

Chapter 2

Isotope-Labeled Amyloids via Synthesis, Expression, and Chemical Ligation for Use in FTIR, 2D IR, and NMR Studies

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Abstract

This chapter provides protocols for isotope-labeling the human islet amyloid polypeptide (hIAPP or amylin) involved in type II diabetes and γ D-crystallin involved in cataract formation. Because isotope labeling improves the structural resolution, these protocols are useful for experiments using Fourier transform infrared (FTIR), two-dimensional infrared (2D IR), and NMR spectroscopies. Our research group specializes in using 2D IR spectroscopy and isotope labeling. 2D IR spectroscopy provides structural information by measuring solvation from 2D diagonal lineshapes and vibrational couplings from cross peaks. Infrared spectroscopy can be used to study kinetics, membrane proteins, and aggregated proteins. Isotope labeling provides greater certainty in the spectral assignment, which enables new structural insights that are difficult to obtain with other methods. For amylin, we provide a protocol for $^{13}\text{C}/^{18}\text{O}$ labeling backbone carbonyls at one or more desired amino acids in order to obtain residue-specific structural resolution. We also provide a protocol for expressing and purifying amylin from *E. coli*, which enables uniform ^{13}C or $^{13}\text{C}/^{15}\text{N}$ labeling. Uniform labeling is useful for measuring the monomer infrared spectrum in an amyloid oligomer or fiber as well as amyloid protein bound to another polypeptide or protein, such as a chaperone or an inhibitor. In addition, our expression protocol results in 2–2.5 mg of amylin peptide per 1 L cell culture, which is a high enough yield to straightforwardly obtain the 2–10 mg needed for high resolution and solid-state NMR experiments. Finally, we provide a protocol to isotope-label either of the two domains of γ D-crystallin using expressed protein ligation. Domain labeling makes it possible to resolve the structures of the two halves of the protein in FTIR and 2D IR spectra. With modifications, these strategies and protocols for isotope labeling can be applied to other amyloid polypeptides and proteins.

Key words Two-dimensional infrared spectroscopy, 2D IR, FTIR, hIAPP, Amylin, Amyloid, Isotope labeling, Expressed protein ligation, Native chemical ligation, γ D-crystallin, NMR spectroscopy

1 Introduction

Infrared spectroscopy is one of the most commonly used techniques for assessing if a peptide or protein has formed amyloid fibers. The amide I mode of proteins is created by the stretching motions of the backbone carbonyl groups (with a little CN stretch). Amyloid fibers exhibit a very sharp and characteristic peak at

1620 cm^{-1} due to strong vibrational coupling resulting from the carbonyl groups vibrating in unison across the strands [1]. While useful for identifying amyloid fibers and other secondary structures, infrared spectra are too congested to assign structure to specific residues in any but the smallest sequences. α -Helices or β -sheets can be identified and their relative abundance quantified [2, 3], but the residues that contribute to the structure cannot be identified. Isotope labeling overcomes this limitation. Individual residues can be resolved with ^{13}C and/or $^{13}\text{C}/^{18}\text{O}$ isotopes of the backbone carbonyl atoms incorporated into the sequence via Fmoc synthesis of the polypeptide [4–6]. ^{13}C labeling produces a 40 cm^{-1} shift [7] while $^{13}\text{C}/^{18}\text{O}$ produces a 66 cm^{-1} shift [8]. 66 cm^{-1} is far outside the spectral width of all natural amide I bands and lies in a region of the spectrum largely absent of side-chain absorbance [9]. Using 2D IR spectroscopy, the secondary structure and solvation of the labeled residue can be deduced from its frequency, cross peaks between labeled and unlabeled modes, and 2D lineshape. The kinetics of amyloid formation can also be followed, residue by residue, either in neat solution or catalyzed by membranes [10–12]. By doing so, we recently identified an on-pathway, β -sheet intermediate in the FGAIL region of amylin that is ultimately disrupted to form the loop in the final fiber [13]. This intermediate may explain why aggregation is so sensitive to mutations in this region.

Another isotope labeling strategy is the expression of amylin in *E. coli*. Expression allows all of the residues to be ^{13}C or $^{13}\text{C}/^{15}\text{N}$ labeled simultaneously by carrying out expression in isotope-enriched growth media. This approach has uses in FTIR, 2D IR, and NMR spectroscopies. For 2D IR spectroscopy, it has two uses. First, it enables the monomer structure of amylin to be studied even when aggregated with many other amylin molecules [7, 14]. As stated above, amide I vibrations become delocalized across multiple polypeptides when the coupling is strong enough. As a result, the infrared spectra become insensitive to the structure of the individual monomers. Due to the frequency difference, isotope labeling prevents delocalization. Thus, by mixing in a small amount of isotope-labeled protein with a larger portion of unlabeled protein, the labeled portion of the spectrum will be dominated by the structure and couplings inherent to the monomer. We have used this fact to determine the number of strands that each monomer contributes to amyloid fibers made from γD -crystallin and to determine that $\text{K}_2\text{Q}_{24}\text{K}_2$ adopts an antiparallel hairpin rather than a beta-turn in its fibers [7, 14]. Second, uniform labeling allows mixtures of different proteins to be studied. In unpublished work, we have mixed isotope-labeled amylin with unlabeled αB -crystallin, which is a chaperone protein that is known to bind to amyloid fibers. Amylin is well resolved from the crystallin, which is enabling us to study its structure and binding to αB -crystallin.

A third strategy is to label pieces of proteins, such as domains, using native chemical ligation (a variant of expressed protein ligation) [15–17]. One domain can then be resolved from the other, enabling independent structural kinetics. γ D-crystallin has two domains, each formed from very similar Greek key motifs and connected by a flexible linker (*see* Fig. 1). By ^{13}C labeling the C-terminal domain, we discovered that it formed the β -sheet core

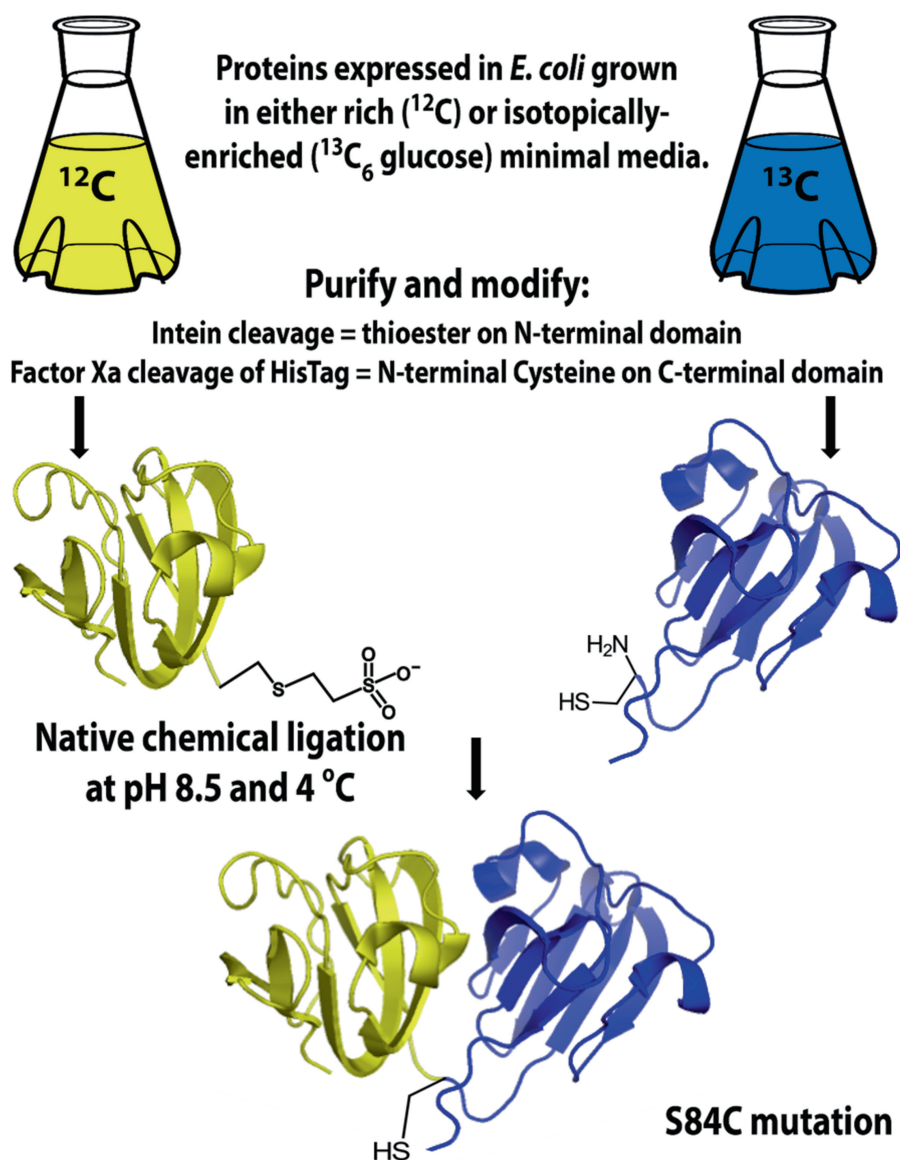


Fig. 1 Isotope labeling and native chemical ligation of γ D-crystallin. The N-terminal domain is expressed in ^{12}C media and purified with intein-mediated cleavage. The C-terminal domain is expressed in ^{13}C media and purified with Ni affinity column. The His tag is cleaved with Factor Xa before ligation. The ligated protein contains a mutation S84C which does not change the structural and chemical property of the protein

of the amyloid fibers, not the N-terminal domain as originally thought from fluorescence and other studies [7, 18]. Mixtures can also be studied as described above, but now with domain-specific resolution.

In this chapter, we provide very specific protocols that have been developed, tested, and used over the course of several years. First, we describe a protocol for ^{18}O exchanging Fmoc-protected amino acids, although other methods also exist [19, 20]. Second, a protocol for amylin expression is given. Amylin has been expressed before [21–24], but our protocol produces C-terminally amidated amylin at a higher yield. Third, we provide a protocol for domain labeling γD -crystallin by expressing the two domains separately and ligating them at position 84 with a serine-to-cysteine mutation (S84C).

How exactly does one obtain precise structural information from these three labeling schemes? When should one use one labeling strategy over another? Is 2D IR required or is FTIR good enough? What additional information does one obtain from 2D IR spectroscopy? These questions and others are addressed in a recent review about vibrational couplings, infrared spectroscopy, and isotope labeling [25]. 2D IR spectroscopy is coming of age. In just the last few years the theoretical underpinnings of the technique have become well enough understood and the experimental methods well enough established that it can now be applied to sophisticated problems in biophysics and structural biology [26–30].

2 Materials

All solutions are prepared using ultrapure 18.2 M Ω water, HPLC-grade reagents, and analytical grade chemicals without further purification, unless specified otherwise. Restricted waste disposal protocols should be followed when disposing of biochemical material and organic solvents.

2.1 Synthesis of $^{13}\text{C}/^{18}\text{O}$ -Labeled hIAPP Using Fmoc Chemistry

2.1.1 $^{18}\text{O}/^{16}\text{O}$ Exchange of Fmoc-Labeled Amino Acids

1. $1\text{-}^{13}\text{C}$ -labeled amino acid (^{13}C isotope label on the backbone carbonyl) with or without Fmoc-protecting groups (*see Note 1*).
2. Reaction solvent: Dioxane and ^{18}O -water in 1 g aliquots.
3. Oven-dried glasswares (assembled as shown in Fig. 2) including a separation funnel, a branched adapter, a small round-bottom flask, a condenser, round-bottom flask with one sidearm, a stopcock as shown in the middle of the figure, and a long needle. Before starting the reaction, leave the needle out of the round-bottom flask with the amino acids.
4. A lyophilizer.
5. Schlenk line (optional) or balloons that are filled with N_2 gas to put on top of the condenser and the separation funnel.

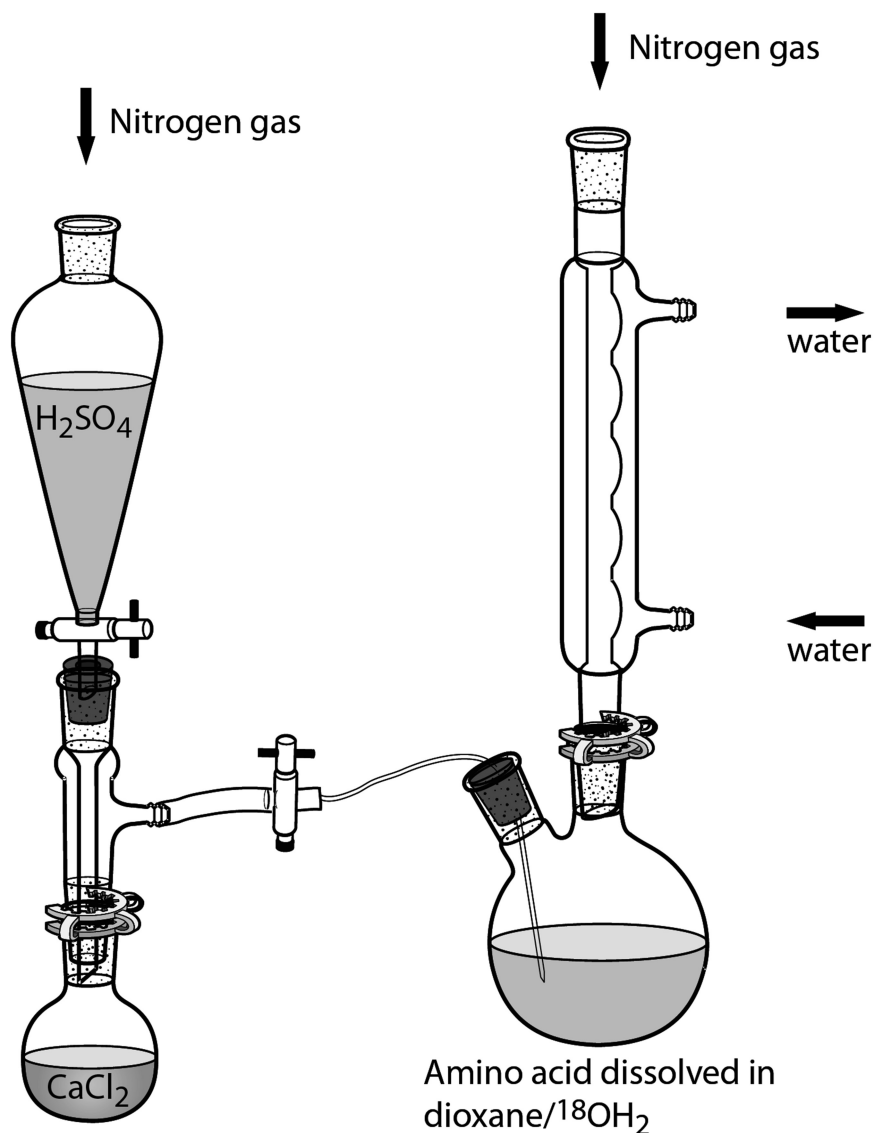


Fig. 2 Experimental setup for ^{18}O exchange of amino acids. All parts should be dried in oven before use and assembled in a fume hood. The unit on the *left* should be assembled first. Allow the reaction between H_2SO_4 and CaCl_2 proceed for at least 2 min before inserting the syringe into the side-armed flask on the *right*. The nitrogen gas source can be a Schlenk line or simply balloons filled with N_2 gas

6. Hydrogen chloride gas-generating reagents: Anhydrous CaCl_2 , concentrated H_2SO_4 .
7. Centrifuge capable of $5000 \times g$ RCF.

2.1.2 Solid-Phase Synthesis for 0.1 mM Scale

1. Solid-phase synthesizer (*see Note 2*).
2. Solid-phase synthesis resin: Fmoc-protected PAL-PEG-PS (*see Note 3*) with loading capacity 0.16 mmol/g (0.16 free amine

for coupling per 1 g of beads). For 0.1 mM-scale synthesis, weigh out 0.625 g of resin.

3. Deprotection solution: 600 mL of 20 % piperidine in dimethylformamide (DMF) with 0.1 M hydroxybenzotriazole (HOBt). Dissolve 103.3 g of HOBt in 480 mL of DMF and mix well with 120 mL piperidine (*see Note 4*).
4. Activation solution: 0.5 M HBTU in DMF. Dissolve 15.2 g of HBTU in 80 mL DMF.
5. Activator base solution: Mix 17 mL of *N, N-diisopropylethylamine* (DIEA) with 30 mL of *N*-methyl-2-pyrrolidone (NMP) to make total 47 mL of reagent.
6. Amino acids: 0.2 M in DMF. Weigh out proper amount of each amino acid and dissolve in DMF in individual tubes. Five times excess of amino acid is used for each coupling reaction (*see Note 5*).
7. Kaiser test reagent A: Dissolve 0.5 g ninhydrin in 10 mL ethanol.
8. Kaiser test reagent B: 0.005 mM KCN in pyridine. Dissolve 65.12 mg of potassium cyanide (KCN) in 10 mL of H₂O to make 0.1 M stock solution. Add 4 μ L of KCN stock solution to 20 mL of pyridine.

2.1.3 Cleavage of Peptide Off the Resin

1. Cleavage cocktail: 90 % trifluoroacetic acid (TFA), 5 % ethanedithiol (EDT), 2.5 % thioanisole, and 2.5 % anisole. In a centrifuge tube, add 9 mL of TFA, 500 μ L thioanisole, 300 μ L of EDT, and 200 μ L of anisole (*see Note 6*).
2. Ether: Chill on ice for at least 30 min.

2.1.4 HPLC Purification

1. HPLC solvent A: 0.046 % HCl in H₂O. Dissolve 5 mL of 37 % HCl in 4 L of H₂O in a fume hood. Mix well.
2. HPLC solvent B: 80 % Acetonitrile in H₂O with 0.046 % HCl. Mix 3.2 L of acetonitrile with 0.8 L of H₂O. Add 5 mL of 37 % HCl and mix well.

2.2 Expression and Purification of Uniform ¹³C hIAPP from *E. coli*

1. BL21 DE3 *E. coli* cells transfected with a PTXB1 plasmid coding for hIAPP fused to chitin-binding domain.
2. Ampicillin plate: Dissolve 5.0 g of tryptone, 2.5 g of yeast extract, and 5.0 g of NaCl in 0.5 L H₂O. Autoclave with liquid cycle at 121 °C for 30 min. Prepare a 60 °C water bath to cool the solution after autoclaving. When the solution is cooled to 60 °C, add 0.5 mL of 100 mg/mL ampicillin solution. Mix the solution well and pour it into sterile disposable petri dishes. Cool at room temperature for 2 h and then store at 4 °C.
3. Minimal media buffer: Dissolve 1.25 g of (NH₄)₂SO₄, 4.5 g of KH₂PO₄, 3.0 g of K₂HPO₄, 0.25 g of citric acid monohydrate

in 0.5 L H₂O. Adjust the pH of solution to 6.6 using KOH. Autoclave buffer at 121 °C for 30 min. Store at room temperature if not used immediately.

4. Trace element solution: Dissolve 16.2 g of FeCl₃·6H₂O, 2.4 g of ZnSO₄·7H₂O, 4.2 g of CoCl₂·6H₂O, 4.2 g of Na₂MoO₄·2H₂O, 4.8 g of CuSO₄·5H₂O, 1.2 g of H₃BO₃, 3.0 g of MnSO₄, and 30 mL of 37 % HCl into 570 mL of H₂O. Autoclave at 121 °C for 30 min [31].
5. Vitamin solution: Dissolve 0.4 g of each of the following ingredients: pantothenic acid (calcium salt), choline chloride, folic acid, nicotinamide, pyridoxal hydrochloride, and thiamine hydrochloride in 800 mL of H₂O. In the same solution, dissolve 0.8 g of myoinositol and 0.04 g of riboflavin. Adjust solution to pH 7.2 and filter with sterile filter. Aliquot the solution, cover the outsides of the tubes with aluminum foil, and place in -80 °C freezer for long-term storage.
6. Stock MgSO₄ solution: Weigh 10 g of anhydrous MgSO₄ and dissolve in 200 mL of H₂O. Autoclave this solution.
7. Minimal media: In 0.5 L of autoclaved minimal media buffer, add 1 g of ¹³C₆-glucose, 10 mL of 0.05 g/mL MgSO₄ (autoclaved separately), 500 µL of trace element solution, 325 µL of vitamin solution, 35 mg of thiamine HCl, and 500 µL of 100 mg/mL ampicillin solution (*see Note 7*).
8. French pressure cell press (used in this protocol) or sonicator for cell lysis.
9. hIAPP column buffer: 20 mM HEPES, 0.1 mM EDTA, 50 mM NaCl, 2 M urea, pH 8.0. Dissolve 3.182 g of HEPES, 30.4 mg of EDTA, 2.337 g of NaCl, and 96.1 g of urea in 800 mL H₂O. Adjust pH to 8.0 at 4 °C with HCl. Store at 4 °C.
10. hIAPP cleavage buffer: 100 mM DTT, 2 M ammonium bicarbonate. In 50 mL hIAPP column buffer, dissolve 0.73 g of dithiothreitol (DTT) and 6.4 g of ammonium bicarbonate (*see Note 8*). The cleavage buffer should be prepared fresh every time.
11. Chitin resin column.

2.3 γ D-Crystallin

2.3.1 Expression of γ D-Crystallin Domains for Native Chemical Ligation

1. The expression of γ D-crystallin N-terminal domain uses the same material as hIAPP expression described in Subheading 2.2 except different column and cleavage buffer.
2. BL21 DE3 *E. coli* cells transfected with a PTXB1 plasmid coding for the γ D-crystallin N-terminal domain fused to chitin-binding domain.
3. γ D-crystallin column buffer: 20 mM HEPES, 200 mM NaCl, pH 8.5. Dissolve 3.81 g of HEPES and 9.36 g NaCl in 800 mL of H₂O. Cool to 4 °C and adjust pH to 8.5 with NaOH.

4. γ D-crystallin cleavage buffer: Column buffer with 50 mM MESNA. Dissolve 0.32 g of MESNA in 40 mL γ D-crystallin column buffer. Adjust pH to 8.5 at 4 °C.
5. γ D-crystallin storage buffer: 5 mM Bis-Tris, pH 6.5, 250 mM NaCl.
6. BL21 DE3 *E. coli* cells transfected with a modified pet16b plasmid coding for the C-terminal domain of γ D-crystallin fused to a His tag and a factor Xa cleavage site IEGR. The actual protein sequence is MHHHHHHHXXXIEGRYYYY (XXX stands for the sequence of ligation site, YYYY stands for the actual protein sequence starting from position 85). Factor Xa cleaves after IEGR and leaves a cysteine at the N-terminus of the protein.
7. Ni column resuspension buffer (NiRB): 50 mM Na_2HPO_4 , 500 mM NaCl, 50 mM imidazole, pH 7.5. Dissolve 5.68 g of Na_2HPO_4 , 23.38 g of NaCl, and 2.72 g of imidazole in 800 mL of H_2O . Adjust pH to 7.5 with HCl.
8. Ni column elution buffers (NiEB): 50 mM NaH_2PO_4 , 500 mM NaCl, 500 mM imidazole, pH 7.5. Dissolve 5.68 g of Na_2HPO_4 , 23.38 g of NaCl, and 27.23 g of imidazole in 800 mL of H_2O . Adjust pH to 7.5 with HCl.
9. Factor Xa enzyme: Purchase before protein expression. Factor Xa does not have a very long shelf life time. Store at -80 °C before use.
10. Factor Xa cleavage buffer: 10 mM Tris, 20 mM NaCl. Dissolve 0.78 g of Tris base and 5.8 g of NaCl in 500 mL of H_2O . Adjust pH to 7.5 with HCl. To make 5 L buffer, dissolve 7.8 g of Tris base and 58.4 g of NaCl in 5 L of H_2O . Adjust pH to 7.5 with HCl.
11. CaCl_2 stock solution, 1.0 M: Dissolve 2.22 g anhydrous CaCl_2 in 20 mL of H_2O .

2.3.2 Native Chemical Ligation of Segmentally Labeled γ D-Crystallin and Purification

1. Ion-exchange chromatography (IEC) column: A column with 16 mm inner diameter and 200 mm bed height is packed with Q Sepharose Fast Flow preswollen beads (prepacked columns are also available through several vendors).
2. IEC buffer A: 20 mM Tris, pH 8.5 at 4 °C. Dissolve 2.42 g of Tris base in 1 L of H_2O . Adjust pH to 8.5 at 4 °C using HCl.
3. IEC buffer B: 20 mM Tris, 1.0 M NaCl, pH 6 at 4 °C. Dissolve 2.42 g of Tris base and 58.44 g of NaCl in H_2O . Adjust pH to 6 at 4 °C using HCl.
4. Size-exclusion chromatography (SEC) column: A column with 16 mm inner diameter and 600 mm bed height is packed with Superdex 75 prep grade gel filtration medium.
5. SEC buffer: 20 mM Na_2HPO_4 , pH 5.5 at 4 °C. Dissolve 0.14 g of Na_2HPO_4 in H_2O . Adjust pH to 5.5 using HCl 4 °C.

3 Methods

3.1 Synthesis and Purification of hIAPP

3.1.1 Synthesis of Specific Amino Acid-Labeled hIAPP Using Fmoc Chemistry

1. Routine maintenance of the solid-phase synthesizer is necessary before any synthesis. Personal protective equipment, such as lab coats, gloves, and goggles, are required for Fmoc peptide synthesis. The solvents used in solid-phase synthesis could dissolve plastic, so it is recommended to use glass vessels, pipets, and flasks. If plastic is inevitable, make the contact time as short as possible. If an isotope-labeled amino acid is used in the synthesis, a small-scale cleavage is recommended to check the synthesis efficiency before adding the isotope-labeled amino acid. Note that in Fmoc solid-phase synthesis, the peptide is synthesized from the C-terminus to the N-terminus, which is opposite from the direction of peptide expression.
2. The sequence of hIAPP is KCNTA TCATQ RLANF LVHSS NNFGA ILSST NVGSN TY (from N-terminus to C-terminus). The underlined amino acids can be replaced by pseudoproline derivatives to facilitate the solid-phase synthesis [32].
3. Swell resin in 7 mL of DMF for 1–2 h at room temperature before synthesis (this step is usually programmed into the synthesizer).
4. Use double coupling for the first amino acid because it is hard to add onto the resin. The following procedure is programmed in the synthesizer. However, the same procedure can be followed for manual synthesis (*see Note 9*).
5. Wash resin with 7 mL of DMF. Drain. Add 7 mL of deprotection solution. Incubate with 40 W microwave power at 75 °C for 3 min. Drain.
6. Repeat **step 5**.
7. Add 2.5 mL of amino acid solution (0.2 M in DMF). Add 1 mL of activation solution and 0.5 mL of activator base solution. Microwave coupling method at 75 °C for 5 min. Drain. Wash with 7 mL DMF.
8. Repeat **step 7**. Wash with additional 7 mL DMF.
9. Use single coupling at 75 °C for 5 min for most of the amino acids. Use 20 W microwave power. Two amino acids, cysteine and arginine, required special treatments. Cysteine is added at 50 °C and incubated for 2 min without microwave power, followed by 4-min incubation with 25 W microwave power. The different temperature is used to prevent the degradation of cysteine. Arginine is usually added with double coupling at 75 °C because its side chain can decrease coupling efficiency. Incubate arginine for 25 min without microwave power and another 5 min with 25 W microwave power. Same washing, deprotecting, and activating method is used as described in **steps 5–8**.

10. Use Kaiser test to monitor the coupling reaction for isotope-labeled amino acids. When synthesizing a peptide by hand, perform Kaiser test after coupling every amino acids. If a solid-phase synthesizer is used, a “pause” should be programmed in the sequence to allow for sampling of the resin.
11. From the reaction vessel, take out a few resin beads and wash with ethanol in a glass test tube. Rinse well and dry the resin until it becomes powder.
12. Add 100 μL each of Kaiser test reagent A and B. Mix well.
13. Heat this solution on a heating block at 100–115 $^{\circ}\text{C}$ for 5 min. A dark blue color suggests an unfinished coupling reaction. If the reaction is complete, the color of the solution should be pink.
14. After all the amino acids are added to the sequence, the last step should be deprotection (as described in **steps 5** and **6**) to produce a free NH_3 at the N-terminus. Resin beads can be rinsed with dichloromethane, dried, and stored at -20°C before cleavage (*see* **Note 10**).

3.1.2 Cleavage of Peptide from the Resin

1. Measure out about 300 mg of resin beads with peptides into a glass test tube (*see* **Note 10**).
2. In a fume hood, add 6 mL of cleavage cocktail to the resin. Let cleavage reaction proceed at room temperature for 4 h and stir the solution every 30 min. The cleavage solution color should change from yellow to brown.
3. Prepare a Teflon filter and fit it tightly inside of a syringe or an empty column. The material of the syringe or column should be tested for chemical compatibility before use (*see* **Note 11**). A vacuum can be applied to help remove the solvent by assembling the syringe as shown in Fig. 3. A needle is attached to the bottom of the syringe. The needle goes through a septum that is on a clean side-arm Erlenmeyer flask.
4. Carefully transfer the cleavage cocktail into the syringe. Pull a vacuum to withdraw all the solution from the syringe. Add the rest of the peptide cleavage cocktail to rinse the resin again. Pull a vacuum once more to obtain all the solution.
5. Disassemble the setup and dry the solution in the Erlenmeyer flask with N_2 gas (*see* **Note 12**).
6. Add 5–10 mL of cold ethyl ether to the flask. Break down and scrape the precipitate in the flask with a spatula to recover as much peptide as possible.
7. Transfer the ethyl ether and the precipitate into a 50 mL centrifuge tube (with known weight to calculate the yield). Rinse the flask with another 5 mL of ethyl ether. Vortex for 30 s and leave on ice for 45 min.

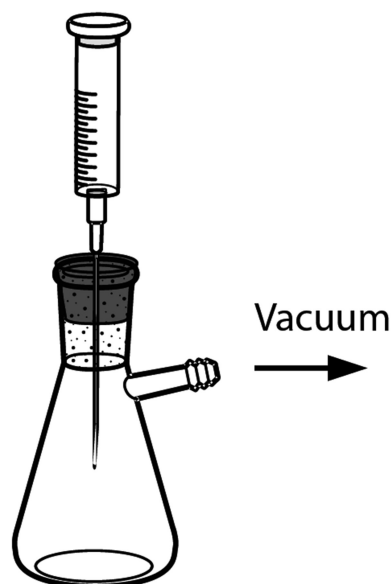


Fig. 3 Experimental setup for peptide cleavage from resin. A Teflon filter should be fitted tightly at the bottom of the syringe

8. Spin down in a centrifuge at $5000 \times g$ for 10–20 min. Decant the ether.
9. Repeat **steps 6–8**. Dry the precipitate under gentle N_2 gas until it forms a white powder.
10. Dissolve precipitate in 15–25 mL of acetic acid (use 25 mL acetic acid for every 100 mg of peptide) and sonicate for 30 min. Lyophilize this solution overnight. Dissolve the lyophilized power in dimethyl sulfoxide (DMSO) (1 mL DMSO per 2 mg of power) and let it sit at room temperature for 48 h.

3.1.3 Purification of the Peptide with HPLC

1. Mix 10 μ L of hIAPP in DMSO and 10 μ L 20 % acetic acid in H_2O . Load this solution onto a C18 analytical HPLC reverse-phase column. Use a gradient of 10–60 % HPLC solvent B for 50 min at 1 mL/min and collect all peaks separately and lyophilize each peak. Identify the peak that corresponds to hIAPP by MALDI. The correct peak should be eluted around 40 % solvent B.
2. For large-quantity purification, mix 2 mL of hIAPP in DMSO and 2 mL 20 % acetic acid in H_2O right before HPLC injection. Load this solution onto a C18 preparation HPLC reverse-phase column (*see Note 13*). The peak containing hIAPP should be eluted around the same percent of solvent B as on the analytical reverse-phase column.
3. Lyophilize the solutions that contain hIAPP. Dried hIAPP powder can be dissolved in hexafluoroisopropanol (HFIP) and sonicated for 4 h. This HFIP solution can be stored at $-20^\circ C$.

4. A second round of purification is recommended if the resolution of the peaks is not ideal. Mix 1 mL of peptide dissolved in HFIP into 3 mL of H₂O and purify with a C18 preparation column using the same gradient as in **step 2**.
5. Redissolve hIAPP powder into HFIP or HFIP-d to make a 1 mM stock and sonicate for 4 h before use. Exact concentration of the stock solution should be measured by nano-drop UV-Vis spectrometry. Aliquot 2 μ L of hIAPP in HFIP or HFIP-d, dry under N₂ gas, and redissolve in H₂O before the concentration is measured. The final concentration is determined as the mean of three measurements.

3.2 Expression of ¹³C Uniformly Labeled hIAPP

3.2.1 First Day

1. Streak the stock cells on an ampicillin plate and incubate at 37 °C overnight (*see Note 14*).

3.2.2 Second Day

1. Pick a round-shaped colony that is well separated from the others. Grow the colony in 10 mL of minimal media or LB broth overnight at 37 °C (*see Note 15*).

3.2.3 Third Day

1. Add 5 μ L of the cell culture from second day to another 10 mL of minimal media and incubate at 37 °C overnight (*see Note 16*).

3.2.4 Fourth Day

1. Add 10 mL of the overnight cell culture from **step 3** to 0.5 L of minimal media and incubate at 37 °C until OD_{600 nm} = 0.6–0.8. Induce with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and incubate for 6–8 h (*see Note 17*).
2. Harvest cells by centrifugation for 15 min at 5000 $\times g$. Resuspend the cell pellets in hIAPP column buffer on ice and immediately crush with French pressure cell press at 4 °C. Centrifuge the homogeneous solution at 50,000 $\times g$ for 45 min to obtain cell lysate as the supernatant. Load the cell lysate onto a 10 mL chitin column at 0.5 mL/min and then wash with hIAPP column buffer for at least 15 column volumes at 1 mL/min.
3. Flush column with 3 volumes of hIAPP cleavage buffer and incubate for 12–24 h at 4 °C (*see Note 18*). The use of ammonium bicarbonate in the hIAPP cleavage buffer leads to the production of a C-terminally amidated cleavage product.

3.2.5 Fifth Day

1. Elute the column with 2.5 volumes of hIAPP column buffer to harvest MhIAPP (*see Note 19*).
2. Dialyze the MhIAPP with 1000 MWCO membrane in 5 L baskets. Dialyze MhIAPP against deionized water in the first round and 0.3 % HCl in the second and third rounds each for 2–4 h (*see Note 20*).

3. Add 150 mg of cyanogen bromide to 30 mL MhIAPP in 0.3 % HCl solution (5 mg cyanogen bromide per mL solution). Allow cleavage of methionine residue at room temperature for 24 h in the dark (*see Note 21*).
4. The cleavage process can be monitored by MALDI. Samples can be mixed with sinapic acid matrix directly. The MhIAPP peak shows up around 4206.3 *m/z*, while the hIAPP peak shows up around 4070.1 *m/z*. Multiple other MALDI peaks might be detected since the chitin bead affinity purification is not perfect.

3.2.6 Sixth Day

1. Freeze the hIAPP solution (methionine residue is now cleaved) and lyophilize. Lyophilization time can be significantly reduced when the solution is partitioned into several aliquots (*see Note 22*).

3.2.7 Seventh Day

1. Dissolve lyophilized residues in 2.5 mL of DMSO (*see Note 23*). Purify the peptide with C18 reverse-phase columns as described in Subheading 3.1.3.

3.2.8 Eighth Day

1. Dissolve hIAPP powder in D₂O. Measure the concentration using nano-drop UV–Vis spectrometry. Aliquot and lyophilize the solutions (*see Note 24*).
2. To redissolve and use hIAPP powder, follow the same steps as described in Subheading 3.1.3, **step 5**.

3.3 Expression of γ D-Crystallin N-Terminal Domain

1. The schematic flowchart is shown in Fig. 1, in which the C-terminal domain is ¹³C isotope labeled. The reversely labeled protein (¹³C N-terminal domain and ¹²C C-terminal domain) can be made by switching the growth conditions of *E. coli*.
2. To grow ¹²C γ D-crystallin N-terminal domain, ¹²C₆-glucose is used instead of ¹³C₆-glucose in the minimal media.
3. The growth of γ D-crystallin N-terminal domain follows Subheadings 3.2.1–3.2.4 using γ D-crystallin cleavage buffer instead of hIAPP cleavage buffer. In **step 3** of Subheading 3.2.4, the incubation time is longer for the γ D-crystallin N-terminal domain because MESNA is used instead of DTT, which cleaves much faster. Take an aliquot every 12 h for up to 3 days to examine the cleavage progress using MALDI.
4. After at least 48 h of incubation, elute the N-terminal domain protein with 3 column volumes of γ D-crystallin cleavage buffer. Use a spin column (5000 Da MWCO) to obtain 1 mL of solution (protein concentration greater than 1 mM) with high protein concentration.
5. Use freshly cleaved protein for ligation to maximize the yield. Protein can be dialyzed into γ D-crystallin storage buffer and

stored at -80°C if it will not be used right away. Frozen samples should be dialyzed back into γD -crystallin cleavage buffer prior to ligation.

3.4 Expression of γD -Crystallin C-Terminal Domain

The growth of γD -crystallin C-terminal domain follows **steps 1–3** in Subheading 3.2 for the first 3 days (*see Note 25*).

3.4.1 Days 1–3

3.4.2 Fourth Day

1. Add 10 mL of the overnight cell culture to 0.5 L of minimal media and incubate at 37°C until $\text{OD}_{600\text{ nm}} = 0.6\text{--}0.8$. Induce with 0.5 mM IPTG and incubate for 6–8 h.
2. Harvest cells by centrifugation for 15 min at $5000\times g$. Resuspend the cell pellets in NiRB on ice. Crush cells with French pressure cell press at 4°C and centrifuge at $50,000\times g$ for 45 min to obtain cell lysate. Load the cell lysate onto a 10 mL Ni column immediately with 0.3 mL/min and wash with NiRB for at least 15 column volumes at 1 mL/min.
3. Elute with 3 column volumes of NiEB. To obtain purer proteins, use a gradient elution starting from 100 mM imidazole and increase the imidazole concentration with steps of 50 mM. Purest protein is eluted around 200 mM imidazole (*see Note 26*).
4. Dialyze protein-containing eluate against 5 L of Factor Xa cleavage buffer three times (3, 3, and 4 h).

3.4.3 Fifth Day

1. Use spin column (5000 Da MWCO) to spin down the protein solution to 4 mL (to obtain protein concentration greater than 1 mM) and transfer to a centrifuge tube. Add 20 μL of Factor Xa (in glycerol) and 5 μL CaCl_2 stock solution (1.0 M) slowly while shaking to make 1.0 mM in the final concentration.
2. Incubate the solution in the dark overnight (*see Note 27*).

3.4.4 Sixth Day

1. Load the protein solution through a Ni column. Collect the solution that does not bind to the column (the flow through).
2. Dialyze the flow through (γD -crystallin C-terminal domain with N-terminal cysteine) against γD -crystallin column buffer.

3.5 Native Chemical Ligation and Purification of Segmentally Labeled γD -Crystallin

1. Add both N-terminal domain (with MESNA) and C-terminal domain (with N-terminal cysteine). When mixing the two domains with 1:4 volume ratio, the final buffer will have 10 mM MESNA, which is the condition suitable for ligation. Incubate the solution at 4°C overnight.
2. Take 5 μL of solution for MALDI every 4 h after the first 8 h if possible.

3. The ligation reaction should be tested without ^{13}C isotope labels first.
4. Purify the ligated protein with IEC using IEC buffer A and B with a gradient of 0–50 % of buffer B in 2 h at 1 mL/min flow rate (the retention time depends on the column). Collect all peaks and identify ligated protein peak using SDS-PAGE gel.
5. Purify the IEC peak of ligated protein with SEC using SEC buffer. The retention time is around 30 min on our column.
6. Use MALDI to verify the mass of the ligated protein.
7. Aliquot the ligated protein and store at $-80\text{ }^{\circ}\text{C}$. Only thaw the sample before use. Frequent freeze-and-thaw cycles can cause precipitation of the protein.

3.6 Infrared Spectroscopy Measurement of hIAPP

1. Dry 2 μL hIAPP (1 mM) in HFIP-d under N_2 in a fume hood. A speed vacuum (concentration centrifuge) is also recommended.
2. Aggregation is initiated by dissolving hIAPP in 2 μL of 20 mM Tris D_2O buffers, pH 7.4 to make 1 mM final concentration of peptides (*see Note 28*).
3. Place 2 μL of peptide solution immediately between two calcium fluoride (CaF_2) windows after dissolving in buffer. Use a 56 μm spacer to fix the path length (*see Note 29*).
4. For FTIR, the kinetics of stationary-phase measurements can be set up with the instrument settings. It is important to collect a background spectrum without solution and a D_2O buffer background spectrum before measuring the peptide sample. A HeNe beam can be used to ensure that light is going through the sample.
5. For 2D IR spectroscopy, the same sample cell and assembly can be used in both our FTIR and 2D IR setups. Before measuring the sample, the laser setup is optimized using a standard calibration molecule, *N*-acetyl-proline (NAP). The peptide sample is placed where the pump and probe pulses are overlapped and focused. We collect frequency domain data on the probe axis with a mercury cadmium telluride (MCT) detector. With a pulse shaper, time t between the two pump pulses is scanned (time domain data) and a Fourier transformation gives us the pump frequencies. 2D IR spectroscopy with a pulse shaper is suitable for kinetics measurements of protein aggregation because a single spectrum can be collected in less than 1 min.

4 Notes

1. This protocol mainly applies to labeling the amino acids with hydrophobic side chains (Ala, Gly, Ile, Leu, Phe, and Val). Many side chains require protection for Fmoc synthesis.

The side chain-protecting groups are acid labile (removable by acid) and so will be removed by the protocol given here for ^{18}O labeling the amino acids, which is catalyzed by acid. The Fmoc group is base labile, so it will not be affected during ^{18}O labeling. If amino acids without the Fmoc group are purchased, the Fmoc group can be added before ^{18}O labeling.

2. The following recipes are used for a particular hIAPP synthesis. The final numbers presented in this protocol are chosen to have 5 % excess over what is actually needed. These numbers are given for a 0.1 mM-scale synthesis. However, the actual amount needed in any particular synthesis will depend on the solid-phase synthesizer being used; use the software provided by the synthesizer company to calculate the right amount of reagents for your instrument. Alternatively, the correct amount of reagents can be calculated based on the concentration and volume required for each step. The concentrations given here are provided to aid manual synthesis of shorter peptides.
3. We typically use Fmoc-PAL-PEG-PS resin for hIAPP synthesis because it produces an amidated C-terminus. The resulting peptide contains the C-terminal amide and free NH_3 , which gives positive charge. The loading capacity of this resin can vary depending on the supplier.
4. HOBT is classified as an explosive and must be stored following special requirements. We recommend that you contact the safety department of your institute for proper chemical storage procedures. Special permission is needed to purchase piperidine because it is a restricted substance. It is recommended to start the approval process early to avoid unnecessary delays in research.
5. If a solid-phase synthesis is used, the amount needed should be calculated. A small amount of excess can be used to ensure a successful synthesis.
6. Other cleavage cocktails are also available. The one presented here is the one that works the best for hIAPP according to our results. All cleavage or synthesis should be performed in a fume hood. This particular cleavage cocktail uses thioanisole, ethanedithiol, and anisole as scavengers; therefore a strong thiol smell will be generated. Gloves and all other wastes should be sprayed with bleach and left in the fume hood overnight before disposal. Another commonly used recipe uses 9 mL TFA, 800 μL ethanedithiol, and 200 μL H_2O for a total volume of 10 mL. This amount of cleavage cocktail is used to cleave about 300 mg of resin (20–25 % of the total resin used in a 0.1 mM scale). We do not recommend cleaving all the resin at once.
7. Another option is to make a nutrient stock solution by dissolving 1 g of $^{13}\text{C}_6$ -glucose into 10 mL of 0.05 g/mL MgSO_4

(autoclaved separately) and add 500 μL of trace element solution, 325 μL of vitamin solution, and 35 mg of thiamine HCl. This can then be added directly to 0.5 L of minimal media buffer, followed by addition of 500 μL ampicillin solution (100 mg/mL).

8. It takes time for the ammonium bicarbonate to dissolve. Add ammonium bicarbonate first and then add DTT. Prepare the solution on ice in the fume hood. Constantly release pressure if the solution is prepared in a closed tube or flask because ammonium bicarbonate will release gas as it dissolves.
9. The first amino acid is usually added with double coupling because it is hard to add onto the resin due to steric hindrance. If peptide is synthesized manually, the microwave-coupling step can be replaced by bubbling with N_2 gas for at least 1 h. The deprotection step is performed twice for every amino acid to increase yield.
10. The amount of resin beads is measured out to obtain the best yield. When the peptides are still attached to the beads, they are stable and can be stored for many months. However, when the peptides are cleaved from the resin, they are susceptible to deamidation [33] even when stored at -20°C . We typically cleave 300 mg (about 25 % of the total resin) at a time, which yields 50 mg of hIAPP peptide that is sufficient for dozens of 2D IR measurements.
11. We use a 30 mL syringe with chemical resistance to the cleavage cocktail. The Teflon filter is placed inside of the syringe without the plunger.
12. It takes 5–12 h to completely dry the solvent. It is important that the solvent is completely dried to obtain good peptide with high yield. You would expect to see pale yellow precipitate if the synthesis is successful. Use a gentle flow of N_2 gas.
13. DMSO is used to form disulfide bonds for synthesized hIAPP. Mixing DMSO and acetic acid can cause breakdown of the disulfide bonds. Thus, it is important that the two solutions are mixed immediately prior to injection. DMSO goes through the reverse-phase column with little interaction. A large saturation peak of DMSO will show up after the dead time of the column. You can start with a larger range of the gradient, for example 10–60 % solvent B in 40 min, and narrow down the range to obtain a better peak resolution.
14. You can grow the overnight cell culture from a frozen stock of cells that has been stored in -80°C in glycerol. However, cells that grow from a single colony are healthier than those from a frozen stock. By screening the cell stock on a plate, you can also select for the antibiotic resistance and the preferred morphology of the colonies.

15. The minimal media can be stored at 4 °C overnight. You can also make a small amount of minimal media fresh using nutrient stock as described in **Note 7**. Add 200 μ L of nutrient stock to 10 mL of minimal media buffer to make a small amount of minimal media in a Falcon tube. 10 μ L of ampicillin (100 mg/mL) should be added to the solution before adding the cell colony.
16. This step is optional. If ^{12}C peptide is desired, this step should be skipped. When making ^{13}C peptide, this step is recommended for a higher labeling efficiency.
17. The doubling time for cell concentration is longer in minimal media than in LB broth. For B121 DE3 cells in minimal media, the doubling time is about 1–1.5 h.
18. There will be bubbles generated in the process. Open columns are recommended. Leave the column open to air to release pressure during overnight incubation in cleavage buffer.
19. The start codon codes for methionine in most protein expressions. Matured IAPP in humans does not contain methionine at the N terminus because it is cut during posttranslational modification. There is no other methionine in the hIAPP sequence, so cyanogen bromide can be used to cleave the N terminus methionine. When designing the expression of other peptides that might contain methionine in other positions of the sequence, different approaches are needed.
20. Since ammonia bicarbonate buffer was used, dialyzing against HCl during the first round of dialysis will generate CO_2 gas and potentially break the dialysis tubing. The smell of DTT might still be detected after three rounds of dialysis. This does not seem to affect the following steps.
21. Cyanogen bromide is highly volatile, light sensitive, and toxic. Cyanogen bromide readily hydrolyzes into hydrogen cyanide, a powerful hemotoxin. Cyanogen bromide is also skin permeable. Work involving cyanogen bromide should be conducted in a fume hood and should not be conducted alone.
22. The solution takes around 12–24 h to lyophilize. The remaining residue is usually yellow and gooey which dissolves completely in DMSO.
23. When working with hIAPP that is synthesized on a solid-phase synthesizer, the peptides that are cleaved from resin beads need to sit in DMSO for 48 h before purification with HPLC to form a disulfide bond between Cys2 and Cys7. Expressed hIAPP contains the disulfide bond, so the 2-day incubation period can be skipped.
24. You can also dissolve the hIAPP powder directly into d-HFIP. Dissolving hIAPP in D_2O makes it easier to aliquot and improves the H/D exchange efficiency.

25. The C-terminal domain (with an N-terminal cysteine) is relatively stable because it does not have a leaving group like MESNA. It should be made before making the N-terminal domain protein.
26. Because some impurities bind to Ni column at low imidazole concentration, purer target protein can be obtained by a gradient elution. You can load the 10 μL of the elution from each concentration step onto an SDS-PAGE gel to determine which fraction of protein is the purest.
27. Different condition of Factor Xa can be used. Incubation at 37 °C is the optimal working condition for Factor Xa when working with proteins that are not stable. Incubation overnight at room temperature works well for the γD -crystallin C-terminal domain.
28. Other D_2O buffers can also be used. Different salt content, concentration, and pH value will affect the kinetics of the aggregation, as well as the final fiber stability [34].
29. Different thicknesses of spacers are available: 12, 25, 56, 75, and 100 μm . A 56 μm spacer works best for our experiments in D_2O buffer. However, a thinner spacer can be used to reduce scattering when working with lipids and micelles.

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