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Discovery of a Novel Fluorescent Probe for the Sensitive Detection of β -Amyloid Deposits

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Abstract

Here we reported the development of the first photoinduced electron transfer (PeT) probe (**1**) to directly locate β -amyloid aggregates ($A\beta$ plaques) in the brain without the need of post-washing procedures. The probe showed a high affinity for $A\beta$ aggregates with a K_d value of 3.5 nM. It is weakly emissive by itself with its fluorescence quenched by electron transfer from PeT donor to the excited fluorophore. But selective binding to $A\beta$ plaques would attenuate the PeT process and restore the fluorescence, therefore facilitating the tracking of $A\beta$ plaques. The probe is advantageous in that its fluorescence is environment-less-sensitive and no washing procedure is required to provide high contrast fluorescent signal when applied to stain brain tissues. As a proof of concept, its application has been exemplified by staining $A\beta$ plaques in slices of brain tissue from double transgenic (APP/PS1) mice of Alzheimer's disease.

Keywords

fluorescent probe; imaging; $A\beta$ plaques; Alzheimer's disease; photoinduced electron transfer

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Appendix

Synthesis of probe **1** and control compounds, their characterization data and NMR traces, photophysical property measurement methods, supporting figures, can be found in the supplementary information.

CCDC 1057219 (**1**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

1. Introduction

Alzheimer's disease (AD) is one of the leading threats to people's wellbeing, especially in the background of global aging. Although the precise molecular mechanisms causing AD remain unclear, the accumulation and deposition of β -amyloid (A β) peptides in the brain is generally regarded as the driven force of AD progression, according to the amyloid hypothesis (Glenner and Wong, 1984; Hardy and Higgins, 1992; Hardy and Selkoe, 2002). A β is the major component of extracellular plaques found in post-mortem brains of AD patients. It is reported that A β plaques develop many years before the onset of dementia and are present in the cortical gray matter in all AD cases (Goedert and Spillantini, 2006). As one of the principal hallmarks of AD (Panza et al., 2014), A β plaques are also viewed as a good diagnostic and predictive biomarker (Nesterov et al., 2005; Zhang et al., 2013; Cui, 2014; Hintersteiner et al., 2005; Harada et al., 2014; Qin et al., 2010). Their detection would be a powerful strategy for the early diagnosis and the progression prediction of AD, as well as the efficient monitoring of novel anti-A β drugs (Ono et al., 2012; Cui et al., 2014). Numerous researches have been devoted to the development of radiolabeled A β imaging probes over the past decade for positron emission tomography (PET) or single photon emission computed tomography (SPECT), which resulted in the approval of Florbetapir, Florbetaben and Flutemetamol (all three labeled with positron emitter ^{18}F) by the FDA and EMA as A β imaging probes, and several others under intense clinical trials (Camus et al., 2012; Choi et al., 2009; Yang et al., 2014; Koole et al., 2009; Catafau and Bullich, 2015). As an alternative to isotope imaging, fluorescent imaging is nonradioactive and low cost, but exhibits comparable sensitivity and can also track biomolecules in living organisms in real-time. Fluorescent imaging therefore has attracted great research interest in recent years (Guo et al., 2014; Escobedo et al., 2010; Weissleder et al., 1999; Cui et al., 2014; Kim et al., 2015). Indeed several A β fluorescent probes have been developed and utilized for *in vitro* and *in vivo* A β detection during the past decade (Fig. S1, Table S1) (Raymond et al., 2008; Lee et al., 2014; Voropai et al., 2003; Okamura et al., 2011). These probes were generally designed by the covalent conjugation of an electron-deficient fluorophore with a styryl bearing an electron donating *N*-methylamine group. They are usually weakly emissive in aqueous media due to the strong polarization effect in the excited state, a phenomenon known as intramolecular charge transfer (ICT) (Boens et al., 2012; Rurack and Resch-Genger, 2002). After binding to the hydrophobic pockets of aggregated amyloid plaques, their fluorescent intensity will increase remarkably as a result of the restriction of conformation mobilization. Although being sensitive with the fluorescent intensity enhancement, the downside of this kind of ICT-based probes is that their fluorogenic properties are environment-sensitive and all hydrophobic environments could trigger on their fluorescence, which means that elaborate washing procedures are necessary in order to minimize background signals. Therefore new probes with novel sensing mechanisms are still under urgent demand.

Photoinduced electron transfer (PeT), in which electron transfer from the PeT donor to the excited fluorophore attenuates the fluorescence of the latter, is another widely applied mechanism for developing small-molecular fluorescent probes. For a given fluorophore, quenching efficiency by PeT is usually governed by the oxidation potential of the PeT donor

and the distance between the donor and the fluorophore (Singh et al., 2000; Le et al., 2000; Lewis et al., 1997). Since PeT efficiency is less sensitive to surrounding polarities, high contrast signal between the target and its background can usually be guaranteed. PeT therefore represents a more desirable mechanism for the development of fluorescent probes to stain biomolecules. Herein, we describe the discovery of the first PeT-based probe **1** for the sensitive staining of A β plaques without washing procedures. The probe is weakly emissive by itself, whereas binding to A β aggregates would restore its fluorescence and therefore "shed light on" the precise location and even quantification of this AD biomarker. Moreover, the quenched emissive property of the probe before binding to A β plaques is insusceptible to the nature of its surroundings, a feature superior to all currently available A β fluorescent probes because it makes staining brains tissues without washing feasible.

2. Materials and methods

2.1. Chemistry

Information on the synthesis of probe **1** and other control compounds was detailed in the supporting information.

2.2. Biology

2.2.1. A β aggregates preparation—A β_{1-42} peptide (1 mg) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), and aliquoted to 10 samples. The samples were placed at room temperature for 12 h until HFIP evaporated. Then they were dried up in vacuum for 2 h and stored at -20°C . A 50 μM stock solution was made by re-dissolving the peptide (0.1 mg) with 11 μL DMSO, followed by 432 μL phosphate buffer solution (PBS, pH=7.4). A β aggregates were prepared by diluting the stock solution to 25 μM with PBS and shaking for 24 h at 37°C . The samples were confirmed structurally by transmission electron microscopy (TEM) (Fig. S2).

2.2.2. K_d measurement—Thioflavin T test was employed to measure the K_d of probe **1** (Ran et al., 2009; Zhang et al., 2013; Zhu, et al., 2015; Malisauskas, et al., 2015). The fluorospectrometer was kept at: Ex slit =10 nm, Em slit =10 nm, PMT Voltage =700, Excitation = 420 nm, Emission= 450–800 nm. Stock solutions were prepared as follows: A β aggregates stock solution in PBS (25 μM) was prepared as above mentioned; Thioflavin T (ThT) was dissolved in PBS to make 25 μM stock solution; probe **1** was dissolved in DMSO to make 0.5 μM stock solution.

Record 1.0 mL PBS emission spectrum (blank). To the above PBS solution, add 10 μL ThT (final ThT conc. 250 nM) and record the emission spectrum of the above solution. Then to the ThT solution, add 10 μL A β aggregates (final A β Conc. 250 nM), record the emission spectrum of the above solution. To the above ThT+A β solution, add 1 μL probe **1** solution and stir the mixture with pipette. Record the emission spectrum of the above solution when the spectrometer provides a steady reading. To the ThT+A β +probe **1** (0.5 nM) solution, add 2 μL probe **1** solution and stir the mixture with pipette. Record the emission spectrum. Repeat the process by adding another 2, 2, 3, 10, 20 μL probe **1** sequentially and record the emission spectrum after each addition to get the emission spectra of the ThT+A β +probe **1**

solution with the concentrations of probe **1** being (2.5, 3.5, 5.0, 10, 20 nM). Read the fluorescent intensity (FI) at 505 nm. Since the binding of A β plaques by probe **1** decreased the amount of A β plaques available for Thioflavin T, the A β plaques-binding-induced fluorescent intensity enhancement of Thioflavin T at 505 nm decreased accordingly (λ_{ex} 420 nm, which is fortunately a weak excitation band for probe **1**). Calculate these changes and plot FIs *versus* probe **1** concentration. Calculate K_d with PRISM software (non-linear regression, one site-binding).

2.2.3. In vitro fluorescent staining of A β plaques in transgenic mouse brain sections—Paraffin-embedded brain tissue from double transgenic mice (APPsw/PS1De9, 12 months old, male) were used for *in vitro* fluorescent staining. The brain sections were roasted at 60°C for 60 min and then deparaffinized with 3 $\times$ 10 min washes in xylene, 2 $\times$ 3 min washes in 100% ethanol, 1 min wash in 95% ethanol/H₂O, 1 min wash in 75% ethanol/H₂O, 1 min wash in 50% ethanol/H₂O, 2 $\times$ 2 min washes in dd water and 2 $\times$ 2 min washes in PBS (pH=7.4).

For washing & no washing assay, the brain sections were incubated with 100 μ M probe **1** or BODIPY acid **2** for 10 min. Next, the sections treated with **2** were washed with 20% ethanol for 5 min followed by washing with PBS for another 5 min. Finally, all the brain sections were incubated with anti-quenching agent and covered with coverslips. Fluorescent observation was performed using IX81 FV1000 Lase Confocal Microscope (Olympus).

For co-localization assay, the brain sections were immersed in citric acid solution (pH = 6.0) and then heated to boil in microwave oven. The boil was kept for 10 min. Then the brain sections were moved to the fume for cooling and washed with 3 $\times$ 5 min PBS. Next, the brain sections were blocked for 30 min and incubated with primary A β antibody ab2454 (Cell Signaling, dilution ratio 1:500) at 4°C overnight. The sections were washed with 3 $\times$ 5 min PBS and incubated with secondary antibody with fluorophore Alexa 546 (Invitrogen, dilution ratio 1:200) for 60 min. After washes with 3 $\times$ 5 min PBS, the experiment was continued according to the steps in washing & no washing assay.

3. Results and discussion

3.1. Probe design and synthesis

Most current available fluorescent probes for staining A β plaques are environment-sensitive π -extended dipolar dyes and their quantum yields are highly dependent on the surrounding polarity. They are poorly fluorescent in aqueous solution because of energy decay through nonradiative pathway. While after binding to the hydrophobic pockets of aggregated amyloid plaques, their conformation mobilization is greatly restricted and nonradiative energy decay is thereby restricted, accompanied by the increase of their quantum yields. Since brightening is the result of the hydrophobic environment, other hydrophobic proteins may trigger nonspecific staining and post washing procedures are necessary to ensure high signal-to-background ratios. To address this problem and develop environment-less sensitive probes, we designed probe **1** (Fig. 1.). We reasoned that 1) borondipyrromethene (BODIPY) fluorophore may bind to A β plaques with potent affinity attributing to its hydrophobic skeleton, as it has been viewed that hydrophobic planarized π system is an important design

feature to obtain amyloid plaques markers (Nesterov et al., 2005); 2) since the quantum yields of BODIPY dyes are less environment-sensitive (Loudet and Burgess, 2007), so would be probe **1**; 3) electron-rich aniline in the *meso*-position of BODIPY core would quench its fluorescence *via* photoinduced electron transfer from the donor to the BODIPY core (Boens, 2012); 4) Since PeT efficiency is greatly dependent on the donor-acceptor distance (Ratner, 1990; Wasielewski, 1992; Lewis et al., 1997; Anderson et al., 2003), a C3 alkyl linker is then employed to adjust PeT efficiency before and after probe **1** binding to A β plaques.

3.2. Probe synthesis

As shown in Scheme 1, probe **1** was facily synthesized starting with BODIPY acid **2** through a 3-step procedure. Reduction of **2** using borane in THF gave alcohol **3**. Oxidation of **3** using pyridine sulfur trioxide in DMSO/DCM gave aldehyde **4**. Borch reduction of **4** with *N*-methylaniline yielded probe **1** as a red powder. Structure of probe **1** was confirmed by NMR, MS spectra as well as X-ray crystallography.

3.3. Binding affinity of probe **1** to A β aggregates

With probe **1** in hand, we first tested its binding affinity towards A β aggregates and K_d measurement based on Thioflavin T test was employed (Ran et al., 2009; Zhang et al., 2013; Zhu, et al., 2015; Malisauskas, et al., 2015). As shown in Fig. S3, probe **1** exhibited a K_d value as low as 3.5 nM, ensuring its potential application to stain A β aggregates.

3.4. Photophysical properties of probe **1**

Having confirmed the high affinity of probe **1** for A β aggregates, we next examined its photophysical properties. As shown in Fig. 2a, its UV-vis spectrum showed a strong absorption band centred at 497 nm, comparable to its parent compound aldehyde **4**. While unlike **4**, which emits strongly at 505 nm, probe **1** remains gloomy under the excitation of 485 nm (Fig. 2b), and the decrease of solvent polarity would not help to increase its emission intensity (Fig. 2c), indicating the effective quenching of BODIPY fluorophore by PeT from the electron-rich aniline group.

3.5. Probe **1** could stain A β in PBS

Next, we evaluated whether binding to A β_{1-42} aggregates would trigger a fluorogenic switch-on response of probe **1**. As shown in Fig. 2d, significant increase of fluorescent intensity was observed for probe **1** treated with A β_{1-42} aggregates and the increase was positively dependent on the concentration of A β_{1-42} aggregates. Since it has been confirmed that decrease of environment polarity would not help to increase the fluorescent intensity of probe **1**, we reasoned that the fluorogenic response of probe **1** towards A β_{1-42} aggregates was due to binding-induced conformation change of the probe, and therefore the distance between the aniline group and the BODIPY core was extended a little more to exceed the distance threshold for efficient PeT process.

3.6. Detection mechanism study

It has been well-recognized that quenching efficiency in PeT greatly depends on the distance between the fluorophore and the donor (Le et al., 2000; Lewis et al., 1997). When the donor is covalently linked to the fluorophore *via* a flexible linker, the distance between them is mainly determined by the steric effects. Based on these facts, we hypothesized that increase of steric hindrance between the BODIPY core and the aniline would in turn elongate their distance, and the elongation, though tiny, would be good enough to dramatically decrease the PeT efficiency (Le et al., 2000; Lewis et al., 1997). To test this hypothesis, we studied the impact of increased steric hindrance between BODIPY and the aniline on the fluorescent intensity. Since it is not so chemically straightforward to prepare BODIPY fluorophores with branched substituents due to their challenging synthesis, we turned to prepare a series of anilines with substituents of various size (Fig. 3). As hypothesized, the quenching effect decreased as the steric hindrance increased, and significant fluorescent intensity remained for compound **8** bearing two branched *iso*-propyl groups. This result supports our argument that the fluorogenic response of probe **1** towards A β aggregates was due to binding-induced conformation change of the probe. In detail, the distance between BODIPY core and the aniline in freely-existing probe **1** is near enough for efficient electron transfer from the electron rich aniline to the excited BODIPY fluorophore to quench the latter. While in the presence of A β plaques, BODIPY would bind to A β plaques with high affinity, increasing the steric hindrance between the A β -binding-BODIPY and the aniline, and therefore elongating their distance beyond the threshold for efficient PeT, which is accompanied by a dramatic fluorescent intensity enhancement of probe **1**.

3.7. Probe **1** could stain A β plaques in brain tissues

Having confirmed the fluorescent switch-on response of probe **1** to A β aggregates, we then tested its feasibility to stain A β plaques in slices of brain tissues from double transgenic mice (APPsw/PS1De9, 12 months old, male), for whom A β plaque deposition starts at approximately six weeks of age in the neocortex (Radde et al., 2006). As shown in Fig. 4a, high-contrast fluorescent spots were observed after staining cerebral cortex with the probe. Those spots were confirmed to be A β plaques by co-localization assay with A β primary antibody ab2454 and secondary antibody with fluorophore Alexa 546 (Fig. 4b, 4c, co-localization in prefrontal cortex and hippocampus was shown in Fig. S4, S5 in Supporting Information).

To further ensure that the fluorescents witch-on response of probe **1** in slices of brain tissue was exclusively due to its binding to A β plaques but not to other biomolecules, negative control experiments were conducted by staining brain slices from wild-type mice where A β plaques are absent. It turned out that no spots were observed on the brain sections from wild-type mice, as compared to the obvious spots observed on the brain tissues from the double transgenic mice (Fig. S6). Moreover, other species involved in AD pathogenesis, including Zn²⁺, Cu²⁺, Fe²⁺ and Fe³⁺ ions (Budimir, 2011) or reactive oxygen species (Uttara et al., 2009; Opazo et al., 2002) such as H₂O₂, hydroxyl radical which are common suspects for AD development were tested (Fig. S7). It turned out that none of these species were able to trigger on the fluorescence of **1** as A β plaques did. All these results revealed the great specificity of probe **1** for A β plaques.

More importantly, unlike other A β staining agents that need subsequent washing procedures to minimize background signals, as illustrated by green fluorescent dye Thioflavin-S (ThS) (Fig. 4d) (Lewis et al., 2001) and NIR fluorescent dye CRANAD-58 (Fig. 4e) in our control experiments, probe **1** is unique in that high signal-to-background ratios were still observed even without washing procedures, indicating that our method is much more convenient for staining of A β plaques *in vitro* than ThS and CRANAD-58 which resulted only in undistinguishable signals without washing (Fig. 4d, 4e). Interestingly, BODIPY acid **2** could also be used to stain A β plaques, but only after careful washing (Fig. 4f), suggesting that BODIPY core itself, as hypothesized in the design section, can bind A β . All these results proved the simplicity of probe **1** to stain A β plaques and its advantage over environment-sensitive probes.

4. Conclusions

In conclusion, we have discovered a novel probe for the specific staining of A β plaques. The probe has following features: (1) high-affinity binding to A β plaques, (2) fluorescent switch-on after binding, and (3) being environment-less-sensitive and no washing procedures required when applied to stain brain tissues with high signal-to-background ratios, as guaranteed by its PeT-based fluorescence quenching mechanism. The feasibility of the probe in practical application has been exemplified by staining A β plaques in slices of brain tissue from double transgenic mice and its unique quality of no washing stain has been well illustrated in comparison with ThS and CRANAD-58. The focusing on bathochromic shifting the emission wavelength of probe **1** to NIR region on the premise of retaining its high A β binding activity is undergoing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Structure of probe **1** and our design rationale.

Fig. 2.

a) Absorption spectra of probe **1** (5 μM) and aldehyde **4** (5 μM) in ethanol; b) Fluorescent spectra of probe **1** (5 μM) and aldehyde **4** (5 μM) in ethanol, excitation at 485 nm, slit width: 3/3 nm; c) Fluorescent intensity at 505 nm of probe **1** (0.5 μM) and aldehyde **4** (0.5 μM) in different solvents. Excitation at 485 nm, slit width: 3/3 nm; d) Fluorescent spectra of probe **1** (5 μM) upon interaction with Aβ₁₋₄₂ aggregates in PBS (pH 7.4), excitation at 485 nm, slit width: 3/5 nm.

Fig. 3.

a) Derivatives of BODIPY dyes for steric effect study; b) Fluorescent spectra of **5–8** (5 μM) in ethanol, excitation at 485 nm, slit width: 3/3 nm; c) Fluorescent intensity at their emission maxima of **5–8** (5 μM) in ethanol.

Fig. 4.

Fluorescent staining of A β plaques in transgenic brain sections (cerebral cortex) from double transgenic mice (APPsw/PS1De9, 12 months old, male) by laser confocal microscope. (a) Staining with probe **1** without washing; (b) Staining primary with A β antibody ab2454 then secondary antibody with fluorophore Alexa 546; (c) Merged images of a and b; Magnification: left –10 $\times$, right –40 $\times$ showing the region outlined in 10 $\times$ image. (d) Staining with ThS by washing (right) and no washing (left) assays; (e) Staining with CRANAD-58 by washing (right) and no washing (left) assays; (f) Staining with BODIPY acid **2** by washing (right) and no washing (left) assays. Washing and no washing pictures were taken under the same instrument parameters. Scale bar: 200 μ m.

Scheme 1.

Synthesis of probe 1: a) $\text{BH}_3 \cdot \text{THF}$, 0°C , 30 min, 26%; b) $\text{Py} \cdot \text{SO}_3$, TEA, DMSO, ambient temperature, 45 min, 37%; c) *N*-methylaniline, NaBH_3CN , CH_3COOH (cat.), ambient temperature, 6 h, 65%.