Detection of Oligomeric Amyloid Aggregates in Real-Time Using Metal Complexes

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1. Experimental Section

Purification of $A\beta_{1-42}$: $A\beta_{1-42}$ was purchased from 21st Century Biochemicals. For HPLC purification, desalted $A\beta_{1-42}$ was dissolved in 0.1% TFA in water by vortex 1 minute. HPLC purification was performed using a reverse phase C-18 peptide column with a gradient elution of water-acetonitrile (both containing 0.1% TFA by volume) changing from 70:30 to 10:90 over 25 minutes with a flow rate of 1.5 mL/min at 60 °C. Purified Aβ was lyophilized and stored at -20 °C.

Preparation of Aβ oligomers and fibrils: A basic stock solution of monomeric Aβ was prepared by dissolving one tube of purified Aβ in 100 µL of 25 mM NaOH solution. Then the solution was filtered through a 0.2 µm centrifuge filter tubes (VWR) to remove any large aggregates. The solution was diluted to 400 µL and the concentration was measured by using an UV-Visible spectrometer (Shimadzu 2450) at 292 nm (ε = 2132 $M^{-1}cm^{-1}$). Stock Aβ basic solution was diluted by adding phosphate buffer solution (PBS) to a final concentration of 100 µM. The final concentration of PBS was 25 mM containing 0.02% NaN_3 (w/v) and the pH was set at 7.4. For Aβ oligomers formation, the solution was kept at room temperature without any agitation. For fibrils incubation, the solution was incubated at 37 °C with stirring at 700 rpm.

Synthesis of $[Ru(bpy)_2(dpqp)]^{2+}$: The complex was synthesized by modifying the method by Sun et al.[1] Briefly, *cis*-$Ru(bpy)_2Cl_2$ and dpqp ligand were refluxed for 4 h in a mixture 1:1 (Ethanol: water). After this time the mixture was allowed to reach room temperature and concentrated in half, then a saturated aqueous NH_4PF_6 solution was added to precipitate the PF_6 complex. The purification of this compound was realized by successive precipitations in acetone/KPF_6(aq) where the aqueous soluble phases were extracted with dichloromethane (3 x 10 mL), evaporated to dryness and the solid was washed with diethyl ether yielding a red solid attributed to $[Ru(bpy)_2(dpqp)](PF_6)_2$ (73% yield). In order to make the complex soluble in water, tetrabutylammonium chloride was used to convert the PF_6 complex to chloride salt by mixing it with the complex in acetone.

Real-time assay for Aβ oligomer detection: 130 µL aliquots (100 µM Aβ oligomers, 25 mM phosphate buffer with 0.02% w/v NaN_3) were taken at different incubation time spots and centrifuged at 1844 g for 5 minutes to precipitate insoluble pellets. The supernatant (100 µL) was mixed with $[Ru(bpy)_2(dpqp)]Cl_2$ (final concentration of 1 µM) for the photoluminescence anisotropy measurement. The rest 30 µL solution was mixed with ThT (final concentration of 20 µM) for fluorescence emission measurement. The fluorescence anisotropy of $[Ru(bpy)_2(dpqp)]Cl_2$ was measured 10 times with 480 nm excitation and 605 nm emission (14 nm slit for both). The fluorescence emission of ThT was measured in the range of 450-550 nm with 440 nm excitation. The emission intensity of 480 nm was choose to do the plotting.

Western blot: 20 µL aliquots (100 µM Aβ oligomers, 25 mM phosphate buffer with 0.02% w/v NaN_3) with different incubation time spots were taken and diluted with phosphate buffer to a final concentration of 50 µM. After adding the crosslinker (0.05% glutaraldehyde) to react with Aβ oligomers, a stop solution (1 M Tris-HCl buffer) was added within 5 seconds. Then the samples were mixed with sample buffer (NuPage LDS 4X) and water for a final concentration of 25 µM Aβ. For control experiments, non-crosslinked samples were prepared by the same method but without adding glutaraldehyde. All the samples were heated at 70 °C for 5 minutes and then loaded into Nu-PAGE gel (Bis-tris gel, 4-12%). The gel was running with MOPS 1X buffer at 120 V for 120 minutes and then blocked with 5% milk in TBS buffer (containing 0.1 % tween). Primary antibody 6E10 (Covance), was added into 5% milk TBS buffer in a 1:2,000 volume ratio and incubated with the gel overnight. Sheep anti mouse was used as secondary antibody in a 1:10,000 volume ratio. Finally, the gel was developed with prime western blotting detection reagent (Amersham™ ECL™) and imaged by Chemidoc imaging system (Bio-Rad) every 60 seconds for 10 minutes.

Cell Viability Assay: N2a cells (mouse neuroblastoma cells) were cultured at 10,000 cells/well for 24 hours. Cells were treated for 24 hours with different forms of Aβ, namely monomers, oligomers formed after 24 and 48 hours, and fibrils at a final concentration of 10 µM of each. Cell viability was assessed using standard MTT protocol.

SDS-PAGE: During the real-time assay, at each time spot 20 µL aliquot (centrifuged) was taken and immediately flash frozen in liquid nitrogen. At the end of real-time assay, 10 µL of each aliquot was mixed with NuPAGE LDS loading buffer and loaded into Bio-Rad 4-20% precast gel. The gel was run in Tris-Glycine-SDS buffer at 130 V for 45 minutes and then stained by standard silver staining protocol.

DLS: 1 mL of Aβ solution (100 µM Aβ, 25 mM phosphate buffer with 0.02% w/v NaN_3) was prepared as previously described. Dynamic light scattering measurements were performed immediately after preparation and after 3, 6, 12, 24, and 48 hours to track the oligomer size over time. DLS measurements were performed using a Malvern Zen 3600 Zetasizer with a disposable polystyrene cuvette. Plotted hydrodynamic diameters were the average of 100 scans.

Binding Assay: A constant concentration of 50 µM $Aβ_{1-42}$ oligomers (72 hours incubation) was mixed with different concentrations of $[Ru(bpy)_2(dpqp)]Cl_2$ (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 µM) to prepare a series of solutions. The photoluminescence anisotropy of each sample was measured 10 times in single point mode (480 nm excitation and 605 nm emission).

Purification of α-synuclein: Recombinant human wild-type α-synuclein was prepared as reported previously[2] (Paik et al. 1997, ABB 344, 325-334). Briefly, α-synuclein cloned in pRK172 was over-expressed in *Escherichia coli* BL21 (DE3) and purified with the steps of cell lysate heat treatment, DEAE-Sephacel anion-exchange, Sephacryl S-200 size-exclusion and S-Sepharose cation-exchange chromatography. Then, α-synuclein was dialyzed against excessive 20 mM Mes at pH 6.5, and stored at -80°C in aliquots.

Kinetics of α-synuclein amyloid formation: α-Synuclein (1 mg/ml) in 20 mM Mes at pH 6.5 was incubated at 37 °C with orbital shaking at 200 rpm. Aliquots of α-synuclein (7 μM) were mixed with ThT (2 μM) to a final volume of 200 μl. Then, ThT-binding fluorescence was monitored at 482 nm (3 nm slit) with excitation at 450 nm (3 nm slit) by using luminescence spectrophotometer (FluoroMax-3, Horiba).

Detection of α-synuclein oligomers: During incubation of α-synuclein (1 mg/ml) in 20 mM Mes at pH 6.5 with agitation at 200 rpm, α-synuclein aliquots were combined with $[Ru(bpy)_2(dpqp)]Cl_2$ to final concentration of 35 μM for the protein and 1 μM for the dye. Anisotropy fluorescence of $[Ru(bpy)_2(dpqp)]Cl_2$ was assessed at 620 emission (3 nm slit) and 480 nm excitation (3 nm slit) by using luminescence spectrometer (FluoroMax-3, Horiba).

2. Supplementary Figures

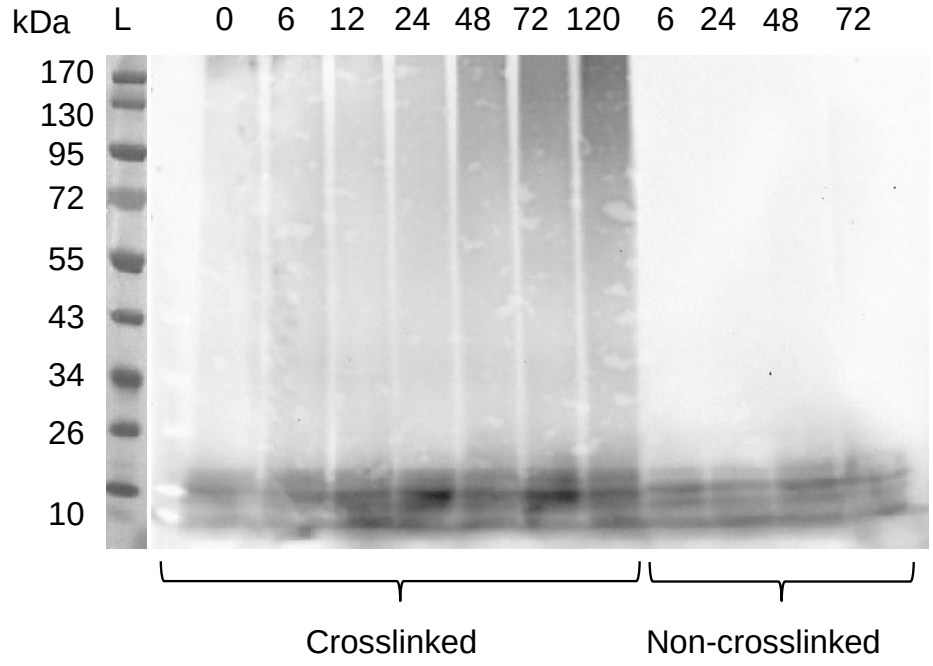

Figure S1. Western-Blot of Aβ oligomers crosslinked with glutaraldehyde.

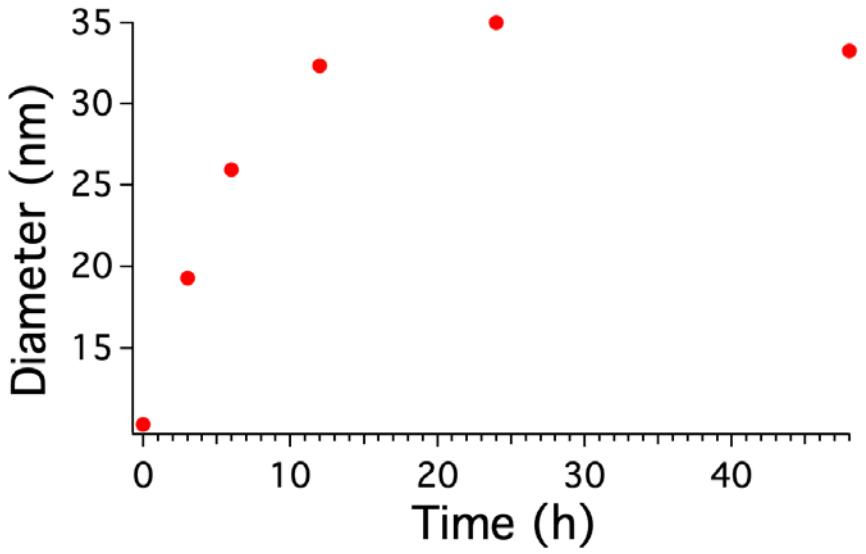

Figure S2. Determination of the size of Aβ oligomers using Dynamic Light scattering.

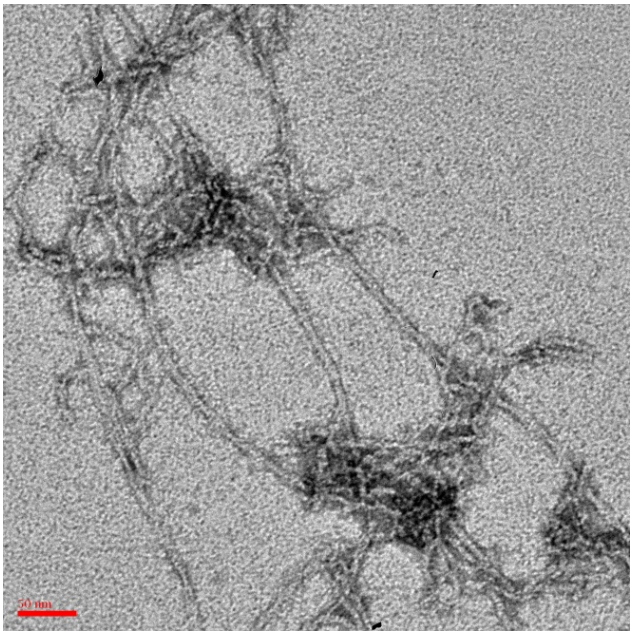

Figure S3. Aβ straight fibrils formed after incubation of the 72h mixture at 600 rpm and 37 °C. Long fibrils can be seen together with short curved fibrils formed during the quiescent incubation.

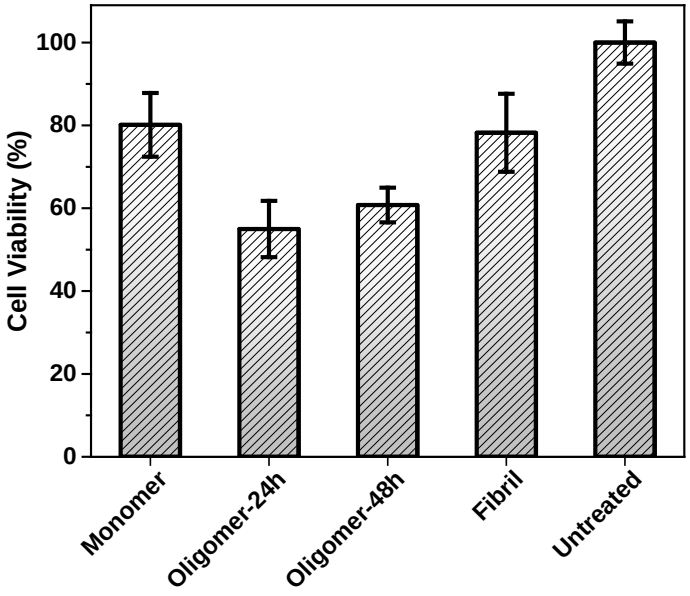

Figure S4. Aβ neurotoxicity assay. N2a cells were incubated for 24 h with Aβ in different conformations and cell viability performed using MTT. Values and error bars are average and standard deviation of three measurements.

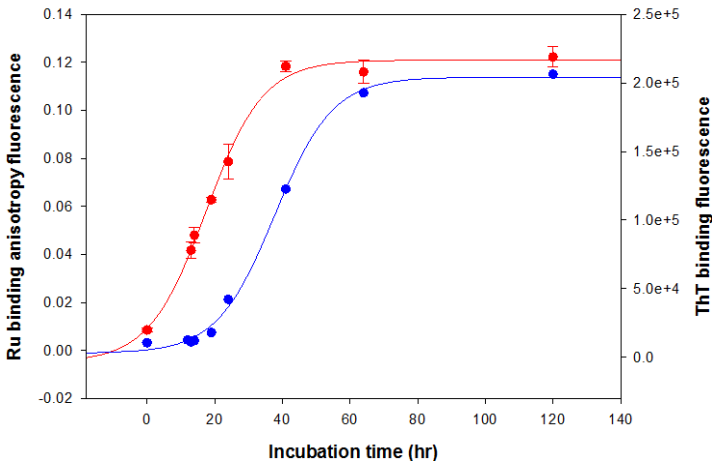

Figure S5. Real-time monitoring of αS oligomers using photoluminescence anisotropy (red squares) and the emission of ThT (blue circles).

References:

1. Sun, Y.; Collins, S. N.; Joyce, L. E.; Turro, C. *Inorg. Chem.* **2010**, *49*, 4257-62.
2. Paik, S. R.; Lee, J. -H.; Kim, D. -H.; Chang, C. -S.; Kim, J. *Archives of Biochemistry and Biophysics*, **1997**, *344*, 325-334.