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# Cyclodextrins promote protein aggregation posing risks for therapeutic applications

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ABSTRACT

The presence of neurofibrillary tangles (NFTs) is a hallmark feature of various neurodegenerative disorders including Alzheimer's (AD) and Niemann-Pick type C (NPC) diseases. NFTs have been correlated with elevated cholesterol levels and a cholesterol-scavenging compound, cyclodextrin, effectively modulates and traffics cholesterol from cell bodies in NPC disease models. Cyclodextrins are also used as drug carriers to the blood–brain barrier (BBB) and other tissues. While cyclodextrins have potential value in treating brain diseases, it is important to determine how cyclodextrins affect natively unfolded proteins such as beta-amyloid (Aβ) whose aggregation has been correlated with AD. We show that cyclodextrins drastically alter Aβ aggregation kinetics and induce morphological changes to Aβ that can enhance toxicity towards SH-SY5Y human neuroblastoma cells. These results suggest that care must be taken when using cyclodextrins for BBB delivery or for treatment of brain disease because cyclodextrins can promote toxic aggregation of Aβ.



Delivery of therapeutic agents to the brain is a significant problem for treating a wide variety of brain diseases due to the difficulty in getting the agents across the tightly regulated blood–brain barrier (BBB). The endothelial cells of the BBB form a tight junction that prevents many toxic chemicals from entering the brain [1–3]. To facilitate delivery of therapeutics to the brain, compounds such as cyclodextrins that can increase membrane permeability have been utilized as delivery vehicles [3–6]. Cyclodextrins are cyclic oligosaccharides that contain a lipophilic core and hydrophilic outer surface. The solubility of hydrophobic drugs can also be enhanced by complexing the drug with the lipophilic core of cyclodextrins [7,8]. A cyclodextrin/drug complex increases both the drug bioavailability and also improves drug absorption across the membrane by extracting cholesterol, phospholipids and proteins to increase membrane permeability [4,5,7,8].

In addition to use as drug delivery vehicle, cyclodextrins have also been studied as potential therapeutics for various diseases such as Niemann-Pick type C (NPC) disease and Gaucher disease [9,10]. NPC disease is a form of progressive neurodegenerative disorder characterized by the intracellular accumulation of cholesterol due to the lack of functional NPC1 protein in the cells [9], while Gaucher disease is a lysosomal storage disorder affecting the liver, spleen bone marrow and brain [10]. Cyclodextrins can sequester lipids in the hydrophobic core of their cup-like structure, enabling them to bind and traffic cholesterol away from cell bodies [9,11]. Because of this capability, a derivative of β-cyclodextrin known as 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) has recently been approved by the food and drug administration (FDA) for the treatment of NPC disease [12]. Treatment with HP-β-CD was shown to be effective in delaying neurological symptoms of NPC [2] and administration of HP-β-CD successfully transported cholesterol away from cell bodies animal model studies [11,13]. Promising results from the long-term clinical trials paved the way for the FDA's approval of the compassionate use of cyclodextrins in treating NPC disease [14].

Methyl-β-cyclodextrin (M-β-CD), another derivative of β-cylodextrin, was shown to lower cholesterol levels in transgenic α-synucleinopathy mice and in cells overexpressing α-synuclein (αS), resulting in a decrease in oligomeric αS accumulation *in vivo*, which was concluded to reduce the risk of Parkinsonism [15]. Interestingly, an elevated level of cholesterol is also a risk factor for Alzheimer's disease (AD), and regulation of cholesterol levels in AD cell model using M-β-CD decreased amyloid precursor protein processing, essentially inhibited beta-amyloid (Aβ) formation, thereby suggesting a link between cholesterol metabolism and Aβ production [16].

AD affects 4.5 million Americans and over 26 million worldwide [17] and is characterized by the extracellular deposition of amyloid plaques and intracellular accumulation of neurofibrillary tangles (NFTs) in the brain [18]. NFTs are pathological aggregates of hyperphosphorylated tau protein [19] and characteristics of tauopathies

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observed in AD, NPC disease [20], progressive supranuclear palsy (PSP) [21] and dementia with Lewy bodies (DLB) [22]. Many studies have focused on the role of Aβ in the pathogenesis and progression of AD [23,24]. While initial *in vitro* and *in vivo* studies indicated that fibrillar Aβ could cause neuronal cell death either directly or by triggering apoptosis [25,26], recent studies suggest that smaller soluble oligomeric forms of Aβ are the relevant cytotoxic species [26–28]. A variety of anti-Aβ therapeutic agents have been studied as a therapeutic approach for treating AD including polyphenols [29] and other small molecules [30].

Natively unfolded proteins such as Aβ contain hydrophobic regions that may interact with the hydrophobic core of cyclodextrins, facilitating accumulation and aggregation of these proteins. Since cyclodextrins have been approved by the FDA for treatment of NPC disease, and can lower cholesterol levels which are beneficial for treating numerous neurodegenerative diseases including AD and PD, it is important to determine whether cyclodextrins also affect aggregation of neuronal proteins such as Aβ. Here we show that aggregation and cytotoxicity of Aβ are altered by several cyclodextrin derivatives that have been previously studied for NPC disease treatment [2,9] and for BBB transport applications [8]: α-, β-, and 2-hydroxypropyl-β-cyclodextrin (α-CD, β-CD and HP-β-CD, respectively).

## Materials and methods

*Aβ peptides.* Aβ40 was synthesized in the Proteomics and Protein Chemistry Lab at Arizona State University. Lyophilized Aβ was dissolved in DI water at stock concentrations of 5 mg/mL and stored at −20 °C until use.

*Cyclodextrin.* α-, β-, and 2-hydroxypropyl-β-cyclodextrins (α-CD, β-CD and HP-β-CD, respectively) were purchased from Sigma–Aldrich (Sigma–Aldrich, MO) and stocks were prepared in 1× PBS buffer (10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, 140 mM NaCl, and 2.7 mM KCl, pH 7.4) and stored at room temperature until use.

*In vitro Aβ aggregation.* Aβ stock was sonicated for 15 min before use and was diluted to a concentration of 50 μM in 1× PBS (pH 7.4). Cyclodextrins were dissolved in 1× PBS to concentrations of 20 mM and 200 mM, respectively. Because of the poor solubility of β-cyclodextrin in aqueous solution, only the lower β-CD concentration (20 mM) was used. The final concentration of Aβ was 25 μM and of each cyclodextrins was either 10 mM or 100 mM. Next, an equivolume of each cyclodextrin was added to a 50 μM Aβ sample and incubated at 37 °C without shaking for 21 days. A control sample containing Aβ with an equal volume of 1× PBS was carried out in parallel. Aliquots were removed at selected time points throughout the 21-day incubation period for analysis.

*Thioflavin T (ThT) fluorescence.* Aggregation of Aβ with or without cyclodextrin was monitored using ThT fluorescence intensity at various time points throughout the 21-day incubation period as previously described [31]. The maximum fluorescence intensity value obtained with the Aβ alone sample was set to 100 (A.U.) and subsequent fluorescence intensities of all other samples were normalized to this value.

*Atomic force microscopy.* The morphology of Aβ at different incubation periods over a 21-day time course was evaluated using a Nanoscope IIIa AFM (Veeco, Santa Barbara, CA). A 5 μL aliquot from each incubated sample was deposited on a piece of freshly cleaved mica for 5 min, rinsed with DI water, dried with $N_2$ gas and imaged with AFM. OTESPA AFM tips (Veeco, Santa Barbara, CA) were used for AFM imaging using a scan rate of 2.5 Hz and 512 pixels resolution. Images were subjected to 2nd or 3rd order polynomial flattening as needed to reduce the effects of bowing and tilt. Representative images were selected for comparative studies.

*Cell culture.* SH-SY5Y human neuroblastoma cells were maintained and grown as previously described [31].

*Cytotoxicity assay.* The cytotoxicity of each sample towards SH-SY5Y cells was measured using a lactate dehydrogenase (LDH) assay as described elsewhere [32]. Reduction of p-iodonitrotetrazolium by LDH enzyme was measured at 490 nm and 650 nm using a Wallac 1420 plate reader (Perkin Elmer, USA). Cytotoxicity of each sample was calculated by subtracting $A_{490}$ from $A_{650}$, normalized to the control (Aβ alone) and expressed as a percentage of the control value. Experiments were repeated in triplicate.

*Cell viability assay.* Cell viability was determined using a resazurin reduction assay [33,34]. Following treatment for 48 h, culture media was removed and replaced with 1× PBS and resaruzin (20 μM final concentration) was added to each well and incubated at 37 °C for 2 h. Fluorescence of resofurin was measured using a Gen5 BioTek plate reader at excitation and emission wavelengths of 530 nm and 560 nm, respectively. Cell viability of each sample was normalized to that of the PBS control. Experiments were repeated in triplicate.

*Data and statistical analysis.* Data was presented as mean ± SE from at least three independent experiments unless otherwise specified. Cytotoxicity and cell viability data were fitted to a linear-log logistics dose-response curve [35] to determine $LD_{50}$ values for each sample. Statistical analysis was performed using a two-tail Student's *t*-test with the PBS treated samples being the control. A *p*-value of $<0.05$ was considered significant.

## Results and discussion

### *Cyclodextrin effects on Aβ aggregation*

We monitored how the different cyclodextrins influence fibrillar Aβ aggregation using ThT fluorescence (Fig. 1). In the absence of cyclodextrins, Aβ aggregation follows a classical nucleation polymerization mechanism with three distinct phases: nucleation (0–7 days), elongation (7–10 days) and a plateau phase where fibril formation reaches steady state (>10 days). Co-incubation with 10 mM of either β-CD or HP-β-CD accelerates Aβ fibril formation, reducing the time to reach the maximum ThT fluorescence value from 10 to 5 days. Unlike the Aβ alone sample, the ThT fluorescence value of Aβ co-incubated with cyclodextrins decreases after reaching a maximum value, indicating a decrease in fibrillar β-sheet contents (Fig. 1). Co-incubation of Aβ with 100 mM α-CD completely inhibits Aβ fibril formation, as determined by ThT fluorescence (Fig. 1). The cyclodextrins alone did not alter the ThT fluorescence signal.

ThT results suggest that Aβ aggregation is differentially affected by cyclodextrins depending on characteristics which may include cavity size, concentration, surface charge, hydration forces and hydrophobicity of the cyclodextrins. The 7-glycopyranose unit β-CDs (β-CD and HP-β-CD) promote Aβ fibrillogenesis at the lower concentrations studied but inhibit fibrillogenesis at the higher concentrations. The smaller 6-glycopyranose unit α-CD decreases Aβ fibrillogenesis at the low concentration and completely inhibits it at the higher concentrations compared to Aβ alone. Previous studies indicate that polyols and sugars can stabilize protein structures by forming hydrogen bonds with water molecules and preferentially allowing water to be excluded from the hydrophobic surfaces [36]. Since Aβ is natively unfolded, the cyclodextrins studied here may facilitate association of hydrophobic surfaces between different Aβ proteins promoting aggregation to oligomeric and fibrillar aggregates. Since the aggregation state of Aβ determines its cytotoxicity [25,27], we studied how different cyclodextrins alter Aβ morphology by AFM.

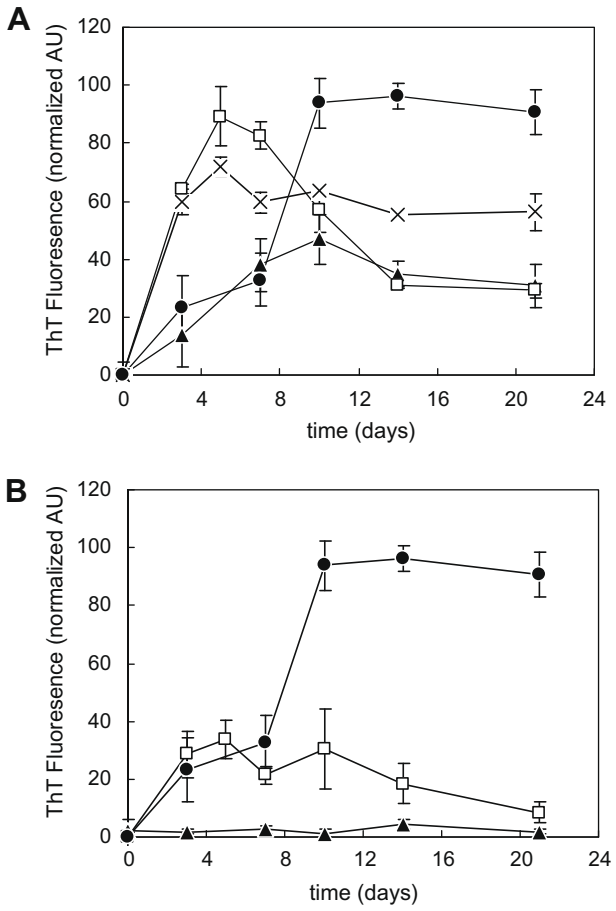


**Fig. 1.** Effects of cyclodextrins on Aβ aggregation. Aβ aggregation with 10 mM (A) or 100 mM (B) of each cyclodextrin was monitored using ThT fluorescence assay. Aβ alone (●), Aβ + α-CD (▲), Aβ + β-CD (×) and Aβ + HP-β-CD (□). The observed fluorescence was normalized Aβ alone sample at its maximum value. Data are presented as mean ± SE from four sets of experiments.

### *Cyclodextrins induce morphological changes in Aβ*

We assessed the effects of the different cyclodextrins on Aβ morphology by AFM (Fig. 2). Since the cyclodextrins alter the rate of Aβ aggregation, we compared morphologies of Aβ aliquots based on respective maximum ThT values rather than corresponding time points. The AFM images show that co-incubation of Aβ with different cyclodextrins induces substantial morphological changes in Aβ structure compared to the Aβ alone sample (Fig. 2A). For example, co-incubation of Aβ with α-CD promotes formation of large oligomeric Aβ species (Fig. 2B and C), β-CD promotes formation of short, thick Aβ fibrils and HP-β-CD promotes formation of fibrils similar to those generated by Aβ alone though substantially shorter (Fig. 2D–F). We also studied how Aβ morphology changes throughout the 21-day time course with and without co-incubation with cyclodextrins. Time course AFM images of Aβ alone and Aβ + 10 mM β-CD are shown in Fig. 3 and all others are shown in the Supplementary material. When incubated alone, Aβ fibrillogenesis followed a typical seeded-nucleation aggregation kinetics, proceeding from oligomers to protofibrils and fibrils, as indicated by ThT fluorescence and AFM image (Fig. 1B and A). Co-incubation with 10 mM β-CD showed considerable oligomeric formation in 3 days and extensive fibril formation after just 5 days. The fibrils remained through the 21-day incubation period (Fig. 3B). The presence of Aβ fibrils did not correspond with the ThT value (Fig. 1B), suggesting that either the fibrils are not typical β-sheet stacked structures or that the ThT fluorescence staining of the fibrils is shielded by the HP-β-CD. In either case, cyclodextrins clearly alter Aβ aggregation, promoting formation of small spherical oligomers, protofibrils, short rigid and short intertwining fibrils. Since Aβ morphology greatly influences its cytotoxic characteristics, we studied how the different Aβ aliquots affect cell viability.

### *Co-incubation of Aβ with cyclodextrins affects cell viability*

The connection between the aggregation state of Aβ and cytotoxicity has been widely studied [25,27]. Increasing evidence suggests that small soluble oligomeric Aβ species are the relevant neurotoxic species in AD [27,28]. Therefore, we determined the toxicity of the various Aβ-CD induced aggregates using the neuroblastoma cell line, SH-SY5Y. The baseline toxicity of each cyclodextrin on SH-SY5Y cells was determined and β-CD was the most toxic followed by α-CD and HP-β-CD with $LD_{50}$ values of 2 mM, 11 mM and 147 mM, respectively in a cell culture density of $10^5$ cells in a 24-well plate (data not shown). $LD_{50}$ values are all well above the concentrations used in Aβ toxicity studies, indicating the cyclodextrins alone do not induce toxicity. When aggregated without added cyclodextrins, Aβ induced a significant decrease in cell viability after aggregation for 3 and 5 days, respectively, but was not toxic to cells at earlier and later aggregation time points (Fig. 4), consistent with other studies indicating toxicity of oligomeric but not monomeric or fibrillar Aβ [27,28]. When incubated with 10 mM α-CD, Aβ induced significant toxicity towards SH-SY5Y cells at 10 days and longer time points (Fig. 4A). At the higher concentration of α-CD (100 mM), Aβ-induced toxicity was observed at earlier time points (Fig. 4B). No toxicity was observed at the studied time points when Aβ was co-incubated with 10 mM HP-β-CD, although a significant decrease in cell viability was observed when Aβ was co-incubated with 100 mM HP-β-CD for 5 days or longer (Fig. 4B). Co-incubation of Aβ with 10 mM β-CD did not affect cell viability at the low concentration for the time points studied here (Fig. 4A), and the higher concentration studies could not be performed due to the poor solubility of β-CD in aqueous solutions.

While cyclodextrins hold great therapeutic promise as drug delivery vehicle and for regulating cholesterol levels, their lipophilic core can alter aggregation of natively unfolded proteins such as Aβ. Since the aggregation states of Aβ is crucial for cytotoxicity, it is critically important to determine how cyclodextrins affects Aβ protein aggregation. Here we show that different cyclodextrins influence Aβ aggregation and promote formation of oligomeric and fibrillar species. Based on AFM and ThT studies, α-CD promotes formation of oligomeric Aβ species, which is reflected in the increased cytotoxicity observed with the α-CD co-incubated samples. The two different β-CD variants both promote formation of short fibrillar Aβ aggregates at low concentration (10 mM), however at the higher concentration (100 mM) HP-β-CD also stabilizes oligomeric Aβ aggregates which are toxic to SH-SY5Y cells. While formation of fibrillar aggregates has been suggested to be a protective mechanism that can sequester toxic oligomeric species [37], other studies have shown that promoting fibril formation may also increase cytotoxicity [38].

Formation of NFTs has been linked to high cholesterol levels in cells, particularly in lipid rafts on cell membranes. Lipid rafts, comprised mainly of cholesterol and sphingolipids can also promote pathogenesis of AD by serving as a deposition site for the accumulation and aggregation of Aβ [16]. Since natively unfolded neuronal proteins such as Aβ and αS are known to associate with lipid rafts [15,16], and cyclodextrins can interact with cholesterol on lipid rafts, the local concentrations of cyclodextrins and neuronal proteins on the lipid rafts can be much higher than elsewhere. While the ability of cyclodextrins to scavenge and traffic cholesterol away from cells shows great therapeutic promise prompting the FDA to

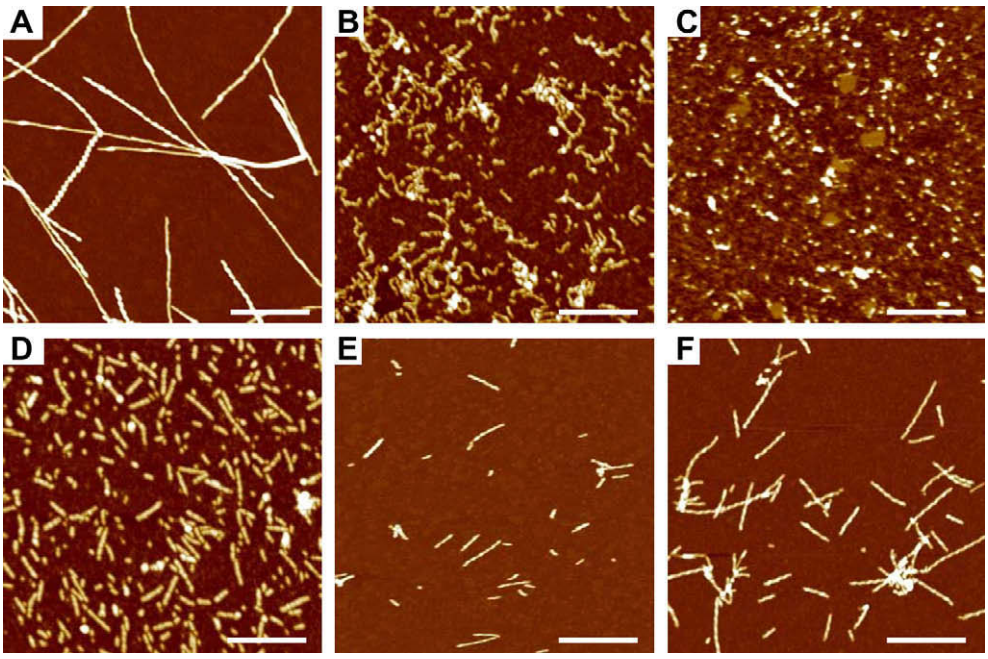


**Fig. 2.** AFM images of Aβ samples with and without co-incubation with cyclodextrins at maximum ThT fluorescence. Aβ alone (A), Aβ + 10 mM α-CD (B), Aβ + 100 mM α-CD (C), Aβ + 10 mM β-CD (D), Aβ + 10 mM HP-β-CD (E) and Aβ + 100 mM HP-β-CD (F). AFM imaging was carried out in air using TMAFM. Scale bar = 500 nm.

approve the use of HP-β-CD as a treatment for NPC disease in children [12], the potential effects of cyclodextrins on aggregation of natively unfolded proteins such as Aβ, tau and α-synuclein and the subsequent long-term effects on cell toxicity are not known. Here we show that cyclodextrins interact with Aβ and significantly alter the aggregation kinetics, morphology, and toxicity of Aβ towards neuronal cells. While cyclodextrins have value as treatments for NPC disease and Gaucher disease, and as delivery vehicles to transport drugs to tumors [6] or to cross the BBB [2,8], the concentration of the cyclodextrins utilized, clearance rates from the brain

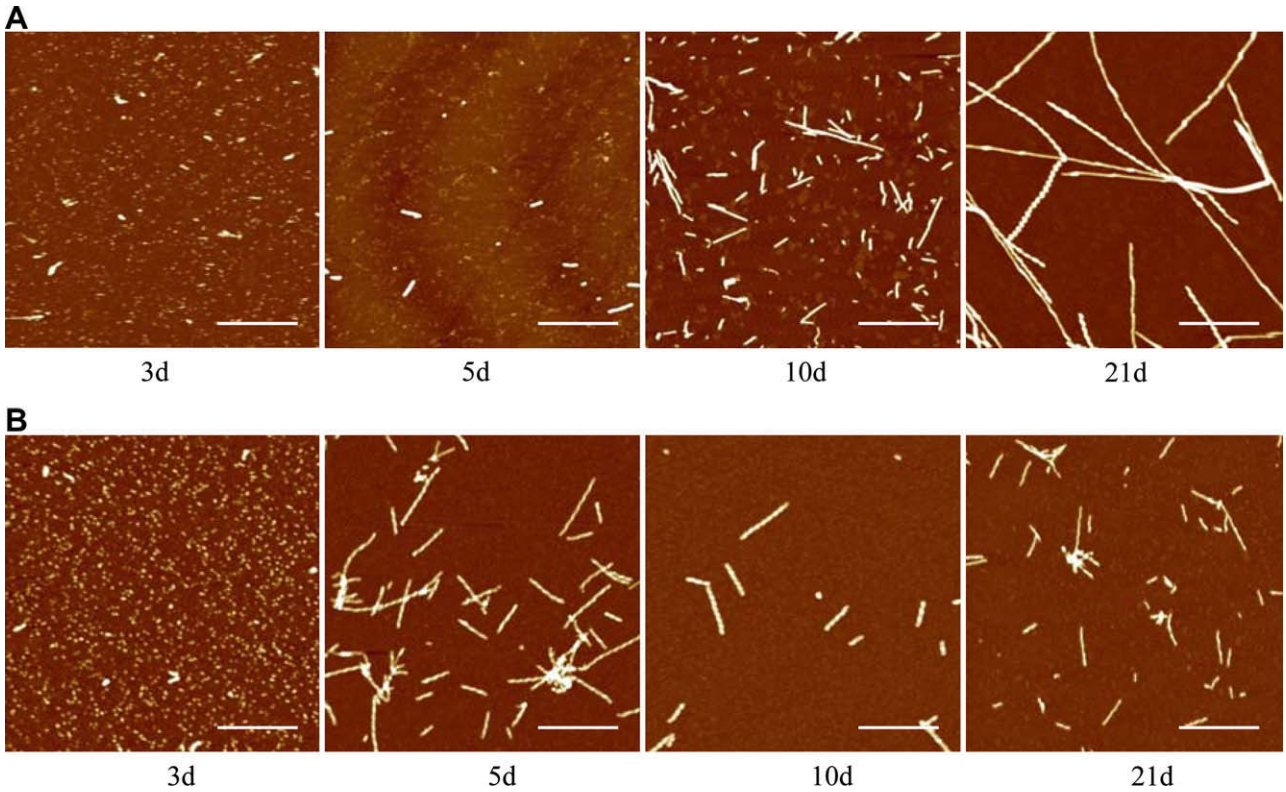


**Fig. 3.** Morphological changes of Aβ with time as monitored by AFM. Aβ alone (A) and Aβ + 10 mM β-CD (B). AFM imaging was carried out in air using TMAFM. Scale bar = 500 nm.

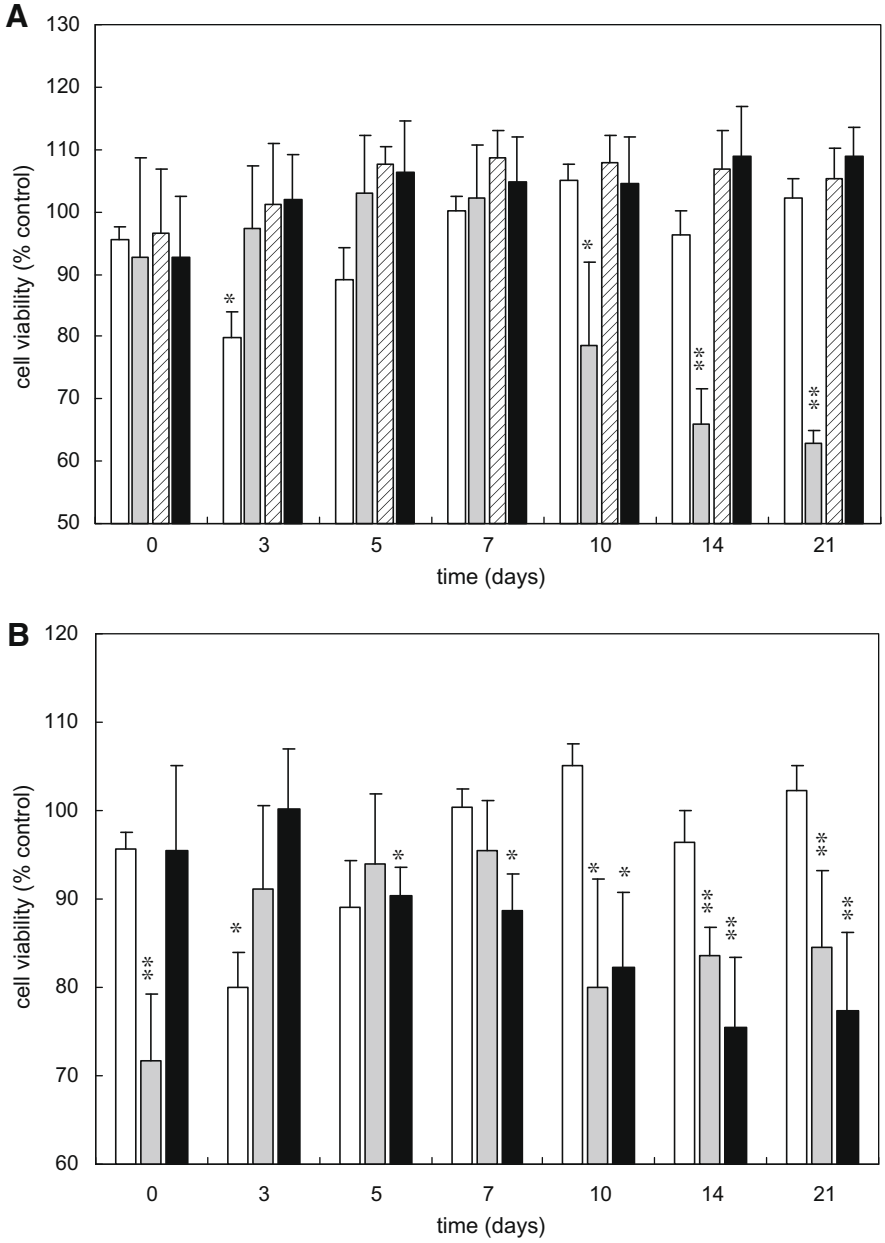


**Fig. 4.** Co-incubation of Aβ with cyclodextrin affects viability of SH-SY5Y cells. Aβ was pre-incubated with 10 mM cyclodextrin (A) or 100 mM cyclodextrin (B) at 37 °C for 21 days. Aβ co-incubated with Aβ alone (□), Aβ + α-CD (▩), Aβ + β-CD (▨) and Aβ + HP-β-CD (■). The pre-incubated Aβ samples at each time point from each set were added to SH-SY5Y cells and incubated at 37 °C for 48 h. Cell viability was measured using a resazurin reduction assay and normalized to PBS control. Data were presented as mean ± SE. $^{*}p < 0.05$; $^{**}p < 0.01$ compared with the control.

and local concentration effects especially around lipid rafts should be carefully studied to ensure the long-term safety of cyclodextrins.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.077.

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