Chapter 2

Application of Photochemical Cross-linking to the Study of Oligomerization of Amyloidogenic Proteins

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Abstract

Assembly of amyloidogenic proteins into toxic oligomers and fibrils is an important pathogenic feature of over 30 amyloid-related diseases. Understanding the structures and mechanisms involved in the assembly process is necessary for rational approaches geared at inhibiting formation of these toxic species. Here, we review the application of photo-induced cross-linking of unmodified proteins (PICUP) to two disease-related amyloidogenic proteins (1) islet amyloid polypeptide (IAPP), whose toxic oligomers are thought to cause the demise of pancreatic β-cells in type-2 diabetes mellitus and (2) α-synuclein, which aggregates into toxic oligomers and precipitates in Lewy bodies in Parkinson's disease. PICUP is an effective method allowing chemical "freezing" of dynamically changing oligomers and subsequent study of the oligomer size distribution that existed before cross-linking. The method has provided insights into the factors controlling early oligomerization, which could not be obtained by other means. We discuss sample preparation, experimental details, optimization of parameters, and troubleshooting.

Key words: PICUP, Cross-linking, IAPP, α-Synuclein, Oligomers, Protein assembly

1. Introduction

Parkinson's disease (PD) and type-2 diabetes mellitus (T2DM) belong to a group of diseases characterized by amyloid formation and hence termed amyloidoses (1). In all of these diseases, one or more proteins that are part of normal physiology respond to genetic, environmental, or yet unknown stimuli by self-assembly leading to the formation of toxic oligomers and amyloid fibrils.

Islet amyloid polypeptide (*IAPP*). IAPP is the major component of the pancreatic islet amyloid associated with the development of T2DM. IAPP, also known as amylin, is a 37-amino acid residue polypeptide hormone (2, 3) that belongs to the calcitonin gene-related family (4). It is one of the most amyloidogenic polypeptides

Einar M. Sigurdsson et al. (eds.), *Amyloid Proteins: Methods and Protocols*, Methods in Molecular Biology, vol. 849,
DOI 10.1007/978-1-61779-551-0_2,

known (5–8). A number of studies have shown that IAPP-mediated degeneration of β-cells does not require amyloid formation (9–12). Rather, prefibrillar oligomers of IAPP have been shown to be more cytotoxic than IAPP fibrils and to cause membrane disruption (13–15). Similar findings have been reported for most other amyloidogenic proteins (16).

Although intensively studied, little is known about the size distribution or structure of early IAPP oligomers. The characterization of early oligomeric species of IAPP is difficult because the oligomers exist as metastable, heterogeneous mixtures comprising a wide range of molecular sizes. Multiple analytical methods have been used to study IAPP oligomer mixtures, each one with its unique advantages and limitations (17).

A useful method for the characterization of oligomer size distribution in vitro is photo-induced cross-linking of unmodified proteins (PICUP). PICUP is a well-established method, first developed to analyze stable proteins complexes (18) and later applied to quantitative study of metastable amyloid protein assemblies, including amyloid β-protein (Aβ) (19), prion (20), and α-synuclein (α-syn) (21).

A major problem found in studies involving amyloidogenic proteins is the significant differences in assembly kinetics and toxicity observed using proteins or peptides from different sources or even using different lots from the same source (22, 23). This irreproducibility likely results from the presence of preexisting aggregates in the peptide stocks. Such aggregates serve as seeds for fibril formation and therefore must be removed or dissociated to improve experimental reproducibility. Here we use 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) treatment (24, 25) as a method for dissociating IAPP aggregates. Importantly, in the case of IAPP, HFIP treatment does not remove preformed aggregates entirely (26). However, we found that for efficient cross-linking, IAPP must be treated with HFIP. This finding suggests that the C-terminal tyrosine, which is the most active side-chain in IAPP in the PICUP chemistry, likely, is buried in IAPP aggregates and becomes exposed upon HFIP treatment.

In our hands, attempts to cross-link IAPP without treatment with HFIP yielded mainly monomer and dimer bands, which were observed also in uncross-linked samples (Fig. 1a). A trimer band also was apparent but its low abundance suggested that low efficiency cross-linking took place. We interpreted these observations as indicating that the presence of preformed aggregates in the lyophilized IAPP powder and rapid aggregation upon dissolution of IAPP attenuated the cross-linking reaction. Attempts to remove preformed aggregates using size exclusion chromatography (SEC) or filtration through 10,000 Da molecular-weight cutoff filters yielded solutions with IAPP concentrations below the detection limit of the UV detector of the SEC system or of silver staining. In contrast, treatment with HFIP was found to yield IAPP in a state that was amenable to photo-cross-linking. When following HFIP treatment,

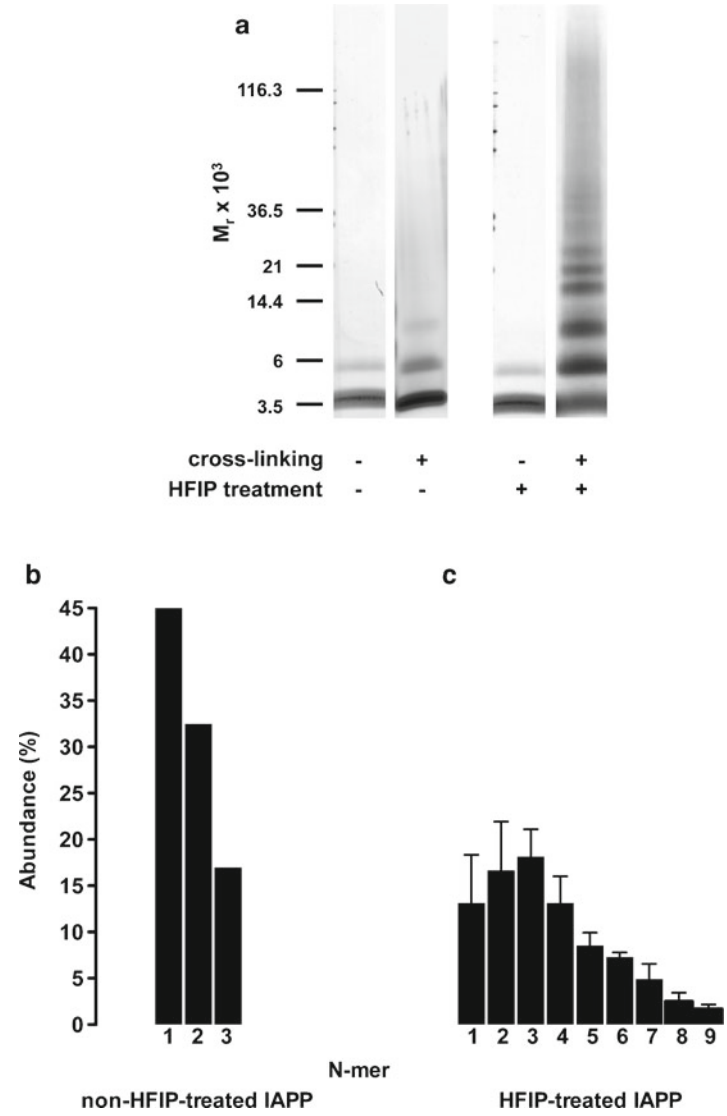

Fig. 1. SDS-PAGE analysis of IAPP following PICUP. HFIP-treated or untreated samples were subjected to PICUP. (**a**) The resulting mixtures were analyzed by SDS-PAGE using a 10–20% gradient Tris-tricine gel and silver stained. The mobilities of molecular mass markers are shown to the left. (**b** and **c**) Densitometric analysis of gels bands for HFIP-untreated IAPP (**b**) and HFIP-treated IAPP (**c**). The abundance of each band is normalized to the entire lane.

the dry peptide film was solubilized in phosphate buffer (see below) and cross-linked immediately, it produced an oligomer distribution comprising monomer through nonamer (Fig. 1), which was consistent with a theoretical distribution under high-efficiency conditions, as described previously (27).

The oligomer size distribution of HFIP-treated IAPP suggested the existence of metastable oligomers. The abundance of IAPP

monomer through tetramer diverged significantly from an exponential curve (Fig. 1c), similar to oligomer size distributions observed previously for Aβ(1–40) and calcitonin (27). This suggests that IAPP monomer, dimer, trimer, and tetramer are in quasi-equilibrium in the initial steps of the self-assembly process.

α-*Syn*. A protein with poorly defined cellular roles, α-syn is known mostly for its association with neurodegenerative diseases. α-Syn self-assembly into neurotoxic oligomers and fibrillar aggregates is thought to be causative in a group of diseases called "synucleinopathies," such as Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy (28). Aggregated α-syn is the major component of the hallmark pathological lesions in PD, Lewy bodies and Lewy neurites (29). In the past, research has focused on the fibrillar form of α-syn. Current research indicates that oligomeric α-syn is the form of the protein most likely to cause neuronal death (30, 31). The role of α-syn in cell death still is unclear as is the relationship between assembly state and toxicity. Prefibrillar α-syn can take a variety of forms, including spherical oligomers, annuli, and protofibrils (32). The characterization of these species and their relative toxicity is of considerable importance for understanding the mechanisms of α-syn-induced neuronal loss in Parkinson's disease and other synucleinopathies.

A PICUP study of α-syn was reported by Li et al. who showed that solutions of recombinant α-syn contained a mixture of monomers, dimers, and trimers (21). The authors suggested that the amphipathic N-terminal region was required for dimerization and trimerization of α-syn and that the later aggregation of α-syn originated from dimeric and trimeric seeds.

We applied PICUP to α-syn at concentrations ranging from 2 to 100 μM (data not shown). Protein concentration of 2 μM produced the best resolution of individual bands without compromising sensitivity. At higher concentrations (20–100 μM), when fractionated by SDS-PAGE, the cross-linked mixture produced a smear in which individual oligomers could not be identified. Control, uncross-linked α-syn samples showed a band with an apparent mobility corresponding to dimer in addition to the monomer band (Fig. 2). This band likely represents an SDS-induced artifact similar to that seen in uncross-linked Aβ42 (33–35). In our hands, application of PICUP to freshly prepared solutions of α-syn yielded oligomer size distributions in which oligomers higher than trimer clearly were observed (Fig. 2). However, the experimental reproducibility of the relative abundance of each oligomer was low as a result of large batch-to-batch variability (Fig. 2). This variability could not be attributed to other causes because the oligomer size distribution was unaffected when the same sample was incubated for several days and cross-linked at different time points.

Two representative α-syn oligomer size distributions are shown in Fig. 2. In some experiments, we observed bands corresponding

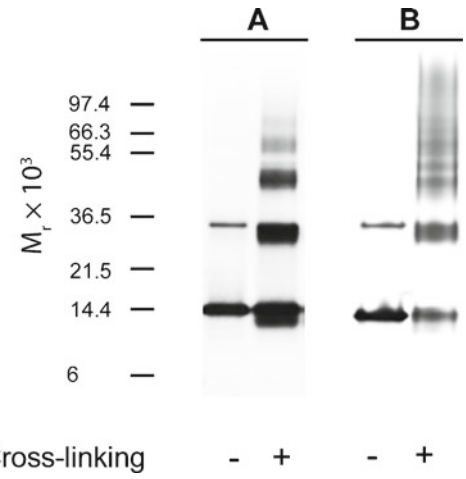

Fig. 2. PICUP analysis of α-syn. α-Syn (2 μM) was cross-linked, fractionated by SDS-PAGE and silver stained. Two representative cross-linking patterns (A and B) are shown.

to monomer through pentamer (Fig. 2, pattern A). In others, a complex pattern was found, wherein bands corresponding to individual oligomers up to a heptamer were observed in addition to larger species that could not be resolved by SDS-PAGE (Fig. 2, pattern B). These results demonstrate the importance of the source of α-syn used in PICUP experiments. One way of improving reproducibility in experiments using α-syn is by measuring the concentration of the protein immediately after dissolution[1] using a molar extinction coefficient $\varepsilon_{274\ nm} = 5{,}600\ M^{-1}/cm^{-1}$ (36) and using exactly the same concentration for all experiments. Using this approach, batch-to-batch variation can be reduced, though not eliminated, in PICUP experiments. Thus, we caution researchers using PICUP to study α-syn oligomerization and suggest that they must characterize the protein structure by complementary means and preferably use the same batch in all experiments.

2. Materials

1. Orbital shaker (INFORS AG, Labotron, Bottmingen, Switzerland).
2. Water-bath sonicator (Branson 1510, Branson Ultrasonic, Danbury, CT).
3. Light source: Dolan-Jenner 200 W incandescent lamp.

[1] Measuring the concentration immediately after dissolution is essential because as proteins aggregate, the absorbance values vary compared with the freshly dissolved proteins. Hence, for aggregating proteins, it is recommended to measure the concentration immediately after dissolution in appropriate buffer.

4. Reaction apparatus allowing controlled exposure and positioning of samples a fixed distance from the light source (see Note 1). An inexpensive, yet highly reliable and flexible apparatus may be constructed using any 35-mm, single lens release (SLR) camera body and an attached bellows.
5. 0.2-mL, clear, thin-walled plastic PCR tubes (Eppendorf).
6. 1.8-mL glass vial (Kimble Chromatography).
7. 1.7-mL, clear, silicon-coated tubes (Denville Scientific, INC).
8. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (TCI America, Portland, OR).
9. Tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate (Ru(Bpy)), $M_r = 748.63$ g/mol (Sigma).
10. Ammonium persulfate (APS, $M_r = 228.2$ g/mol) (Sigma).
11. Quenching reagent: 5% (v/v) β-mercaptoethanol (Sigma) in 2× SDS-tricine sample buffer (Invitrogen) or 1 M dithiothreitol (DTT, $M_r = 154.5$ g/mol) (Fisher) in water (see Note 1).

3. Methods

The most important factors that must be considered when designing a PICUP experiment are the reagent stoichiometry, irradiation time, and sample preparation procedure. The former two issues require empirical optimization, whereas the latter largely affects interpretation of the experimental data. For amyloidogenic proteins in particular, determination of size distributions of metastable oligomers requires using aggregate-free starting preparations. PICUP can be used to generate stable, soluble protein oligomers which, following fractionation and purification, may be used for structural studies, cytotoxicity assays (37), oligomerization-inhibition studies (38, 39), and/or as targets for the development of molecular-recognition tools (40).

The background, mechanism, instrumentation, protocol, optimization, scope, modifications, applications, and limitations of PICUP were discussed in previous publications (19, 25, 41, 42). This chapter focuses on sample preparation and experimental troubleshooting for IAPP and α-syn.

3.1. Preparing HFIP-Treated IAPP for Photo-cross-linking

The following is a method for preparation of aggregate-free IAPP by treating the lyophilized peptide with HFIP, drying the solution, and resolubilizing the resulting peptide film initially in dilute NaOH to increase solubility and decrease de novo peptide aggregation, which can account for poor reproducibility among experiments (43). The method illustrated below also can be applicable to other amyloidogenic proteins.

(a) Weigh out ~300–360 μg of lyophilized peptide using a microbalance and transfer into labeled, silicon-coated, low-adsorbent tubes.

(b) Before dissolving the peptide in HFIP, chill the HFIP container on ice inside a fume hood wearing adequate protection (HFIP is volatile and toxic). Cooling of a 250-mL bottle typically requires 10–15 min. Also, it is advised to chill all the tubes used.

(c) Add HFIP to prechilled tubes containing peptide lyophilizates to obtain a nominal peptide concentration of 0.1 mM.

(d) Sonicate the peptide solutions in a water-bath sonicator for 5 min at room temperature.

(e) Incubate the tubes for 30 min at room temperature with agitation using an orbital shaker at 200 rpm.

(f) Chill the tubes on ice (for 1 min), and divide the solutions into 100–120-μL aliquots in labeled silicon-coated low-adsorbent tubes (see Note 2).

(g) Remove HFIP by leaving the tubes open in the fume hood overnight. Place the open tubes in a rack and cover them with a large sheet of Kimwipe to prevent dust contamination.

(h) Exsiccate the remaining HFIP in vacuo in a lyophilizer or a centrifugal concentrator for 2 h, or in an exsiccator attached to a vacuum inlet for 4 h. The final product will be a peptide film at the bottom of the tubes. If properly exsiccated, the tubes can be stored airtight for extended periods (months) at −20 or −80°C.

3.2. Solubilizing the HFIP-Treated IAPP Films

Before solubilizing the HFIP-treated peptides for cross-linking reactions, one needs to prepare the cross-linking and quenching reagents.

(a) Prepare the Ru(Bpy) solution in 10 mM sodium phosphate, pH 7.4, using a vortex to mix the solution until particulate material is no longer observed. The Ru(Bpy) solution is sensitive to light and should be protected from ambient light. A simple method is to use aluminum foil to wrap the tube (see Notes 3 and 5).

(b) Prepare the APS solution in 10 mM sodium phosphate, pH 7.4. Mix the solution using a vortex until particulate material is no longer observed (see Notes 4 and 5).

(c) For SDS-PAGE analysis following cross-linking, a convenient quenching reagent is 5% β-mercaptoethanol in 2× SDS-PAGE sample buffer. Alternatively, 1 M DTT in deionized water or a suitable buffer can be used.

(d) HFIP-treated peptide films now can be dissolved in dilute NaOH first and then sodium phosphate buffer is added to get ~10 μM peptide concentration as follows.

(e) Add 60 mM NaOH followed by deionized water into the tube containing the peptide film such that NaOH and water constitute 10 and 45% of the final volume, respectively (see Note 6).

(f) Gently mix the peptide film by pipetting up and down and sonicate for 1 min in a water-bath sonicator (see Note 7).

(g) Add 45% 20 mM sodium phosphate, pH 7.4, and mix gently by pipetting. The peptide solution is ready. It should be kept on ice and used immediately after preparation.

3.3. α-Syn Sample Preparation

The aggregation kinetics of α-syn is considerably slower than that of IAPP and does not require HFIP treatment. Rather, freshly dissolved protein is used for cross-linking.

(a) Dissolve lyophilized α-syn at 20 μM in 10 mM phosphate buffer and incubate at 37 °C with mechanical agitation.

(b) Measure the solution's absorbance using a UV spectrometer at $\lambda = 274$ nm, subtract the buffer's absorbance, and calculate the protein concentration using $\varepsilon_{274} = 5{,}600\ M^{-1}/cm^{-1}$ (36).

(c) Remove ~2 μL aliquots of the aggregating solution and dilute tenfold in 10 mM phosphate buffer for cross-linking.

3.4. PICUP Reaction

(a) Adjust the camera shutter delay to 1 s. At longer irradiation times, extensive radical reactions may cause protein degradation. Irradiation time may need to be optimized when using the method with a new protein. Wind the camera shutter.

(b) A typical PICUP reaction is performed in a 20-μL reaction volume. Transfer 18 μL of the protein solution into a thin-walled, clear, 0.2-mL PCR tube.

(c) Add the PICUP reagents in this order: 1 μL Ru(Bpy) followed by 1 μL APS, and mix the reagents by pipetting.

(d) Quickly place the reaction tube inside a 1.8-mL glass vial. Place the vial inside the bellows attached in front of the camera body. Attach the lens protector, turn on the light, and press the shutter so that the sample is irradiated for 1 s inside the bellows. Turn off the light to avoid heating.

(e) After sample irradiation, quickly take the vial out of the bellows and the PCR tube out of the vial. Quickly quench the reaction by adding 1 μL DTT or 10 μL reducing PAGE sample buffer. Repeat the reaction for each peptide aliquot.

(f) The reaction mixtures can now be frozen at −20°C for storage for no longer than 7 days or kept on ice before analysis by SDS-PAGE and silver staining.

3.5. SDS-PAGE and Silver Staining of Cross-linked Peptide Products

(a) Routine SDS-PAGE and silver staining are performed to visualize cross-linked peptides.

(b) Load 100–150 pmol of protein per lane.

(c) Include similar amounts of uncross-linked proteins for comparison. Also include a standard protein ladder for visual approximation of molecular weight of peptide bands.

(d) Run the gel using a standard gel electrophoresis apparatus. We use the XCell SureLock Mini-Cell system from Invitrogen and perform silver staining according to Invitrogen publication IM-1002, Novex Pre-Cast Gel Electrophoresis Guide.

4. Notes

1. The technical details of PICUP were addressed in a previous edition of Amyloid and Proteins Methods and Protocol Part I, In Vitro Assays, Chapter 2. Determination of Peptide Oligomerization State Using Rapid Photochemical Cross-linking (41).
2. HFIP is volatile. Therefore, this step should be done on ice to avoid evaporation.
3. It is important to consider the protein:Ru(Bpy) ratio for optimization of the experimental system. Based on our experience, we recommend for IAPP: A reaction volume of 18 μL, 10 μM IAPP with 1 μL of 2 mM Ru(Bpy). For α-syn: The same reaction volume, 2 μM α-syn and 1 μL of 1 mM Ru(Bpy).
4. The Ru(Bpy):APS concentration ratio should be kept 1:20.
5. The APS and Ru(Bpy) solutions should be prepared fresh and, in case of a series of experiments, can be stored at room temperature and used for up to 48 h following preparation.
6. When working with IAPP, it is advised to prechill all the solutions on ice prior to use and always keep the tubes on ice to avoid aggregation during the sample preparation.
7. Brief sonication is an efficient way to break apart loosely attached aggregates but times longer than 1 min can induce aggregation and should be avoided.

Acknowledgments

This work was supported by grants from American Health Assistance Foundation (A2008-350), the Jim Easton Consortium for Alzheimer's Drug Discovery and Biomarker Development at UCLA, UCLA Center for Gene-Environment Studies in Parkinson's Disease, and the Michael J. Fox Foundation.

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