Template-assited lateral growth of amyloid-β42 fibrils studied by differential labeling with gold nanoparticles

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Supporting Information

Supporting materials and methods

Plasmid construction and protein production

The plasmid codifying for Aβ42 was provided by the laboratory of Dr. Ronald Riek (The Salk Institute for Biological Studies, USA). A pRSETA vector (Invitrogen) coding for the following sequence:

$(H)_6$-$(NANP)_{19}$-RSM-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGL**L**VGGVVIA

1 35 42

The fusion construct comprises an N-terminal hexahistidine affinity tag, a soluble polypeptide segment comprising 19 repeats of the tetrapeptide sequence NANP, a methionine cleavage site (RSM) for cyanogen bromide, and the modified Aβ42 sequence (M35L-Aβ42), which contains a Leu instead of a Met at position 35 (Figure S1). This substitution enables use of cyanogen bromide to cleave Aβ42 protein from the N-terminal fusion protein. Using this plasmid as template, we genetically engineered an Aβ42 variant that contains the sequence GCGGGGGG at its N-terminus, GC-M35L-Aβ42 (Figure S1). These residues act as a spacer between Aβ42 and the label, and the cysteine provides a thiol group for the subsequent labeling. To introduce such a long insertion (eight amino acids) into the template plasmid, we employed a modified version of the protocol described in the QuickChange® Site-Directed Mutagenesis Kit (Stratagene, #200517), based on the work of Wang *et al.*[1] A single primer extension was performed by separately pre-amplifying each primer before the standard protocol was executed.

The protein was produced and purified as previously described.[2, 3] Briefly, sequence verified plasmids were transformed into competent E. coli cells BL21 (DE3), and were then grown in 1 L of TB-Medium to an OD_{600} of 1.0. The cell suspension was centrifuged at 6400*g* and 4 ºC for 10 min, and the resulting pellet was dissolved in 100 mL of buffer G

(100 mM sodium-phosphate, 10 mM Tris·HCl pH = 8.0, 6.0 M GdnCl, 5 mM DTT), and then lysed by sonication (300W) for 6 min on ice. Cell debris was removed by centrifugation at 18000*g* and 4 ºC for 30 min. The supernatant was incubated with 12 mL of Chelating Sepharose Fast Flow resin (GE Healthcare) equilibrated in buffer G for 1 h and subsequently packed into a Econo-Pac Disposable Chromatography Column (BioRad). This resin was then washed with ten column volumes of buffer G, ten column volumes of buffer U (10 mM Tris·HCl pH = 8.0, 8.0 M urea, 5 mM DTT), and four column volumes of buffer A (40 % acetonitrile, 5 mM DTT). The fusion protein was then eluted with buffer E (40 % acetonitrile, 1 M formic acid, 5 mM DTT). Eluates were combined and lyophized, and then stored at -80 ºC until cleavage. The GC-M35L-Aβ42 was quantitatively cleaved by resuspending it at 15 mg/mL in 20% acetonitrile and 50% formic acid in the presence of 50 mg/mL of cyanogen bromide at room temperature for 5 h. The cleavage products were stored at -80 ºC until usage. The cleavage mixture was subsequently diluted with H_2O to a final concentration of formic acid of 0.7% and 5 mM DTT, and then run on a cation-exchange column (Toyopearl SP-650M, Tosoh Bioscience), which enabled separation of charged GC-M35L-Aβ42 from the other uncharged components of the cleavage reaction. The resin was washed with five column volumes of buffer C1 (1% acetic acid, 5 mM DTT, 1 M NaCl in 20% acetonitrile), five column volumes of buffer C2 (1% acetic acid, 5 mM DTT in 20% acetonitrile), and two column volumes of buffer C3 (1% acetic acid, 5 mM DTT in H_2O). GC-M35L-Aβ42 was eluted with 50 mM ammonium hydroxide and 5 mM DTT. The fractions were combined and lyophilized, and then stored at -80 ºC.

Production of NEM-Aβ42 and MPB*-Aβ42

Lyophilized GC-M35L-Aβ42 was resuspended in 6 M guanidinium chloride (GndCl) and loaded onto a Superdex-75 HR 10/30 size-exclusion chromatography (SEC) column (GE Healthcare), previously equilibrated with 50 mM ammonium carbonate. Eluents of the monomeric peak were combined and lyophilized. The protein was then treated with HFIP for 20 min and re-lyophilized. The lyophilized GC-M35L-Aβ42 was then dissolved in 20 mM sodium phosphate pH 6.5, 150 mM NaCl, 1 mM EDTA, 0.01% NaN_3, and the oxidation state of the Cys thiol was confirmed (see section on entitled "Confirmation of the Cys oxidation state"). Either N-ethylmaleimide (NEM) (Aldrich, #04259) or Maleimide-PEG_2-Biotin (MPB) (No-Weigh™ Maleimide-PEG_2-Biotin, Thermo Scientific, #21902) were added at 40-fold molar excess. Conjugation was carried out at room temperature for 2 h without agitation and protecting the sample from light, obtaining NEM-Aβ42 and MPB*-Aβ42 (Figure 1). The reaction mixture was then lyophilized.

The identity of each protein variant was confirmed by high resolution mass spectrometry (HRMS) analysis (LTQ-FT Ultra Thermo Scientific). NEM-Aβ42: HRMS calcd. for $C_{227}H_{346}O_{70}N_{64}S$ 5120.51974, found 5120.53791. MPB*-Aβ42: HRMS calcd. for $C_{244}H_{374}O_{75}N_{68}S_2$ 5520.69778, found 5520.70703.

Fibril growth at low pH and low ionic strength

Preparation of monomeric NEM-Aβ42 and MPB-Aβ42*

Lyophilized NEM-Aβ42 and MPB*-Aβ42 were separately dissolved in 6 M GndCl at 1 mg/mL and then sonicated on ice for 1 to 2 min. Each of the Aβ42 variants was separately loaded onto a SEC column (Superdex 75 HR 10/30) previously equilibrated with 50 mM ammonium carbonate. This step enabled collection of monomeric fractions of each of the Aβ variants as well as removal of any excess of either NEM or MPB. The purification was not performed at pH 2.0 because Aβ aggregation has been described to occur rapidly at

acidic pH.[4] The protein concentration in the eluents of the monomeric peak was estimated again using the Bradford assay. The eluents were then combined and lyophilized. The protein was then treated with HFIP for 20 min and re-lyophilized.

Single-fibril growth assay

Lyophilized NEM-Aβ42 was dissolved in H_2O at pH 2.0 at 7 μM, and the resulting solution was incubated at 37 ºC for 14 days. The resulting NEM-Aβ42 fibrils were sonicated for 10 min in an ice bath. Lyophilized MPB*-Aβ42 was dissolved at 19 μM in H_2O at pH 2.0 and then mixed with an equal volume of sonicated NEM-Aβ42 fibrils. This mixture was incubated at 37 ºC without agitation, and sampled immediately and after 3 days. For both time points, the sample was quenched by deposition onto the electron microscopy (EM) grid. Immunogold labeling was performed on the same EM grids, which had previously been glow discharged. All solutions were filtered (0.22 μm) and were either freshly prepared or stored at 4 ºC for up to one month. The EM grid was placed on top of the following solution drops for the indicated times: sample (3 min); 10% fetal bovine serum (FBS) in 0.1 M PBS (3 x 5 min); anti-biotin antibody (ab1227, Rabbit Polyclonal to Biotin; Abcam, Cambridge, UK) diluted 1:1000 in 5% FBS (30 min, at 4 ºC); 1% FBS (5 x 5 min); anti-rabbit antibody conjugated to gold NP-12 nm (Jackson ImmunoResearch) diluted 1:30 in 5% FBS (30 min, at RT and protected from light); 0.1 M PBS (5 x 5 min); 2% glutaraldehyde (5 min); milli-Q water (3 x 1 min); and 2% uranyl acetate (1 min).

Fibril growth assay at neutral pH and high ionic strength

Preparation of monomeric NEM-Aβ42 and MPB-Aβ42*

Lyophilized NEM-Aβ42 and MPB*-Aβ42 were separately dissolved in 6 M GndCl at 1 mg/mL and then sonicated on ice for 1 to 2 min. Each Aβ42 variant was separately loaded onto a SEC column (Superdex 75 HR 10/30) previously equilibrated with 20 mM sodium phosphate pH 6.5, 150 mM NaCl, 1 mM EDTA, 0.01% NaN_3. This step enabled collection of monomeric fractions of each of the Aβ variant as well as removal of any excess of either NEM or MPB. Protein concentration in the eluents of the monomeric peak was estimated using the Bradford assay. The samples were either immediately used or stored at -20 ºC until use.

Single-fibril growth assay

Monomeric NEM-Aβ42, obtained from the SEC purification at 7 μM, was incubated at room temperature without agitation for two days and a half. The resulting NEM-Aβ42 fibrils were sonicated for 10 min in an ice bath, and then mixed with an equal volume of monomeric MPB*-Aβ42 at 19 μM. The mixture was incubated at room temperature without agitation, and sampled immediately, and after 3 and 8 days. For each time point, the sample was quenched by deposition onto the EM grid. Immunogold labeling was performed as described above for low pH and low ionic strength.

Electron microscopy

For negative staining, samples (5 to 10 μl) were deposited on Formvar carbon-coated copper grids and stained with 2% uranyl acetate. Images were obtained with a Jeol JEM1010 transmission electron microscope operating at 80 kV or with a FEI Tecnai SPIRIT operating at 120 kV. In both cases images were acquired with an SIS Megaview III camera.

Quantification of electron microscopy data

Nanoparticle (NP) counts, distances between NPs and amyloid fibrils, and area measurements were performed using ImageJ software (U.S. National Institutes of Health, http://rsb.info.nih.gov/nih-image). Two macros were created which required that the fibril be drawn. The first macro enabled automatic detection of the NPs and measurement of their distance to the nearest fibril. Since the fibrils were drawn as a line, and NP labeling involved two antibodies and a 12 nm gold NP, NP distance to the fibril was measured in units of 40 nm. The second macro enabled determination of the area available to each of the analyzed distances.

To measure NP distances to fibril ends, only the first 150 nm of each end of the fibril was drawn. The density of NPs per available unit area along the length of the fibril was obtained by dividing the number of NPs found within 40 nm from the fibril, by the area (within this distance) available to the entire fibril. Similarly, the density of NPs at fibril ends was obtained by dividing the number of NPs found within 40 nm from the fibril end, by the area (within this distance) available to the fibril ends. Statistical analysis was performed using Excel software. A two-sided Student's t-test was used to evaluate differences at different significance levels (***$p < 0.01$; **$p < 0.01$; and *$p < 0.05$).

Thioflavin T binding

The ThT binding assay was performed by mixing 10 μM Aβ solution with 10 μM ThT dye (Aβ/ThT ratio = 1:1) and 50 mM glycine–NaOH, pH 8.5 (final concentrations) in Bio-Rad 96-wells well fluorescence plates (assay volume:100 μl/well). The ThT fluorescence of each sample was measured in an FluoDia T70 (Photon Technology International) at excitation and emission wavelengths of 450 and 485 nm, respectively. The samples were

analyzed in duplicate at selected time points. The ThT data for each Aβ variant was normalized to 100% of its maximum fluorescence. Average normalized fluorescence values and standard deviation were plotted.

Confirmation of the Cys oxidation state

Maleimides absorb at 304 nm due to their cyclic conjugated imide moiety.[5] However, when maleimides react with a thiol, they form thioethers, which are unconjugated, and therefore, do not absorb at this wavelength.[6] Thus, the absorbance at 304 nm in these reactions correlates to the amount of free thiol, and therefore, can be exploited to quantify it. The free thiol groups in GC-M35L-Aβ42 were quantified in this manner before being conjugated to either NEM or MPB. A calibration curve was constructed using NEM itself, as it contains the required maleimide moiety, and β-mercaptoethanol. The curve showed a good correlation between the absorbance at 304 nm and thiol concentration ($R^2 = 0.99$). Given that NEM hydrolyzes over time and also as a function of concentration, we prepared fresh solutions of NEM before each experiment.

Supporting Figures

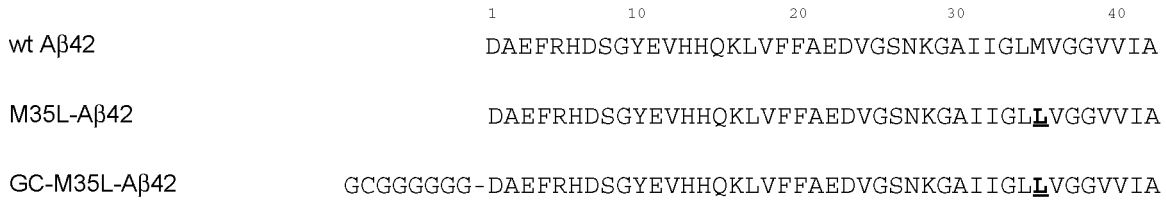

Figure S1. Amino acid sequences of wild type (wt) Aβ42, M35L-Aβ42, and GC-M35L-Aβ42.

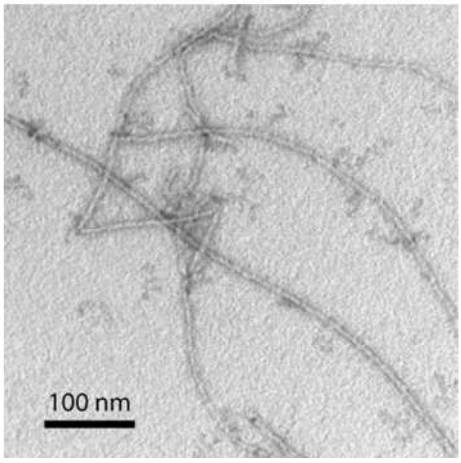

Figure S2. Representative TEM image of wt Aβ42 amyloid fibrils prepared at low pH and low ionic strength conditions: H_2O at pH 2.0 and 37 °C for 17 days.

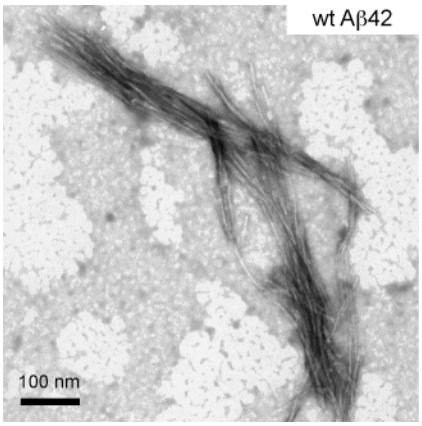

Figure S3. Representative TEM images of wt Aβ42 fibrils prepared at neutral pH and high ionic strength conditions: 20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 0.01% NaN_3 at pH 6.5 for 12 h.

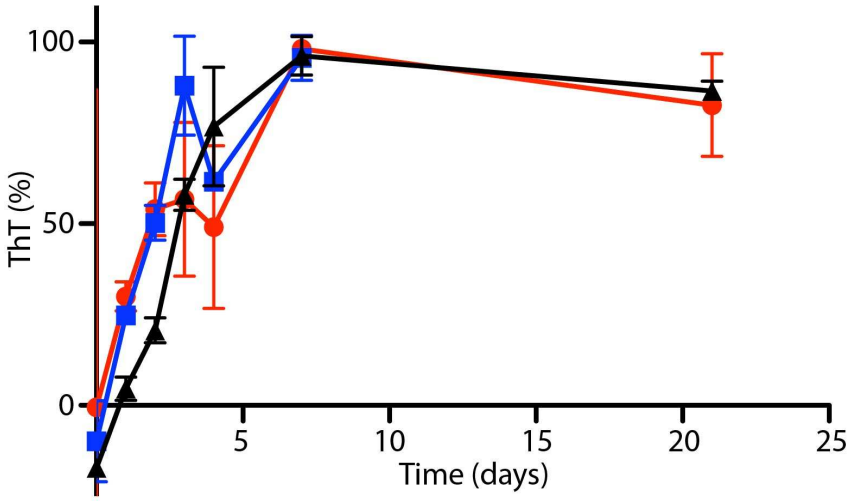

Figure S4. Kinetics of Aβ42 (black), NEM-Aβ42 (red), and MPB*-Aβ42 (blue) amyloid fibril formation at 10 μM at neutral pH and high ionic strength conditions monitored by ThT fluorescence.

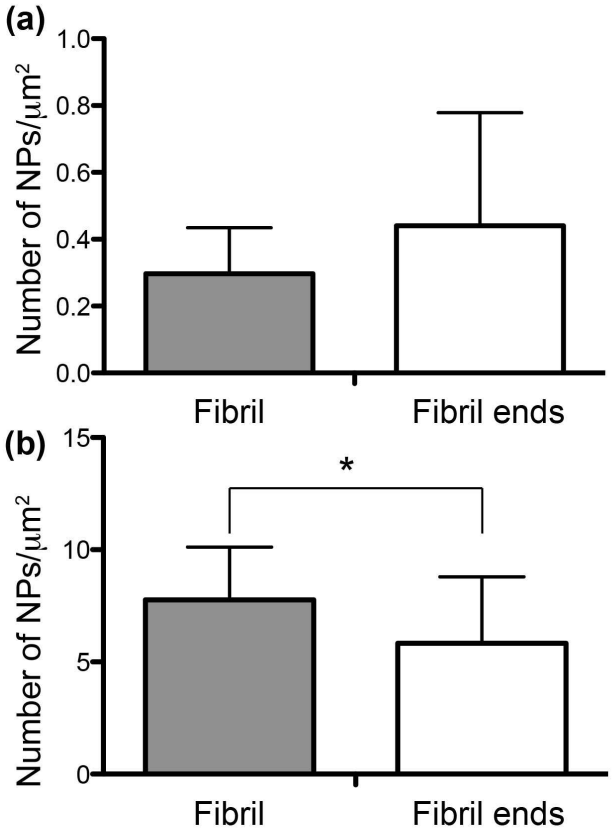

Figure S5. Number of NPs normalized to the available area at the fibril ends and along the entire fibril for samples prepared within 1 minute of mixing NEM-Aβ42 fibrils with MPB*-Aβ42 monomer at (a) low pH and low ionic strength and (b) neutral pH and high ionic strength. Data are presented as Mean-SD; *$p < 0.05$; Student´s t test. For panel A, the data is significantly different with a $p < 0.1$; Student´s t test.

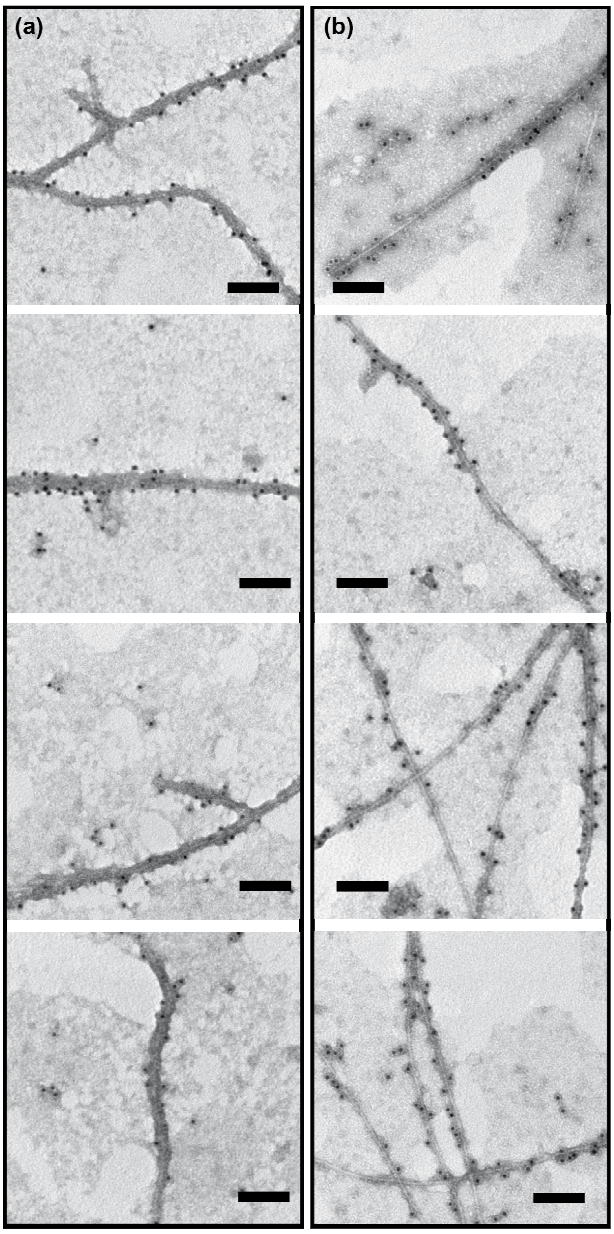

Figure S6. Comparison of NP labeling in (a) immunogold labeled MPB*-Aβ42 fibrils prepared at low pH and low ionic strength with that in (b) immunogold labeled samples obtained after 3 days incubation of NEM-Aβ42 fibrils with MPB*-Aβ42 monomer at low pH and low ionic strength. Scale bars represent 100 nm.

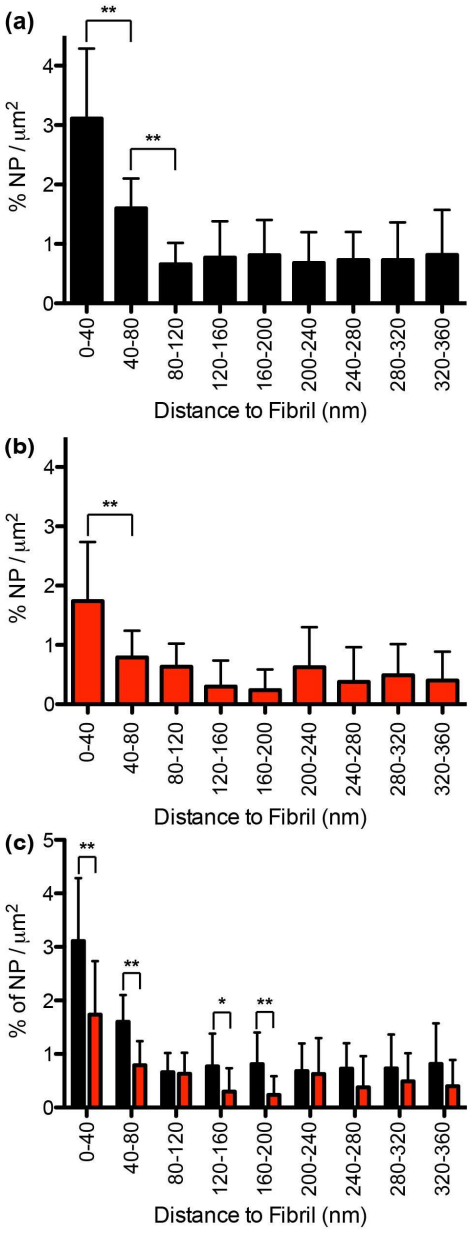

Figure S7. Percentage of NPs normalized to the available area as a function of its distance to the closest fibril, measured within 1 minute of mixing NEM-Aβ42 fibrils with monomeric MPB*-Aβ42 at (a) neutral pH and high ionic strength (black), (b) low pH and low ionic strength (red), and (c) comparison of both conditions. Data are presented as Mean-SD; **$p < 0.01$;*$p < 0.05$; Student´s t test.

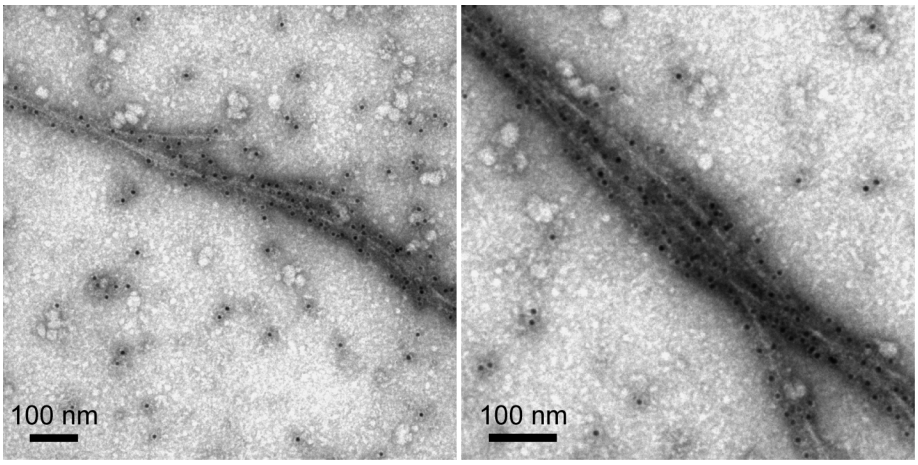

Figure S8. Representative TEM images of immunogold labeled samples of NEM-Aβ42 fibrils incubated with monomeric MPB*-Aβ42 for 3 days at neutral pH and high ionic strength.

Supporting references

(1) Wang, W., and Malcolm, B. A. (1999) Two-stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using QuikChange Site-Directed Mutagenesis. *Biotechniques 26*, 680-682.

(2) Dobeli, H., Draeger, N., Huber, G., Jakob, P., Schmidt, D., Seilheimer, B., Stuber, D., Wipf, B., and Zulauf, M. (1995) A biotechnological method provides access to aggregation competent monomeric Alzheimer's 1-42 residue amyloid peptide. *Biotechnology 13*, 988-993.

(3) Luhrs, T., Ritter, C., Adrian, M., Riek-Loher, D., Bohrmann, B., Dobeli, H., Schubert, D., and Riek, R. (2005) 3D structure of Alzheimer's amyloid-beta(1-42) fibrils. *Proc. Natl. Acad. Sci. U.S.A. 102*, 17342-17347.

(4) Teplow, D. B. (2006) Preparation of amyloid beta-protein for structural and functional studies. *Methods Enzymol. 413*, 20-33.

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(6) Shafer, D. E., Inman, J. K., and Lees, A. (2000) Reaction of Tris(2-carboxyethyl)phosphine (TCEP) with maleimide and alpha-haloacyl groups: anomalous elution of TCEP by gel filtration. *Anal. Biochem. 282*, 161-164.