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Determination of Peptide Oligomerization State Using Rapid Photochemical Crosslinking

Sabrina S. Vollers, David B. Teplow, and Gal Bitan

Summary

The assembly of the amyloid β -protein ($A\beta$) into neurotoxic oligomers and fibrils is a seminal pathogenic process in Alzheimer's disease (AD). Understanding the mechanisms of $A\beta$ assembly could prove useful in the identification of therapeutic targets. Owing to the metastable nature of $A\beta$ oligomers, it is difficult to obtain interpretable data through application of classical methods, such as electrophoresis, chromatography, fluorescence, and light scattering. Here, we apply the method Photo-Induced Crosslinking of Unmodified Proteins (PICUP) to the study of $A\beta$ oligomerization. This method directly produces covalent bonds among unmodified polypeptide chains through *in situ* generation of peptide free radicals. PICUP provides a snapshot of the native oligomerization state of proteins and can be used for assembly state analysis of a wide variety of peptides and proteins.

Key Words: Crosslinking; oligomer; oligomerization state; protein assembly; amyloid β -protein.

1. Introduction

Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, prion diseases, and Alzheimer's disease (AD) are all associated with pathologic protein folding and amyloid formation (1,2). For example, the amyloid β -protein ($A\beta$) plays a key role in the etiology of Alzheimer's disease (3). Recent findings support the hypothesis that oligomeric forms of $A\beta$ are the proximate effectors of neurotoxicity (4,5). Oligomerization also appears to be involved in the assembly and cytotoxicity of a variety of other amyloidogenic proteins, including PrP^{Sc} (6), α -synuclein (7), and huntingtin (8). Therefore, elucidating pathways of oligomerization and assembly of $A\beta$ should not only facilitate the development of therapeutic strategies for AD, but also should be of value for understanding and treating other diseases.

Biophysical studies of A β oligomerization have been difficult owing to the metastable nature of the oligomers and have not yielded a consensus regarding the oligomer size distribution. Size exclusion chromatography (SEC) (**9**), polyacrylamide gel electrophoresis (PAGE) (**10**), dynamic light scattering (DLS) (**11**), fluorescence resonance energy transfer (FRET) (**12**), analytical ultracentrifugation (**13**), and nuclear magnetic resonance (NMR) spectroscopy (**14**) have been used to study A β oligomerization. Low resolution, peptide denaturation, and induced aggregation often have complicated the interpretation of the data. We have shown that stabilization of A β oligomers through covalent chemical crosslinking allows quantitative analysis of the A β oligomer size distribution (**15**). This approach is a useful, general tool for studying metastable protein oligomers, and in addition, produces results which readily distinguish natively associated protein oligomers from oligomers formed through random collisions in solution (**Fig. 1**).

Photo-Induced Crosslinking of Unmodified Proteins (PICUP) (**16**) is a powerful method for forming covalent bonds between polypeptides utilizing photolysis of a light-harvesting catalyst (Ru(Bpy)) in the presence of an electron acceptor (**Fig. 2**). PICUP offers several advantages relative to other crosslinking methods. It requires a very short reaction time (≤ 1 s), no *pre facto* peptide modifications or insertions, and produces high yields (approx 80%) of cross-linked material. The method is applicable across a wide range of pH and minimizes potential damage to proteins and other biological molecules by employing visible rather than UV light. In this chapter, we discuss the use of PICUP to study peptide and protein oligomerization, both for analytical and preparative purposes.

2. Materials

1. Light source: Dolan-Jenner (Lawrence, MA) 200 W incandescent lamp (*see Note 1*).
2. Reaction apparatus allowing controlled exposure and positioning of samples a fixed distance from the light source. An inexpensive, yet highly reliable and flexible apparatus, may be constructed using any 35 mm SLR (single lens reflex) camera body and an attached bellows (**Fig. 3**).
3. 0.2 mL Clear, thin-walled plastic polymerase chain reaction (PCR) tubes (Eppendorf, Westbury, NY).
4. 1.8 mL glass vial (Kimble Chromatography, Vineland, NJ).
5. 35 \times 10 mm Plastic petri dishes (Falcon, Franklin Lakes, NJ).
6. Tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate (Ru(bpy)) (Sigma, St. Louis, MO), 1 mM, in 10 mM sodium phosphate, pH 7.4 (*see Notes 2 and 3*).
7. Ammonium persulfate (APS) (Sigma), 20 mM, in 10 mM sodium phosphate, pH 7.4.
8. Low molecular weight A β (*see Note 4*).

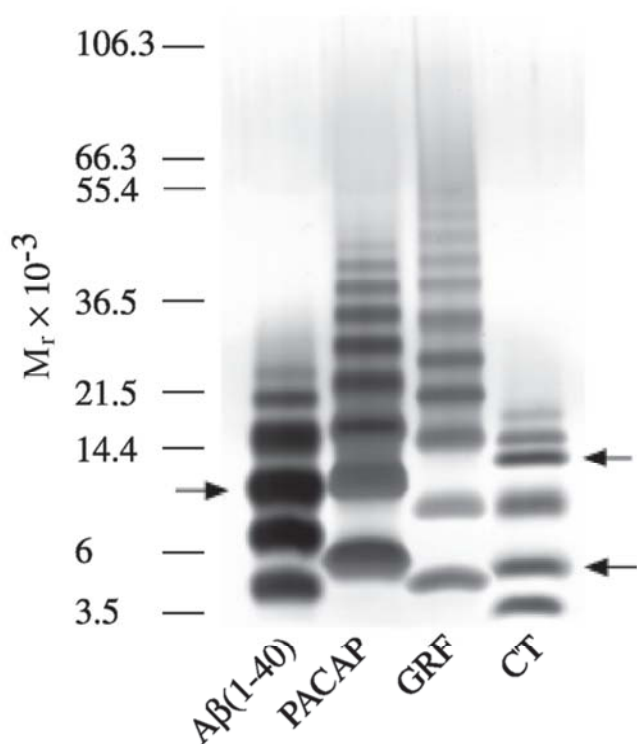


Fig. 1. PICUP results for amyloidogenic and non-amyloidogenic proteins analyzed by SDS-PAGE (adapted with permission from **ref. 15**). The non-amyloidogenic proteins pituitary adenylate cyclase-activating peptide (PACAP) and growth hormone-releasing factor (GRF) show continuous oligomer distributions whose elements decrease in intensity as molecular weight increases. The amyloidogenic proteins $A\beta$ and calcitonin (CT) display restricted oligomer distributions with non-monotonic intensity changes. Arrows indicate nodes in the intensity distributions of these latter proteins.

9. Quenching reagent: 5% (v/v) β -mercaptoethanol (Sigma) in 2X SDS-tricine Sample Buffer (Invitrogen, Carlsbad, CA), or 1 M dithiothreitol (DTT) (Fisher, Fair Lawn, NJ) in water (*see Note 5*).

3. Methods

The methods described in the following detail crosslinking for analytical purposes and for preparative purposes. Factors such as the amount of sample available and the intended purpose of crosslinking determine which method is most appropriate. The method can be customized to satisfy special conditions or parameters (*see Note 6*).

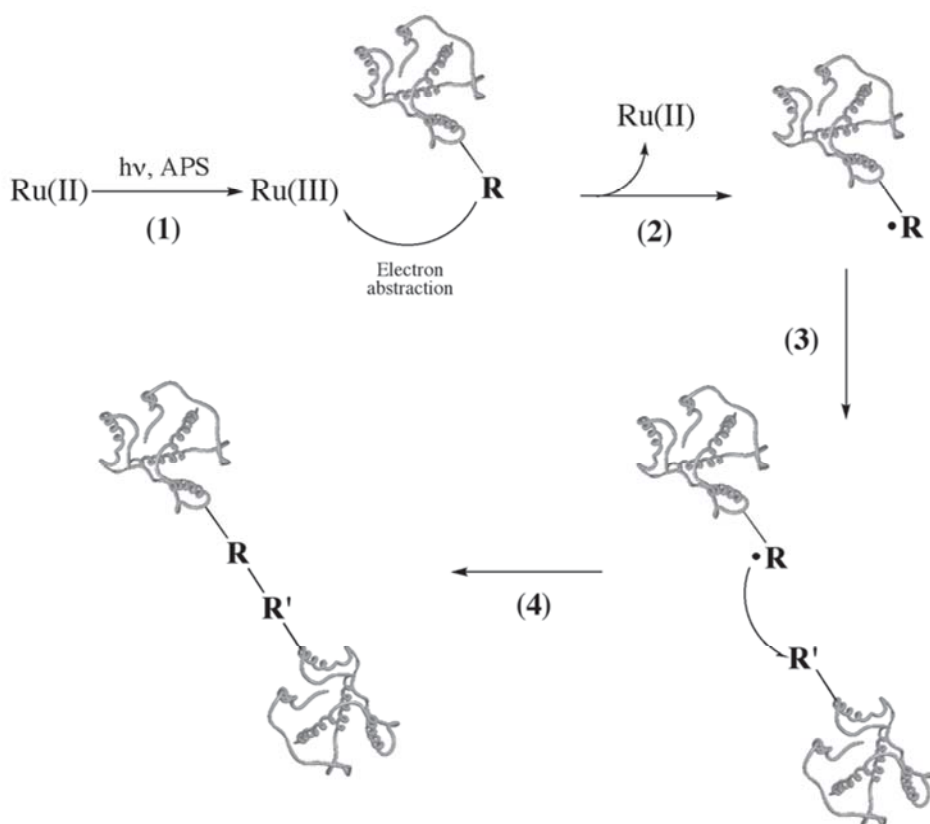


Fig. 2. Simplified reaction mechanism. (1) A Ru(II) complex is photooxidized, producing Ru(III)—a powerful one-electron abstraction agent. (2) The Ru(III) complex abstracts an electron from a nearby reactive group, R, creating a radical intermediate. (3) Adjacent reactive side-chains, R' (e.g., His, Tyr, Met) may react with the radical. (4) When the reaction is intermolecular, this produces a covalent complex.

3.1. Analytical Crosslinking

The analytical method is employed for small scale (final sample volume: 30–180 μL) studies on samples of concentration approx 25 μM (see **Note 7**).

1. Isolate the peptide or protein sample as appropriate. Here, for example (**Fig. 1**), LMW A β was isolated according to published procedures (see **ref. 10** and Chapter 6).
2. Transfer an 18 μL aliquot to a PCR tube.
3. Add 1 μL of Ru(bpy) and 1 μL of APS and mix by drawing up and expelling solution from a pipet tip.
4. Place in the illumination chamber (bellows) and irradiate for 1 s (see **Note 8**).

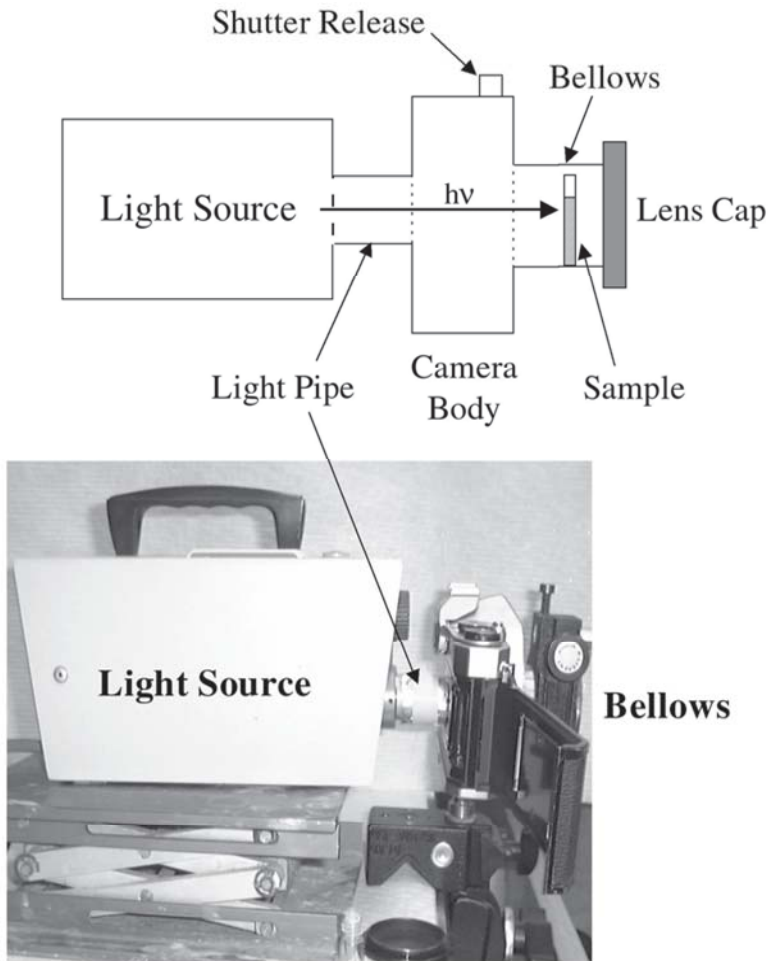


Fig. 3. Reaction apparatus scheme and actual setup. A light source is positioned directly behind the open film chamber of a 35-mm SLR camera. The sample tube is inserted in a bellows attached to the lens aperture and the end of the bellows then is capped. Activation of the shutter release allows light to enter the bellows for a predetermined interval (set using the camera body's exposure time mechanism).

5. Quench immediately by mixing either 10 μL $\beta\text{-ME}$ in sample buffer or 1 μL DTT with the sample (see **Note 9**).

3.2. Preparative Crosslinking

The preparative crosslinking method has been used on a large scale for up to 10 mL of a 2 mg/mL solution. It is appropriate when separation and purification of individual oligomers is desired.

1. Isolate the peptide or protein sample as appropriate. Here, LMW A β was prepared using 7 mg of peptide at 2 mg/mL in 10 mM sodium phosphate, pH 7.4, by filtration (see Chapter 1).
2. Place the protein sample in a 35 \times 10 mm Petri dish.
3. Place the Petri dish on a flat surface 10 cm below a miniature 50 W halogen lamp.
4. Add 700 μ L each of APS and Ru(bpy). Swirl gently to mix after each addition.
5. Irradiate for 8 s.
6. Quench with 700 μ L of DTT (see **Note 10**).

4. Notes

1. Both a 150 W Xe lamp and a 200 W incandescent lamp have been used successfully (**15,17**). Other sources of light can be used. In these cases, exposures must be adjusted empirically to maximize crosslinking efficiency. Other groups have employed the method of filtering the light beam through distilled water to prevent sample overheating (**17**). We have found this to be unnecessary when short (≤ 8 s) exposure times are used.
2. Studies show that other combinations of water-soluble metal-ion complexes and electron acceptors are possible, such as palladium (II) polyphorins and/or cobalt (III) pentamine chloride (**17**). The combination of Ru(III) and ammonium persulfate (APS) may not be ideal, depending on the experimental parameters. When using proteins containing cysteine and methionine, which are particularly susceptible to oxidation, it may be preferable to use Co(III) as the electron accepting reagent during longer incubations. For studies involving living cells, it should be noted that APS is not cell-permeable.
3. Ru(bpy) requires vortexing until the solution is transparent to the eye. The Ru(bpy) solution is light-sensitive and should be protected from ambient light. A simple method is to use aluminum foil to wrap the tube containing the Ru(bpy). The APS and Ru(bpy) reagent solutions can be used for up to 48 h following preparation.
4. The studies discussed here have used low molecular weight A β (**10**). However, the method is readily applied to the analysis of other peptides and proteins. The most important factors that must be considered are the reagent stoichiometry, irradiation time, and sample preparation procedure. The former two issues may require empirical optimization experimentation. The latter issue largely determines how the experimental data are to be interpreted. For amyloidogenic proteins in particular, native or time-dependent oligomerization states can only be determined if aggregate-free starting preparations can be produced.
5. The choice of quenching reagent depends upon the purpose of the crosslinking experiment. Samples analyzed using PAGE are quenched with the appropriate sample buffer containing 5% β -mercaptoethanol. Samples analyzed by chromatography or other methods may be quenched with 1 M DTT.
6. Cell media or extracts can be cross-linked using either the analytical or preparative method, provided the reagent concentrations are adjusted. Crosslinking of these types of samples requires higher concentrations of reagents, up to 1 M APS and

100 mM Ru(bpy). Upon addition of reagents to the sample, some precipitate forms. This precipitate does not appear to interfere with crosslinking and can be removed by centrifugation or dissolved upon addition of sample buffer after the procedure is complete.

7. The concentrations of reagents in this procedure are intended to be used with peptide samples of concentration approx 25 μ M. For less concentrated samples, the crosslinking reagents should be diluted proportionately to maintain the specified molar stoichiometry (1:2:40; protein:Ru(bpy):APS). Under these conditions, longer irradiation may be necessary for the same crosslinking yield to be obtained.
8. The PCR tube containing the sample is placed in a glass vial in order for it to be free-standing when positioned in the bellows.
9. For the analytical crosslinking procedure, the sample, reagent, and quencher volumes can be increased up to six times without affecting reaction efficiency.
10. Crosslinked samples may be stored in a -20°C freezer for several days prior to analysis. Longer storage of samples will result in decreased resolution on a gel.

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