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Amplified voltammetric characterization of cleavage of the biotinylated peptide by BACE1 and screening of BACE1 inhibitors

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ABSTRACT

Cleavage of amyloid precursor protein (APP) by the β-site APP cleaving enzyme 1 (BACE1) is a key step in the formation of amyloid beta (Aβ) peptide, the main component of amyloid plaques in Alzheimer's disease (AD). Suppression of BACE1 activity has thus become an efficient way for the treatment of AD. In this study, BACE1 in the absence or presence of BACE1 inhibitors was exposed to the biotinylated peptide substrate-modified electrode. This step was followed by the attachment of ferrocene (Fc)-capped gold nanoparticle/streptavidin conjugates. Due to the blockage of the BACE1 activity by select inhibitors, well-defined voltammetric peaks of high signal intensity were obtained. However, featureless voltammogram was obtained upon initiating the cleavage reaction. The proposed method is simple, sensitive, and suitable for monitoring of BACE1 activity and screening of BACE1 inhibitors.

1. Introduction

Alzheimer's disease (AD), being a degenerative disorder of brains, is characterized by the presence of extracellular senile plaques and intracellular neurofibrillary tangles (Melnikova, 2007; Selkoe, 2004). Amyloid beta (Aβ) peptide is the major component of the senile plaques and its accumulation plays a vital role in the pathogenesis of AD (Hardy and Selkoe, 2002; Selkoe, 2001, 1999). Aβ is produced via sequential proteolytic cleavage of the amyloid precursor protein (APP) by β-secretase (i.e., BACE1) and γ-secretase in which the rate-limiting step is governed by the BACE1 activity (Lin et al., 2000; Sinha and Lieberburg, 1999). As a result, suppression of BACE1 activity has thus become a potential therapeutic strategy for the treatment of AD (Ghosh et al., 2002; Mancini et al., 2011).

Typically, monitoring of BACE1 activity and screening of BACE1 inhibitors could be easily conducted by the fluorescence resonance energy transfer (FRET) method (Choi et al., 2012; Garino et al., 2006; Kennedy et al., 2003; Xu et al., 2010). For example, FRET pair based on the donor europium and the acceptor allophycocyanin has been fabricated for measuring BACE1 activity and large-scale screening of peptide inhibitors (Kennedy et al., 2003). In the absence of BACE1 or in the presence of BACE1 and select inhibitors, FRET occurred in which the quenched 620-nm emission was accompanied by the increased 665-nm emission. The cleavage of the 18-amino-acid APP Swedish-synthetic peptide by BACE1 at the scissile bond then disrupted the FRET process. The FRET-based system involving the assembly of CdSe/ZnS quantum dots and gold nanoparticles was reported by Song and coworkers (Choi et al., 2012). However, the choice of the donor/acceptor pairs and labeling of the peptide substrate on both ends impose limitations on the FRET-based assays. Other methods such as sensitive 96-well HPLC biochemical assay and homogeneous electrochemiluminescence assay have been demonstrated for the evaluation of BACE1 inhibition (Pietrak et al., 2005). BACE1-like activity has also been identified using mass spectrometry (Fiona et al., 2000). As an alternative, improved microtiter plate-based fluorescence and colorimetric assays for screening of BACE1 inhibitors were performed (Mancini et al., 2007). Recently, sensitive and continuous screening of BACE1 inhibitors with antibody-amplified signal enhancement at single surface plasmon resonance chips has been reported by our group (Yi et al., 2013). Two inhibitors were screened and the renewable chip enabled multiple assays to be continuously performed.

Electrochemical methods are simple, sensitive, and do not involve complicated instrumentation (Bard and Faulkner, 2001). By electrochemistry, sensing for caspase 3 activity and inhibition amplified by quantum dots was monitored (Zhang et al., 2011). Electrochemical measurement of proteolytic activity of trypsin and

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α-thrombin enzymes toward Fc-labeled peptides was also reported (Adjemian et al., 2010). Using electrochemical impedance spectroscopy, detecting HIV-1 protease and screening for its inhibitors were carried out (Mahmoud and Luong, 2008).

Using Fc-capped gold nanoparticle/streptavidin conjugates, amplified voltammetric detection of the pre-S gene of hepatitis B virus, miRNA in sera of glioma patients, and wild-type p53 protein from normal and cancer cell lysates has been carried out by our group (Wang et al., 2003, 2008, 2012). In this study, the same conjugates-based voltammetric method for monitoring of BACE1 activity and screening of select BACE1 inhibitors has been developed. The gold electrode was modified with the biotinylated peptide substrate terminated with a cysteine residue. The cleavage of the peptide by BACE1 resulted in detachment of the biotinylated fragments (biotin-KTEEISEVNL) from the electrode surface, leading to the attachment of less conjugates. Consequently, smaller or negligible voltammetric signals were obtained. However, suppression of the BACE1 activity by potential inhibitors produced larger voltammetric responses. The sensing protocol is thus advantageous for assessment of BACE1 activity and screening of BACE1 inhibitors.

2. Experimental

2.1. Chemicals and materials

Biotinylated peptide substrate terminated with a cysteine residue (biotin-KTEEISEVN*LD*AEFRHDKC) was purchased from ChinaPeptides Co., Ltd. (Shanghai, China). The peptide substrate was a segment of APP with the Swiss mutation (KM→NL) (Mullan et al., 1992) and the cleavage sites were denoted by residues in italics. For screening of BACE1 inhibitors, three compounds, which have been reported in our previous work (Yi et al., 2013), were assayed. KTEEISEVN-Sta-VAEF (compound 1) and Ph-LL-4,5-dehydro-L-CHO (compound 2) were purchased from Anaspec Inc. (Fremont, CA) and BaChem Inc. (Torrance, CA), respectively. Compound 3 with a sequence of DPDNEAYEMPSEEG was synthesized in house on an automatic peptide synthesizer (Symphony Quartet, Protein Technologies, Inc., Tucson, AZ). BACE1, gold nanoparticle/streptavidin conjugates, 6-ferrocenyl-1-hexanethiol, KH_2PO_4, K_2HPO_4, 1-hexanethiol (HT), and tris (2-carboxyethyl)-phosphine hydrochloride (TCEP) were acquired from Sigma-Aldrich. Anhydrous sodium acetate was obtained from Alfa Aesar. Acetonitrile (CH_3CN), trifluoroacetic acid (TFA) and dimethyl sulfoxide (DMSO) with HPLC/Spectro grade were obtained from Tedia Company, Inc. (USA). The stock solution of 2 mM peptide substrate was prepared with PBS buffer (10 mM phosphate, pH 7.4) in which 50 μM TCEP was added. TCEP was utilized for effectively reducing the disulfide bonds in proteins and peptides. BACE1 was prepared with HAc–NaAc buffer (pH 4.5). The three compounds tested were dissolved in DMSO and then diluted with the acetate buffer. The preparation and characterization of ferrocene (Fc)-capped gold nanoparticle/streptavidin conjugates have been reported previously (Wang et al., 2003).

2.2. Instruments

The electrochemical determination was performed on a CHI 832 electrochemical workstation (CH Instruments, Austin, TX). A three-electrode configuration was involved in which a 2-mm-diameter gold electrode (CH Instruments, Austin, TX) was used as the working electrode. A platinum wire and a Ag/AgCl electrode were used as the auxiliary and reference electrodes, respectively. HPLC separation and detection were performed with a Shimadzu LC-20A system (Shimadzu, Japan) equipped with a C_{18} column (4.6 mm × 250 mm, 5 μm) from Phenomenex (Torrance, CA) and a SPD-20A UV–vis detector. The mobile phases were 0.1% trifluoroacetic acid in water (v/v, mobile phase A) and 0.1% trifluoroacetic acid in acetonitrile (v/v, mobile phase B). The isocratic elution was conducted with 30% of mobile phase B for 20 min at a flow rate of 1 mL min^{-1}.

2.3. Procedures

Gold electrode was carefully polished with alumina slurry down to 0.3 μm on a polishing cloth, followed by sonication in water and ethanol for 5 min. The above electrode was then cleaned electrochemically in 0.5 M H_2SO_4 via scanning the electrode potential between 0 and 1.4 V at 0.1 V s^{-1} until a stable cyclic voltammetric signal was obtained. The as-treated electrode was then exposed to 1 μM peptide substrate solution overnight and the peptide terminated with the cysteine residue was anchored onto the electrode through the Au–S bond. This step was followed by rinsing the electrode with PBS buffer and distilled water, correspondingly. The resultant electrode was treated with 0.1 mM HT for 5 min, and then 1% BSA for 1 h to block the empty sites on the surface.

The cleavage of the peptide substrate by BACE1 was carried out by exposing the peptide-covered electrode to different concentrations of BACE1 at 37 °C for 45 min. For screening of BACE1 inhibitors, the peptide-modified electrode was exposed to the mixed solution of BACE1 and select inhibitors in which the concentration of BACE1 was maintained at 10 nM. Finally, the electrode was exposed to Fc-capped gold nanoparticle/streptavidin conjugates for 1 h. The electrochemical determination was carried out in 0.1 M $KClO_4$.

3. Results and discussion

3.1. Principle of the voltammetric assay

Schematic representation of the cleavage of the biotinylated peptide substrate by BACE1 and screening of BACE1 inhibitors is shown in Fig. 1. First, self-assembled monolayers of the cysteine-terminated peptide substrate were formed onto the gold electrode. After blocking with HT and BSA, the peptide-covered electrode was then treated with BACE1 in the absence and presence of a BACE1 inhibitor. This step was followed by attachment of Fc-capped gold nanoparticle/streptavidin conjugates. The cleavage of the biotinylated peptide substrate by BACE1 detached the biotinylated fragments (biotin-KTEEISEVNL), resulting in attachment of less conjugates on the electrode surface (top panel). However, if the BACE1 activity was suppressed by the inhibitors, more conjugates were incorporated onto the surface (bottom panel). Thus, the sensing protocol could be utilized for monitoring of BACE1 activity and screening of BACE1 inhibitors.

3.2. Voltammetric characterization of the cleavage of the biotinylated peptide substrate

The feasibility of the proposed method for monitoring of BACE1 activity and screening of BACE1 inhibitors has been demonstrated (Fig. 2). Voltammetric response at electrodes modified with 10 μM biotinylated peptide, followed by blocking with HT/BSA and attachment of Fc-capped gold nanoparticle/streptavidin conjugates is shown in curve a. A pair of well-defined redox waves with high signal intensity and peak potential separation of 20 mV was observed. Owing to the relatively large mass of the conjugates, the Fc tags were positioned in close proximity to the electrode surface (Wang et al., 2003). Thus, facile electron transfer reaction could occur. When the biotinylated peptide-covered electrode was

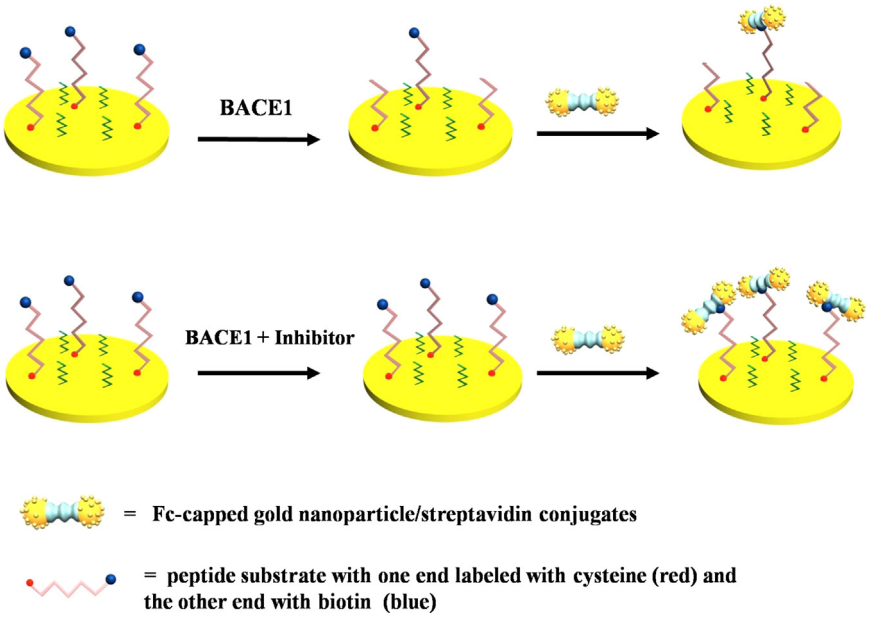

Fig. 1. Schematic representation of electrochemical screening of inhibitory compounds for BACE1 by using the peptide substrate with one end labeled with cysteine and the other end with biotin. The cysteine-tagged end is tethered onto the electrode surface, followed by blocking with HT and BSA. BACE1 in the presence of a noninhibitor or in the absence of an inhibitor cleaves the peptide substrate and the detachment of the biotin-tagged segment at the surface prevents the Fc-capped gold nanoparticle/streptavidin conjugates from attached to the electrode surface (top panel). In the presence of a potent inhibitor, blockage of the BACE1 active sites inhibits the enzymatic activity. Consequently, attachment of the Fc-capped gold nanoparticle/streptavidin conjugates to the intact peptide substrate produces a larger signal (bottom panel).

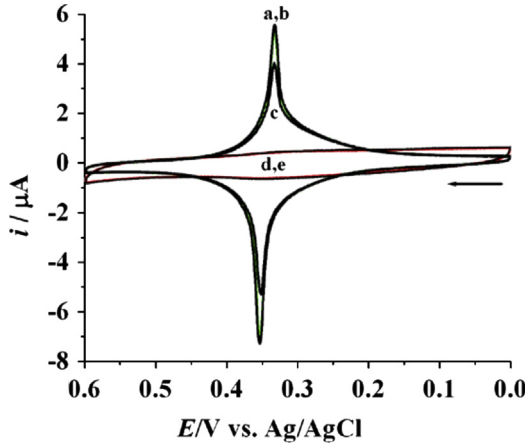

Fig. 2. Cyclic voltammograms (CVs) acquired at electrodes covered with 10 μM biotinylated peptide substrate when exposed to (a) the acetate buffer, (b) 10 nM BACE1 mixed with 120 nM compound 1, (c) 10 nM BACE1 mixed with 6 μM compound 2, (d) 10 nM BACE1 only, and (e) 10 nM BACE1 mixed with 120 nM compound 3, followed by attachment of Fc-capped gold nanoparticle/streptavidin conjugates. The cleavage of the peptide substrate by BACE1 in the absence or presence of select inhibitors was carried out in HAc–NaAc buffer at 37 °C for 45 min. The scan rate was 0.1 V s^{-1} and the arrow indicates the scan direction.

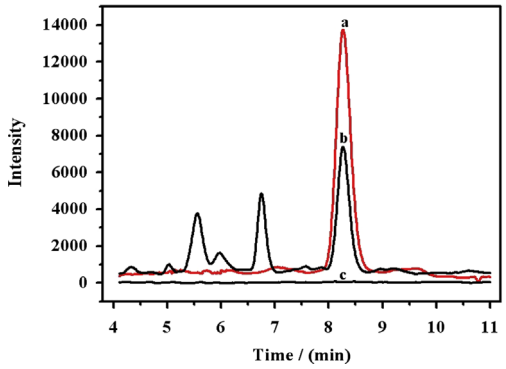

Fig. 3. Chromatograms depicting the separation and UV–vis spectrometric detection at 220 nm of 50 μM biotinylated peptide (a), the mixture of 50 nM BACE1 and 50 μM biotinylated peptide (b), and 50 nM BACE1 (c). The cleavage reaction between BACE1 and the biotinylated peptide was carried out in HAc–NaAc buffer at 37 °C overnight.

exposed to 10 nM BACE1 at 37 °C for 45 min, featureless voltammetric signal was obtained (curve d). This indicates that the peptide substrate has been clipped by BACE1 at the cleavage site, detaching the biotinylated fragments (biotin-KTEEISEVNL) from the electrode surface. The absence of any discernible peaks in curve d also indicates that the nonspecific adsorption of the conjugates was negligible. As a proof-of-concept experiment for screening of BACE1 inhibitors, three selected compounds were assayed. As shown in curve e, when 10 nM BACE1 was mixed with 120 nM compound 3, the electrochemical signal was the same as that acquired at electrodes exposed to 10 nM BACE1 only (curve d), indicating that compound 3 was not a BACE1 inhibitor. However, when the peptide-modified electrode was exposed to the mixed solution of 10 nM BACE1 and 6 μM compound 2, a decrease of 27% in the anodic peak current was observed (curve c). Such a decrease could be ascribed to the incomplete or partial inhibition of BACE1 activity by compound 2 at a concentration of 6 μM. When the peptide-modified electrode was exposed to the mixture of 10 nM BACE1 and 120 nM compound 1 (curve b), the peak current remained the same as that in curve a, which suggests that the BACE1 activity has been suppressed entirely. The inhibition process involves the binding of a hydroxyl or hydroxyl-like moiety of BACE1 inhibitors to the two catalytically active aspartic acids of BACE1 (Bursavich and Rich, 2002). We also conducted an experiment in which the peptide-covered electrode was exposed to the solution containing only the BACE1 inhibitor, followed by treatment with the conjugates, the electrochemical response was found not to be influenced by the inhibitors assayed (data not shown).

3.3. HPLC characterization

The cleavage of the peptide substrate by BACE1 was also characterized by HPLC (Fig. 3). The biotinylated peptide exhibited

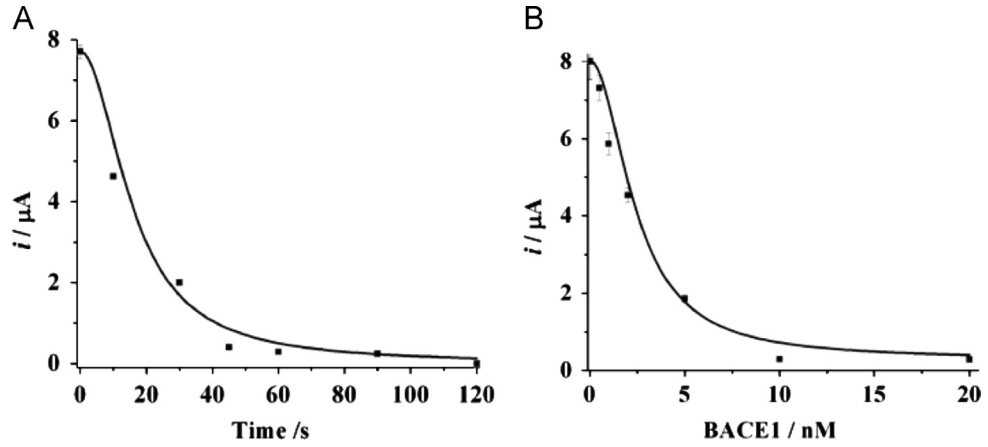

Fig. 4. Dependence of the anodic peak current on the cleavage time (A) and BACE1 concentration (B). In panel A, the cleavage time examined are 0, 10, 30, 45, 60, 90, and 120 min. In panel B, the concentrations of BACE1 are 0, 0.5, 1.0, 2.2, 5.0, 10, and 20 nM. Other experimental conditions are the same as those in Fig. 2. Each point was averaged from three replicate measurements and the absolute errors were shown as the error bars.

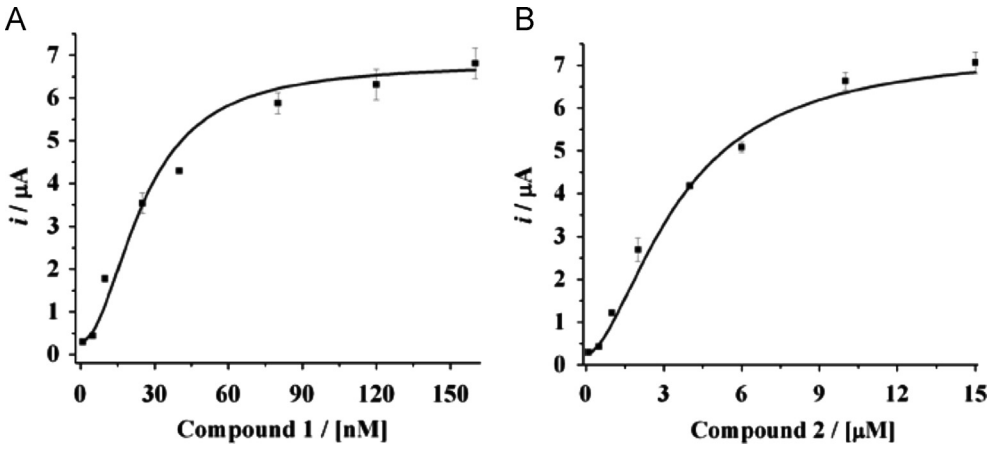

Fig. 5. Dependence of the anodic peak currents on the concentrations of compound 1 (A) and compound 2 (B). The concentrations of compounds 1 and 2 examined are 1, 5, 10, 25, 40, 80, 120, and 160 nM and 0.1, 0.5, 1, 2, 4, 6, 10, and 15 μM, respectively. Other experimental conditions are the same as those in Fig. 2.

a large absorption peak at 8.27 min (curve a). However, the cleavage reaction of the peptide by BACE1 resulted in two other peaks at 5.56 min and 6.75 min (curve b). On the other hand, the original peak of the peptide at 8.27 min in curve b decreased correspondingly in comparison with that in curve a. These peaks all corresponded to the absorption of the amide groups in the peptide. Note that BACE1 essentially exhibited no absorbance over the time scale examined, as shown in curve c. Thus, we can conclude that the two peaks at 5.56 min and 6.75 min in curve b resulted from the fragments (biotin-KTEEISEVNL and DAEFRHDKC) formed via cleavage of the peptide at the specific site.

3.4. Optimization of the experimental conditions

Cleavage time has a profound influence on the cleavage reaction. As shown in Fig. 4A, the anodic peak currents decreased sharply with the cleavage time less than 45 min, suggesting that sufficient reaction time is beneficial to the complete cleavage reaction. Beyond 45 min, the peak currents began to level off and approached to a much smaller value, which indicates that almost all of the immobilized peptide substrate has been clipped by BACE1 at the cleavage site. Thus, the optimal cleavage time was fixed at 45 min for higher cleavage efficiency. We should note that the biotinylated peptide substrate possesses higher stability in HAc–NaAc buffer (pH 4.5) over the time scale examined and higher BACE1 activity is maintained at acidic pH value. The influence of BACE1 concentration on the cleavage reaction has also been examined (Fig. 4B). With a similar trend with Panel A, the anodic peak currents decreased precipitously with BACE1 concentrations lower than 10 nM. The plateau beyond 10 nM indicates a complete cleavage reaction.

3.5. Voltammetric assay of BACE1 inhibitors

Finally, the dependence of the anodic peak currents on the concentrations of compounds 1 and 2 was assessed (Fig. 5). The increase in the concentrations of compounds 1 (Panel A) and 2 (Panel B) both led to the increase in the anodic peak currents, suggesting that compounds 1 and 2 are effective BACE1 inhibitors. IC_{50}, defined as the half maximal inhibitory concentration of compounds 1 and 2, were determined to be 35 nM and 3.6 μM, respectively, being in agreement with the reported values of 30 nM (Tung et al., 2002) and 3.0 μM (Wagner and Munoz, 1999). The much lower IC_{50} value of compound 1 suggests that compound 1 is a more effective BACE1 inhibitor. Our method thus holds great promise for screening of BACE1 inhibitors.

4. Conclusions

An amplified voltammetric assay has been proposed for monitoring of BACE1 activity and screening of BACE1 inhibitors. The biotinylated peptide substrate-modified electrode was exposed to BACE1 solution in the absence or presence of BACE1 inhibitors,

followed by the attachment of Fc-capped gold nanoparticle/streptavidin conjugates. Due to the large number of Fc groups on the gold nanoparticle, the electrochemical signal of the peptide-covered electrode without exposing to BACE1 solution has been greatly amplified. However, the efficient cleavage of the peptide substrate by BACE1 detached the biotinylated fragments (biotin-KTEEISEVNL) from the electrode surface, leading to a remarkable decrease in the electrochemical response. On the other hand, the incorporation of BACE1 inhibitors into BACE1 solution suppressed the BACE1 activity, which yielded a larger voltammetric signal. The IC_{50} of compounds 1 and 2 were determined to be 35 nM and 3.6 μM, respectively, being in agreement with those reported. The proposed method is simple, sensitive, serving as a viable alternative for monitoring of BACE1 activity and screening of BACE1 inhibitors.

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