Chapter 5

In-Cell NMR in Mammalian Cells: Part 2

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Abstract

Delivery of isotope-labeled IDPs into mammalian cells for the purpose of generating suitable in-cell NMR samples can also be facilitated by action of pore-forming bacterial toxins. In the course of this procedure, mammalian cell membranes are permeated for short periods of time in order to enable the influx of exogenous proteins via a concentration gradient between the outside and the inside of the targeted "host" cells. In contrast to CPP-mediated IDP uptake, toxins offer the advantage that cellular protein transduction does not rely on active biological processes like endocytosis, but on simple passive diffusion. Therefore, proteins that are to be delivered into mammalian cells are not required to contain additional "targeting" sequences, and can be employed in their native contexts. The protocol outlined here employs isotope-labeled human α-synuclein, adherent human HeLa cells, and the *Streptococcus pyogenes* endotoxin Streptolysin O (SLO).

Key words: Pore-forming toxins, Streptolysin O, α-Synuclein, HeLa cells

1. Introduction

In the previous chapter we outlined a protocol for cellular IDP transduction via the HIV-derived cell penetrating peptide, Tat. Here, we present an alternative method for IDP delivery that exploits the cell permeating properties of the pore-forming toxin Streptolysin O (SLO). Cellular uptake of exogenous protein material by action pore-forming toxins offers the possibility to deliver isotope-labeled purified IDPs into the cytoplasm of live cells without the need to first couple the protein to a carrier molecule (i.e., a cell penetrating peptide).

Streptolysin O is a bacterial endotoxin produced by *Streptococcus pyogenes.* The biological function of this and other toxins is to perforate mammalian cell membranes in the course of the bacterial infection cycle and to thereby destabilize host cell integrity (1). Pore-forming toxins can be categorized according to the tertiary

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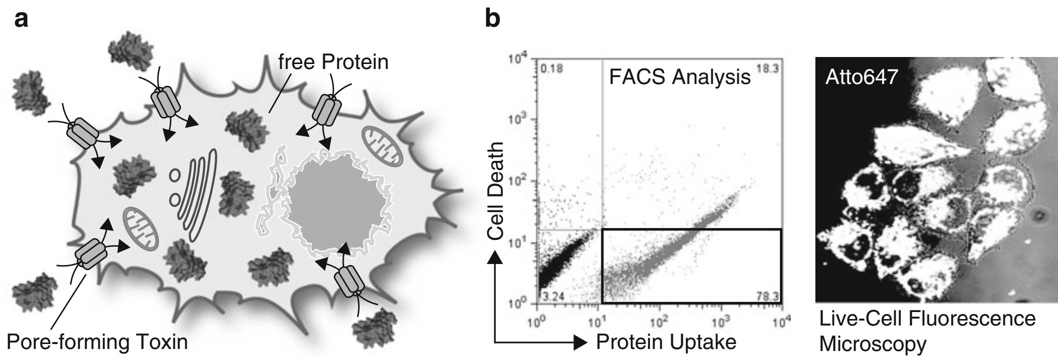

Fig. 1. (**a**) Schematic representation of Streptolysin O (SLO)-mediated protein delivery into mammalian cells. SLO forms homooligomeric, amphipathic pores in mammalian plasma cell membranes, through which proteins can enter into the cytoplasm along a concentration gradient. After protein delivery, cell membranes are resealed with Ca^{2+}-containing buffer solutions. (**b**) Assessment of cellular α-synuclein (AS) uptake and overall cell viability for the SLO-mediated protein delivery protocol by Flow Cytometry (FCM) (*left image*). Scatter plot analysis of untreated HeLa cells (*black*) and of 50 ng/mL SLO-, 200 μM AS- (Atto647-labeled) treated HeLa cells (*gray*). 78 % of the cells contain AS and are viable (*lower right quadrant, boxed*). 18 % of the cells contain AS but are not viable (*upper right quadrant*). 3 % of viable cells (*lower left quadrant*) and 0.2 % of dead cells (*upper left quadrant*) do not contain AS. The overlay of transmission and fluorescence live cell microscopy images of HeLa cells treated with 50 ng/mL SLO and 200 μM AS (Atto647-labeled, *bright white color*) is *shown on the right*.

protein structures into which they fold upon plasma membrane insertion, and based on their different membrane binding characteristics (2, 3). SLO is a member of the cholesterol-dependent cytolysin (CDC), β-pore forming toxin family (4). It is secreted in a monomeric form and assembles into barrel-like structures upon contact with cholesterol-containing membranes (5). The resulting homooligomeric, amphipathic pores extend well into the host membrane and lead to severe cellular leakage (6). In essence, these toxin pores produce an open passage between the intracellular and extracellular compartment, through which molecules may move in a concentration gradient-dependent manner (Fig. 1a) (7). At high concentrations, pore-forming toxins cause irreversible membrane damage and cell death by lysis. When applied at subcytolytic concentrations, cells are typically able to recover from such injuries through a series of homeostatic responses that quickly reseal the perforated membranes (8). Upon mild SLO exposure for example, cellular recovery is accomplished in less than 1 h and via mechanisms that also involve cell-surface patching with membranes from intracellular organelles such as lysosomes, endosomes, and in some instances the Golgi apparatus (9). It is therefore the sizes of the pores formed by SLO (diameters between 25 and 30 nm) and the ability to promote membrane recovery through the re-establishment of cellular calcium homeostasis that enables this toxin to be exploited for the delivery of different biological macromolecules (10–14). The treatment of mammalian cells with even small amounts of a pore-forming toxin like SLO does, however, bear some risks. For the purpose of

generating a suitable mammalian in-cell NMR specimen, a fine balance between efficient protein transduction and cellular toxicity has to be found (Fig. 1b). Additionally, aberrant activation events of intracellular signaling pathways, as well as changes in epigenetic modification states, have to be considered. Both types of unwanted "side-effects" have been reported for SLO treated cells (15–18). Therefore, the SLO delivery method described here may be less suitable for in-cell NMR analyses of biological events that require more unperturbed cellular settings. While this protocol focuses on SLO-mediated protein delivery, a range of other bacterial pore-forming toxins have been employed for controlled cell permeabilization (2, 19, 20). We detail the delivery of α-synuclein into adherent human HeLa cells, but as this method can be adapted to transduce different IDPs to adherent or suspension cells, we have first specified a set of useful experiments (suitable for either cell type) that help to optimize the overall efficiency of protein uptake.

2. Materials

1. Equipment: The following protocol assumes that standard laboratory equipment for recombinant protein production and purification, including a fast protein liquid chromatography (FPLC) system, is available. In addition, cell culture equipment for maintaining and manipulating mammalian cells, including a sterile workbench, a CO_2 incubator, and a basic tissue culture microscope are needed. For optimizing SLO-mediated uptake efficiencies and to determine cell viability and SLO toxicity, a Flow Cytometer (FCM) and a confocal fluorescence microscope are required. Access to high-field (>500 MHz) solution-state NMR spectrometers must be available.
2. The protein to be delivered should be available in an appropriately isotope-labeled form for the envisaged in-cell NMR experiments. Detailed protocols for recombinant protein expression and purification, as well as for stable-isotope labeling for NMR purposes, are described elsewhere (21, 22).
3. Streptolysin O from *S. pyogenes* (Sigma Aldrich, USA).
4. Suitable cell culture medium for HeLa cells: Complete DMEM (low Glucose, 5 mM Glutamine, 10 % Fetal Bovine Serum, (FBS) (PAA Laboratories, Canada)).
5. Phosphate buffered saline (PBS), cell culture grade, without Calcium/Magnesium (PAA Laboratories, Canada).
6. 6-Well cell culture plates/T175 cell culture flasks.
7. 0.25× Trypsin-EDTA (PAA Laboratories, Canada).

8. 20 mM Phosphate buffer pH 7.4.
9. HBSS buffer: 137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 0.1 % (w/v) D-glucose, 30 mM HEPES, pH 7.2.
10. Membrane resealing buffer: HBSS + 2 mM $CaCl_2$.
11. N-Hydroxysuccinimide fluorescence dye (i.e., Atto647 NHS succinimide ester, Sigma Aldrich, USA).
12. Sephadex G-25 column (GE Healthcare, USA).
13. Trypan Blue 0.4 % (Sigma Aldrich, USA).
14. Lactate dehydrogenase (LDH) Assay Kit (Abcam, USA).
15. *Optional.* Low melting agarose (USB Affymetrix, USA).
16. Redigrad™ solution (GE Healthcare, USA).
17. In-cell NMR buffer: Suspension cell growth medium containing 20 % D_2O and 30 % Redigrad™ or serum free DMEM, 10 % D_2O.

3. Methods

3.1. IDP and SLO Stock Solutions

1. Before starting the procedure, carefully assess the required number of mammalian cells to prepare the in-cell NMR sample (see Note 1), the target volume of the final in-cell NMR sample (see Note 2) and the target concentration of the isotope-labeled protein inside the cells (see Note 3).
2. Produce isotope-labeled IDP stock solution in 20 mM Phosphate, 150 mM NaCl buffer (pH 7.4) at a concentration of ~1 mM in 1 mL (see Note 4).
3. *Optional* For optimization trials, the IDP to be delivered into cells may be labeled with a fluorescence dye for direct intracellular detection by fluorescence microscopy or Flow Cytometry (see Note 5). The protein of interest should be purified in the appropriate buffer for the dye-coupling reaction. Below, we describe labeling of human α-synuclein with a lysine-conjugated dye. Whenever fluorescence labeling is not desired proceed to step 9.
4. Prepare 100–200 μL of 2 mg/mL of the desired dye in DMSO (see Note 6).
5. Adjust pH with fresh $NaHCO_3$ (1 M) according to the manufacturers' instructions for optimal coupling efficiency (for most dyes this is pH 7.8).
6. Mix 2–5 mg of the protein in 500 μL of 20 mM Phosphate buffer, pH 7.8 with a two-fold molar excess of the dye.
7. Incubate at room temperature for 1–2 h on a shaker plate.

8. Separate the labeled product from nonreacted dye using a Sephadex G-25 column and measure the protein concentration of the fluorescent product by UV/VIS spectrophotometry (see Note 7).
9. Dissolve lyophilized SLO powder in a suitable protein buffer (0.2–1 mg/mL) (see Note 8). This stock is further diluted to 20–50 ng/μL in HBSS buffer. 50 μL aliquots are frozen and stored at −20 °C (avoid repeated freeze–thaw cycles). These aliquots constitute 1,000× stocks for the recommended concentrations to be directly added to mammalian cells (i.e., 20–50 ng/mL) (see Note 9).

3.2. Protein Delivery into Adherent Cells

The goal of this section is to determine the concentrations of SLO, and of SLO–protein mixtures that can safely be applied to mammalian cells for efficient protein delivery and without adverse effects on cell viability (see Note 10). In a first step, the maximum tolerable SLO concentration is determined.

1. Seed 2.5–3 × 10^5 cells/well in a 6-well plate to test multiple SLO concentrations in parallel.
2. Incubate the cells for 24 h in complete DMEM at 37 °C in a CO_2 incubator. The cells should be ~80 % confluent at the beginning of the experiment (~5–6 × 10^5 cells).
3. Wash the cells twice with 3 mL/well of prewarmed PBS to remove FBS (which contains Ca^{2+} and inhibits SLO pore formation).
4. Wash the cells twice with 3 mL/well of ice-cold HBSS (see Note 11).
5. Add SLO concentrations ranging from ~20 to 50 ng/mL in 500 μL/well of HBSS to the cells and incubate for 30 min at 4 °C (see Note 12).
6. Remove non-incorporated, excess SLO by washing the cells twice with 3 mL/well of ice-cold HBSS.
7. Add 500 μL/well of fresh HBSS and incubate the cells for 30 min at 37 °C in a CO_2 incubator. Once, the optimal SLO concentration has been determined (step 5) the protein of interest to be delivered into the cells is added to the HBSS solution at this point (see Note 13).
8. Check for successful SLO pore-formation. This is best done with a lactate dehydrogenase (LDH) assay, which, similar to the Trypan Blue staining procedure described in the previous chapter, is a simple test for cell membrane integrity. Specifically, this assay is used to "quantify" successful membrane perforation by measuring the amount of intracellular LDH that is released into the culture medium following SLO treatment (see Note 14).

This step can be omitted once a suitable SLO concentration has been found.

9. Wash the cells once with 3 mL/well of ice-cold HBSS.
10. Add ice-cold membrane resealing buffer (HBSS/Ca^{2+}, 500 μL/well), incubate for 30 min at 4 °C (see Note 15).
11. Remove HBSS/Ca^{2+}, wash the cells twice with 3 mL/well of prewarmed PBS.
12. At this point, check for overall cell viability. This can be done by a second LDH assay (see above), Trypan Blue staining, or by alternative means (see Note 16). Cell viability may also be tested after the following recovery step (see Note 17). Once the above routines are executed with the target IDP in place (added in step 7) it is suggested to additionally verify cellular protein uptake by fluorescence microscopy, Flow Cytometry, or other appropriate methods (see Note 18).
13. Add 2 mL/well of complete DMEM and incubate at 37 °C for 1 h in a CO_2 incubator.
14. Choose optimal SLO concentrations and repeat steps 7–13 including the protein to be delivered. Evaluate protein uptake efficiency and changes in cell viability parameters (see Note 19).

3.3. Protein Delivery into Suspension Cells

As the only other published SLO/in-cell NMR application describes the manipulation of non-adherent mammalian cells (human embryonal 293F kidney cells) (14), we also outline a tailored protocol for SLO-mediated protein delivery into suspension cells. Many of the required routines are similar to the ones outlined for adherent cells (see above).

1. To determine tolerable SLO concentrations, collect ~1.0×10^6 cells for every SLO concentration to be tested and sediment cells by centrifugation (~$400 \times g$).
2. Discard supernatant and transfer cell slurry to 2 mL Eppendorf tubes.
3. Perform optimization steps as outlined in Subheading 3.2, reducing the indicated volumes to 100–200 μL for each incubation and wash step (see Note 20).

3.4. In-Cell NMR Sample Preparation Using Adherent Cells

1. For adherent (i.e., HeLa) cells, seed 4–5 $\times 10^6$ cells in a T175 cell culture flask.
2. Incubate in complete DMEM for 24 h at 37 °C in a CO_2 incubator, until the cells are 80 % confluent (0.8–1 $\times 10^7$ cells/T175 flask).
3. Wash the cells twice with 15 mL prewarmed PBS to remove FBS (see Note 21).
4. Wash the cells twice with 15 mL ice-cold HBSS.

5. Add determined concentration of SLO in 4 mL HBSS and incubate the cells for 30 min at 4 °C.
6. Remove non-incorporated, excess SLO by washing the cells twice with 15 mL ice-cold HBSS.
7. Add 4 mL of fresh HBSS containing the amount of IDP that has been shown to yield the best protein uptake results (300–600 μM on average (see Note 22)). (It should be noted that a fluorescent tag is not necessarily desired on the IDP used for the final in-cell NMR sample preparation).
8. Incubate the cells for 30 min at 37 °C in a CO_2 incubator.
9. Wash the cells once with 15 mL ice-cold HBSS.
10. Add ice-cold membrane resealing buffer (HBSS/Ca^{2+}, 4 mL) and incubate for 30 min at 4 °C.
11. Remove HBSS/Ca^{2+}, wash the cells twice with 15 mL pre-warmed PBS.
12. *Optional* Add 30 mL fresh complete DMEM and allow the cells to recover for 1 h at 37 °C in a CO_2 incubator.
13. In order to transfer the manipulated adherent cells to the NMR sample tube, incubate the cells with 4 mL (minimal surface volume) 0.25 % Trypsin/EDTA in prewarmed PBS for 3 min. If a recovery step was performed, wash once with 15 mL pre-warmed PBS to remove FBS.
14. Add 20 mL prewarmed complete DMEM to inactivate Trypsin and transfer cell suspension to a 50 mL centrifugation tube.
15. If testing for cell viability is desired, remove a 20 μL aliquot and proceed with Trypan Blue staining protocol (for details see Chapter 6).
16. Sediment cell suspension by centrifugation (~400 × *g*). Wash cell slurry twice in the final in-cell NMR buffer.
17. Resuspend in 500 μL (or 300 μL, depending on the size of the NMR tube) in-cell NMR buffer.
18. Transfer the cells to the NMR tube and proceed to perform the in-cell NMR experiment.

3.5. In-Cell NMR Sample Preparation Using Suspension Cells

1. Prepare 2×10^7 suspension cells in suitable suspension culture flasks.
2. Collect the cells in 50 mL centrifugation tube and sediment at ~400 × *g*.
3. Wash cell slurry twice with 2 mL prewarmed PBS to remove FBS (which contains Ca^{2+} and inhibits SLO pore formation).
4. Wash the cells once with 2 mL ice-cold HBSS.

5. Discard supernatant and resuspend cell slurry in 1–2 mL HBBS containing the optimized concentration of SLO (~20–40 ng/mL) (see Note 23).
6. Incubate the cells according to optimized conditions on a roller platform to avoid cell settling.
7. Remove nonincorporated, excess SLO by two centrifugation/wash steps with 2 mL ice-cold HBSS.
8. Add 2 mL HBSS containing the amount of IDP that has been shown to yield the best protein uptake results (300–600 μM on average) (see Note 24). (It should be noted that a fluorescent tag is not necessarily desired on the IDP used for the final in-cell NMR sample preparation).
9. Incubate the cells for 30 min at 37 °C in a CO_2 incubator on a roller platform.
10. Wash the cells twice with 2 mL ice-cold HBSS.
11. Add ice-cold membrane resealing buffer (HBSS/Ca^{2+}, 2 mL), incubate for 30 min at 4 °C on a roller platform.
12. Remove HBSS/Ca^{2+}, wash the cells twice with 2 mL pre-warmed PBS.
13. Resuspend cell suspension in appropriate cell culture medium and transfer to suitable cell culture flasks. Allow the cells to recover for 1 h at 37 °C in a CO_2 incubator.
14. Transfer cell suspension to a 50 mL centrifugation tube. Remove a 20 μL aliquot for Trypan Blue staining.
15. Sediment remaining suspension by centrifugation (~400 × *g*).
16. Resuspend in 500 μL (or 300 μL, depending on the size of the NMR tube) in-cell NMR buffer (see Note 25).
17. Transfer the cells to the NMR tube and proceed to in-cell NMR experiments.

4. Notes

1. On average, $1–3 \times 10^7$ cells are needed for a single in-cell NMR sample. This number depends on the kind of cells that are used and on their individual cell volumes, i.e., 2 pL for human HeLa cells (23).
2. The final volume of the in-cell NMR sample is determined by the kind of NMR tube that is to be used. On narrow-bore NMR probes, standard 5 mm (~500 μL), or Shigemi™ NMR tubes (~300 μL) may be employed. Their difference in volume will require different numbers of cells.

3. The effective NMR concentration (C_{NMR}) of the final in-cell NMR sample will be determined by the intracellular concentration of the "delivered" protein (C_{Cell}), the number of cells in the NMR sample (N_{Cell}), their individual cell volume (V_{Cell}), the final NMR sample volume (V_{NMR}), and the corresponding volume dilution factor, VDF ($V_{NMR}/V_{Cell} \times N_{Cell}$). According to $C_{NMR} \times V_{NMR} = C_{Cell} \times (V_{Cell} \times N_{Cell})$ the effective NMR concentration can be calculated as $C_{NMR} = C_{Cell}/$ VDF. For example, in order to obtain a spectrum with an effective NMR concentration (C_{NMR}) of 10 μM of isotope-labeled protein in 300 μL of NMR sample volume (V_{NMR}), for 1×10^7 cells (N_{Cell}) with an average cell volume (V_{Cell}) of 2 pL (i.e., 2×10^{-6} μL) a total intracellular protein concentration (C_{Cell}) of 150 μM of isotope-labeled IDP must be reached (VDF in this case is 15). It is therefore suggested to first determine the minimum protein concentration that is required for suitable in vitro NMR results (i.e., the lower limit of C_{NMR}). This will define the benchmark intracellular protein concentration (C_{Cell}) that has to be reached in a defined number of cells (N_{Cell}) of volume (V_{Cell}), for satisfactory in-cell NMR results.
4. Initial optimization trials may also be performed with non-isotope-labeled IDPs.
5. For direct live cell imaging or Flow Cytometry, fluorescence dyes must be coupled to the IDP. We routinely use Atto647 or Atto488, which are conjugated to recombinant proteins via lysine or cysteine residues. Dyes should be chosen based on desired detection wavelengths and available functional groups for coupling. For details see Chapter 6.
6. Many fluorescence dyes are light sensitive and must be stored protected from light.
7. A correction factor for the specific absorbance of the fluorophore must be applied when the concentration of the conjugated product is determined by UV/VIS spectrophotometry.
8. SLO is usually obtained as a lyophilized powder, prepared from 10 mM Tris, 3 mM Na-azide, 5 mM, EDTA, 1 mM PMSF solutions. In such instances, the SLO stock is prepared by directly resuspending the SLO powder in distilled H_2O, or in distilled H_2O containing 2–5 mM DTT. DTT has been reported to further "activate" the toxin by reducing its single cysteine (10, 24, 25). However, addition of reducing agents does not seem to be strictly required for SLO activity (11, 12, 14).
9. Many vendors refer to SLO's "activity" in terms of enzymatic units (U). Ogino et al. employ 40–80 U/mL SLO in 300 μL to perforate 293F cells (14). Concentrations employed in this protocol correspond to 30–70 U/mL SLO for adherent HeLa cells.

10. As different cell lines possess different tolerance levels for bacterial toxins, suitable SLO concentrations need to be determined first. The aim is to perforate 70–90 % of the cultured cells while maintaining maximum cell viability.
11. Ice-cold HBSS is used to adjust cells to the following SLO incubation at 4 °C (see step 5 and below).
12. Incubation at 4 °C promotes accumulation of SLO on cell membranes and subsequently pore-formation (26). Alternatively, cells can also be treated with SLO at 37 °C, as less robust cells might not tolerate low temperature incubation (12, 24, 25). Choosing this option may improve overall cell viability, but result in less efficient pore formation.
13. During this incubation step, "functional" SLO pores form in the plasma membrane and cellular protein uptake eventually occurs.
14. Several LDH-based toxicity kits are commercially available. They comprise a colorimetric assay for dead or plasma membrane-leaked cells. LDH present in the culture supernatant (due to SLO pore formation) participates in a coupled reaction that converts a yellow tetrazolium salt into a red formazan-class dye, which is measured by absorbance at 492 nm. The amount of formazan is directly proportional to the amount of LDH in the culture, which is in turn directly proportional to the number of SLO pores (and/or dead cells).
15. Alternatively, complete DMEM may be added at this point. FBS contains sufficient amounts of Ca^{2+} to ensure membrane resealing. For less robust mammalian cells, this option is suggested (10, 12, 25).
16. A range of other (commercial) cell viability assays may be employed at this point (see also Chapter 6).
17. For many cell lines, overall viability and resealing efficiency may increase with the following recovery step in complete DMEM. For less robust mammalian cells in particular, this option is suggested.
18. A detailed description of quality control methods to determine cellular protein uptake is provided in Chapter 6.
19. The intracellular accumulation of the delivered IDP is likely to affect overall cell viability (in a concentration-dependent manner). Therefore, it is advisable to carefully adjust SLO- and target protein concentrations for most satisfactory results. In many instances, a compromise between highest tolerable SLO- and highest-possible IDP concentrations will have to be found for the preparation of suitable in-cell NMR samples.
20. The smaller incubation volumes afforded by suspension cells and the concomitant requirements of reduced amounts of IDPs and SLO offer some advantages over adherent cell protocols.

21. Protein delivery into adherent cells can also be conducted under suspension conditions. While this bears several advantages (see previous note), it may not be suitable for all cell types. In addition, problems arise regarding the separation of nonviable from viable cells. When working with adherent cells, this is conveniently achieved in the course of the repeated washing steps. To manipulate adherent cells under suspension conditions, incubate cells from step 3 with 4 mL 0.25 % Trypsin/EDTA in prewarmed PBS for 3 min. Inactivate Trypsin with 20 mL complete DMEM. Continue with IDP delivery protocol **steps 2–16** of Subheading 3.5, which is customized for suspension cells.
22. Assuming an average protein uptake efficiency of 2–5 % (14), incubation with 300–600 μM IDP yields intracellular concentrations in the range of 6–30 μM.
23. This concentration resulted in a 50 % pore forming-, and 70–80 % membrane resealing efficiency in 293F suspension cells (14).
24. Ogino et al., incubated cells with 1 mM thymosin β4 to achieve a reported intracellular protein concentration (C_{Cell}) of 50 μM. Approximately a 5 % protein uptake efficiency (14).
25. Ogino et al. resuspended human embryonal 293F kidney cells in cell culture medium containing 20 % D_2O and 30 % RediGrad. RediGrad is a nontoxic medium, composed of colloidal silica particles, which is covalently coated with silan. This commercially available product is used for density gradient centrifugation. It is also suitable to reduce cell sedimentation in the NMR tube during the NMR experiment (14). An alternative method to avoid cell sedimentation is to use a 0.2 % low melting agarose NMR buffer solution, as described in Chapter 4.

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