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Detection of Aβ plaques in mouse brain by using a disaggregation-induced fluorescence-enhancing probe†

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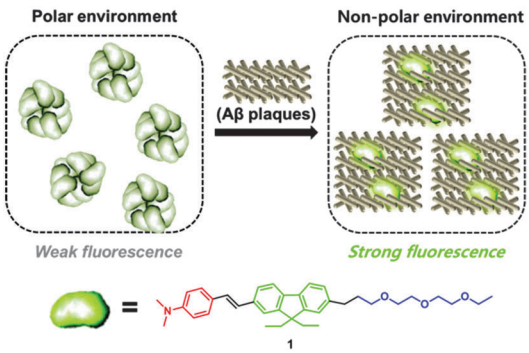
We herein report a fluorescence probe 1 capable of detecting water-soluble oligomeric Aβ aggregates and Aβ fibrils. Upon injection into $Aβ_{42}$-challenged mouse brains, probe 1 shows increased fluorescence intensity, indicating its facile binding to extracellular Aβ fibrils in brain tissues.

Amyloid-β (Aβ) peptide is a primary component of extracellular plaques associated with Alzheimer's disease (AD). Aβ is generated through proteolytic cleavage of the amyloid peptide precursor (APP) by β- or γ-secretase.[1] For some decades, insoluble Aβ peptide plaques have been believed to be the main contributors to the pathogenesis of AD.[2] Therefore, many efforts have been made, such as the development of a wide variety of fluorescent probes to selectively visualize plaques in brain tissues.[3] The visualization is based on the fluorescence of the probe that is "turned on" during its transfer from the polar and random aqueous environment into the hydrophobic and highly ordered amyloid β-sheet structure of plaques.[4] Fluorescence enhancement of the probe may be due to rotational bond restriction of the probes and/or the hydrophobic environment in plaques compared to aqueous media.[5]

Aggregation of hydrophobic fluorescent materials generally results in reduced quantum yield.[6] The fluorescence intensity of the probes, however, can be markedly increased following their disaggregation,[6] *e.g.*, with nonpolar peptide monomers.[7] Moreover, upon interaction of aggregated fluorescent probes with hydrophobic plaques, it is expected that the probes are disaggregated followed by their distribution over the plaque surfaces and homing to binding sites, which results in the increased fluorescence intensity of the probes. To the best of our knowledge, this type of approach has not been reported with regard to the detection of Aβ plaques or water-soluble oligomeric Aβ species in brain tissues.



Scheme 1 Fluorescence enhancement upon binding of probe **1** to amyloid β (Aβ) plaques.

We prepared a new probe, **1**, that showed disaggregation-induced fluorescence enhancement and was capable of detecting both water-soluble oligomeric Aβ species as well as insoluble Aβ aggregates as indicated in Scheme 1. Probe **1** is comprised of three components: (i) an *N*,*N*-dimethylamino styrene unit that is able to bind to β-sheets of Aβ aggregates, (ii) a hydrophilic triethylene glycol unit to enhance the water solubility, and (iii) fluorene as an "Off–On-type" fluorescence signaling unit upon intercalation of the *N*,*N*-dimethylamino styrene unit with oligomeric Aβ aggregates or Aβ fibrils.[8,9]

Probe **1** was prepared according to the synthetic route shown in Scheme 2. Compound **3**, 9,9-diethyl-2-iodofluorene, was synthesized from **2** by adaptation of procedures reported earlier.[9] Then, **3** was converted to **4** by the Sonogashira coupling reaction with 3-(2-(2-ethyoxyethoxy)ethoxy)-1-propyne (35% yield). Subsequently, hydrogenation of **4** using Pd/C in ethanol gave **5** in 86% yield. Iodination of **5** with I_2/HIO_4 provided **6** in 59% yield. Finally, the Pd-catalyzed Heck reaction of **3** with *N*,*N*-dimethylaminostyrene gave probe **1** in 28% yield. The identities of all newly synthesized compounds were confirmed by ^{1}H NMR, ^{13}C NMR, and ESI-MS (ESI†).

Probe **1** in phosphate-buffered saline (PBS) exhibited absorption and emission bands with maxima at 412 and 462 nm, respectively, as shown in Fig. 1a. The fluorescence spectral properties changed

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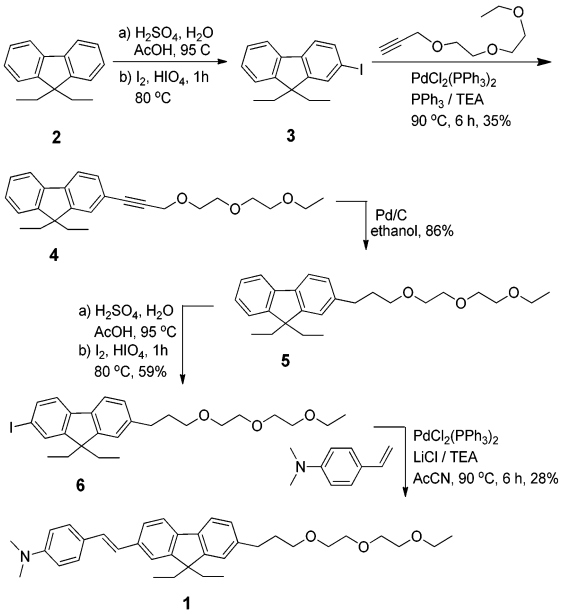

Scheme 2 Route for the synthesis of probe **1**.

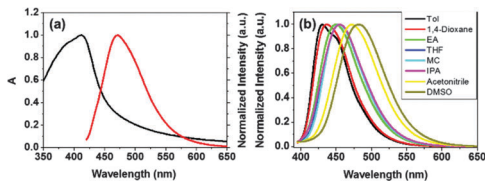

Fig. 1 (a) Absorption and fluorescence spectra of probe **1** (2 μM) in 0.2% (v/v) DMSO in PBS (pH 7.4), excitation at 412 nm, slit width = 5/5 nm. (b) Fluorescence spectra of probe **1** in different solvents.

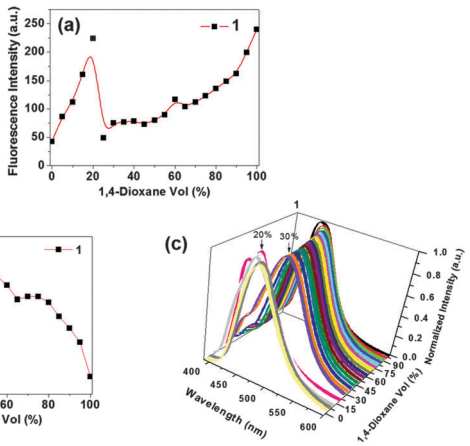

Fig. 2 (a) Plot of the fluorescence intensity of probe **1** at λ_{em} = 449 nm. (b) The maximum wavelength (λ_{max}) of probe **1** in aqueous 1,4-dioxane solutions, excitation at 367 nm. (c) The normalized fluorescence spectra of probe **1**, 3D view. [probe] was 1 μM.

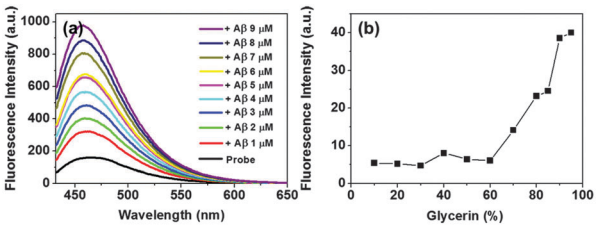

Fig. 3 (a) Fluorescence intensity of probe **1** (1.0 μM) upon interaction with $A\beta_{42}$ oligomers in 0.2% (v/v) DMSO in PBS (pH 7.4) at λ_{ex} = 412 nm, slit width: 3/5 nm. (b) Emission intensity of probe **1** in water containing varying amounts of glycerol (v/v, %).

significantly in different solvents (Fig. 1b), *i.e.*, the intensity increased with decreasing solvent polarity (Table S1, ESI†), resulting in a significant blue shift. This blue shift is the result of a solvatochromic shift that is due to an intramolecular charge transfer between the donor (aniline) and the acceptor (fluorene) across the ethylene spacer.

To understand the photophysical properties of probe **1** due to the solvent species used, the spectra of probe **1** were measured in water containing varying amounts of 1,4-dioxane, and fluorescence intensity, maximum wavelength (λ_{max}) and the normalized spectra are shown in Fig. 2. As shown in Fig. 2a, the profile of 1,4-dioxane percentage in water *versus* the probe's fluorescence intensity has three phases: (i) 0–20%, where it increases; (ii) 20–25%, where it rapidly decreases; and (iii) 30–100%, where it gradually increases. Similarly, the profile of λ_{max} in the solution (Fig. 2b) displays three phases in the percentage ranges of 1,4-dioxane, each of which shows a blue, red, and blue shift, respectively. A similar three phasic behaviour of the solvatochromic fluorescence properties of the probe was found using acetonitrile (Fig. S1, ESI†). The blue shifts of the spectra with increasing intensity found in the 0–20% and 30–100% ranges of the 1,4-dioxane may be attributed to the reduced solvent polarity.[7]

The initial two phases in the 0–25% range of 1,4-dioxane shown in Fig. 2a and b reveal quite dramatic changes in the intensity and λ_{max}. These changes occur due to the self-aggregation nature of probe **1** in 100% aqueous solution. The aggregation of probe **1** is increased and a blue shift of the emission has been observed by up to 20% of 1,4-dioxane due to its decreased polarity. Then it was solubilized in the solution showing a dramatic red shift and decreased fluorescence intensity, which has a relatively polar environment compared to that on the inside of the aggregates. These results indicate that the probe exists in an aggregated form with lower fluorescence intensity in aqueous environments and shows strong fluorescence emission upon solubilisation. Similarly, we anticipate disaggregation of probe **1** upon binding to amyloid oligomers or plaques in aqueous environments. To test whether probe **1** can detect water-soluble oligomeric $A\beta_{42}$ aggregates, changes in the fluorescence intensity of probe **1** were investigated in the presence of $A\beta_{42}$ oligomers. As shown in Fig. 3a, we observed a significant increase (6.5-fold) in the fluorescence intensity of probe **1** without a wavelength shift. To understand the origin of the increase in the emission intensity of probe **1** upon binding to $A\beta_{42}$ oligomers, we carried out two independent spectroscopic experiments using probe **1** in solvents of varying polarity and viscosity in the absence of $A\beta_{42}$ oligomers. It was previously reported that the fluorescence wavelength of an Aβ probe is blue-shifted when the probe is embedded inside of $A\beta_{42}$ oligomer aggregates, which represents a non-polar environment.[10] As shown in Table S1 (ESI†),

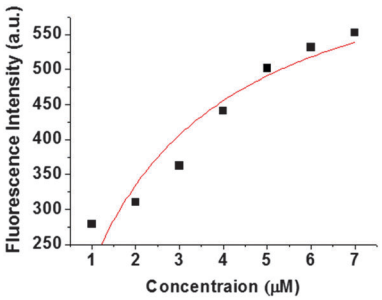

Fig. 4 Plot of the fluorescence intensity at λ_{max} = 467 nm as a function of the concentration of oligomeric $A\beta_{42}$ aggregates in the presence of probe **1** (1.0 μM) in 0.2% (v/v) DMSO in PBS (pH 7.4).

however, we did not observe any noticeable correlation of the increase in the fluorescence intensity of probe **1** with solvent polarity, but noticed wavelength shifts depending on solvent polarity.

Next, with regard to solvent viscosity, the changes in the fluorescence intensity of probe **1** were also investigated in water containing varying amounts of glycerol. It is well known that the fluorescence intensity increases markedly as the viscosity of the solvent increases because the intramolecular rotation of small molecules is considerably restricted.[11] We also found that the fluorescence intensity of probe **1** increases as the solvent viscosity increases (Fig. 3b). In this regard, it is conceivable that the interaction originates from a strong hydrophobic attraction between β-sheets of oligomeric Aβ aggregates and the *N,N*-dimethylaniline ring of the probe, which presumably leads to smoother intramolecular rotations, resulting in the increase in fluorescence intensity.[12]

Titration experiments were also conducted using probe **1** (1.0 μM) and oligomeric $A\beta_{42}$ aggregates in PBS in the concentration range of 1.0–7.0 μM. The results are shown in Fig. 4. From this graph, the binding constant (K_d) of probe **1** toward oligomeric Aβ aggregates was calculated to be 2.25 μM.

To investigate the specificity of probe **1** for oligomeric Aβ aggregates over other biocompatible peptides, probe **1** was tested with bovine serum albumin and lysozyme amyloid, and the corresponding fluorescence intensity changes were investigated. We could rarely find any changes in the fluorescence intensity of probe **1**, which confirms that probe **1** is specific for oligomeric Aβ aggregates, as shown in Fig. S2 (ESI†).

Another important objective was to test the ability of probe **1** to detect insoluble Aβ plaques, observed as changes in the fluorescence intensity. To do this, confocal microscopy was used and probe **1** and thioflavin S (ThS), the standard staining dye for Aβ plaques, were independently applied to HeLa cells. As illustrated in Fig. 5a, in HeLa cells incubated with ThS, ThS is mainly localized around the cell membrane. A previous report showed that ThS is located at the extracellular sites where it interacts with Aβ plaques.[10] In contrast to ThS, fluorescence signals of probe **1** were not detected around the cell membrane or inside the cells (Fig. 5b). On the basis of this observation, we conclude that probe **1** has the potential to interact with Aβ plaques, which are, in general, formed outside the cells.

To test the ability of the probe to detect Aβ plaques in living brain tissues, *ex vivo* experiments were carried out using male ICR mice. Under aseptic conditions, a burr hole (1.0 mm in diameter)

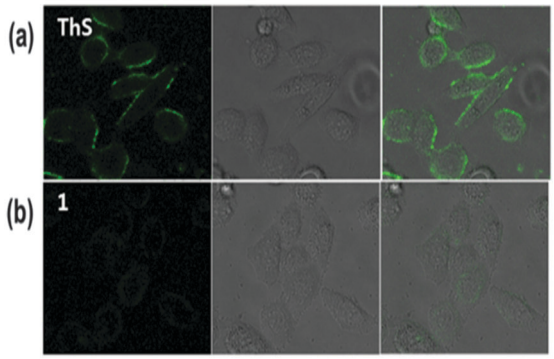

Fig. 5 Confocal microscopy images of HeLa cells treated with 1.0 μM ThS (a) and 1 μM of probe **1**. (b) Cells were incubated with DMEM at 37 °C and for 30 min with DMEM containing ThS and probe **1**, respectively.

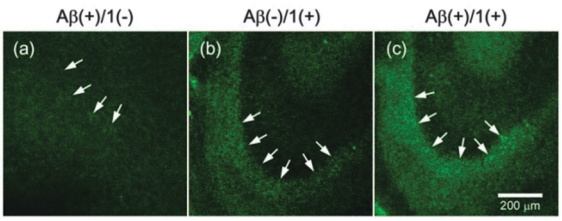

Fig. 6 Representative hippocampal sections of three groups. (a) Mice received a single injection of 3.0 μL of Aβ aggregates; (b) mice received a single injection of 3.0 μL of probe **1**; and (c) mice received consecutive injections of Aβ aggregates and probe **1** into the same region of the hippocampus.

was drilled in the skull (1.5 mm caudal and 2.0 mm lateral to bregma). Then, $A\beta_{42}$ aggregates and probe **1** were injected consecutively into mouse hippocampi. Coronal brain sections (50 μm-thick) were made using a freezing microtome. Incubation with $A\beta_{42}$ aggregates and/or probe **1** for 48 h was expected to cause sufficient accumulation of both $A\beta_{42}$ aggregates and probe **1** in mouse hippocampi. Analysis of the sections revealed that the fluorescence intensity of hippocampal sections increased upon treatment with probe **1** (Fig. 6b), which indicates that the probe is able to intercalate with Aβ aggregates. Subsequent addition of $A\beta_{42}$ aggregates induced a further increase in the fluorescence intensity (Fig. 6c), which again indicates that probe **1** could bind to Aβ aggregates in mouse hippocampi.

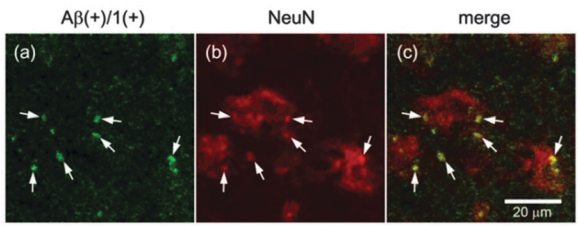

Fig. 7 *Ex vivo* fluorescence observations of sections from mouse brains. (a) After injection of probe **1** and Aβ aggregates; (b) neuronal cells were confirmed by immunohistochemical staining using NeuN; and (c) a fluorescence micrograph representing regions of overlap of fluorescence labeling of probe **1** and NeuN in brain tissues.

To confirm the regional distribution pattern of Aβ aggregates and probe **1** in brain tissues, neuronal cells of tissues treated with Aβ peptides and probe **1** were stained (Fig. 7). The brain sections were fixed and immunostained with NeuN, which is a marker of mature neurons (Fig. 7b). Fibril aggregates stained by probe **1** appeared as a granular smear (2.0 μm^2) throughout the hippocampal sections, as shown in Fig. 7a. Furthermore, the fluorescence signal of probe **1** rarely overlapped with that of NeuN, as shown in Fig. 7c, which indicates again that probe **1** does not enter neuronal cells but binds selectively to Aβ aggregates outside neuronal cells, resulting in increased fluorescence.

In conclusion, we synthesized a new probe, **1**, containing three important components, *i.e.*, an *N*,*N*-dimethylamino styrene unit, a hydrophilic triethylene glycol unit, and fluorene as a fluorescence signaling unit that is "switched on" upon intercalation with oligomeric Aβ aggregates or Aβ fibrils. Probe **1** was shown to be able to bind to water-soluble oligomeric Aβ species as well as insoluble Aβ aggregates. Probe **1** showed significantly increased fluorescence intensity upon binding to oligomeric $A\beta_{42}$ aggregates, which is attributed to the viscous environment of $A\beta_{42}$ oligomers. Probe **1** showed a binding constant (K_d) of 2.25 μM toward oligomeric Aβ aggregates. On the basis of fluorescence images, we found that probe **1** is localized outside the cells to bind to Aβ plaques and not in the cell membrane or cytosol. The results from *ex vivo* experiments showed that the fluorescence intensity observed in mouse hippocampal sections was increased upon treatment with probe **1**, which is due to the binding of probe **1** to Aβ aggregates. In brain tissues stained with NeuN, probe **1** was again confirmed to be distributed outside neuronal cells and to interact with extracellular Aβ fibril aggregates. These findings open a new strategy for the development of small molecules that are able to detect water-soluble oligomeric Aβ aggregates as well as Aβ fibril aggregates mostly found in patients with AD.

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