

Effective screen for amyloid β aggregation inhibitor using amyloid β -conjugated gold nanoparticles

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Abstract: The abnormal aggregation of amyloid β (A β) and its subsequent intra- and extracellular accumulation constitute the disease-causing cascade of Alzheimer's disease (AD). The detection of A β aggregates and senile plaque formation, however, is nearly impossible during early pathogenesis, and the absence of a convenient screen to validate the activity of A β aggregation regulators impedes the development of promising drug targets and diagnostic biomarkers for AD. Here, we conjugated amyloid β 42 (A β 42) peptide to gold nanoparticles (AuNPs) to visualize A β 42 aggregation via A β 42 aggregation-induced AuNP precipitation. AuNP-A β 42 precipitate was quantified by optical density measurements of supernatants and thioflavin T binding assay. Transmission electron microscopy (TEM) analysis also showed reduced interparticle distance of AuNPs and confirmed the A β 42 aggregation-induced AuNP precipitation. Transthyretin, a widely known A β aggregation inhibitor, limited AuNP-A β 42 precipitation by preventing A β 42 aggregation. Finally, according to TEM analysis, A β 42-conjugated AuNPs treated with blood-driven serum revealed the differentiated aggregation patterns between normal and AD. These findings may open a scientific breakthrough in finding a possible diagnostic and prognostic tool for neurodegenerative diseases involving abnormal protein aggregation as their key pathogenesis processes.

Keywords: transthyretin, Alzheimer's disease, diagnosis, amyloid β aggregation, gold nanoparticle

Introduction

Abnormal protein aggregation is often the pivotal step in the disease-generating and propagating cascade of neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's diseases.¹⁻³ In particular, Alzheimer's disease (AD) is the most common neurodegenerative disease, and the atypical polymerization and aggregation of amyloid β (A β) peptide in neuronal inclusions and plaques have long been considered the hallmark of AD pathology.³⁻⁵ The oligomeric form and senile plaques of A β peptide that result from aggregation are the chief causes of neuronal dysfunction, degeneration, toxicity, and, ultimately, brain malfunction, leading to malignant impairments in learning and memory in AD.^{1,3,6,7}

The cleavage of amyloid precursor protein (APP) generates several A β species, predominantly A β 40 and small fragments of A β 42. A β 42 clusters more rapidly than the former in a concentration- and temperature-dependent manner and is prone to link to intra- and extracellular deposits of A β aggregates, which are closely associated with the initiation of AD pathogenesis.^{2,4} Specifically, recent studies have demonstrated that the oligomeric form is more closely linked to neurotoxicity and memory impairment

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compared with the fibrillated form in senile plaques, which appears to be a secondary phenomenon following initiation.^{1,3,6}

In addition to the developing amyloid hypothesis in AD, attention on A β aggregation inhibitors has increased in an attempt to impede aggregation. Evidence suggests that there is an endogenous inhibitor of A β aggregation.^{8,9} Transthyretin (TTR), originally known as thyroid hormone T4 and retinol transporter, acts as an A β aggregation inhibitor by binding and sequestering A β , thereby preventing amyloidogenesis.^{9,10} TTR is a homotetrameric 55-kDa protein that is produced in the liver and choroid plexus and exists in cerebrospinal fluid (CSF) and blood. Increased TTR levels in the Tg2576 mouse, an APP-overexpressing AD animal model, prevented A β plaque formation and neuronal loss for 12 months, despite rising A β levels in the brain.^{10,11} Moreover, decreased TTR concentrations in CSF in human AD patients caused unbound A β peptide to aggregate with A β oligomers and plaques to form, leading to neurotoxicity and synaptic dysfunction.¹² Based on these data, TTR is a potent A β aggregation inhibitor and regulates AD pathogenesis.^{9,12}

The combination of biomedical science and nanotechnology has launched a new era in medicine, particularly with regard to inventing and applying new treatments and diagnostic devices. Gold nanoparticles (AuNPs) have attracted much attention due to their unique properties, including amenability to particle size control, chemical composition, and surface modifications. Moreover, surface plasmon resonance (SPR) is a distinct characteristic that induces the intense red color of AuNP colloidal solutions and can be modulated based on concentration, particle size, and interparticle distance.^{13,14} For 50-nm diameter AuNPs, the SPR peak develops at 520 nm, which causes the red color of this colloidal solution.¹³ Further, the enormous surface area of nanoparticles enhances protein fibrillation by reducing the lag time of nucleation.¹⁵ Much effort has been made to use AuNPs whose surfaces have been modified with nucleotides, antibodies, and drugs to sense and sequence DNA, target cancer, and mediate protein disaggregation.^{14,16,17}

Here, we induced and visualized neuropathogenic A β 42 aggregation optically by precipitating A β 42-conjugated AuNPs. The optical density of free AuNPs (A β 42-conjugated particles that did not precipitate) in supernatant was measured to quantify the precipitation, and thioflavin T (ThT) binding assay verified and quantified A β aggregation-induced AuNP precipitation. AuNP–A β 42 precipitation occurred in a conjugated A β 42 concentration-dependent manner and was blocked by TTR, a well known A β aggregation inhibitor. Moreover, this system differentiated particle distances and

aggregation patterns in blood-derived samples from normal and AD patients.

Materials and methods

Preparation of AuNPs and conjugation of A β 42 to AuNPs

Streptavidin-conjugated AuNPs (40 nm, optical density at 520 nm = 10.3, BBI International, Cardiff, UK) were centrifuged and washed with distilled water 3 times and suspended in phosphate buffer solution (PBS) (pH = 7.2). The streptavidin–AuNP solution was combined with biotin-tagged A β 42 (biotin–A β 42 [H-5642.0500, Bachem, Torrance, CA, USA] dissolved in dimethyl sulfoxide [DMSO]) to a designated concentration and mixed thoroughly. The AuNP–A β 42 solution was incubated for 48 hours at room temperature (RT) to induce aggregation.

Dot blot analysis to confirm A β 42 conjugation to AuNP

Control (AuNPs without A β 42) and AuNP–A β 42 were loaded onto a nitrocellulose membrane (Protran®, Schleicher & Schuell, Keene, NH, USA) immediately after biotin–streptavidin reaction and the extensive washes with distilled water. Anti-A β 42 (epitope: A β 17–24 monoclonal antibody 4G8 [SIG-39220, Covance, Princeton, NJ, USA]) was used to detect A β 42 on the surface of AuNPs, visualized using horseradish peroxidase-conjugated secondary antibody (HRP-anti-mouse IgG, Pierce, Rockford, IL, USA) in an enhanced chemiluminescence reaction (GE Healthcare, Piscataway, NJ, USA). Images were taken on Las-3000 (Fuji Film, Tokyo, Japan).

Optical density measurements and ThT binding assay

The optical density of the supernatant was measured at 520 nm on a spectrophotometer (ND-1000, NanoDrop, Wilmington, DE, USA). For the ThT binding assay, 20 μ L of sample was mixed with 150 μ L of 5 μ mol/L Thioflavin T solution in 50 mmol/L glycine buffer, and the fluorescence was measured within 15 minutes (Infinite M200, TECAN, Switzerland, excitation 437, emission 485 nm).

Blood-derived serum preparation

Blood (3 mL) from normal and AD patients was provided by Samsung Medical Center with approval from the local ethical review board; informed consent was obtained from all subjects. Vacutainers (Vacuette [#450076, Greiner Bio-One, Monroe, NC, USA]) and SST tubes (SSTTMII

Advance [#368972, Becton, Dickinson and Company, Franklin, NJ, USA]) were used to collect blood. Inside the SST tube, serum was separated by serum separation gel, and the upper serum layer was drawn and stored at -80°C .

Preparation and quantification of human serum TTR level

TTR was purchased from Sigma-Aldrich (St Louis, MO, USA), suspended and diluted in PBS (pH = 7.2), and incubated with AuNP–A β 42 solution for 48 hours at RT. Human TTR levels in serum were measured using a human prealbumin detection enzyme-linked-immunosorbent serologic assay (ELISA) kit (Standard Diagnostic, Inc., Kyunggi-do, Korea) according to the manufacturer's guidelines. In brief, human sera were diluted with sample diluents 1:4000 (working on ice), loaded onto plates coated with human TTR-specific antibody (DAKO, Glostrup, Denmark) and incubated for 30 minutes at 37°C . They were then washed five times and incubated with HRP-conjugated human TTR-specific antibody (Standard Diagnostic, Inc., Kyunggi-do, Korea) for 30 minutes at 37°C . After being washed a further five times, stabilized TMB (3,3',5,5'-tetramethylbenzidine) solution was added for 10 minutes at RT, and then stop solution was added. Absorbance at 450 nm was read using a plate reader (POWER-XS, BIO-TEK, Vinooski, VT, USA).

TEM analysis

AuNP–A β 42 was prepared as described above; 1 drop (10 μL) of solution was mounted on a carbon-coated grid (200 mesh, Electron Microscopy Science, PA, USA). Two hours after filtering and drying at RT, TEM images were taken and analyzed on a JEM-1400 (Jeol, Tokyo, Japan).

Statistical analysis

All data were expressed as mean \pm SEM (standard error of the mean) or fold-change of the mean compared with control. Student *t*-test was used for two-group comparisons, and analysis of variance, followed by Tukey-Kramer multiple comparison test, was used to compare three or more groups. Significance was designated as *P*-value < 0.05 .

Results

Conjugation of A β peptide to AuNP and visualization of A β aggregation-induced AuNP precipitation

In this initial step, we hypothesized that it would be possible to visualize protein aggregation if we conjugated A β peptides

to the surface of nanoparticles that had distinct colors or characteristics (Figure 1). AuNPs in a colloidal solution have a unique red color due to SPR; the color is altered by changes in particle size, distance, and concentration.¹⁸ A β 42 peptide was conjugated to AuNP (40 nm) by streptavidin–biotin reaction and then incubated for 48 hours at RT for inducing A β 42 aggregation. The AuNP control was added to an equal amount of DMSO vehicle. A β 42 conjugation to the AuNP was confirmed by dot blot analysis (Figure 2A). The AuNP control (without A β 42 conjugation) failed to generate a signal, whereas AuNP–A β 42 elicited a robust positive signal from anti-A β 42.

Incubation at RT for 48 hours induced a visible purple precipitate and a clear supernatant in the AuNP–A β 42 solution but not for unconjugated AuNP (Figure 2B). AuNPs had a distinct red color in colloidal solution, and AuNP–A β 42 precipitation was observed, as was a clearer supernatant than the AuNP control, due to the decreased concentration of free AuNPs. Changes in interparticle distance in AuNPs in the precipitate caused the color to change from red to purple and induced a thin, tightly packed layer of precipitate on the bottom of the tube.

The optical density of the supernatant at 520 nm (the property of AuNP that confers their red color) was measured and compared as a quantitative indicator of the amount of AuNP–A β 42 precipitation. The supernatant of the AuNP–A β 42 solution had a lower optical density than the unconjugated AuNP control ($P < 0.0001$) (Figure 2C), due to the AuNP–A β 42 precipitate. By ThT binding assay, β -sheet-enriched A β 42 aggregates were detected in the AuNP–A β 42 precipitate, demonstrating that precipitation resulted from A β 42 aggregation ($P < 0.005$) (Figure 2D); the ThT binding assay is routinely and frequently used to measure and quantify A β 42 aggregation.¹⁹ AuNP–A β 42 precipitation caused a 238% increase in ThT values compared with the AuNP control. A time-dependent increase of aggregation and precipitation of AuNP–A β 42 was investigated, and 48-hour incubation was the time point at which to maximize the reaction (Supplementary Figure 1).

A β 42 concentration-dependent increase in AuNP–A β 42 precipitation

We determined whether the amount of AuNP–A β 42 precipitation was proportional to the concentration of A β 42 that was conjugated to AuNPs. Biotin–A β 42, at concentrations ranging from 0 to 300 $\mu\text{mol/L}$, was conjugated to AuNPs and incubated for 48 hours to induce precipitation. Based on the optical density and ThT binding assay results, AuNP–A β 42 precipitation and A β 42

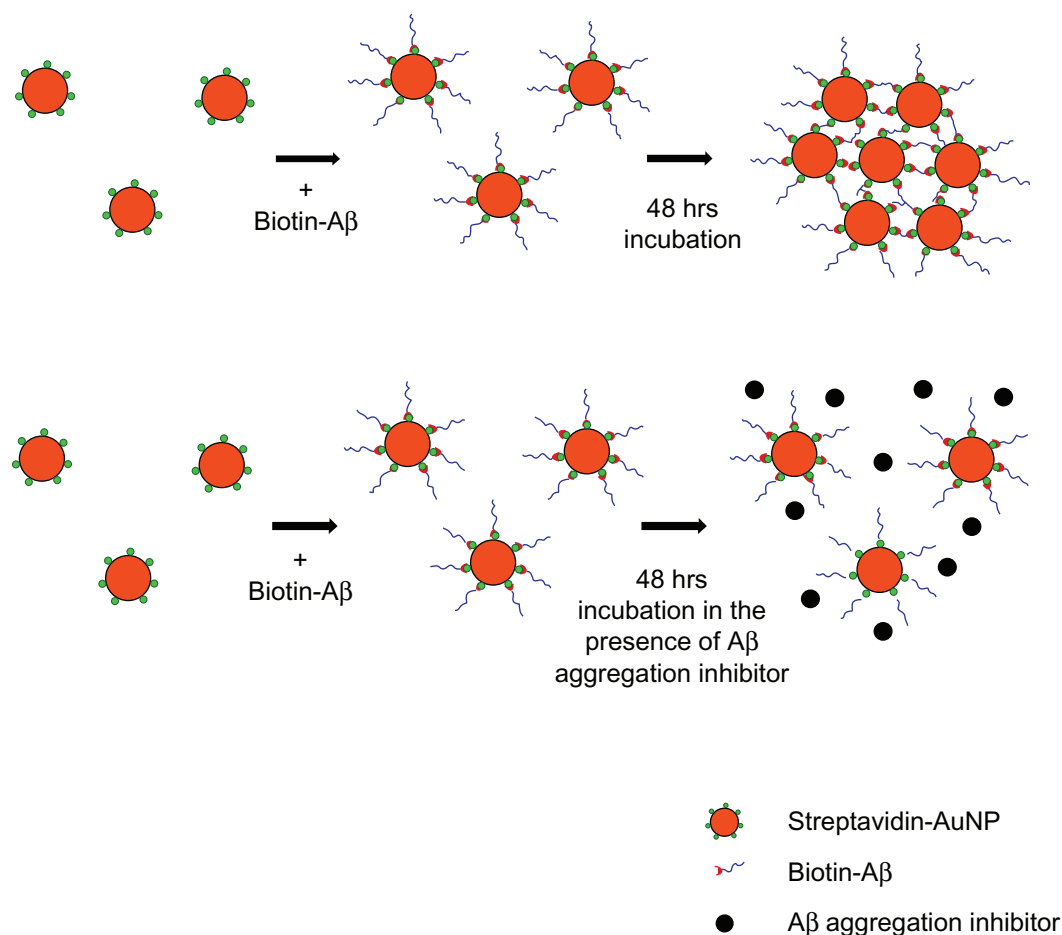


Figure 1 Schematic of sequence of Aβ42 conjugation to AuNP surface via biotin–streptavidin interaction and formation of AuNP–Aβ42 precipitate by Aβ42 aggregation. **Abbreviations:** Aβ, amyloid β; Aβ42, amyloid β42; AuNP, gold nanoparticle.

aggregation increased in an Aβ42 concentration-dependent manner (Figure 3). AuNP–Aβ42 precipitation was visible between 10 and 50 μmol/L Aβ42, consistent with the results of the optical density and ThT binding assays, which demonstrated lower optical density and increased ThT binding, respectively, at 50 μmol/L.

Thus, the simultaneous changes in visual precipitation, optical density, and ThT binding at 50 μmol/L Aβ42 suggest that AuNP–Aβ42 precipitation is caused by Aβ42 aggregation. Above 50 μmol/L, the optical density pattern reversed, despite the increase in ThT binding (100, 300 μmol/L). We speculated that residual-free biotin–Aβ42 in the solution incorporated into AuNP–Aβ42 precipitates during aggregation and expanded the interparticle distance, exceeding that in AuNP–Aβ42 that did not contain extra biotin–Aβ42 (50 μmol/L) but remaining shorter than in the AuNP control. The appearance of AuNP–Aβ42 precipitates in 300 μmol/L biotin–Aβ42 in Figure 3A supports this model, because its aggregation pattern differed compared with 50 μmol/L, developing a dark purple, cloudy, and floating precipitate rather than a tight, thin

layer. We believe that these floating AuNP–Aβ42 aggregate particles influenced the optical density measurements and increased the optical density; thus, the optimal biotin–Aβ42 concentration for AuNP conjugation was 10–50 μmol/L, and we used this range for the remaining experiments, unless noted otherwise.

Inhibition of AuNP–Aβ42 precipitation by cotreatment with the Aβ aggregation inhibitor TTR

Because Aβ42 aggregation led to AuNP–Aβ42 precipitation, we examined whether we could use this system to evaluate the activity of an Aβ42 aggregation inhibitor. Aβ42 aggregation inhibitors prevent and alleviate Aβ42 aggregation, regulating AD pathogenesis.^{8,12} Based on increased Aβ deposits due to TTR mutation or deficiency, TTR is considered an Aβ aggregation inhibitor and governs AD pathogenesis.^{11,20} We incubated AuNP–Aβ42 with or without TTR (3 μmol/L, Sigma) for 48 hours and observed that TTR inhibited AuNP–Aβ42 aggregation, eliciting no visible precipitation and generating

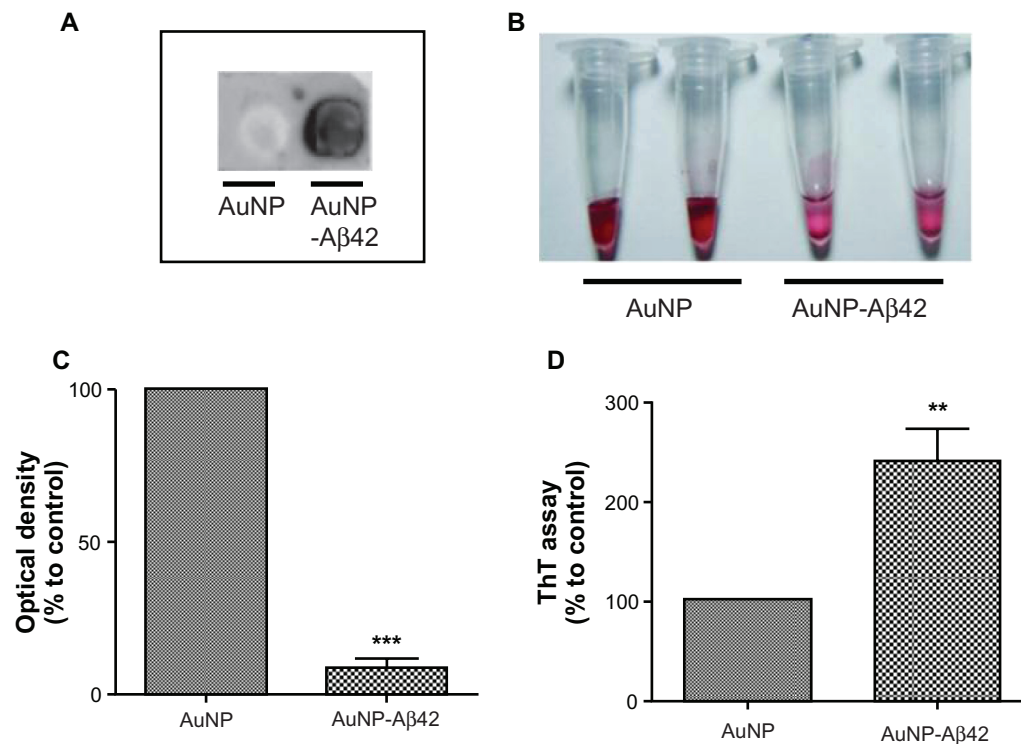


Figure 2 Confirmation of A β 42 conjugation to AuNP and induction of AuNP-A β 42 precipitates by A β 42 aggregation. **A)** Verification of biotin-A β 42 conjugation to streptavidin-AuNP by dot blot analysis. **B)** After 48 hours incubation, visible AuNP-A β 42 precipitates formed in the bottom of the tubes and the supernatant became clear; no precipitate was observed in the AuNP control. **C)** Optical density of supernatant as a quantitative indicator of AuNP-A β 42 precipitation (Paired t-test, *** P < 0.0001). **D)** ThT binding assay to measure and quantify A β 42 aggregation, reflected by significantly increased ThT binding in AuNP-A β 42 (** P < 0.005).

Abbreviations: A β 42, amyloid β 42; AuNP, gold nanoparticle; ThT, thioflavin T.

a completely different color in the supernatant compared with AuNP-A β 42 (Figure 4A). TTR rescued the decrease in optical density and increase in ThT binding in AuNP-A β 42 by inhibiting A β 42 aggregation (Figure 4B and 4C).

By TEM, the particle arrangement of AuNP-A β 42 precipitate was identified, as was its change on TTR inhibition of A β aggregation. The AuNP-A β 42 preparation was the same as in the previous experiment. The same concentrations and volumes of AuNP, free A β 42 (50 μ mol/L, A β 42 without biotin, American Peptide, Sunnyvale, CA), TTR (3 μ mol/L, Sigma) were used for each set. Unconjugated AuNP did not cluster and dispersed homogeneously, whereas AuNP-A β 42 assembled and clustered, distributing itself heterogeneously (Figure 5A). Particle distances fell significantly in AuNP-A β 42 aggregates, compared with AuNP without A β 42 conjugation (P < 0.001) (Figure 5A and 5B). Small gaps between particles in AuNP-A β 42 were possible spaces for conjugated A β 42 to aggregate. Incubation of AuNP-A β 42 with additional free A β 42 led to the same pattern of precipitation as with 300 μ mol/L biotin-A β 42 in Figure 3, which formed floating dark purple granules (Supplementary Figure 2). The distances between particles were greater than in AuNP-A β 42 aggregates but shorter than the AuNP control, and the

background appeared dark and irregular, possibly reflecting the aggregation of free A β 42. Incubation of AuNP-A β 42 with TTR for 48 hours did not cause precipitation or clustering of AuNP-A β 42, and the particles were distributed evenly, as in the control, due to the inhibition of aggregation by TTR (Figure 5A and 5B, Supplementary Figure 2). There was no difference from the AuNP control and a significant increase compared with AuNP-A β 42 (P < 0.001) with regard to particle distance, and background staining did not develop. TTR had no direct effect on AuNPs when they were added with TTR (Supplementary Figure 2). These results suggest that this AuNP-A β 42 system is a potential method for screening candidates of A β 42 aggregation inhibitors. Visualizing AuNP-A β 42 precipitation would be a tremendous advantage in validating inhibitor activities and specificities.

Differential aggregation patterns of AuNP-A β 42 in sera from normal and AD patients

We hypothesized that this system could be used for blood-derived samples to differentiate AD patients from normal individuals by monitoring AuNP-A β 42 aggregation. Serum (1/20 dilution) from four AD patients and four normal controls

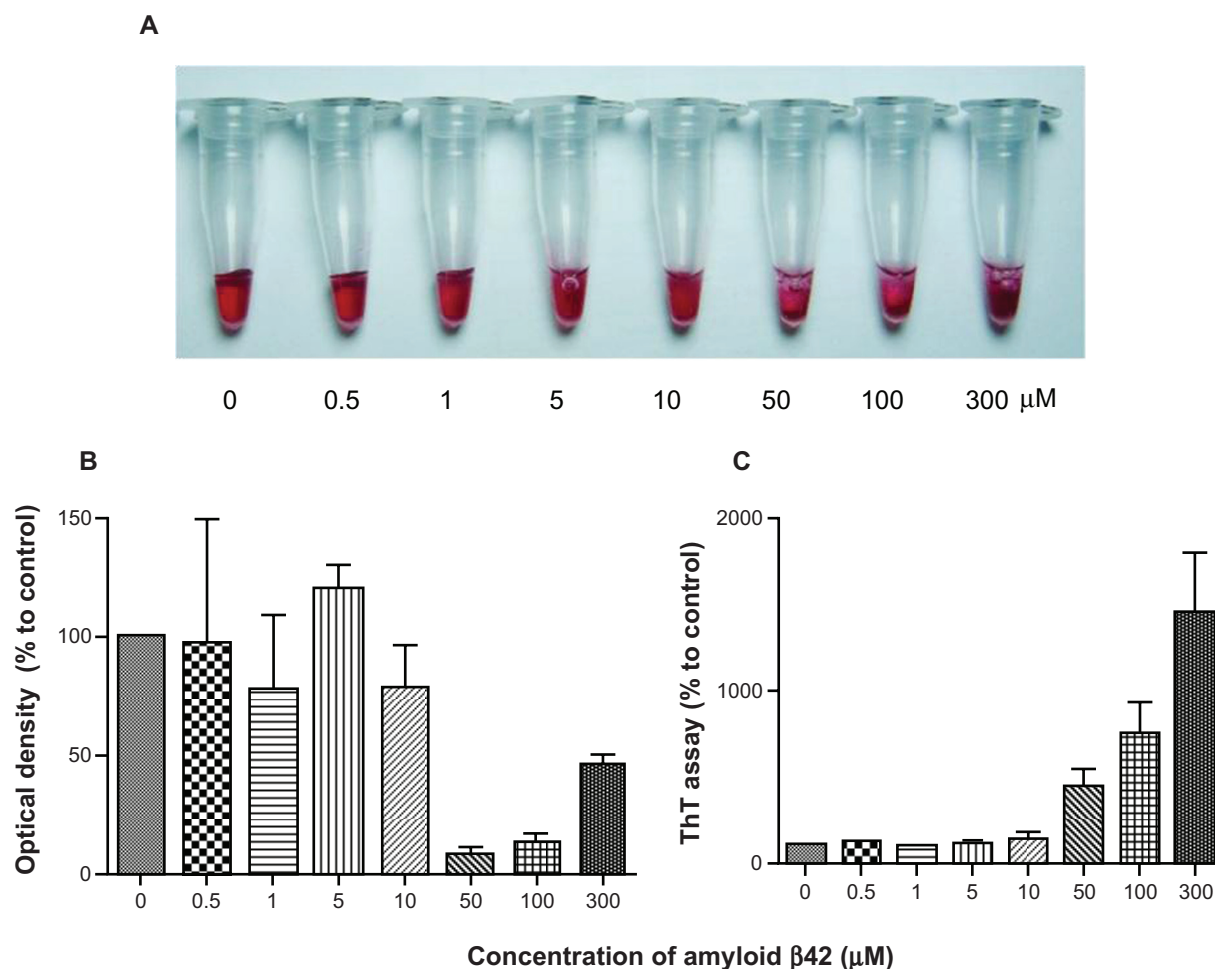


Figure 3 AuNP-A $\beta 42$ precipitates in an A $\beta 42$ concentration-dependent manner. Various concentrations of biotin-A $\beta 42$ (0, 0.5, 1, 5, 10, 50, 100, and 300 $\mu\text{mol/L}$) were added to streptavidin-AuNP to determine the optimal concentration of A $\beta 42$ conjugation. Visible precipitates developed at 50 $\mu\text{mol/L}$ biotin-A $\beta 42$ **A**), accompanied by a decrease in optical density **B**), and increase in ThT binding **C**), indicating that 10–50 $\mu\text{mol/L}$ is the optimal concentration of A $\beta 42$ to saturate AuNP surfaces and induce AuNP-A $\beta 42$ aggregation. Above 50 $\mu\text{mol/L}$, free A $\beta 42$ reverses the optical density despite the increased ThT value.

Abbreviations: A $\beta 42$, amyloid $\beta 42$; AuNP, gold nanoparticle; ThT, thioflavin T.

was tested. The average ages of control and AD subjects were 71 ± 2.00 and 80 ± 3.65 years, respectively. Serum TTR levels were also quantified and compared, showing 1227.25 ± 18.30 in the control and 408.25 ± 75.33 in the AD group, respectively. Although it was hard to tell the clear differences in AuNP-A $\beta 42$ precipitation patterns (Supplementary Figure 3), TEM images showed increased AuNP-A $\beta 42$ aggregation in AD serum and relatively even distribution of particles in the normal controls (Figure 6A and 6B). The background developed irregular patterns of protein aggregation in both normal and AD patients. In normal serum, AuNP-A $\beta 42$ spread evenly, yet AuNP-A $\beta 42$ in AD serum clustered near or above the protein aggregate, reflected by the dark background staining. Moreover, Figure 6B shows differential patterns of AuNP-A $\beta 42$ aggregation between normal and AD individuals, revealing that the interparticle distance was significantly shortened in AD compared with

normal samples. In AD samples, particles clustered near the protein aggregation of dark-stained background, whereas homogeneous dispersion was shown in normal subjects.

Discussion

We have demonstrated that the combination of two distinct properties – the color of visible precipitates of AuNPs and the aggregation-prone nature of A β – makes the visualization of A β aggregation possible. The strong conjugation of A $\beta 42$ to AuNP by biotin-streptavidin interaction, followed by a 48-hour incubation in PBS (pH = 7.2), induced aggregation, which was detected easily as dark purple precipitation.

Kogan et al reported that 10 nm β sheet breaker (PEP)-conjugated AuNP recognized and bound the hydrophobic pocket of A $\beta 42$ and formed small black precipitates after 168 hours at 37°C.¹⁷ Their purpose in inducing hydrophobic aggregation between AuNP-PEP and A β was to use AuNPs

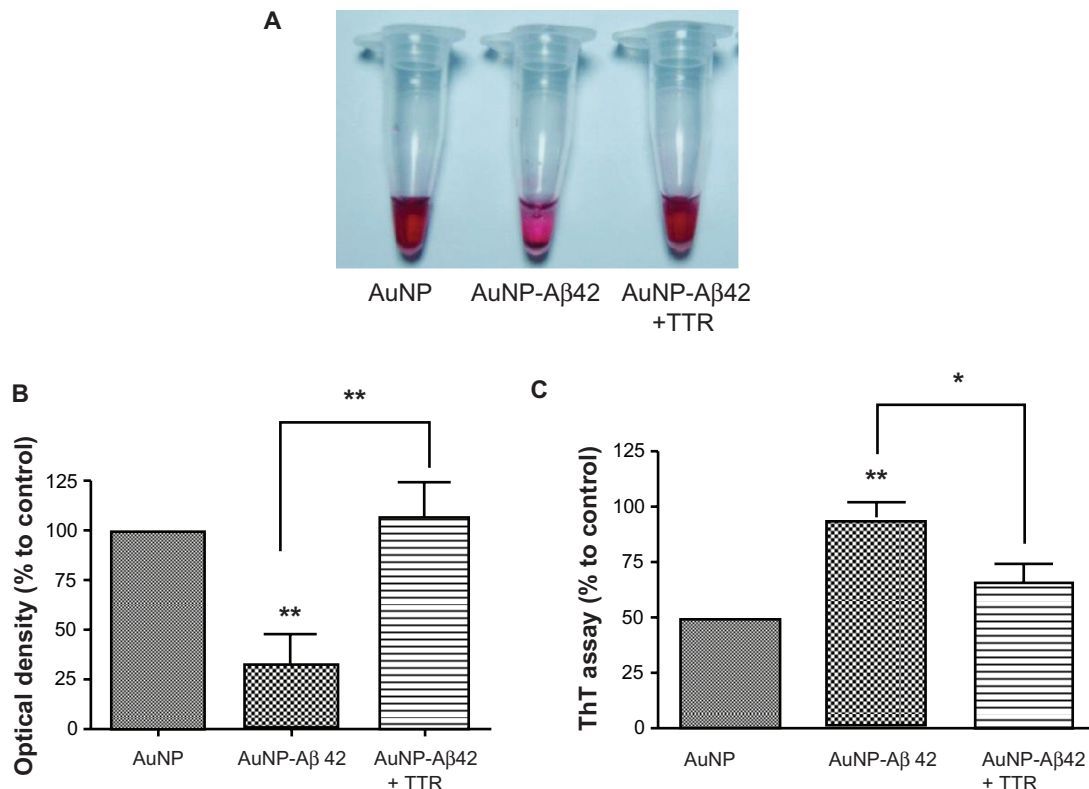


Figure 4 Activity validation of an A β aggregation inhibitor TTR, using AuNP-A β 42 precipitates. In inducing AuNP-A β 42 aggregation, its coincubation with TTR prevented the formation of visible AuNP-A β 42 precipitates and maintained the red color of the colloidal solution, as in the AuNP control **A**). TTR reversed the significant decrease in optical density **B**), and increase in ThT value **C**) of AuNP-A β 42, resulting from the inhibition of both A β aggregation and AuNP-A β 42 and one-way ANOVA (* $P < 0.05$, ** $P < 0.01$). **Abbreviations:** A β , amyloid β ; A β 42, amyloid β 42; AuNP, gold nanoparticle; ThT, thioflavin T; TTR, transthyretin.

to mediate the delivery of irradiation to A β aggregates, demonstrating the dissolution of A β precipitate and the reappearance of the red AuNP solution by irradiation. However, the precipitate was rarely visible, and 168 hours is a long period to wait to induce protein aggregation.

Thus, we conjugated full-length A β 42 directly to AuNP through strong interactions of biotin-streptavidin. This specific conjugation fastened aggregation and precipitation only after 48 hours at RT, allowing us to quantify and compare AuNP-A β 42 precipitates. Protein aggregation and fibril formation have nucleation-dependent kinetics; thus, A β 42 nucleation appears to be the rate-limiting step of aggregation and fibrillation. Nanoparticles may initiate protein nucleation and reduce nucleation lag time.¹⁵

Our results in Figure 2 show that the attachment of A β 42 to nanoparticles induced aggregation in a relatively short time (48 hours), compared with 168 hours in Kogan et al's experiments. Also, it is noted that the appearance of AuNP-A β 42 precipitates induced by full-length A β 42 aggregation in our study differed from the hydrophobic incorporation of small-fragment A β 42 to full-length A β 42. Factors such as size of nanoparticle, types, and length of

A β and conjugation method might affect the appearance of this precipitate.

This visible AuNP-A β 42 precipitate system is useful in screening and validating possible drug targets and diagnostic approaches. TTR, a well known A β 42 aggregation inhibitor, had no direct influence on AuNP itself (Supplementary Figure 2), but it blocked AuNP-A β 42 precipitation by inhibiting A β 42 aggregation (Figure 4). The possible explanation for this phenomenon is that TTR binds to A β 42 and blocks the initial nucleation of A β 42 aggregation, which prohibits further processing. We incubated AuNP-A β 42 and TTR for up to 168 hours and did not observe any difference.

The TEM analysis in Figure 5 details the structure and interparticle distances of AuNP-A β 42 precipitates and TTR activity. AuNP-A β 42+TTR affected a particle distance and distribution that were similar to those in AuNP control, but AuNP-A β 42 alone clustered in an irregular pattern. As discussed, AuNP-A β 42+free A β 42 clustered and precipitated in a pattern that differed from AuNP-A β 42. Additional free A β 42 lengthened the particle distance compared with AuNP-A β 42 aggregates but remained shorter than the AuNP control. Also, the addition of free A β 42 to AuNP-A β 42

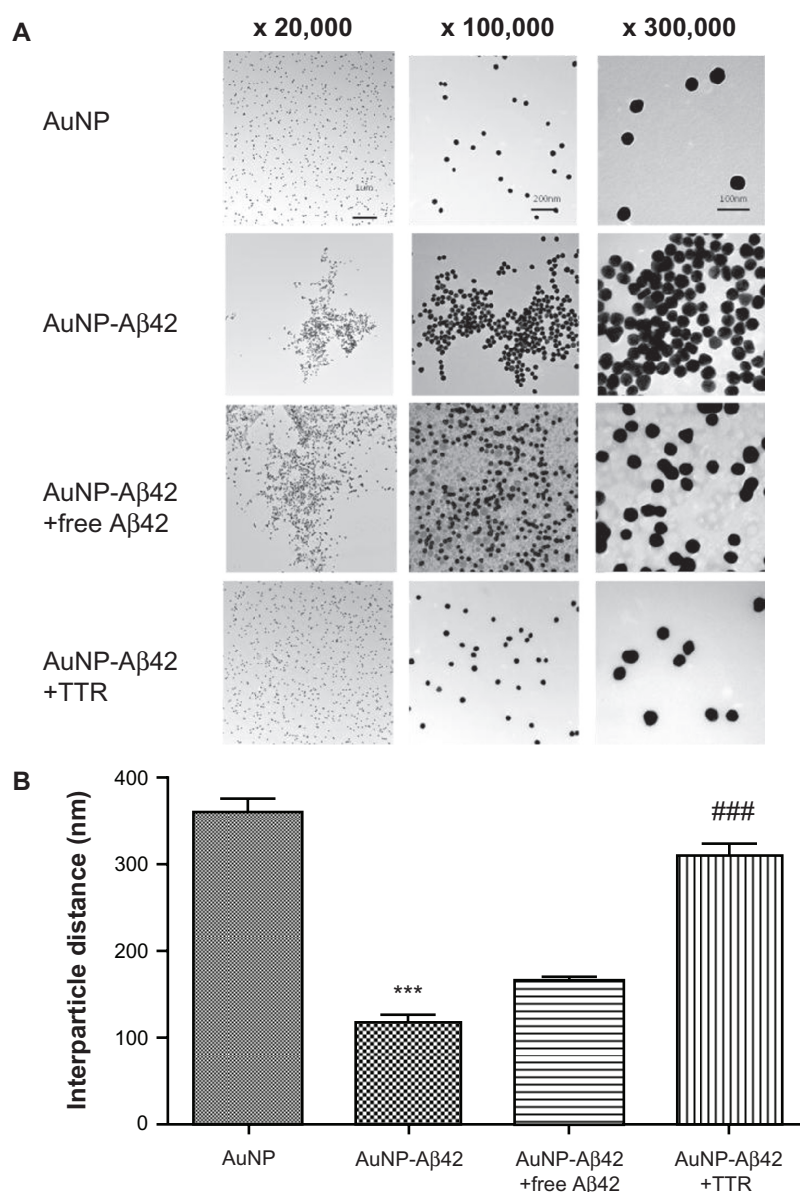


Figure 5 TEM analysis of AuNP-Aβ42 precipitates. **A**) AuNP-Aβ42 formed clusters with short interparticle distances, and AuNP underwent an even distribution of particles. The addition of free Aβ42 resulted in a similar pattern of aggregation as AuNP-Aβ42 but a greater interparticle distance and dark background staining. TTR blocked the formation of AuNP-Aβ42 aggregates and maintained the homogeneous dispersion of particles as evenly as the AuNP control. **B**) Interparticle distances were measured and compared (***) $P < 0.001$ compared with AuNP, ### $P < 0.001$ compared with AuNP-Aβ42).

Abbreviations: Aβ42, amyloid β42; AuNP, gold nanoparticle; TEM, transmission electron microscopy; TTR, transthyretin.

(AuNP-Aβ42+free Aβ42) induced a dark background of protein that did not appear in AuNP-Aβ42 (Figure 5A). This result might have been caused by the intervention of free Aβ42 into AuNP-Aβ42 aggregates during precipitation, and the irregular background staining reflects Aβ42 aggregation between AuNP-Aβ42 and free Aβ42. Also, based on this result, we are reassured that AuNP-Aβ42 precipitate is caused by aggregation of “unfree” biotin-Aβ42 but conjugated Aβ42 to AuNP. We might be able to use these distinct characteristics of AuNP-Aβ42 precipitates in the presence of free Aβ42 to detect and quantify free Aβ42 by adjusting the

proportion of conjugated and free Aβ42 in future studies. At certain concentration ratios – possibly a lower biotin-Aβ42 concentration than what is used to saturate AuNP surfaces (50 μmol/L) – free Aβ42 might lead to aggregation and precipitation that are proportional to the free Aβ42 concentration.

Levels of protein aggregates, appearing as background staining by TEM, peaked in blood-derived serum samples (Figure 6). High concentrations of diverse proteins in serum and the nonspecific intercalation of AuNP-Aβ42 into serum protein aggregates meant that it was not possible to see a clear difference in AuNP-Aβ42 precipitates between normal

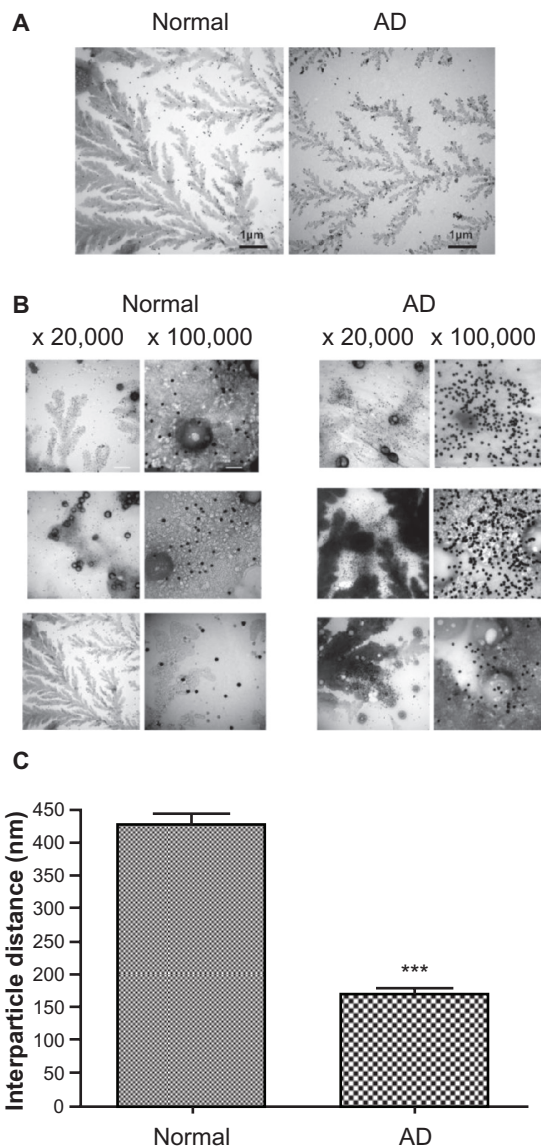


Figure 6 TEM analysis of AuNP-A β 42 incubated with blood-derived serum from normal and AD patients. Sera from normal individuals ($n = 4$) and AD patients ($n = 4$) were incubated with AuNP-A β 42 during aggregation. AuNP-A β 42 in AD patient serum showed aggregation and significantly shorter interparticle distances (**A** right, **B** right, and **C**) ($***p < 0.001$), whereas AuNP-A β 42 in normal serum dispersed evenly and represent regular distances (**A** left, **B** left, and **C**). Sera from both normal and AD patients developed irregular and dark backgrounds of protein aggregation (white scale bar in $\times 20,000$ image: 200 μm , in $\times 100,000$ image: 200 nm).

Abbreviations: AD, Alzheimer's disease; A β 42, amyloid β 42; AuNP, gold nanoparticle; TEM, transmission electron microscopy.

and AD-derived sera, yet TEM analysis clearly showed differential patterns of particle distance and aggregation. Compared with normal samples, AD serum that was incubated with AuNP-A β 42 developed AuNP-A β 42 clusters with significantly short particle distances; most aggregates were concentrated in the dark protein aggregation background stain. This result might be explained by several mechanisms. First, the concentration of A β 42 aggregation inhibitor might be lower in AD blood compared with normal subjects. As shown

in Figure 4, TTR is a known active A β 42 aggregation inhibitor that exists in low concentrations in CSF of AD patients.¹² We confirmed the concentration of TTR in blood-derived serum, quantified by ELISA, and demonstrated significantly lower concentrations in AD subjects compared with the normal group. Besides TTR, the possibility of the existence of AD-related changes of other A β binding proteins could not be excluded. The potential role of metal ions has been reported in A β aggregation and AD pathogenesis. Metal ions, such as copper and zinc, bind to A β 42, and their concentrations are susceptible to change during AD pathogenesis.²¹ Metal ions, especially copper and zinc, bind and induce A β 42 aggregation, and their concentration in blood might change during the pathogenic process and influence A β 42 aggregation.^{22,23} Other factors that might govern A β 42 aggregation should be considered, including biological lipids, which have been reported to generate toxic A β 42 protofibrils.²⁴

Conclusion

We visualized A β 42 aggregation through the conjugation of A β 42 to AuNPs. AuNP-A β 42 precipitate was quantified by optical density measurements of AuNP supernatant and ThT binding assay. With this method, an A β 42 aggregation inhibitor screen was performed and successfully validated the effect of TTR against AuNP-A β 42 aggregation. Our TEM analysis supported these results and further differentiated between aggregation patterns of AuNP-A β 42 in AD blood and normal controls. These findings establish a valuable screening system for A β aggregation regulators and potentiate the development of diagnostic and prognostic tools for neurodegenerative diseases in which abnormal protein aggregation is the critical step in their pathogenesis.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary figures

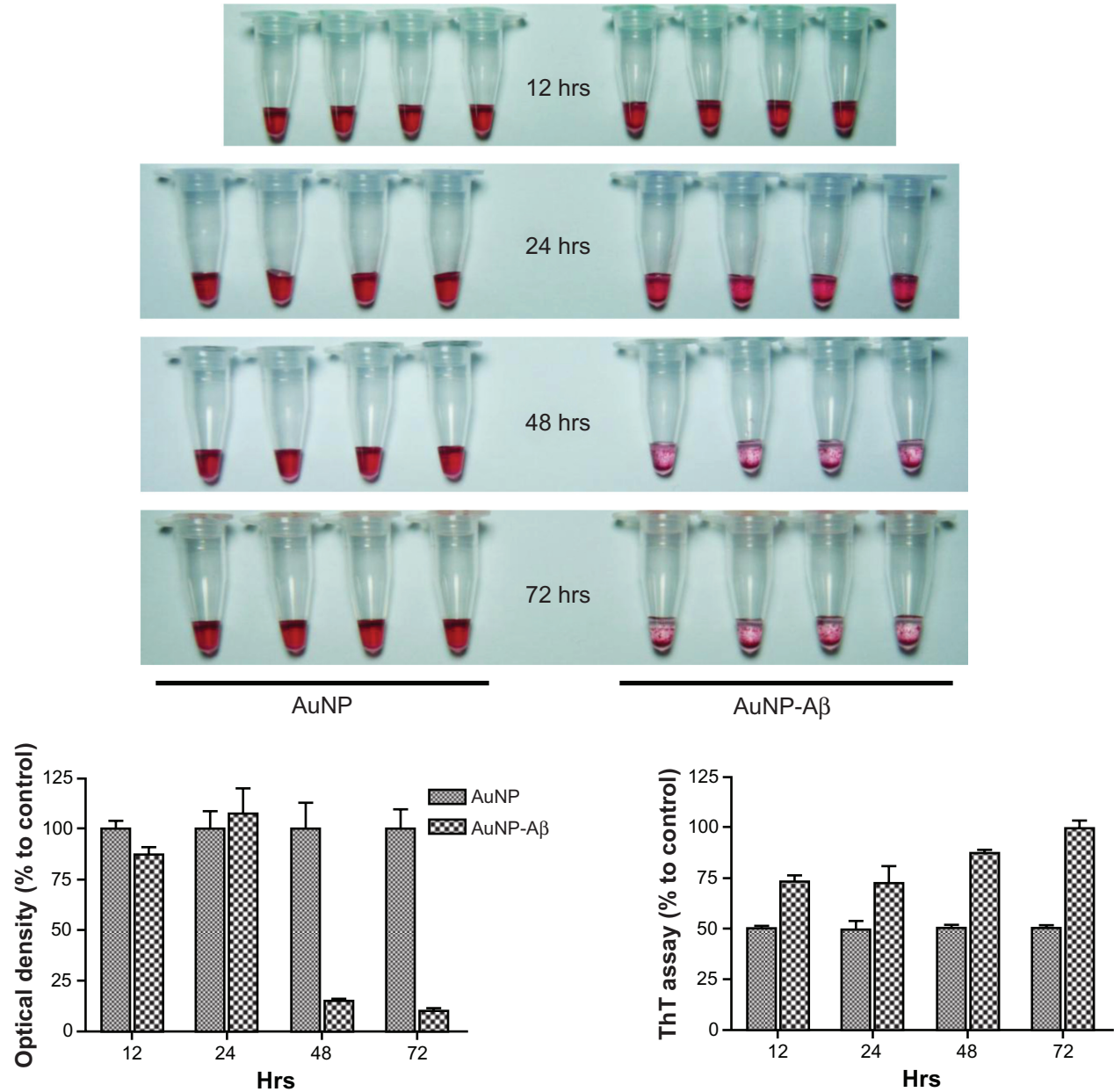


Figure S1 Time-dependent aggregation and precipitation pattern of AuNP-A β 42.
Abbreviations: A β , amyloid β ; A β 42, amyloid β 42; AuNP, gold nanoparticle; ThT, thioflavin T.

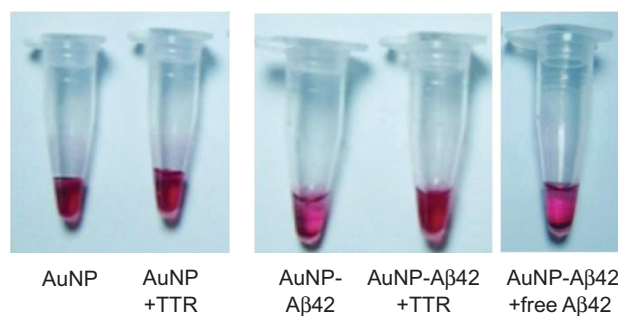


Figure S2 Precipitation pattern of AuNP-A β 42 with or without TTR or free A β 42. Images were taken before the TEM analysis in Figure 5.

Abbreviations: A β 42, amyloid β 42; AuNP, gold nanoparticle; TEM, transmission electron microscopy; TTR, transthyretin.

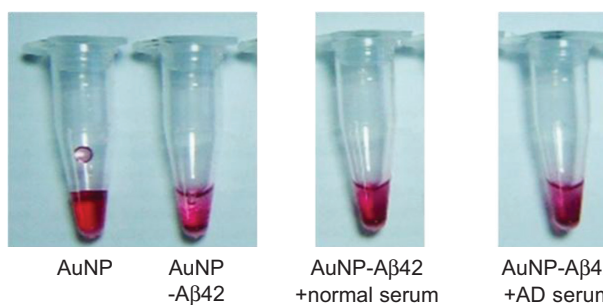


Figure S3 Precipitation pattern of AuNP-A β 42 incubated with sera from normal and AD patients. Images were taken before TEM analysis in Figure 6.

Abbreviations: AD, Alzheimer's disease; A β 42, amyloid β 42; AuNP, gold nanoparticle; TEM, transmission electron microscopy.

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