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Nanoparticle-Chelator Conjugates as Inhibitors of Amyloid-β Aggregation and Neurotoxicity: A Novel Therapeutic Approach for Alzheimer Disease

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Abstract

Oxidative stress and amyloid-β are considered major etiological and pathological factors in the initiation and promotion of neurodegeneration in Alzheimer disease (AD). Insomuch as causes of such oxidative stress, transition metals, such as iron and copper, which are found in high concentrations in the brains of AD patients and accumulate specifically in the pathological lesions, are viewed as key contributors to the altered redox state. Likewise, the aggregation and toxicity of amyloid-β is dependent upon transition metals. As such, chelating agents that selectively bind to and remove and/or "redox silence" transition metals have long been considered an attractive therapeutic target for AD. However, the blood-brain barrier and neurotoxicity of many traditional metal chelators has limited their utility in AD or other neurodegenerative disorders. To circumvent this, we previously suggested that nanoparticles conjugated to iron chelators may have the potential to deliver chelators into the brain and overcome such issues as chelator bioavailability and toxic side-effects. In this study, we synthesized a prototype nanoparticle-chelator conjugate (Nano-N2PY) and demonstrated its ability to protect human cortical neurons from amyloid-β-associated oxidative toxicity. Furthermore, Nano-N2PY nanoparticle-chelator conjugates effectively inhibited amyloid-β aggregate formation. Overall, this study indicates that Nano-N2PY, or other nanoparticles conjugated to metal chelators, may provide a novel therapeutic strategy for AD and other neurodegenerative diseases associated with excess transition metals.

Keywords

Alzheimer disease; amyloid; chelator; drug-delivery; nanoparticle

Introduction

Although the development of Alzheimer disease (AD) is incompletely understood, amyloid-β (Aβ), a 39-43 amino acid peptide, is thought by many [16,17], though not all [8,28], to play a major role in disease pathogenesis. The neurotoxicity of Aβ may result from the formation of

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protease-resistant oligomeric and fibrillar forms of Aβ [45], and blocking Aβ aggregation may provide a valuable therapeutic approach [11].

Metal chelators are among the agents with potential to prevent and reverse Aβ aggregation [13,29,48]. Chelators provide a “three pronged” mode of action. First, since iron and copper are suggested to play an important role in the self-assembly and neurotoxicity of Aβ [2,6,15, 24], not surprisingly Aβ toxicity is markedly attenuated by such chelators [23,37,42,44]. In fact, the ability of Aβ to sequestrate redox metals likely explains conflicting *in vivo* and *in vitro* reports demonstrating Aβ as both oxidant [3] and antioxidant [19,35,36,49]. Second, redox metals, as redox-active centers, lead to free radical generation [4,9,43,50] and oxidative stress, which contribute to the initiation and promotion of neurodegeneration [7,34,39,52]. Third, since oxidative stress, some of which is consequent to metal-mediated processes [43], is associated with increased Aβ [55]—a consequence of the coordinated upregulation of amyloid-β protein precursor (AβPP) [55] and β- and γ-secretases [53,56]—it is also not surprising that treatment of AβPP-overexpressing transgenic mice, a model of AD that displays significant Aβ deposition and oxidative stress [38,51], with chelating agents results in less Aβ deposition [1,10].

Overall, the aforementioned data suggests chelating agents as a potential and powerful therapeutic approach to prevent and/or treat AD. Indeed, metal chelating compounds, such as desferrioxamine, ethylenediaminetetraacetic acid (EDTA), and iodochlorhydroxyquin (clioquinol), have been used to treat patients with AD and provided significant clinical improvement [12,40,41]. Limitations concerning chelator bioavailability such as blood-brain barrier (BBB) penetration and toxic side-effects have hindered further investigation, limiting both the understanding of the pathologic role of metal dysregulation in AD as well as the evaluation of the efficacy and safety of chelation therapy.

Drug delivery using nanoparticles to target the brain has shown promise in improved drug efficacy and reduced drug toxicity [26,27]. Nanoparticles are able to cross the BBB by mimicking low density lipoprotein (LDL), enabling them to interact with the LDL receptor, resulting in their uptake by brain endothelial cells [26,27]. Nanoparticles may also employ transferrin transcytosis for their transport [26,27]. Significantly, our previous studies have suggested that nanoparticles covalently conjugated to chelators may have the potential to deliver chelators into the brain without altering metal chelating capability [30].

Here, we report on the synthesis of new nanoparticle-chelator conjugates and their ability to protect normal human brain cells from Aβ-associated neurotoxicity. These nanoparticle-chelator conjugates can also inhibit Aβ aggregation, a possible mechanism by which the conjugates inhibit this neurotoxicity.

A prototype nanoparticle-chelator conjugate (Nano-N2PY) was synthesized according to earlier studies (Figure 1) [31,32]. Briefly, carboxylic functionalized polystyrene nanoparticles (240 nm diameter; Bangs Laboratories, Indiana) were activated by N-cyclohexyl-N’-(2-morpholinoethyl)carbodiimide methyl-p-toluensulfonate (CMC) and then reacted with the iron chelator, 2-methyl-N-(2’-aminoethyl)-3-hydroxyl-4-pyridinone (MAEHP) in 2-(N-morpholino)ethane sulfonic acid buffer solution (MES). After synthesis, the conjugation yield (> 85%) was determined by measuring the chelator concentrations before and after conjugation spectrophotometrically at λ_{max} 281nm. To confirm the conjugation, nanoparticle samples spread on KCl crystal IR sample cards (Aldrich-Sigma, Wisconsin) were examined using a FT-IR Spectrophotometer (Perkin-Elmer Spectrum 1000). Comparing the carboxylic functionalized nanoparticles with their MAEHP conjugates, the band around 1737 cm^{-1} due to the carbonyl stretch of carboxylic acids was virtually diminished, implying the conversion

of the acids into amides. Because the polystyrene nanoparticles show very strong signals in the spectra, other characteristic bands of carbonyl groups could not be distinguished.

The metal binding of the conjugate was investigated by reaction with iron. Freshly prepared solution of $Fe(NO_3)_3$ was incubated with Nano-N2PY [31,32], the particles washed thoroughly with EDTA solution (5 mM), and stained with Perl's method [47]. The particles changed from white to blue, indicating the presence of iron. Transmission electron microscopy (Philips Tecnai 12 TEM, Eagle, FEI Company) examination at X37,000 magnification confirmed ferric-ferrocyanide granules on the nanoparticle surface, which did not affect the particle size (Figure 2). According to previous studies, the conjugation may convert two MAEHPs (bidentate chelator) into a hexadentate chelator [31,32], with advantages including kinetic stability, concentration independence of iron affinity, and low toxicity [22].

The ability of Nano-N2PY to inhibit Aβ-associated cytotoxicity was evaluated *in vitro* with human cortical neuronal cells (HCN-1A; ATCC, Maryland). Cells (5,000/well) in a 96 well micro-plate were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, California) with 1% fetal bovine serum (FBS; Gibco, California) at 37°C in a 5% CO_2 atmosphere. The conjugate and Aβ (Bachem, California) were added to final concentrations of 2 μM (based on chelators) and 1 μM, respectively [54]. After three days culture, Aβ-induced cytotoxicity was investigated using a Cytotoxicity Detection Kit (LDH) (Roche, Indiana). The conjugate significantly protects cells from Aβ-associated cytotoxicity compared to cells treated with Aβ alone (Figure 3).

In addition, cell proliferation was examined with a Cell Proliferation (Roche, Indiana) after three days-culture. Cells treated with the conjugate/Aβ (2:1 molar ratio) have a similar proliferation value as the control cells, significantly higher than the Aβ-treated cells (Figure 4). Thus, Nano-N2PY is effective at protecting neuronal cells against Aβ-associated cytotoxicity *in vitro* and has no significant adverse effects of Nano-N2PY on cell growth/proliferation.

To investigate the possible mechanism by which the conjugate inhibits Aβ neurotoxicity. Nano-N2PY and Aβ were incubated in Phosphate Buffered Saline (PBS, 10 mM, pH 7.4) at a molar ratio of 2 to 1 at 37°C [54]. To follow Aβ fibril formation, at specific incubation times, Congo red (CR; 3 μM) in PBS (10 mM, pH 7.4) was mixed with an aliquot of the solutions and duplicate samples examined spectrophotometrically. When the CR is bound to Aβ fibrils, the complex formed has a characteristic absorbance, which can be used for detecting Aβ aggregate formation [25]. Aβ aggregate formation observed under fluorescence microscopy (AmScope, X 400 magnification) could be completely prevented by co-incubation with Nano-N2PY (Fig. 5a,b).

In this study, a new prototype nanoparticle-chelator, Nano-N2PY, is shown to protect neuronal cells from Aβ-associated neurotoxicity by inhibiting Aβ aggregation. A derivative of deferiprone, with high affinities for iron, aluminum, copper, and zinc and lacking the ability to bind calcium and manganese [21], makes Nano-N2PY a suitable choice for AD therapy, only depleting excess metals without affecting essential ions.

DFO, an iron chelator approved by the FDA for the treatment of iron overload, has been shown to slow progression of AD [12], however, it has serious side effects including neurotxicity and neurological changes [5] and cannot penetrate the BBB due to its hydrophilic nature [33]. While small molecular weight lipophilic chelators, like bi- or tridentate iron chelators, have the ability to penetrate the BBB, they have considerable neurotoxicity [20] and do not remove iron from the brain despite effectively binding iron [14]. Thus, the use of these chelators is currently limited by their bioavailability and/or toxic side-effects.

However, nanoparticles, such as Nano-N2PY, have the potential to transport iron chelators across the BBB and the metal binding ability of chelators is not affected by conjugation [30]. The lipophilic character of the chelator is attenuated upon conjugation and therefore does not contribute to potential toxicity. This novel approach to chelator therapy could provide a safe and effective chelation treatment strategy in AD and other neurodegenerative diseases. Thus, studies are warranted in AD transgenic animal models with the nanpoarticle-chelator conjugates.

In conclusion, nanoparticle-chelator conjugates can effectively inhibit Aβ aggregate formation and, thereby, protect human brain cells from Aβ-related toxicity. As such conjugates have the potential to cross the BBB and thereafter be actively transported out of the brain, this approach may offer great potential for AD therapeutics. Moreover, this novel approach of nanoparticle chelator delivery could significantly improve the efficacy and reduce the toxicity of chelation therapy. This approach could also provide a valuable tool to uncover the role of metals in AD pathogenesis.

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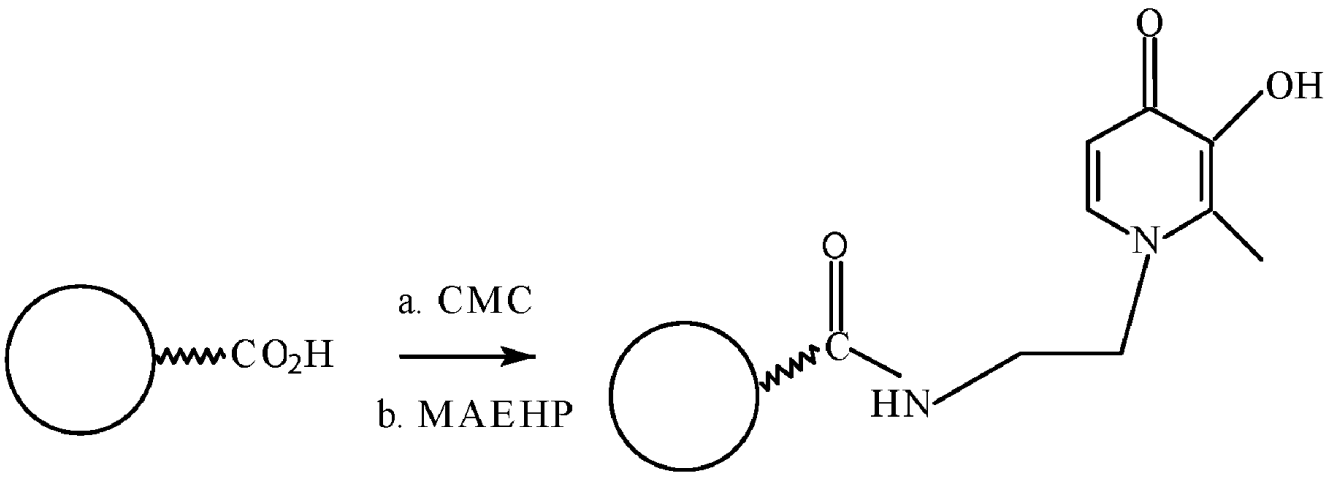

Figure 1.

Synthesis of a nanoparticle-chelator conjugate (Nano-N2PY). (a) Reaction of carboxylic functionalized nanoparticles with CMC in MES buffer solution at room temperature for a half hour. (b) Conjugation of activated carboxylic nanoparticles with excessive MAEHP in MES at room temperature (a half hour).

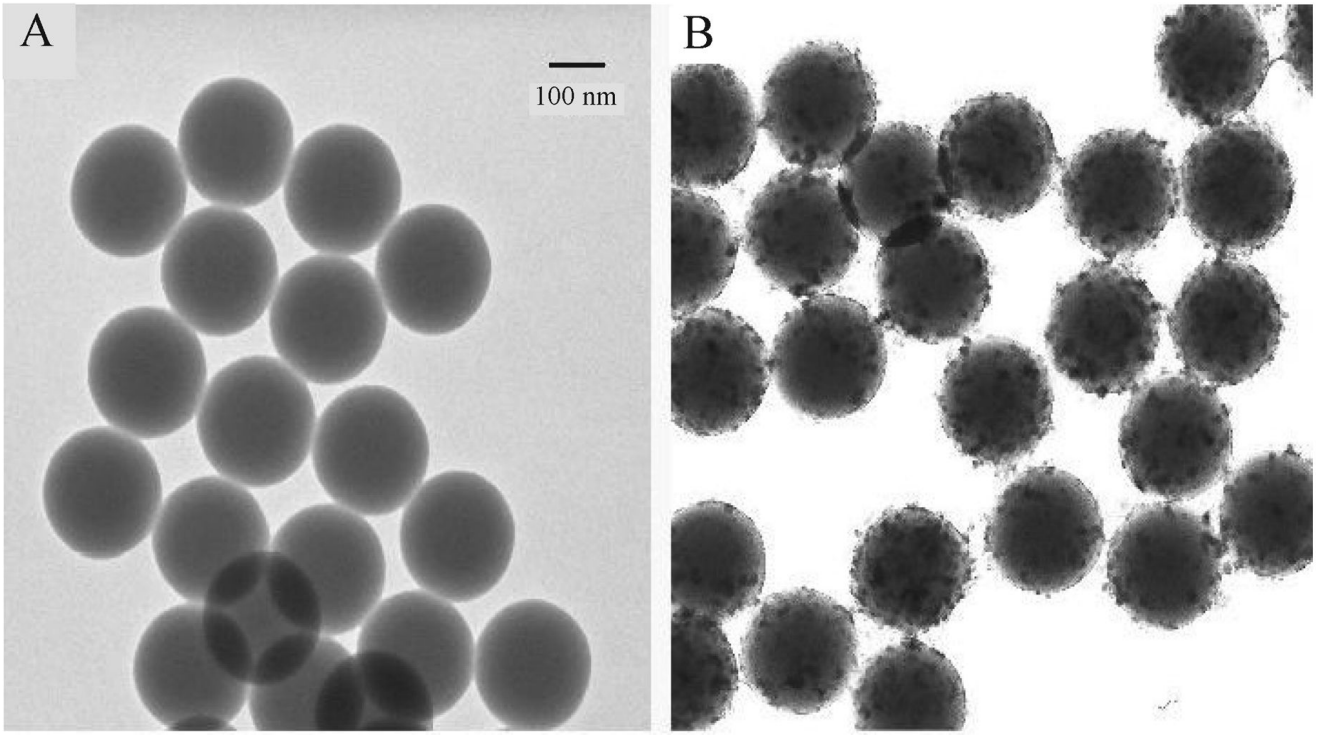

Figure 2.
TEM images of nanoparticle samples. (a) Nanoparticles without chelator conjugation and iron binding. (b) Nanoparticles with both reactions. The samples were dispersed in Milli-Q water, drop-cast onto carbon-coated copper grid and examined via TEM after air dry at room temperature.

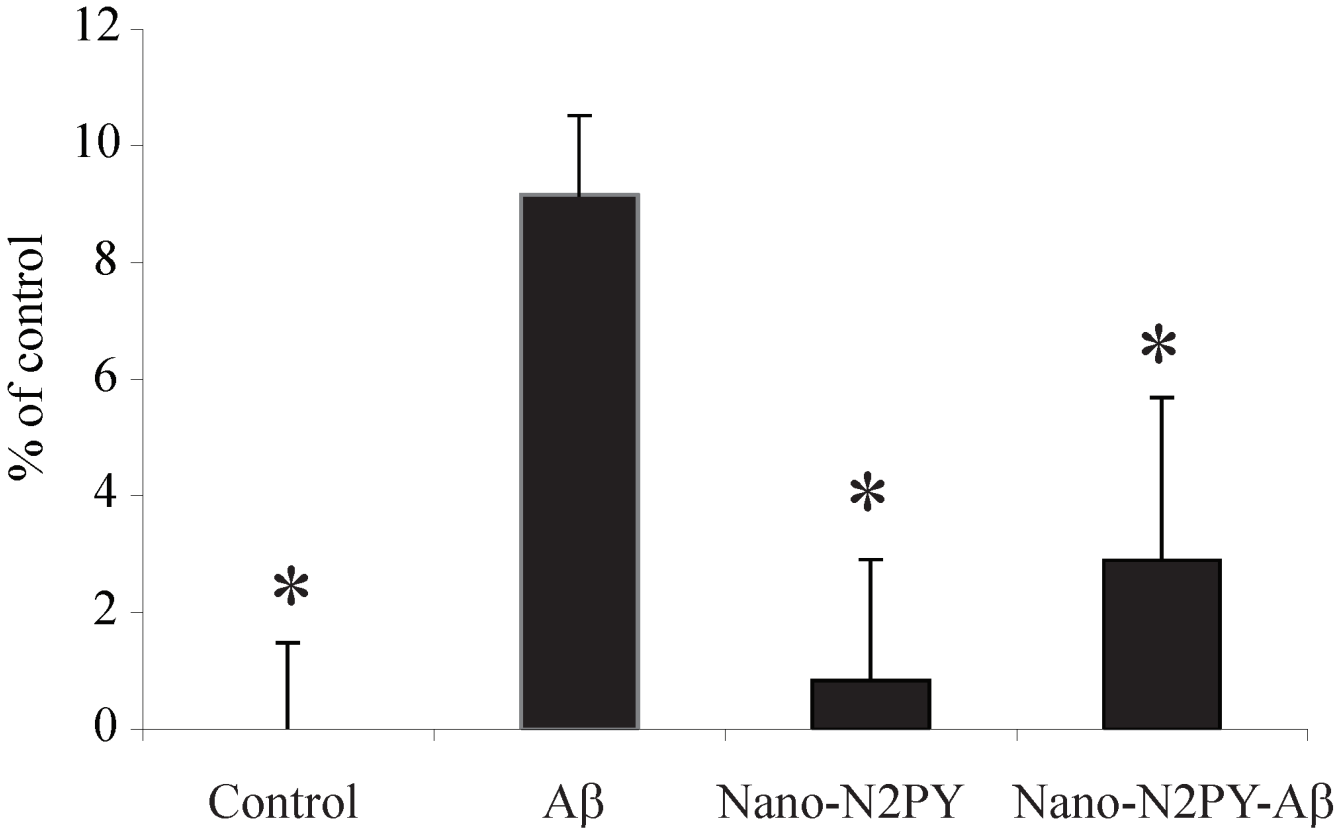

Figure 3.

Cytotoxicity of Aβ, Nano-N2PY and Aβ/Nano-N2PY (compared with control) when incubated with neuron cells as measured by LDH cytotoxicity detection assay. Absorbance wavelength measured in this experiment was 490 nm with a reference at 630 nm. Values were represented as mean ± standard errors (n = 5. *Significantly different from control group at $P < 0.05$).

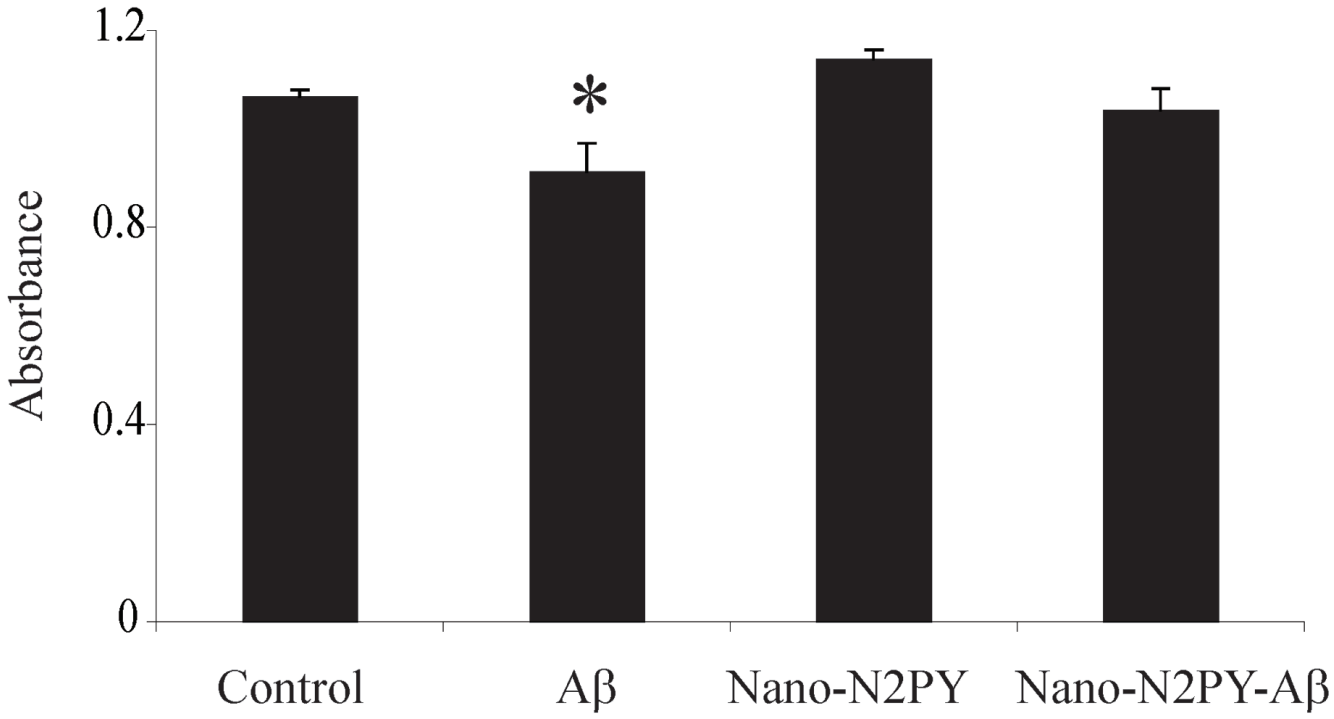

Figure 4.

Effects of Aβ, Nano-N2PY and Aβ/Nano-N2PY on cell proliferation of neuron cells as determined by WST assay. Absorbance wavelength measured here was 450 nm and a reference 600 nm. Results were represented as mean ± standard errors (n = 3. *Significantly different from control group at $P < 0.05$).

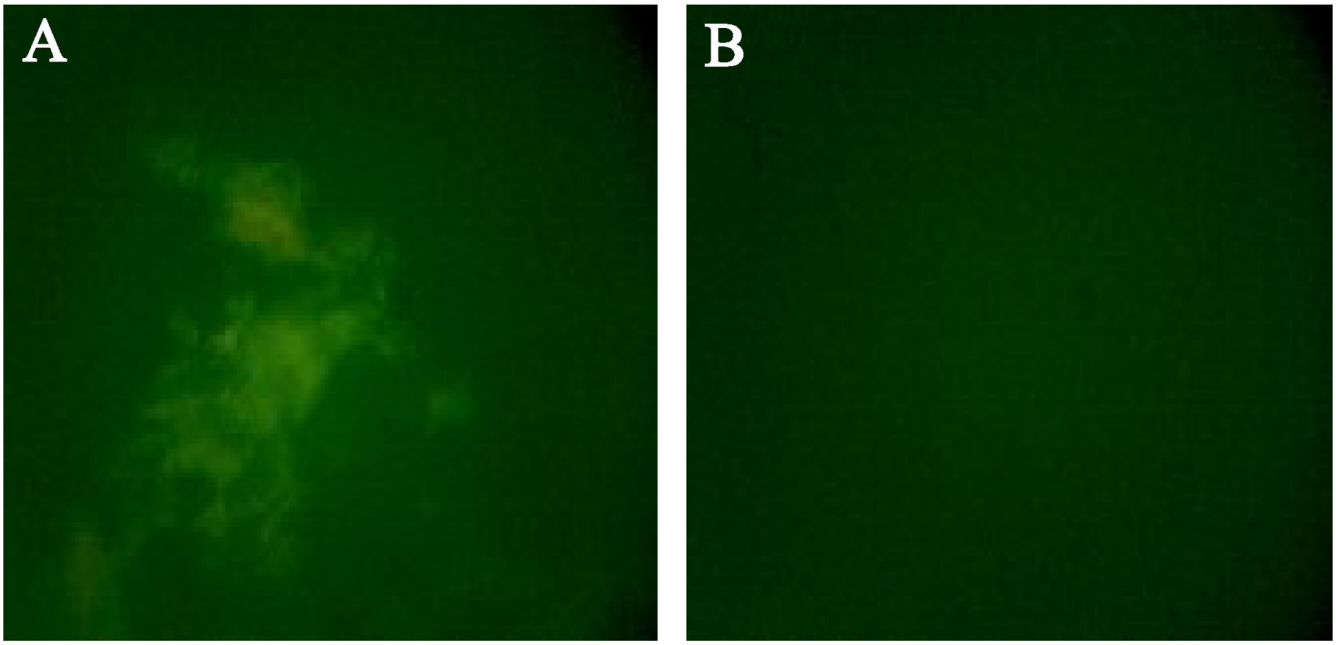

Figure 5.
Fluorescence microscopy images. (a) Precipitates of Aβ aggregates formed in PBS without Nano-N2PY. (b) No such precipitates from PBS containing Nano-N2PY.