhomogeneous broadening is not a dominant factor in E. coli cells. We used 19F NMR spectroscopy and 5FW-labeled GB1 and 3FY-labeled Y3FGB1 to quantify the contribution of homogeneous broadening and inhomogeneous broadening to the width of resonances in oocytes. The widths at half-height (W1/2) were obtained from line shape analysis by using Lorentz/Gauss deconvolution. Contributions from homogeneous broadening (Rin/π) were obtained from Carr–Purcell–Meiboom–Gill (CPMG) experiments. Contributions from inhomogeneous broadening (R(inho)/π) were then estimated from the difference between the 19F line width at half-height (W1/2) and homogeneous broadening (Figure 5). For W43 of GB1, inhomogeneous broadening (13.8 Hz) contributes 15% to the width of resonances in oocytes. For Y45 and Y33 in GB1, inhomogeneous broadening contributes 23 and 9%, respectively, to the width in oocytes. For residues Y45 and Y33, inhomogeneous broadening might be due to the larger size of proteins Y45 and Y33 in GB1, inhomogeneous broadening contributes 60–70% to the line width. This lower intracellular viscosity could be a general property of eukaryotic cells, perhaps as a result of their lower concentration of macromolecules. Our labeling and broadening mechanism efforts in Xenopus oocytes open additional opportunities for protein NMR spectroscopy in this model system.

Experimental Section

Details are given in the Supporting Information.

Acknowledgements

We thank Dengdi Li (School of Life Sciences, Central China Normal University) for help with oocytes preparation. This work is supported by the Ministry of Science and Technology of China (grant 2013CB910200), the 1000 Young Talents Program, and the National Natural Sciences Foundation of China (grants 21173258, 21120102038 and 21221064).

Keywords: in-cell NMR spectroscopy · NMR spectroscopy · proteins · Xenopus laevis oocytes

![Figure 5. Quantification of the homogeneous and inhomogeneous broadening to the 19F resonances from 5FW-labeled GB1 and 3FY-labeled Y3FGB1 in E. coli (red) and Xenopus oocytes (black). Homogeneous broadening was estimated from R(inho)/π. Inhomogeneous broadening (R(inho)/π) was estimated from the difference between the 19F line width at half-height (W1/2) and homogeneous broadening (Figure 5). For W43 of GB1, inhomogeneous broadening (13.8 Hz) contributes 15% to the width of resonances in oocytes. For Y45 and Y33 in GB1, inhomogeneous broadening contributes 23 and 9% in E. coli and about 59 and about 69% in oocytes, respectively.](image-url)


Received: January 21, 2015
Published online on **www.chemeurj.org**

These are not the final page numbers!
Various isotope enrichment and labeling techniques in protein NMR spectroscopy have been evaluated in *Xenopus laevis* oocytes and $^{19}$F NMR spectroscopy proves to be a promising technique for investigating larger proteins. The NMR resonance broadening mechanism is also clarified in *Xenopus laevis* oocytes.