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Kinetic Studies of Inhibition of the A β (1–42) Aggregation Using a Ferrocene-tagged β -Sheet Breaker Peptide

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Abstract

The aggregation of amyloidogenic proteins/peptides has been closely linked to the neuropathology of several important neurological disorders. In Alzheimer's disease (AD), amyloid beta (A β) peptides and their aggregation are believed to be at least partially responsible for the etiology of AD. The aggregate-inflicted cellular toxicity can be inhibited by short peptides whose sequence are homologous to segments of the A β (1–42) peptide responsible for β -sheet stacking (referred to as the β -sheet breaker peptides). Herein a water-soluble ferrocene (Fc)-tagged β -sheet breaker peptide (Fc-KLVFFK₆) is used as an electrochemical probe for kinetic studies of the inhibition of the A β (1–42) fibrillation process and for determination of the optimal concentration of β -sheet breaker peptide for efficient inhibition. Our results demonstrated that Fc-KLVFFK₆ interacts with the A β aggregates instantaneously in solution, and sub-stoichiometric amount of Fc-KLVFFK₆ is sufficient to inhibit the formation of the A β oligomers and fibrils and to reduce the toxicity of A β (1–42). The interaction between Fc-KLVFFK₆ and A β (1–42) follows a pseudo-first-order reaction, with a rate constant of $1.89 \pm 0.05 \times 10^{-4} \text{ s}^{-1}$. Tagging β -sheet breaker peptides with a redox label facilitates design, screening, and rational use of peptidic inhibitors for impeding/altering A β aggregation.

Keywords

amyloid beta; aggregation; inhibition kinetics; ferrocene tag; β -sheet breaker; cytotoxicity

Introduction

The pathology of several neurological disorders has been attributed to one or several of the following processes: misfolding of amyloidogenic peptides/proteins, staking of the β -sheet-rich oligomers, further aggregation of oligomers into larger and higher-ordered aggregates, and the ultimate deposition of filamentous aggregates onto neuronal cells. For Parkinson's

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disease and prion diseases, the culprit proteins are believed to be the α -synuclein protein and the prion protein, respectively [1-4]. Alzheimer's disease, on the other hand, is manifested by aggregated amyloid beta ($A\beta$) peptides in senile plaques and the intracellular aggregates of the τ protein [5, 6]. The $A\beta$ peptides include variants containing 39–43 amino acid residues, with $A\beta(1-42)$ as the major amyloidogenic component. In general, the aggregation pathway of these proteins/peptides is believed to proceed with the transformation of monomers into β -sheet-containing intermediates or nucleating units, which is followed by stacking of these units to form higher-ordered aggregates (e.g., protofibrils and fibrils) [7-9]. Addition of monomers is presumed to be responsible for the elongation and smoothening of the surface of the fibrils [9-11]. By monitoring the aggregation of fluorophore-labelled $A\beta(17-22)$, Lynn and co-workers recently showed that fibril-like amyloid assemblies could emerge from early, micrometer-sized aggregates [12]. The observation suggests that the early $A\beta$ aggregates might be viable targets for therapeutic intervention.

Peptidic inhibitors (also referred to as the β -sheet breaker peptides [13]) are a class of compounds known to be highly potent in ameliorating $A\beta(1-42)$ - or α -synuclein-inflicted cell toxicity [14-22]. Typically the sequences of such peptides are homologous to parts of the hydrophobic segments of the amyloidogenic molecule responsible for the β -sheet formation. To inhibit the $A\beta$ aggregation process, Tjernberg et al. designed an inhibitor of the KLVFF sequence (residues 16-20 of $A\beta(1-42)$) [23] and Soto and co-workers have shown that the LPFFD peptide is highly effective in inhibiting $A\beta$ amyloidogenesis [24]. Murphy and co-workers found that linking a lysine hexamer to the C-terminus of KLVFF improves both the solubility and inhibitory efficacy [25]. In a later work, they investigated the interaction between KLVFFK₆ and $A\beta(1-40)$ and posited that the mode of inhibition was an accelerated assembly of filamentous $A\beta$ intermediates into short fibrils [26]. As a result, the amount of the more neurotoxic $A\beta$ oligomers is greatly reduced in solution.

Despite an extensive effort in developing peptidic inhibitors and analogs, in many cases it is not clear how a given peptidic inhibitor interacts the various protein/peptide forms (e.g., monomers, oligomers, large filaments, and amorphous aggregates) to reduce the $A\beta$ -elicited cytotoxicity [23-26]. These uncertainties are partly caused by a lack of sensitive methods for real-time monitoring of the entire inhibition process. Atomic force microscopy (AFM) and electron microscopy are typically used to image aggregates formed after a relatively long incubation. Other real-time techniques, such as dynamic light scattering or fluorescence spectrometry of β -sheet-intercalating dye molecules (e.g., Thioflavin T) are also more suitable to studies of similarly late events wherein aggregates of certain sizes (a few nanometers or greater) and shapes (e.g., rod-like protofibrils and fibrils) are formed [27, 28]. While circular dichroism (CD) spectroscopy can detect the transition of natively unstructured peptides/proteins into oligomers rich in the β -sheet conformation (the very first step in the aggregation process), the different stages of the aggregation cannot be differentiated. Moreover, some β -sheet breaker peptides themselves are capable of forming β -sheet-containing structures. Consequently, these β -sheet breaker peptides could obscure CD signals given rise by β -sheet formation (or lack of) in the amyloidogenic peptides/proteins. Recently label-free electrochemical methods were explored for studying the aggregation of $A\beta(1-42)$. It was found that the redox activity of the tyrosine-10 (Tyr-10) residue of $A\beta(1-42)$ changes during the $A\beta(1-42)$ aggregation process [29]. However, due to the fact that the Tyr-10-encompassing hydrophilic termini of $A\beta(1-42)$ molecules emanate into solution from the core of the aggregates [30], the difference in the tyrosine redox currents is not significant between unstructured and aggregated $A\beta(1-42)$ species. In other words, the oligomerization and fibrillation (aggregation) processes produce gradually changed Tyr-10 oxidation currents and the electrochemical method consequently cannot differentiate oligomers of different sizes or even oligomers from protofibrils, fibrils or amorphous aggregates. This is in contrast to the commonly used Thioflavin T (ThT) assay in

which the presence of a lag phase is indicative of a nucleation-elongation mechanism [9, 11] and the induced ThT fluorescence signals the formation of fibrils. As for interrogating the inhibition of aggregation by a β -sheet breaker, any uncertainty in recording small current changes can affect the accuracy of the kinetics of the inhibition reaction [31]. Furthermore, β -sheet breakers disrupt A β (1–42) aggregation/fibrillation via interacting with the hydrophobic domain of A β (1–42), which does not significantly block/affect the electrochemical oxidation reaction of Tyr-10, which resides in the hydrophilic domain of A β (1–42). Due to the aforementioned issues, it has been difficult to gauge the effective breaker dosage for inhibiting/disrupting the aggregation/fibrillation. Furthermore, the paucity of knowledge about the kinetics of the inhibitory process makes the investigation on the time-dependent aggregation disruption a largely trial-and-error practice. Typically multiple aliquots of solutions incubated for different times are incrementally sampled and studied. Such a practice is both laborious and sample-consuming.

Tagging various biomolecules and small organic compounds with the ferrocene (Fc) moiety has been widely used for electrochemical studies of a wide range of biomolecular interactions [32–36]. We previously synthesized a ferrocenoyl pentapeptide (Fc-KLVFF) and found it to possess some inhibitory effect on the A β fibril formation [37]. But its solubility in water is rather poor. As a consequence of the poor water solubility, a high Fc-KLVFF concentration in a mixed organic/water solvent had to be used to elicit inhibition of the A β (1–42) aggregation. As a result, kinetic information about the β -sheet breaking and inhibition of aggregation could not be obtained and the optimal dosage for the aggregation inhibition in a physiologically relevant medium could not be determined. It was also not clear whether tagging KLVFF with Fc would compromise the ability of KLVFF in attenuating the cell toxicity of A β (1–42) aggregates. KLVFF is also known to self-aggregate [38, 39] and therefore is not considered as an ideal inhibitor (especially for cytotoxicity or animal model studies). In an attempt to gain insight into the kinetics of the impediment engendered by a β -sheet breaker peptide to the A β (1–42) aggregation, we attached the Fc moiety to the N-terminus of the water-soluble KLVFFK₆ breaker. By monitoring changes in the electrochemical current of the Fc tag, real-time electrochemical monitoring of the inhibition of the A β (1–42) aggregation was accomplished. Inhibition of the A β (1–42) aggregation was found to follow a pseudo-first-order reaction and effective inhibition can be obtained at a breaker concentration that is much smaller than that of A β (1–42). Fc-KLVFFK₆ was found to not self-aggregate, eliminating ambiguities in our previous studies [37]. Remarkably, we found that Fc-KLVFFK₆ completely halts the A β (1–42) fibrillation and its inhibition of the A β (1–42)-inflicted cytotoxicity compares well to that of KLVFFK₆.

Materials and Methods

Materials

A β (1–42) was purchased from American Peptide Co. Inc. (Sunnyvale, CA), and pretreated by the 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) method [40–42]. The treatment of HFIP follows a centrifugation of the solution for 30 min at 13800 rps and the supernatant solution was lyophilized for further sample preparation. Stock solution of A β (1–42) (0.5 mM) was prepared as previously reported [43]. Ferrocene monocarboxylic acid (Fc-COOH) was obtained from Sigma-Aldrich (Milwaukee, WI). Wang resin, Fmoc-protected amino acids, diisopropylcarbodiimide (DIC), pyridine and piperidine were purchased from Anaspec, Inc. (San Jose, CA). SH-SY5Y cell (human neuroblastoma) was obtained from American Type Culture Collection, Inc. (Manassas, VA) and fetal bovine serum (FBS) was from Omega (Tarzana, CA). Both Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 Medium were acquired from Mediatech Inc. (Manassas, VA). The mixture of penicillin and streptomycin for cell culture and cytotoxicity study was purchased from Millipore (Billerica,

MA). The other chemicals were obtained from Thermo-Fisher Scientific Inc. (Pittsburgh, PA).

Peptide Synthesis

Fmoc-K(Boc)LVFFK(Boc)₆ attached to the Wang resin was synthesized through the solid-phase Fmoc chemistry on a Symphony Quartet peptide synthesizer (Protein Technologies, Tucson, AZ). To remove the Fmoc group, the resin was allowed to react with 20% piperidine in 20 mL dimethylformamide (DMF) for 45 min. Filtering the resin and washing it sequentially with DMF, CH₂Cl₂ and CH₃OH produced NH₂-K(Boc)LVFFK(Boc)₆ on the resin. To couple the Fc tag to the peptide, Fc-COOH (0.1 mol) in 20 mL DMF was mixed with DIC, *N*-hydroxybenzotriazole and 4-dimethylaminopyridine (0.1 mole each) for 30 min and the resultant mixture was mixed with the resin covered with NH₂-K(Boc)LVFFK(Boc)₆. The solution was then shaken at room temperature for 6 h. The resin was filtered and washed with DMF and CH₃OH to produce Fc-K(Boc)LVFFK(Boc)₆. Finally, a mixture containing 95% trifluoroacetic acid (TFA) and 5% triisopropylsilane was used to remove the Boc group and to cleave the peptide off the resin. The filtrate was recrystallized with cold ether to yield the crude product of Fc-KLVFFK₆, which was then purified by semi-preparative reversed-phase HPLC (Shimadzu AD, Columbia, MO) using a Jupiter-10-C18-300 column (10 mm i.d. × 250 mm; Phenomenex Inc., Torrance, CA). The eluting solutions were 0.1% TFA in water (mobile phase A) and 0.1% TFA in acetonitrile (mobile phase B). At a flow rate of 4.75 mL/min, an elution gradient of 20–45% phase B lasted for 12 min. The as-purified peptide was characterized by electrospray-mass spectrometry (ES-MS), which showed a single peak at $m/z = 1635.6$ (theoretical $m/z = 1636.9$). Fc-K₆ was synthesized and purified similarly.

Electrochemical Measurements

All electrochemical measurements were carried out with a CHI660B electrochemical workstation (CH Instruments, Austin, TX). The working electrode was a glassy carbon disk with a diameter of 3 mm (Bioanalytical System Inc., West Lafayette, IN). A platinum wire and a Ag/AgCl electrode were used as the auxiliary and the reference electrodes, respectively. Aβ(1–42) and Fc-KLVFFK₆ were dissolved in 100 mM phosphate buffer/50 mM KClO₄ (pH 7.4). The entire experimental setup was lowered into a water bath maintained at 37 °C. For differential pulse voltammetric measurements, the following parameters were used: sample width = 17 ms, pulse amplitude = 50 mV, pulse width = 50 ms, and pulse period = 200 ms.

Size-Exclusion Chromatography

Blue dextran (2000 kD), bovine serum albumin (66 kD), chymotrypsinogen A (25 kD), aprotinin (6.7 kD), and vitamin B12 (1.35 kD) were used to calibrate the retention time of the size exclusion chromatographic columns (GFC 2000 from Phenomenex Inc). Two columns were connected in series and the separation/detection was carried out on a Waters 600 HPLC system (Milford, MA) that is equipped with a photodiode array detector (Model 2996). Phosphate buffer (100 mM, pH 7.4) was utilized as the mobile phase and the flow rate was 0.2 mL/min. Elutions of Aβ species, Fc-KLVFFK₆, and other peptides were monitored at 220 nm. For each assay, a 20-μL aliquot taken from a solution incubated in a 37 °C water bath was injected into the columns.

Atomic Force Microscopic Measurements

Freshly cleaved mica sheets were pretreated with Ni(II) in 10 mM NiCl₂ for 15 min. Prior to imaging, aliquots were taken from incubated solutions containing Aβ(1–42), Fc-KLVFFK₆/Aβ(1–42), KLVFFK₆/Aβ(1–42), or Fc-KLVFFK₆ and cast onto these treated mica sheets.

The mica sheets were then rinsed with water to remove salt residues, and dried with nitrogen prior to imaging. The morphology of the various A β aggregates was characterized with an MFP-3D-SA microscope (Asylum Research, Santa Barbara, CA) using the tapping mode.

Cell Cytotoxicity Assay

SH-SY5Y cells were cultured in a medium of 44.5% DMEM comprising L-glutamine (4 mM), Ham's F12, FBS, and a mixture of penicillin and streptomycin (V:V:V:V = 44.5%:44.5%:10%:1%). The cultured cells were then transferred to a sterile 96-well plate with approximately 20000 cells per well. These cells were allowed to acclimatize overnight in the DMEM/F12 media containing 5% FBS in a humidified incubator under 5% CO₂ at 37 °C. Solutions of Fc-KLVFFK₆, KLVFFK₆, A β (1–42), Fc-KLVFFK₆/A β (1–42) and KLVFFK₆/A β (1–42) were pre-incubated at 37 °C for 24 h and were allowed to react with the SH-SY5Y cells for 24 h. Viability of cells exposed to each solution was determined based on the 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (EMD Inc., Gibbstown, NJ) assay, as described by others and our previous work [44, 45].

Results and Discussion

KLVFF-containing short peptides have been demonstrated to be effective in inhibiting A β aggregation through hydrophobic interaction and salt bridge formation with residues 18 – 23 of A β (1–42), though truncated or shortened fibrils were observed as some of the end products [25]. As reported by Murphy and co-workers, attachment of a hexalysine segment to the C-terminus of KLVFF not only increases the breaker's solubility, but also reduces cell toxicity of the A β aggregates [46]. We envision that, when Fc is attached to the water-soluble KLVFFK₆ peptide and the resultant molecule is allowed to interact with the A β (1–42) oligomers and larger aggregates (e.g., protofibrils and fibrils), diffusion of the soluble oligomers with Fc-KLVFFK₆ incorporated should be retarded. Incorporation of Fc-KLVFFK₆ into and co-precipitation with insoluble and higher-ordered A β (1–42) aggregates would also decrease the Fc-KLVFFK₆ bulk concentration. Both of the above processes will decrease the electrochemical signal, through which the kinetics of the inhibition reaction can be interrogated (cf schematics in Figure 1). As shown in Figure 2, Fc-KLVFFK₆ displays a cyclic voltammogram (CV) with an oxidation peak at 499.2 mV and a reduction peak at 430.6 mV (inset). The oxidation potential is much less positive than that of the tyrosine oxidation, which has been shown by us [43, 47] and others [29, 31] (see also Figure S1 in Supporting Information for comparison) to be around 0.72 V vs. Ag/AgCl. On the basis that the peak potential splitting (ΔE_p) = 68.6 mV and the current ratio between the anodic and cathodic peaks (i_{pa}/i_{pc}) = 1.03 are close to the theoretical values (59.6 mV and 1.00, respectively) and the fact that the oxidation peak current (i_{pa}) is proportional to the scan rate [48], we conclude that the Fc tag undergoes facile electron transfer with the electrode. Figure 2A is an overlay of a series of differential pulse voltammograms (DPVs) of 20 μ M Fc-KLVFFK₆ in the presence of equimolar A β (1–42) collected at different incubation times. The DPV oxidation peak decreases with incubation time, confirming that the Fc tag can serve as a sensitive probe for studying the interaction between a potential β -sheet breaker peptide and A β (1–42). Our final consideration of using Fc as a tag is that Fc derivatives have been shown to be innocuous to many cell lines [49] and consequently the Fc tag would not compromise the ability of KLVFFK₆ of alleviating the cell toxicity inflicted by A β (1–42) aggregates.

By plotting the electrochemical currents of Fc-KLVFFK₆ as a function of time and overlaying the curves obtained in solutions of different amounts of A β (1–42), kinetic information about the interaction between Fc-KLVFFK₆ and A β (1–42) can be garnered. As shown in Figure 2B, the precipitous drop in the black curves at the beginning suggests that Fc-KLVFFK₆ binds to A β (1–42) instantaneously. In the absence of A β (1–42), a steady

DPV current was maintained in an Fc-KLVFFK₆ solution for 24 h (red curve), suggesting that Fc-KLVFFK₆ does not undergo self-aggregation (unlike the water-insoluble KLVFF [38, 39]). We also monitored the current of Fc-K₆, an Fc-tagged hexalysine peptide, at various incubation times in the presence of Aβ(1–42). The essentially constant current (blue curve) suggests that Fc-K₆, which lacks the KLVFF domain, does not interact with Aβ(1–42).

Continuous electrochemical monitoring of the interaction between Fc-KLVFFK₆ and Aβ(1–42) at different stoichiometric ratios all produced plateaus at about 6 h and beyond. We also centrifuged samples incubated for at least 6 h and recorded DPV currents in both the supernatants and the residual solutions. The currents from the supernatants (data not shown) are essentially congruent with the respective curves in Figure 2B, whereas those in the residual solutions did not produce noticeable Fc oxidation currents. Thus, the plateaus in Figure 2B correspond to excess Fc-KLVFFK₆ remaining in solution. We found that all the decay curves in Figure 2B can be fitted with a pseudo-first-order reaction rate equation:

$$i = i_0 - Ae^{-k_a t} \quad (1)$$

where i_0 and i represent the current values at the beginning and time t of an incubation, respectively. A is a constant and k_a is the forward reaction rate constant in the following reaction:

The rate constants deduced from these simulations (black curves in Figure 2B) are within a relatively narrow range of $1.26\text{--}1.89 \times 10^{-4} \text{ s}^{-1}$ (Table 1), and the half-life of the conjugate formed between Fc-KLVFFK₆ and the Aβ(1–42) aggregates is $4547.4 \pm 705.8 \text{ s}$. Notice that the fits are excellent even at $[\text{Fc-KLVFFK}_6]/[\text{A}\beta(1-42)] < 1$, indicating that a significant amount of free Fc-KLVFFK₆ has remained in solution. This observation suggests that a single Fc-KLVFFK₆ molecule is associated with aggregates comprising multiple Aβ(1–42) molecules. Another possible explanation is that the binding affinity between Fc-KLVFFK₆ and the Aβ(1–42) aggregates is in the μM range, which establishes an equilibrium between free Fc-KLVFFK₆ in solution and Fc-KLVFFK₆ bound to the aggregates.

As mentioned above, the decrease in the DPV peak currents in Figure 2B could be ascribed to two possible processes—(1) a decrease of the bulk Fc-KLVFFK₆ concentration resulted from the Fc-KLVFFK₆ incorporation in the precipitates of large and insoluble Aβ(1–42) aggregates and (2) a slower diffusion due to its association with soluble Aβ(1–42) oligomers. To pinpoint the specific form of Aβ(1–42) (soluble oligomers or large aggregates) to which Fc-KLVFFK₆ is bound, we conducted size exclusion chromatography (SEC) of mixtures of Aβ(1–42) and Fc-KLVFFK₆ incubated for various times. As shown in Figure 3A, under our chromatographic condition, the Aβ(1–42) monomer elutes at ca. 32 min. During the Aβ(1–42) sample injection and its migration across the column (ca. 50 min), the freshly prepared Aβ(1–42) sample plug has already produced a discernible amount of dimers and a small amount of soluble oligomers (cf. the pentamer peak at 25 kD). The time for forming dimers and pentamers is consistent with those reported in some studies [50, 51]. Aβ pentamers and hexamers are the predominant oligomeric species, consistent with findings from previous reports [52]. Incubation of an Aβ(1–42) solution for 2–3 h converts monomers and small oligomers into larger oligomers (cf. the peak identified as a 160 mer at 740 kD). Very few monomers and oligomers remained in Aβ(1–42) solutions that had been incubated for 6 h or longer, as evidenced by the disappearance of the Aβ(1–42) monomer

and dimer peaks. The decrease in the concentrations of monomers and soluble oligomers is due to the formation of insoluble, higher-ordered aggregates, which are not amenable to the SEC separation. We should note that the small peak at ca. 40 min with a size of 1.3–2 kD was verified by electrospray mass spectrometry to be an impurity in the synthetic A β (1–42) sample.

The SEC separation of an Fc-KLVFFK₆-only (20 μ M) solution did not show any elution peaks (cf. Figure S2A in Supporting Information), because of the small molar extinction coefficient of Fc-KLVFFK₆. We also confirmed with SEC that Fc-KLVFFK₆ alone does not aggregate throughout the incubation process (Figure S2B). When Fc-KLVFFK₆ was present in the A β (1–42) solution, no soluble oligomers larger than the A β (1–42) dimer were observed (Figure 3B). In addition, the monomer concentration at the inception of the incubation is significantly greater (two times as high as the combined area of the monomer and dimer peaks in Figure 3A). Unlike in Figure 3A, it is more difficult to resolve the monomer and dimer peaks in Figure 3B, though peaks in the latter chromatogram appear to be of higher abundance than those in the former. Since Murphy and coworkers have shown that KLVFFK₆ does not directly interact with the A β (1–40) monomer [26], we believe that the large and broad peak in Figure 3B does not contain A β (1–42) monomers conjugated with Fc-KLVFFK₆. Therefore, the much enhanced monomer/dimer peak must be resulted from the alteration of the A β (1–42) aggregation process, which stabilizes both the monomer and dimer at the initial stage of the incubation. We posit that, instead of directly interacting with A β (1–42) monomers and soluble oligomers, Fc-KLVFFK₆ alters the A β (1–42) aggregation pathway by interacting with larger, intermediate A β (1–42) aggregates, thereby preventing further attachment of A β (1–42) monomers to such aggregates. To observe insoluble aggregates, we conducted the following atomic force microscopic measurements.

Figure 4 are atomic force microscopy (AFM) images obtained from A β (1–42) solutions in the absence (row A) and presence of Fc-KLVFFK₆ (row B). In the absence of Fc-KLVFFK₆, few insoluble globular aggregates were observed at the beginning of the incubation. The abundance of the globular aggregates became much greater at 3 h. At 6 h and later times, the predominant aggregate is the A β (1–42) fibril. In contrast, in the presence of Fc-KLVFFK₆, large aggregates with heights around 20 nm and diameters in sub-micrometers were observed in the first 3 h and are predominately of the amorphous morphology (*cf.* the cross-sectional contours). The abundance of the amorphous aggregates became greater at 6 h, and after 24 h, the amorphous aggregates were the predominant end product. We also collected AFM images from a mixture of KLVFFK₆ (the soluble β -sheet breaker peptide without the Fc tag) and A β (1–42) at various incubation times. As shown in row C of Figure 4, the aggregates observed at 3 h and 6 h have essentially the same morphology (amorphous aggregates) as those shown in row B. The slight difference is that after extensive incubation (24 h), the mixture of A β (1–42) and KLVFFK₆ produced a mixture of short fibrils and amorphous aggregates, whereas in row B, the solution was populated with amorphous aggregates. We conclude that the mode of inhibition by Fc-KLVFFK₆ and KLVFFK₆ should be quite similar. The small difference in the final product(s) between the Fc-KLVFFK₆- and KLVFFK₆-containing solutions (largely amorphous aggregates vs. a mixture of short fibrils and amorphous aggregates) can be rationalized by the presence of the Fc moiety in the former. The additional steric hindrance rendered by the Fc tag perhaps contributes to the overall breakage of the stacked, β -sheet-rich A β (1–42) oligomers. Consequently, the more disordered amorphous aggregates, instead of shortened fibrils, are produced.

AFM also showed that Fc-KLVFFK₆ does not self-aggregate when incubated alone (row D). This is consistent with our electrochemical results (*cf.* red curve in Figure 2B). This again demonstrates the advantage and greater biochemical relevance of using a water-soluble p-

sheet breaker peptide over its aggregation-prone counterpart (i.e., Fc-KLVFF [37]). Therefore, the amorphous aggregates shown in rows B and C of Figure 4 must be produced from altered A β (1–42) aggregation pathway resulted from the interaction between A β (1–42) aggregates and the β -sheet breaker peptides. Taken together, the mode of inhibition by KLVFFK₆ (or its Fc-tagged analog) is to interact with the aggregation intermediates, thereby diverting the A β (1–42) aggregation away from the main aggregation (i.e., fibril-producing) pathway. This point is in line with the finding by Murphy and co-workers who showed that KLVFFK₆ can interact with linear aggregate of A β (1–42) or protofibril-like aggregate [26].

It appears that Fc-KLVFFK₆, analogous to KLVFFK₆, completely eliminates soluble A β (1–42) oligomers and fibrils in solution. The soluble oligomers (sometimes referred to as amyloid-derived diffusible ligand [53]) are known to be the most cytotoxic among all of the A β (1–42) aggregates [54]. To assess whether the amorphous aggregates formed in the Fc-KLVFFK₆/A β (1–42) mixture are less pernicious to cells, SH-SY5Y human neuroblastoma cells were exposed to various solutions. As shown in Figure 5, both Fc-KLVFFK₆ and KLVFFK₆ are largely benign to the SH-SY5Y cells (viability values are 91.4 and 96.7%, respectively). However, the cell viability upon exposure to a pre-incubated A β (1–42) solution dropped to 53.7%. As shown by the AFM images in row A of Figure 4, an A β (1–42) solution incubated for 24 h produced a mixture of fibrils, protofibrils, and globular aggregates, with mature fibrils being the most abundant. Thus, these aggregates have collectively exerted toxicity to the SH-SY5Y cells. However, when A β (1–42) was pre-incubated with Fc-KLVFFK₆ and then added into the SH-SY5Y cell media, the cell viability was rescued to 92.4%. When a pre-incubated KLVFFK₆/A β (1–42) mixture was added into the SH-SY5Y cell media, the final cell viability was determined to be 82.9%. Because the error associated with the former is 5% and that with the latter is 7%, the difference between the two cases is statistically insignificant. Any slight difference is most likely contributed by the existence of a small number of short fibrils present in the KLVFFK₆/A β (1–42) mixture (cf. AFM image at 24 h in row C). Thus, tagging an Fc moiety to the N-terminus of the KLVFFK₆ peptide does not alter the inhibitory effect of KLVFFK₆ but facilitates kinetic studies of the breakage/disruption of the stacking of β -sheet-containing A β (1–42) aggregates.

Conclusions

By tagging a water-soluble β -sheet breaker (KLVFFK₆) with an Fc moiety, we demonstrate that disruption of the A β (1–42) aggregation/fibrillation process can be monitored in real time by electrochemical methods. We found that a sub-stoichiometric amount of Fc-KLVFFK₆ is sufficient to completely prevent the A β (1–42) oligomers and fibrils from being formed, an observation confirmed by size exclusion chromatographic and atomic force microscopic experiments. For the first time, we show that the aggregation inhibition is a pseudo-first-order reaction. Our study confirms that the inhibitory effect is realized through a strong interaction between Fc-KLVFFK₆ and the A β (1–42) aggregation intermediates, similar to that for KLVFFK₆. As a consequence, cytotoxicity caused by A β (1–42) aggregates is abolished extensively. The method described herein is of general appeal, as β -sheet breaker peptides have been touted as a potential therapeutic remedy to treat AD, PD, and other related neurological disorders. Kinetic information about the inhibition of the aggregation of amyloidogenic proteins/peptides and knowledge of the effective dosage of β -sheet breaker molecule are essential for rational design and high-throughput screening of potential therapeutic drugs for treating these neurological disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AD	Alzheimer's disease
PD	Parkinson's disease
Aβ	amyloid beta
Fc	ferrocene
AFM	atomic force microscopy
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
TFA	trifluoroacetic acid
Fc-COOH	ferrocene monocarboxylic acid
DMF	dimethylformamide
DIC	diisopropylcarbodiimide
FBS	fetal bovine serum
DMEM	Dulbecco's Modified Eagle Medium
ES-MS	electrospray-mass spectrometry
MTT	3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
CV	cyclic voltammogram
DPV	differential pulse voltammogram
SEC	size exclusion chromatography

Figure 1.

Schematic of the use of an Fc (yellow sphere)-tagged β -sheet breaker peptide (Fc-KLVFFK₆) to investigate the kinetics of its interaction with A β (1–42) and to estimate the binding stoichiometry between Fc-KLVFFK₆ and A β (1–42) aggregates. A hexamer is used to represent the soluble oligomer (top) and insoluble aggregate are represented by the amorphous precipitate (bottom). KLVFF is depicted as the stick and the K₆ segment is shown as a wavy tail. Incorporation of Fc-KLVFFK₆ into these aggregates leads to a decreased electrochemical signal.

Figure 2.

(A) Differential pulse voltammograms of 20 μM Fc-KLVFFK₆ in the presence of 20 μM A β (1-42) at different incubation times: 0 h (black curve), 0.5 h (red), 2 h (blue), 3 h (green), 6 h (magenta), and 24 h (pink). Inset: a cyclic voltammogram of 20 μM Fc-KLVFFK₆. The arrow indicates the initial scan direction. (B) Current-time responses of 20 μM Fc-KLVFFK₆ at different [Fc-KLVFFK₆]/[A β (1-42)] ratios. Each data point is the average of

three replicates and the curves are simulated using the pseudo-first-order rate law. The black line curves correspond to simulated data. The colored curves correspond to the response of 20 μM Fc-KLVFFK₆ in the absence of A β (1–42) (red) and 20 μM Fc-K₆ in the presence of 20 μM A β (1–42) (blue).

Figure 3.

Size exclusion chromatograms of (A) 80 μM Aβ(1–42) incubated for different times, with the monomer (~4.5 kD), dimer (~9 kD), pentamer (~25 kD), and a soluble oligomer (~740 kD) identified; (B) the same as (A) but co-incubated with 20 μM Fc-KLVFFK₆.

Figure 4.

Atomic force microscopic images of aggregates formed in incubated solutions of (A) 80 μM Aβ(1–42), (B) 80 μM Aβ(1–42)/20 μM Fc-KLVFFK₆, (C) 80 μM Aβ(1–42)/20 μM KLVFFK₆, and (D) 20 μM Fc-KLVFFK₆. The incubation times are indicated in the images. The scale of each image is 5 μm × 5 μm. The cross-sectional contours are shown for the representative aggregates identified by the green bars.

Figure 5. SH-SY5Y cell viabilities in the following media (from left to right): no additives, 20 μ M Fc-KLVFFK₆, 20 μ M KLVFFK₆, 80 μ M A β (1-42), 80 μ M A β (1-42) mixed with 20 μ M Fc-KLVFFK₆, and 80 μ M A β (1-42) mixed with 20 μ M KLVFFK₆. All the peptide-containing solutions had been pre-incubated for 24 h at 37 °C. The cell viabilities were determined 24 h after the addition of the various solutions. The error bars correspond to relative standard deviation values of measurements conducted on three different days.

Table 1
Pseudo-first-order rate constant and half-life ($t_{1/2}$) values

[Fc-KLVFFK ₆]:[A β (1–42)]	k_a (s ⁻¹)	$t_{1/2}$ (s)
1:0.25	$(1.49 \pm 0.15) \times 10^{-4}$	4651.0
1:0.5	$(1.43 \pm 0.08) \times 10^{-4}$	4846.2
1:1	$(1.26 \pm 0.07) \times 10^{-4}$	5500.0
1:2	$(1.71 \pm 0.05) \times 10^{-4}$	4052.6
1:4	$(1.89 \pm 0.05) \times 10^{-4}$	3687.0