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Ac-LVFFARK-NH_2 conjugation to β-cyclodextrin exhibits significantly enhanced performance on inhibiting amyloid β-protein fibrillogenesis and cytotoxicity

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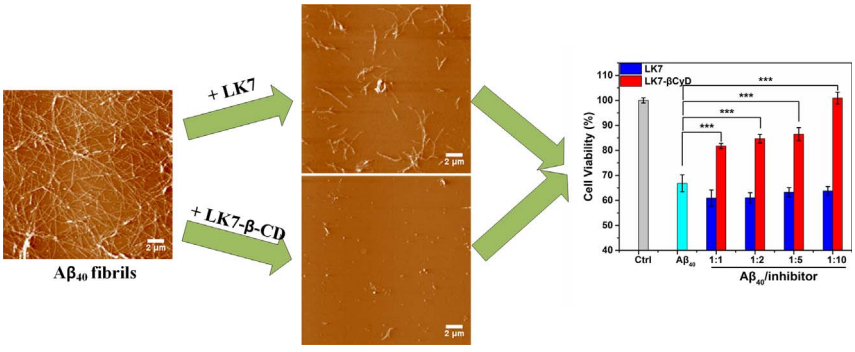
HIGHLIGHTS

- β-Cyclodextrin (βCyD) conjugation markedly changed the conformation of LVFFARK (LK7).
- βCyD conjugation increased the hydrophobic interactions between LK7 and β-amyloid (Aβ).
- βCyD conjugation distinctly improved the inhibition effect of LK7 on Aβ fibrillation.
- LK7-βCyD significantly reduced the cytotoxicity of Aβ aggregates.

GRAPHICAL ABSTRACT



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ABSTRACT

Inhibition of amyloid β-protein (Aβ) aggregation is of significance for the potential treatment of Alzheimer's disease. We have herein conjugated heptapeptide Ac-LVFFARK-NH_2 (LK7) to β-cyclodextrin (βCyD) and studied the inhibitory effect of the LK7-βCyD conjugate on Aβ aggregation. The conjugation significantly improved the peptide solubility and suppressed the self-assembly propensity. This led to 30% increase of the binding affinity of LK7 for Aβ in the conjugate due to increased hydrophobic interactions. Thus, LK7-βCyD suppressed the conformational transition of Aβ and showed stronger inhibitory effect on Aβ fibrillation than LK7. Thus, LK7-βCyD exhibited protective effect on $Aβ_{40}$-induced cytotoxicity, and the cells completely survived at 10 molar excess of LK7-βCyD (from 67% to 100%). By contrast, LK7 showed only a moderate inhibition on Aβ fibrillation, and could not inhibit the amyloid cytotoxicity. The research proved that conjugation of hydrophobic peptide to βCyD was promising to increase its inhibition potency against Aβ aggregation.

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1. Introduction

Alzheimer's disease (AD) is one of the most populated chronic neurodegenerative disorders, which has caused worldwide health problem and huge economic loss in the word [1–3]. It has been recognized that senile plaques in the brain parenchyma are the hallmark of AD [4]. Moreover, the accumulation of amyloid β-protein (Aβ) is responsible for senile plaques, which disrupts membrane functions and causes primary neuronal dysfunction [4]. Many studies have proved that soluble Aβ oligomers [5] and protofibrils [6] are the most toxic species, responsible for neuronal dysfunction and death. Aβ is produced by the sequential proteolytic cleavages of the transmembrane amyloid precursor protein (APP) by β- and γ-secretases [7,8]. Thus, decreasing Aβ production, preventing Aβ aggregation, neutralizing or removing cytotoxic species are suggested for the treatment of AD [9]. By considering the essential physiological functions of the secretases and APP, however, inhibition of Aβ aggregation is considered as a promising strategy for the treatment or delaying the onset of AD [10].

Recently, many inhibitors, such as small molecules [11–13], antibodies [14–16], peptides and peptide mimetics [17–19], proteins [20] and nanoparticles [21,22], have been studied to prevent Aβ aggregation and reduce its associated cytotoxicity. Among them, peptide inhibitors have been acquired extensive interesting due to their easy synthesis and broad bioavailability [23,24]. Previous experiments have demonstrated that Aβ fragments, such as $Aβ_{17–21}$ [25,26], $Aβ_{30–34}$ [27], $Aβ_{32–37}$ [28,29], $Aβ_{38–42}$ [30], display a protective effect against Aβ-mediated neurotoxicity. Based on the sequence of $Aβ_{16–21}$, a heptapeptide (Ac-LVFFARK-NH_2, LK7) [19] was designed. Biochemical and biophysical experiments demonstrated that LK7 was more effective in inhibiting Aβ fibrillogenesis than other peptide analogous derived from the same hydrophobic core ($Aβ_{16–21}$). However, the shortcomings of the peptide, such as poor solubility, propensity to self-assembly and the strong cytotoxicity of its aggregates, limited its utilization. In order to improve the anti-aggregation effects of peptide-based inhibitors or to obtain additional functions on the therapy of AD, nanoparticles [19,31] and dendritic macromolecules [32] have been utilized to modify peptides. However, most of the nanoparticle inhibitors are too large to traverse the blood–brain barrier to arrive the focus location.

Cyclodextrins (CyDs), cyclic oligosaccharide compounds, have been used successfully in the treatment of various types of neurodegenerative disorders to gain higher therapeutic effect with lower dosage and to reduce side effects [33]. Numerous researches demonstrated that CyDs were useful in improving the effectiveness of drugs, mainly to the compounds of poor solubility [34]. More importantly, as a functional host, CyDs were proved to be nontoxic and to possess high water solubility [33]. All these features make them suitable for functional modifications. Previously, βCyD was attempted to reduce Aβ fibrillogenesis [35], but the binding affinity between βCyD and Aβ was too weak to make it an effective inhibitor.

Herein, we synthesized LK7-βCyD conjugate by coupling the C-terminal of LK7 to βCyD. It is expected the conjugation could improve its solubility and enhance its inhibitory efficiency against Aβ aggregation. The physiochemical properties and inhibitory effects of LK7-βCyD on Aβ fibrillation were investigated by multiple approaches, including thioflavin T (ThT) fluorescence assay, atomic force microscopy (AFM), circular dichroism (CD) spectroscopy and cytotoxicity assay. Isothermal titration calorimetry (ITC) was also conducted to explore the molecular interactions between the inhibitor and Aβ.

2. Materials and methods

2.1. Chemicals and reagents

1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), ThT, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Human neuroblastoma SH-SY5Y cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12 (DMEM/F12) and fetal bovine serum (FBS) were purchased from Gibco Invitrogen (Grand Island, NY, USA). All other chemicals were the highest purity available from local sources.

2.2. Synthesis of peptides and LK7-βCyD

$Aβ_{40}$, LK7 and LK7-βCyD (all > 95% in purity) were obtained from GL Biochem (Shanghai, China). The peptide synthesis was following the Fmoc (9-fluorenylmethoxycarbonyl) chemistry by solid phase synthesis method carried out in the reverse order (from C-terminal to N-terminal). The cleavage of resin-bound peptide unit from the resin bead was done in nitrogen atmosphere using a cocktail mixture of trifluoroacetic acid (TFA)/water/triisopropylsilane/phenol (95/3/1/1).

As for LK7-βCyD synthesis, the peptide part was synthesized as described above, while its conjugation to βCyD was done as follows. Details include the acetylation of the amino group of leucine (L) and coupling of 6-amino-6-deoxy-β-CyD (NH_2-βCyD) to the carboxyl group of lysine (K) by incubating with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (Pybop) and *N*,*N*-diisopropylethylamine (DIEA) for 2 h (Scheme 1). Then, the crude product was precipitated from the solution by addition of citric acid. The precipitate was collected by filtration, and the Fmoc groups on the side chains of arginine (R) and lysine (K) were removed by reaction with 86% TFA. Finally, the reaction solution was diluted with diethyl ether to obtain a precipitate of LK7-βCyD, which was then collected by filtration and purified. The purity of LK7-βCyD was confirmed by reverse phase HPLC using a Venusil MP C18–5 column (250 × 4.6 mm I.D.) by linear gradient elution with aqueous acetonitrile solution containing 0.1% TFA. Finally, the product was identified by mass spectrometry (MS) in combination with nuclear magnetic resonance spectroscopy (^{1}H NMR).

2.3. $Aβ_{40}$ preparation

$Aβ_{40}$ monomer solutions were prepared as reported previously [36,37]. In brief, fresh produced $Aβ_{40}$ powder was first dissolved in HFIP at concentrations of 1.0 mg/mL. The peptide solution was in quiescence at room temperature for 2 h, followed by sonication for 30 min to monomerize the existing oligomers and then centrifugation at 15000 ×*g* to remove the existing Aβ aggregates. Thereafter, the peptide solution was freeze-dried overnight in a lyophilizer (Labconco, America). Before the experiment, the obtained white cotton-shaped sample was stored at −20 °C.

$Aβ_{40}$ stock solutions (275 μM) were prepared by dissolving of lyophilized peptide in 20 mM NaOH, and sonicating in a cold-water bath for 15 min. Then the stock solution was diluted in phosphate buffer (100 mM sodium phosphate, 10 mM NaCl, pH 7.4) to a final concentration of 25 μM. LK7-β-CyD was dissolved in PBS and mixed with $Aβ_{40}$ at different molar ratios (1:1, 2:1, 5:1 and 10:1). For comparison, the $Aβ_{40}$ samples in the presence of LK7 were also prepared. Finally, all the samples were incubated at 37 °C by continuous shaking at 150 rpm for 48 h.

2.4. ThT fluorescence assay

Each $Aβ_{40}$ sample was incubated (37 °C, 150 rpm) with LK7/LK7-βCyD in different molar ratios (1:1, 1:2, 1:5 and 1:10) for 48 h. ThT fluorescence intensity was examined by mixing 200 μL aliquot from each test sample with 2 mL of ThT buffer (25 μM ThT in PBS). ThT fluorescence assay was performed using a fluorescence spectrometer (Perking Elmer LS-55, MA, USA) at excitation and emission wavelengths of 440 and 480 nm, respectively. Inhibition experiments were also done with LK7 and βCyD at an equimolar condition. The fluorescence intensity of the solution without $Aβ_{40}$ was subtracted as