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Facile Quantification of Alzheimer's Disease Amyloid-β Based On Aggregation-Induced Emission

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ABSTRACT

A novel, facile amyloid beta (Aβ) peptide detection method was developed based on an aggregation-induced emission (AIE) dye using a modified amyloid fibrillization-promoting peptide (AFPP) probe. The AFPP portion of the probe plays a role in accelerating Aβ fibrillization, and the AIE portion of the probe plays a role in signal emission. Since the probe molecules were uniformly dispersed in buffer solution without Aβ, the fluorescent intensity was weak. While Aβ was added to the probe solution, the aggregation of Aβ was promoted and the fluorescence was enhanced within 3 hrs. The limit of detection with this method is 4.2 nM. The limit of detection value is insufficient for the quantification of Aβ found in bodily fluids; however, the present method has the advantage of a simple "add-and-measure" procedure without the need for a high level of laboratory skills and sophisticated equipment.

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia and is a neurodegenerative disorder characterized by progressive memory loss and decline in cognitive function that eventually leads to death [1]. The clinical diagnosis of AD is mainly based on neurological and neuropsychological observations that often lead to an inaccurate diagnosis [2].

AD pathogenesis is believed to be triggered by the formation of toxic aggregation of amyloid beta (Aβ) peptides. Thus, Aβ and the aggregated oligomers and fibrils may serve as useful biomarkers for the early detection of AD. Based on this concept, current pathological diagnosis for clinical or preclinical AD patients is made by *in vivo* and *in vitro* imaging of Aβ plaques using magnetic resonance imaging [3-6], positron emission tomography [7-11], and single photon emission computed tomography [12-16], while definitive pathological diagnosis is only obtained from autopsy examination of the brain for amyloid plaques. These imaging techniques for Aβ plaques require expensive medical equipment and radioactive probes.

Recently, novel methods were developed for the determination of Aβ monomers and oligomers using enzyme-linked immunosorbent assay [17,18] and mass spectrometry [19]. Although these methods are accurate, they are time-consuming, technically demanding, and costly. Thus there is a great need for an alternative rapid, simple, cost-effective, and non-invasive detection method.

Many fluorescence-based detection techniques have been developed using fluorescent dyes that function as highly sensitive visualizing probes. Congo red [20-23] and thioflavin T [23-25] fluorescence have been used for Aβ fibril detection; however, these dyes have several shortcomings such as non-specific binding [22, 25], concentration quenching [24], and false results in the presence of exogenous compounds [24]. In addition, traditional fluorescent dyes exhibit concentration quenching or aggregation-caused quenching (ACQ), because the traditional fluorescent probes are unsuited for the detection of the assembled aggregates. Recently, Takahashi and Mihara reported [26] the detection of Aβ fibrils using Förster resonance energy transfer between cyan fluorescent protein and yellow fluorescent protein. Though these fluorescent proteins did not show appreciable self-quenching [27], this method was quite effective for the detection of mature Aβ fibrils. The technique required long incubation times to detect monomeric and oligomeric Aβ because of the slow aggregation process of Aβ. Moreover, the fluorescent protein probe seems to be a huge molecule with respect to the target Aβ fibrils. To counteract this intrinsic shortcoming, aggregation-induced emission (AIE) dyes may be potential candidates to overcome ACQ. The AIE phenomenon is exactly opposite to ACQ, and AIE dyes have high fluorescence emissions in the aggregated state, thus they can open a new way for the detection of aggregated materials.

Our goal was to overcome the slow aggregation of Aβ. In our previous report, we demonstrated that the Aβ fibrillization-promoting peptide (AFPP) (DAEFHDKLVFFYEVHHQK) had an efficient ability to accelerate amyloid fibril formation [28]. In the presence of AFPP, the slow aggregation process of Aβ was stimulated and the aggregates were observed within 3 h. Thus, in the present report we chose AFPP for AIE-based Aβ detection.

In this paper, we describe the preparation of AFPP-AIE conjugates, and use these conjugates to determine Aβ 1-40 amounts and the degree of Aβ aggregation in the absence or presence of the amyloidogenic protein, amylin.

2. Experimental Methods

2.1 Materials and General Methods

Most of the reagents and solvents were purchased from Wako Pure Chemical Industries, Ltd., Tokyo Kasei Kogyo Co. and Sigma-Aldrich Co. (St. Louis, MO, USA) and used without further purification. Fmoc-Lys(Boc)-Alko-PEG resin and other Fmoc-derivative natural amino acids were purchased from Watanabe Chemical and used without further purification. Amyloid β-peptide (human, 1-40) was obtained from Peptide Institute, Inc. Distilled-deionized water was used for peptide synthesis. The products from the organic synthesis were isolated by column chromatography on silica gel (Wakogel C-300 or C-200) or preparative TLC on silica gel (Wakogel B-5F). AFPP and AIE-modified AFPPs were prepared by solid-phase synthesis based on Fmoc protocol and purified by high-performance liquid chromatography using a Waters XBridge C18 reverse-phase column (4.6 × 150 mm). ^{1}H NMR spectra were recorded on a JEOL JNM-ECA 500 spectrometer at 500.16 MHz using CDCl3 or CD3CN (both purchased from Cambridge Isotope Laboratories) as a solvent and tetramethylsilane as an internal standard. J values are given in Hz. ESI mass spectra were measured with a Waters Micromass ZQ 2000 under the following ionizing conditions: electrospray, cone voltage 20-60 V; capillary voltage 3.50 kV; extractor voltage 4 V; RF lens voltage 0.3 V; source

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temperature 150 °C; desolvation temperature 350 °C. Matrix-assisted laser desorption ionization time-of-flight masses of the AFPP and AIE-modified AFPP were recorded on a Bruker Autoflex III Amartbeam. The concentration of peptides solution was determined by UV measurement using a Jasco V-630 spectrophotometer. Fluorescence spectra and circular dichroism (CD) spectra were measured using a Jasco FP-6200 spectrofluorometer and Jasco J-820 spectropolarimeter, respectively.

2.2 Preparation of 1-(4'-Hydroxyphenyl)-1,2,2-Triphenylethylene

Seven milliliters of degassed toluene was added to the mixture of 1-bromo-1,2,2-triphenylethylene (0.674 g, 2.01 mmol), 4'-(4,4,5,5-tetramethyl-1,3,2-dioxaborolane-2-yl)phenol (0.221 g, 1.00 mmol), potassium carbonate (0.690 g, 5.00 mmol) and tetrakis(triphenylphosphine)palladium(0) (0.0575 g, 0.0498 mmol). The solution was stirred at reflux temperature for 8 h under nitrogen atmosphere. Then the reaction mixture was passed through a silica gel (upper) and celite (lower) bilayer pad and the pad was rinsed with ethyl acetate. After the subsequent removal of volatile compounds, the residue was purified by silica gel column chromatography using hexane/chloroform (1/4) as an eluent to give 1-(4'-hydroxyphenyl)-1,2,2-triphenylethylene (TPE-OH) as a white solid (0.231 g, 66.4% yield), ^{1}H-NMR($CDCl_3$, 500 MHz) δ(ppm) 4.62 (s, 1H, Ph-O*H*), 6.56 (d, *J* =8.5, 2H, *m-Ph*-OH), 6.89 (d, *J* = 8.0, 2H, *o-Ph*-OH), 7.00-7.10 (m, 15H, *Ph*-); m/z (ESI) 349 (M + H$^+$, 100%).

2.3 Preparation of TPE(A)

Potassium carbonate (0.346 g, 2.51 mmol) was added to a solution of TPE-OH (0.174 g, 0.500 mmol) in 10 mL of acetone. After the mixture was stirred at 50 °C for 30 min, ethyl bromoacetate (0.138 mL, 1.25 mmol) was added and stirred for another 3 h at 50 °C. Then the remaining potassium carbonate was filtered and the filtrate was evaporated. The residue was purified by column chromatography with chloroform and hexane. The solvent was removed using suction, and 20 mL of ethanol and 15 mL of 1N sodium hydroxide was added to the residue and stirred overnight at room temperature. After the reaction was complete, the reaction mixture was neutralized with 1 N hydrochloric acid and extracted with ethyl acetate. Then the extract was evaporated to dryness, and the solid was purified by reprecipitation using chloroform and hexane to obtain TPE(A) as a white solid (0.160 g, 78.7% yield), m/z (ESI) 407 (M + H$^+$, 66%), 429 (M + Na$^+$, 100) and 445 (M + K$^+$, 43).

2.4 Preparation of TPE(B)

Potassium carbonate (0.317 g, 2.30 mmol) was added to a solution of TPE-OH (0.201 g, 0.576 mmol) in 7 mL of DMF. After the mixture was stirred at 50 °C for 30 min, ethyl bromohexanoate (0.203 mL, 1.15 mmol) was added and stirred for another 22 h at 50 °C. After the reaction was complete, the reaction mixture was extracted by chloroform and washed with distilled water. The solvent was removed using suction and the residue was purified by column chromatography with chloroform and hexane (1:1 ratio). After evaporation of the fraction to dryness, the mixed solvent (THF/ethanol/1 N sodium hydroxide) was added and stirred for 36 h at ambient temperature. Then the solution was neutralized by 1 N hydrochloric acid, and the target compound was precipitated. The precipitation was dissolved in ethyl acetate and washed with distilled water, and the organic phase was evaporated to give TPE(B) as a pale yellow oil (0.178 g, 66.7% yield), m/z (ESI) 463 (M + H$^+$, 58%), 485 (M + Na$^+$, 100).

2.5 Preparation of 1-(4'-Formylphenyl)-1,2,2-Triphenylethylene

One hundred milliliters of degassed toluene was added to the mixture of 1-bromo-1,2,2-triphenylethylene (4.00 g, 11.9 mmol), 4'-(4,4,5,5-tetramethyl-1,3,2-dioxaborolane-2-yl)phenol (2.32 g, 10.0 mmol), potassium carbonate (4.17 g, 30.2 mmol) and tetrakis(triphenylphosphine)palladium(0) (0.245 g, 0.212 mmol). The solution was stirred at reflux temperature for 24 h under nitrogen atmosphere. Then the reaction mixture was passed through a silica gel (upper) and celite (lower) bilayer pad and the pad was rinsed with ethyl acetate. After the subsequent removal of volatile compounds, the residue was purified by silica gel column chromatography using hexane/chloroform (2:1 ratio) as an eluent. The solvent was evaporated, and methanol and water was added to the residue, and the precipitate was collected by suction filtration to give 1-(4'-formylphenyl)-1,2,2-triphenylethylene (TPE-CHO) as a white solid (2.81 g, 78.1% yield), ^{1}H-NMR($CDCl_3$, 500 MHz) δ(ppm) 7.00-7.04 (m, 6H, *m-Ph*), 7.10-7.13 (m, 9H, *o- and p-Ph*), 7.19 (d, *J* = 8.0, 2H, *o-Ph*-CHO), 7.62 (d, *J* = 9.0, 2H, *m-Ph*-CHO) and 9.90 (s, 1H, -C*H*O); m/z (ESI) 361 (M + H$^+$, 85%), 383 (M + Na$^+$, 100).

2.6 Preparation of 4-Methyl-1-(1'-Carboxypentan-5-yl)Pyridin-1-ium (Py-C5-COOH)

Ethyl-6-bromohexanoate (2.50 mL, 14.1 mmol) was added to a solution of 4-methylpyridine (1.61 mL, 16.5 mmol) in 15 mL acetonitrile, and stirred overnight at 50 °C. The solvent and unreacted 4-metylpyridine were removed under reduced pressure, then 30 mL of ethanol and 50 mL of 1 N sodium hydroxide were added to the residue and stirred for 1.5 h at room temperature. The mixture was neutralized by the addition of 50 mL of 1 N hydrochloric acid and evaporated. The residue was purified by silica gel column chromatography with acetone/methanol (2:1 ratio) to yield a white solid. The solid was washed with ethyl acetate and dried to give Py-C5-COOH (2.96 g, 72.9% yield), ^{1}H-NMR(CD_3OD, 500 MHz) δ (ppm) 1.37-1.43 (quin, *J* = 7.8, 2H, -C_2H_5*CH_2*C_2H_5-), 1.63-1.69 (quin, *J* = 7.6, 2H, -*CH_2*CH_2COOH), 1.97-2.03 (quin, *J* = 7.6, 2H, N$^+$-CH_2*CH_2*-), 2.31 (t, *J* = 7.0, 2H, -CH_2*CH_2*COOH), 2.67 (s, 3H, *CH_3*), 4.56 (t, *J* = 7.5, 2H, N$^+$-*CH_2*CH_2-), 7.92 (d, *J* = 6.5, 2H, *3-Py*-CH_3) and 8.81 (d, *J* = 7.0, 2H, *2-Py*-N$^+$); m/z (ESI) 208 (M$^+$, 100%).

2.7 Preparation of TPE(C)

Piperidine (0.500 mL, 5.06 mmol) was added to a suspension of TPE-CHO (0.233 g, 0.645 mmol) and Py-C5-COOH (0.320 g, 1.11 mmol) in 15 mL of acetonitrile and stirred for 2 h at 110 °C. Then the solvent was removed and the residue was purified by column chromatography with chloroform/methanol (10:1 to 1:2 ratio). The solvent was removed using suction, and the solid was reprecipitated by chloroform and hexane. The resulting yellow solid was collected by suction filtration to obtain TPE(C) as a white solid (0.101 g, 24.8% yield), ^{1}H-NMR(CD_3OD, 500 MHz) δ(ppm) 1.35-1.41 (quin, *J* = 7.5, 2H, -C_2H_5*CH_2*C_2H_5-), 1.63-1.69 (quin, *J* = 7.4, 2H, -*CH_2*CH_2COOH), 1.97-2.03 (quin, *J* = 7.5, 2H, N$^+$-CH_2*CH_2*-),2.17 (t, *J* = 7.3, 2H, -*CH_2*COOH), 4.50 (t, *J* = 7.0, 2H, Py-*CH_2*CH_2-), 6.99-7.13 (m, 17H, TPE and *m-Ph*-CH=CH-Py), 7.33 (d, *J* = 16, TPE-*CH*=CH-Py), 7.49 (d, *J* = 8.0, 2H, *o-Ph*-CH=CH-Py), 7.83 (d, *J* = 16, TPE-CH=*CH*-Py), 8.10 (d, *J* = 6.5, 2H, -CH-*Py*) and 8.75 (d, *J* = 5.5, 2H, *Py*-N$^+$); m/z (ESI) 550 (M$^+$, 100%) and 572 (M -H$^+$ + Na$^+$, 6).

2.8 Preparation of AFPP-TPE Probes

AFPP-TPE probes were prepared using Fmoc solid-phase synthesis with *O*-(7-aza-1H-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate and 1-hydroxy-1H-benzotriazole, anhydrous as coupling reagents. The AIE dyes were connected at the *N*-terminus of AFPP in a similar manner to the solid synthesis of peptides. The obtained probes were purified by reversed-phase high-performance liquid chromatography. The purified products were characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry; m/z (MALDI-TOF) for AFPP-TPE(A); found 2835.5 (calculated 2832.3), for AFPP-TPE(B); found 2891.5 (calculated 2888.4), AFPP-TPE(C); found 2976.7.5 (calculated 2976.5).

3. Results and Discussion

To test the promoting effect of AFPP-AIE probes on the aggregation of Aβ (1-40), the CD spectra of the mixture of 10 μM Aβ (1-40) and 50 μM AFPP-TPE (A, B, or C) probes were observed at hourly intervals. After 3 h incubation, the CD analysis of Aβ and the TPE(A) probe displayed a random coil secondary structure. This result meant that the steric hindrance of the TPE framework of the probe prevented the formation of a β-sheet structure. Therefore, the TPE(B) probe with the longer linker between AFPP and TPE was used. In this case, the CD signal due to the β-sheet was initially observed, but it gradually disappeared, and some fluorescent precipitates were observed after 24 h. The possible cause of these results was possibly the strong hydrophobicity of TPE and the linker moiety. Thus, the next approach we used was the introduction of hydrophilic functional groups in the TPE frameworks, such as TPE(C). Immediately after mixing of 10 μM Aβ (1-40) and 50 μM AFPP-TPE(C), the CD spectra displayed a random coil structure in the N-ethyl morpholine (EM) buffer (20 mM EM, 150 mM NaCl, pH 7.0), changing to β-aggregate with an ellipticity minimum at 220-225 nm after 3 h (Fig. 1). This result indicates that the AFPP-TPE(C) probe was capable of inducing the aggregation of Aβ (1-40) similar to AFPP itself [28]. Thus, AFPP-TPE(C) was used for subsequent investigations.

Fig. 2 shows the fluorescence spectra of 0.25 μM AFPP-TPE(C) and 0-5 μM Aβ (1-40). Since the fluorescent signal intensity was increased with increasing concentrations of Aβ (1-40), this AIE-based probe was applicable in principle to quantify Aβ (1-40).

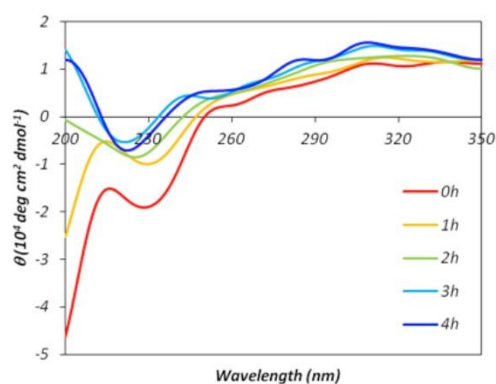


Fig. 1 Time-dependent changes in CD spectra of 50 μM TPE(C)-AFPP with 10 μM Aβ.

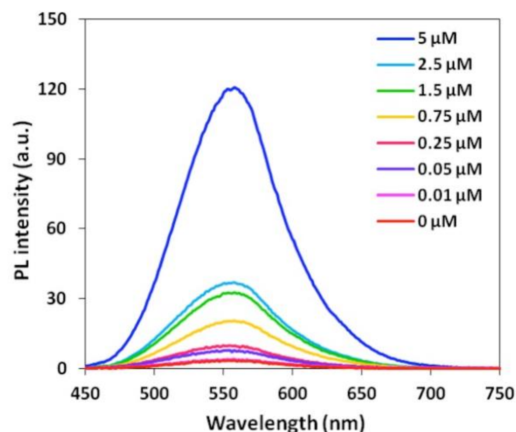
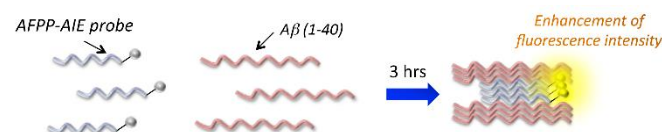


Fig. 2 Fluorescent spectra of a mixture of 0.25 μM AFPP-TPE(C) and 0-5 μM Aβ (1-40). Each sample was left standing for 3 h at 25 °C in EM buffer (pH 7.0) and then measured.

The principle of Aβ (1-40) detection is illustrated in Scheme 1. Without target Aβ (1-40), AFPP-TPE(C) probes were uniformly dispersed in buffer solution; therefore, the fluorescence was weak. However, when Aβ (1-40) was added to the AFPP-TPE(C) solution, the aggregation of Aβ (1-40) was promoted with the action of AFPP, and the fluorescence was enhanced with the simultaneous aggregation of AIE dyes.



Scheme 1 Schematic image showing the principle of Aβ detection with AFPP-AIE.

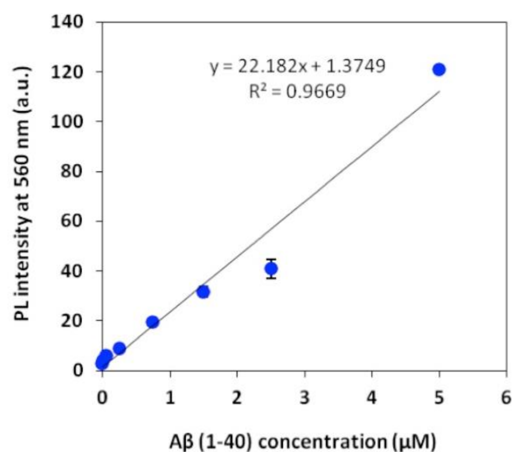


Fig. 3 The relationship between Aβ (1-40) concentration and fluorescent intensity. The standard deviation of the blank (σ) was 0.353. Error bars indicate the standard error of the mean for n = 3.

According to the fluorescence measurements, the fluorescent intensity increased linearly with a linear increase in Aβ (1-40) concentration in the range of 0-5 μM. The LOD, estimated by three times the standard deviation of the blank (3σ), was 47.7 nM for the target Aβ (1-40) (Fig. 3). Unfortunately, the obtained LOD value was insufficient for the quantification of Aβ (1-40) in cerebrospinal fluid (8-11.9 ng/mL) or plasma (213-290 pg/mL) [17], so different concentrations of probe were investigated to enhance detection sensitivity.

To improve the sensitivity of the fluorescence-based analysis, the fluorescent probe concentration was increased. However, in this case, increased probe concentration caused aggregation of the probe itself and consequently the background fluorescence increased. Thus, the use of higher concentrations of the probe was found not suitable for Aβ detection. Because of these considerations, the fluorescent spectra were measured at lower Aβ (1-40) concentrations (0-500 nM) in the presence of 0.5 μM AFPP-TPE(C) probe.

Fig. 4 shows the fluorescence spectra and the relationship between Aβ (1-40) concentration and fluorescence intensity. As shown in Figure 4b, the fluorescence intensity shows a good linear correlation (R^2 = 0.9785) with the Aβ (1-40) concentration in the range of 0-75 nM. The LOD value of 4.2 nM was improved and was better than those of a previously reported capillary electrophoresis assay (0.5 μM) [29], electrochemical assay (0.75 mg/mL) [30], and our previous report (18 nM) [28]. Though there are some methods with better LOD, such as a sol-gel-derived optical array biosensor (2.5 ng/mL) [31] and an electrochemical immunoassay (5 pM Aβ (1-42)) [32], the present "simple-add-and-measure" methodology has several advantages with regard to speed, simplicity, and cost effectiveness, and does not require highly skilled laboratory technicians and sophisticated equipment.

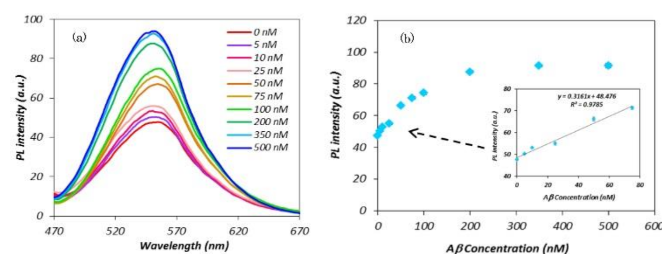


Fig. 4 Fluorescence spectra (a) and the relationship between Aβ and fluorescence intensity (560 nm) (b) of the mixture of 0.5 μM AFPP-TPE(C) and 0-500 nM Aβ (1-40). Each sample was left standing for 3 h at 25 °C in EM buffer (pH 7.0) and then measured. Error bars indicate the standard error of the mean for n = 3.

To investigate the selectivity of the AFPP-TPE(C) probe, we used amylin instead of Aβ (1-40). Amylin is a 37mer peptide that forms amyloid fibrils. Fig. 5 shows the relationship between peptide concentration and fluorescent intensity. The fluorescent intensities of Aβ (1-40) and amylin were both increased with increased peptide concentration; however, the rate of increase was larger for Aβ (1-40) than amylin. Thus, the AFPP-TPE(C) probe exhibited moderate selectivity.

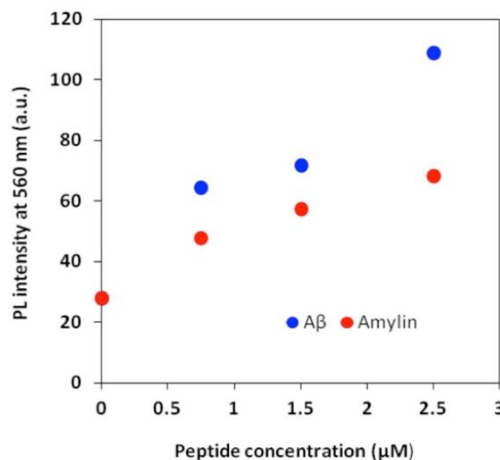


Fig. 5 The relationship between peptide concentration and fluorescent intensity. Each sample was left standing for 3 h at 25 °C in EM buffer (pH 7.0) and then measured.

4. Conclusion

In the present report we show a novel Aβ (1-40) detection method based on aggregate-induced emission. The procedure consists of just two steps: 1) adding an AFPP-TPE(C) probe solution to the sample containing Aβ (1-40) and incubating for 3 h; 2) measuring fluorescent intensity. This method provides a simple, rapid, sensitive and cost-effective assay for Aβ (1-40). The LOD value (4.2 nM) is insufficient with levels of Aβ (1-40) found in cerebrospinal fluid or plasma; however, the simplicity of the procedure is of great advantage. Thus, we believe that this novel Aβ detection method will be valuable for the convenient diagnosis of AD, but further improvement in Aβ sensitivity is need.

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