Video Article

Photo-Induced Cross-Linking of Unmodified Proteins (PICUP) Applied to Amyloidogenic Peptides

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Abstract

The assembly of amyloidogenic proteins into toxic oligomers is a seminal event in the pathogenesis of protein misfolding diseases, including Alzheimer's, Parkinson's, and Huntington's diseases, hereditary amyotrophic lateral sclerosis, and type 2 diabetes. Owing to the metastable nature of these protein assemblies, it is difficult to assess their oligomer size distribution quantitatively using classical methods, such as electrophoresis, chromatography, fluorescence, or dynamic light scattering. Oligomers of amyloidogenic proteins exist as metastable mixtures, in which the oligomers dissociate into monomers and associate into larger assemblies simultaneously. PICUP stabilizes oligomer populations by covalent cross-linking and when combined with fractionation methods, such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or size-exclusion chromatography (SEC), PICUP provides snapshots of the oligomer size distributions that existed before cross-linking. Hence, PICUP enables visualization and quantitative analysis of metastable protein populations and can be used to monitor assembly and decipher relationships between sequence modifications and oligomerization[1]. Mechanistically, PICUP involves photo-oxidation of Ru^{2+} in a tris(bipyridyl)Ru(II) complex (RuBpy) to Ru^{3+} by irradiation with visible light in the presence of an electron acceptor. Ru^{3+} is a strong one-electron oxidizer capable of abstracting an electron from a neighboring protein molecule, generating a protein radical[1,2]. Radicals are unstable, highly-reactive species and therefore disappear rapidly through a variety of intra- and intermolecular reactions. A radical may utilize the high energy of an unpaired electron to react with another protein monomer forming a dimeric radical, which subsequently loses a hydrogen atom and forms a stable, covalently-linked dimer. The dimer may then react further through a similar mechanism with monomers or other dimers to form higher-order oligomers. Advantages of PICUP relative to other photo- or chemical cross-linking methods[3,4] include short (≤1 s) exposure to non-destructive visible light, no need for *pre facto* modification of the native sequence, and zero-length covalent cross-linking. In addition, PICUP enables cross-linking of proteins within wide pH and temperature ranges, including physiologic parameters. Here, we demonstrate application of PICUP to cross-linking of three amyloidogenic proteins the 40- and 42-residue amyloid β-protein variants (Aβ40 and Aβ42), and calcitonin, and a control protein, growth-hormone releasing factor (GRF).

Protocol

1. Peptide preparation

1. Weigh out ~100–200 µg of lyophilized peptide using a microbalance and transfer into labeled, silicon-coated, low-adsorbent microfuge tubes. Here, we use the human sequences of Aβ40, Aβ42, calcitonin, and GRF.
2. Here, we use peptides pre-treated with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to obtain homogeneous, aggregate-free preparations. This step is necessary because pre-formed aggregates induce rapid aggregation of amyloidogenic proteins, which result in poor reproducibility among experiments[5]. Other methods such as filtration and SEC also can be used to obtain aggregate-free preparations for PICUP[6].
3. To treat the peptides with HFIP, pre-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. Add HFIP to pre-chilled tubes containing peptide lyophilizates to obtain a nominal peptide concentration of 0.5 mM.
4. Sonicate the peptide solutions in a water-bath sonicator for 5 min at room temperature.
5. Vortex gently and incubate the tubes for 30 min at room temperature.
6. Chill the tubes on ice (for 1 min), and divide the solutions into 10–50-µl aliquots in labeled 0.6-ml low-adsorbent tubes.
7. Remove HFIP by evaporation under a gentle stream of nitrogen, or leave the tubes open in the fume hood overnight. For overnight evaporation, place the open tubes in a rack and cover them with a large sheet of Kimwipes to prevent dust and particulate contamination.
8. Exsiccate the remaining HFIP *in vacuo* in a lyophilizer, or a centrifugal concentrator for 30 min, or in an exsiccator attached to a vacuum inlet for 2 h. The final product will be a peptide film at the bottom of the microfuge tubes. If properly exsiccated, the tubes can be stored airtight for extended periods (months) at −20 or −80 °C.

2. Solubilizing the HFIP-treated peptides and photo cross-linking

1. Before solubilizing the HFIP-treated peptides for cross-linking reactions, one needs to prepare the cross-linking and quenching reagents.
2. Weigh out ammonium persulfate (APS, Mr 228.2 g/mol) and prepare a 20 mM solution in 10 mM sodium phosphate, pH 7.4. Mix using a vortex until the solution is clear.
3. Prepare 1 mM solution of Tris(2,2-bipyridyl)dichlororuthenium(II) hexahydrate (RuBpy, Mr 748.63 g/mol) in 10 mM sodium phosphate, pH 7.4. Mix using a vortex and verify complete dissolution. Protect the tube from light using aluminum foil.
4. For SDS-PAGE analysis following cross-linking, a convenient quenching reagent is 5% β-mercaptoethanol in 2× SDS-PAGE sample buffer. Alternatively, 1 M dithiothreitol (DTT, Mr 154.5 g/mol) in deionized water or a suitable buffer can be used.
5. HFIP-treated peptide films are dissolved in dilute NaOH first and then sodium phosphate buffer is added to get ~30 µM peptide concentration. 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

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the final volume, respectively. Scrape the peptide film off the inside walls of microfuge tube using the tip, mix by pipetting up and down, and sonicate for 5 min in a water-bath sonicator.

6. Add 45% 20 mM sodium phosphate, pH 7.4, mix by pipetting, and centrifuge at 16,000 *g* for 10 min. Set aside aliquots of uncross-linked peptides mixed with the quenching reagent for negative controls.
7. Adjust the camera shutter-delay to 1 s. At higher irradiation times, extensive radical reactions may cause protein degradation. Load the camera shutter. Irradiation time may need to be optimized when using the method with a new protein.
8. A typical PICUP reaction is performed in a 20 µl reaction volume. Transfer 18 µl of the peptide solution into a thin-walled, clear, 0.2-ml PCR tube.
9. To the peptide solution, add 1 µl RuBpy followed by 1 µl APS and mix the reagents by pipetting (the final concentration of RuBpy and APS in the reaction mixture will be 0.05 and 1 mM, respectively).
10. 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 and press the shutter so that the sample is irradiated for 1 s inside the bellows.
11. 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.
12. 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. SDS-PAGE and sliver-staining of cross-linked peptide products

1. Routine SDS-PAGE and silver-staining are performed to visualize cross-linked peptides.
2. Load 5 µl of the reaction mixture per lane to obtain ~130 pmol of peptide per lane.
3. Include similar amounts of uncross-linked peptides for comparison. Also include a standard protein ladder for visual approximation of molecular weight of peptide bands.
4. 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.

Part 4: Representative Results (Figure 1)

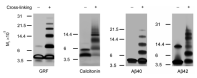

Figure 1: Silver-stained polyacrylamide gels showing uncross-linked (−) and photo-cross-linked (+) GRF, calcitonin, Aβ40, and Aβ42

SDS-PAGE and silver-staining analyses of PICUP-generated Aβ40 oligomers show approximately similar band intensities of monomer through tetramer, followed by a sharp decrease in the abundance of higher oligomers[7]. Uncross-linked Aβ40 migrates with an Mr consistent with that of monomer[7]. Aβ42 shows a distinct oligomer size distribution. Aβ42 oligomers comprise 2–3 groups[8]. The first group, monomer through trimer, displays decreasing intensity with increasing oligomer order. In the second group, a Gaussian-like distribution is observed between tetramer and heptamer, with a maximum at pentamer and hexamer. The third group, which is not shown here, contains oligomers of Mr ~30,000–60,000 Da[8]. These higher Mr oligomers typically are observed using SEC-isolated LMW Aβ42 but not peptide prepared by filtration or HFIP treatment[6]. Uncross-linked Aβ42 produces predominantly two bands, a monomer band and a broad trimer/tetramer band, which is an artefact induced by SDS[5,9]. Calcitonin yields an oligomer size distribution that strongly diverges from a monotonous exponential decrease in the region monomer through tetramer, suggesting pre-existence of dimers, trimers, and tetramers[7]. The control polypeptide, GRF, produces a monotonic oligomer distribution ranging from dimer through hexamer such that the apparent relative amounts of oligomers decrease exponentially with increasing molecular mass. In other experiments, higher oligomers also could be visualized[7]. The exact number and relative intensities of oligomer bands may vary somewhat among experiments depending on the actual protein concentration, the amount of protein loaded on the gel, and the time used for the development step in the silver-staining protocol.

Discussion

PICUP was developed originally to study stable protein complexes[2]. The method was applied later to quantitative study of metastable amyloid protein assemblies, including Aβ[10], prion and disease-associated PrP^{Sc} [11], and α-synuclein[12]. 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 may require empirical optimization, whereas the latter issue 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. The background, mechanism, instrumentation, protocol, optimization, scope, modifications, applications, and limitations of PICUP were covered in previous publications[1,2,13]. PICUP can be used to generate stable, soluble protein oligomers that following fractionation and purification, could be used for structural studies, cytotoxicity assays, oligomerization-inhibition studies, and as targets for development of molecular-recognition tools.

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