Advances in development of fluorescent probes for detecting amyloid-β aggregates

Ming-ming XU1, #, Wen-ming REN2, #, Xi-can TANG1, You-hong HU2, *, Hai-yan ZHANG1, *

1CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Science, Shanghai 201203, China; 2State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China

With accumulating evidence suggesting that amyloid-β (Aβ) deposition is a good diagnostic biomarker for Alzheimer’s disease (AD), the discovery of active Aβ probes has become an active area of research. Among the existing imaging methods, optical imaging targeting Aβ aggregates (fibrils or oligomers), especially using near-infrared (NIR) fluorescent probes, is increasingly recognized as a promising approach for the early diagnosis of AD due to its real time detection, low cost, lack of radioactive exposure and high-resolution. In the past decade, a variety of fluorescent probes have been developed and tested for efficiency in vitro, and several probes have shown efficacy in AD transgenic mice. This review classifies these representative probes based on their chemical structures and functional modes (dominant solvent-dependent mode and a novel solvent-independent mode). Moreover, the pharmaceutical characteristics of these representative probes are summarized and discussed. This review provides important perspectives for the future development of novel NIR Aβ diagnostic probes.

Keywords: Alzheimer’s disease; diagnostic biomarker; amyloid-β; optical imaging; near-infrared fluorescent probes

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Introduction

Alzheimer’s disease (AD) is a devastating and progressive neurodegenerative disease that leads to progressive cognitive decline, functional impairment and loss of independence; thus, AD represents a significant and increasing clinical challenge[1-3]. AD is characterized by two main hallmarks in the brain: the extracellular deposition of the amyloid-β (Aβ) in senile plaques and the appearance of intracellular neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau protein[2]. Today, more than 35 million people worldwide live with dementia and this number is expected to be more than triple to 115 million by 2050[3]. The heavy social costs and caregiving burden of AD have raised the attention for increasing research and potential therapeutics. However, the pathological mechanism of AD is elusive, and currently Food and Drug Administration (FDA)-approved medications, including four acetylcholinesterase inhibitors and one N-methyl-D-aspartate (NMDA) receptor antagonist, only provide temporary relief from symptoms. Additionally, none of these treatments halts the progression of this terminal disease. Therefore, early diagnosis seems to be a hopeful and promising approach to further the understanding and clinical treatment of this disorder. Currently, memory and behavioral tests are widely used for late-stage AD diagnosis[4], and a definitive diagnosis is achieved only by postmortem examination to show the presence of Aβ plaques and NFTs. Early-stage and asymptomatic diagnoses remain a challenge.

As a widely studied pathological hallmark of AD, the aggregation of fragmented Aβ proteins into Aβ plaques is closely connected with AD pathogenesis. Many risk factors in sporadic AD are associated with Aβ metabolism, and mutations of Aβ-related genes in familial AD lead directly to AD pathogenesis[5-19]. Clinical research[20-25] supports the hypothesis that there is a lengthy temporal lag between the appearance of Aβ plaques and the emergence of clinical symptoms. Sperling et al[26] proposed a biomarker model of the preclinical stage of AD in which Aβ accumulation first becomes abnormal and a substantial Aβ load accumulates before the appearance of clinical symptoms. As a consequence, the process of Aβ plaque formation, namely Aβ accumulation, has attracted growing attention as an important biomarker for early AD diagnosis. Moreover, Bruggink et al[27]
recently discovered an age-dependent Aβ oligomer increase in the cortex and hippocampus of transgenic mice (APP/PS1) along with elevated levels of soluble Aβ oligomers in human AD brain tissue compared with controls. Therefore, increasing evidence suggests that Aβ oligomers are the proximal toxicant forms underlying the AD pathogenesis that eventually leading to cognitive and behavioral disorders. Central Aβ oligomers, rather than Aβ plaques, are predicted to be a better potential marker for early AD diagnosis.

Based on the role of Aβ accumulation in AD diagnosis, the past decade has seen great progress in the development of imaging probes for the non-invasive detection of Aβ, mainly relying on techniques such as positron emission tomography (PET), single-photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI)\(^{28, 29}\). In particular, several PET probes have shown efficacy for imaging Aβ in AD brains. Among them, Florbetapir (\(^{18}F\))\(^{30, 31}\) was launched by Lilly in 2012 and Flutemetamol (\(^{18}F\))\(^{32, 33}\) was launched by GE Healthcare in 2014. However, factors such as low sensitivity of MRI probes, high cost, narrow isotope availability, the short-lived isotopes of PET probes and the radioactivity of SPECT probes have restricted their clinical use. Currently, clinically available PET imaging requires expensive instruments, highly skilled personnel, time-consuming data analysis and unavoidable radiation exposure. The cost of one PET examination is approximately $1000, which is a relatively high burden for the average family in developing countries.

Therefore, alternative technologies that are relatively inexpensive and easily applicable have recently attracted increasing attention. Optical imaging, especially near-infrared fluorescence (NIRF) imaging, when compared to PET, SPECT and MRI, is highly promising because it is real-time, inexpensive, not radioactive and of high-resolution both in vivo and ex vivo\(^{34}\). Additionally, unlike PET, NIRF imaging instrument costs are much lower, the data analysis takes less time, and highly skilled personnel are not needed. Optical imaging is an imaging technique using visible, ultraviolet, and infrared light for imaging. In particular, NIRF probes are favorable for Aβ detection in vivo because emission wavelengths in the NIR range (650–900 nm) are distinct from the autofluorescence of biological matter, which is especially suitable for in vivo applications. During the last decade, a variety of Aβ fluorescent probes have been developed and used to detect Aβ in vitro and in vivo. The current trend in technical developments indicates that Aβ fluorescence imaging is a potentially applicable technique that in the future could be feasible and widely used clinically.

**What makes an ideal Aβ fluorescent probe?**

In principle, an ideal fluorescent probe for Aβ should have the following properties\(^{35-38}\): (1) a high selectivity and a binding affinity for Aβ; (2) a high quantum yield and a significant change in fluorescence upon binding to Aβ; (3) a suitable emission wavelength greater than 450 nm to minimize background fluorescence from brain tissue, ideally between 650 nm and 900 nm for in vivo detection; (4) the ability to rapidly cross the blood-brain barrier (BBB); (5) a high metabolic stability; (6) fast washout kinetics from normal brain regions; (7) low toxicity; and (8) straightforward synthesis.

**Aβ fluorescent probes with classical push-pull architecture**

In general, the emission spectra and quantum yield of fluorophores are profoundly influenced by the environment and the solvent used\(^{39}\), especially the solvent polarity\(^{40}\). Most fluorophores are polar molecules, which contain both an electron-donating and an electron-accepting group\(^{41, 42}\) and usually display a large sensitivity to solvent polarity. Typically, fluorophores have a larger dipole moment in the excited state than in the ground state. The polar solvent can be more relaxed around the excited state than the ground state, which lowers the energy of the excited state\(^{43-45}\). The above-mentioned solvent effects are used to determine any probe-macromolecule interactions. When the fluorophore binds to the macromolecule, the emission spectra or quantum yields change after dissolution in solvents of different polarity\(^{46-48}\).

The existing Aβ fluorescent probes largely share a common structure, the classical push-pull architecture with terminal electron donor and acceptor moieties that are interconnected by a highly polarizable bridge. These probes are highly sensitive to solvent polarity and are essentially nonfluorescent in aqueous solutions, but highly fluorescent in nonpolar solvents or when bound to Aβ. In the following section, we introduce the different scaffold features of this mode.

**Thioflavin-T and its derivatives**

First introduced in 1959, the fluorescent dye thioflavin-T (ThT) (Figure 1, 1) has become a widely used “gold standard” for selectively staining and identifying amyloid fibrils both in vivo and in vitro\(^{49}\). When bound to amyloid fibrils, ThT shows enhanced fluorescence and a characteristic red-shift of the excitation and emission maxima from 350 and 438 nm to 450 and 482 nm\(^{50}\). The molecule of ThT consists of a benzothiazole (acceptor) and a benzylamine ring (donor) freely rotating around a shared C-C bond (bridge). The hindrance in free rotation of the acceptor and donor is thought to suppress quenching and result in a high quantum yield of fluorescence when bound to Aβ, which is rich in β-sheet structures\(^{51}\). However, due to its charge and an emission wavelength less than 650 nm, the in vivo usage of ThT is limited. A variety of derivatives of ThT have been developed. Although many of these derivatives are radiolabeled for PET/SPECT imaging, there are some fluorescent probes that show high affinity for Aβ aggregates in vitro.

Compound 12a (2) is a benzothiazole Schiff-base and has a high affinity for AD brain homogenates with a \(K_d\) value of 4.38 nmol/L. This compound can label senile plaques, as confirmed by staining with thioflavin-S (ThS) in adjacent sections of AD brain tissue\(^{52}\). Jung et al designed and synthesized a series of ThT analogs. One of these analogs, compound 2 (3), showed high affinity for Aβ fibrils (\(K_d=3.27\) μmol/L), a significant fluorescence increase (\(F_{ex}/F_0=36.1\)) and a Log \(P\) value (3.7) suitable for potential BBB penetration. 3 was able to stain...
intracellularly aggregated Aβ fibrils in cultured cells\textsuperscript{[53]}. However, the fluorescence spectra of the two above-mentioned probes are still in a range that limits their potential use in vivo.

Ono et al synthesized two push-pull benzothiazole derivatives (PP-BTA-1 (4) and PP-BTA-2 (5)) with either benzothiazole or styrylbenzothiazole as the highly polarized bridge, a dimethylamino group as the donor and a dicyanomethylene group as the acceptor. When exposed to Aβ aggregates, the fluorescence intensity of 4 and 5 increased with increasing concentrations of Aβ aggregates. Results of inhibition assays on the binding to Aβ aggregates indicated that 4 and 5 may occupy a binding site on Aβ aggregates similar to that of 1. Meanwhile, the apparent IC\textsubscript{50} values for 4 and 5 were 0.12 and 0.11 μmol/L, respectively, indicating a high binding affinity for Aβ aggregates. 4 and 5 clearly stained Aβ plaques in brain sections of both transgenic mice and human AD patients\textsuperscript{[54]}. Because the maximal emission wavelengths of 4 and 5 are approximately 600 nm and 550 nm, respectively, further red-shifting of the emission wavelengths is necessary before these two probes are applicable for in vivo Aβ imaging.

**Derivatives of stilbene**

Stilbene is a hydrocarbon consisting of a trans ethene double bond substituted with phenyl groups on each carbon atom of the double bond. This structure was introduced because it is half of Chrysamine-G, which is a very close analog of Congo red, one of the most used compounds for staining Aβ plaques in brain slices\textsuperscript{[55]}. The double bond in stilbene is the bridge connecting the electron donor and acceptor. Stilbene derivatives constitute a large family of radiolabeled Aβ probes. Based on the PET imaging probe SB-13, Hong et al synthesized 32 stilbene derivatives. Among them, compound 42 (Figure 2, 6) showed high fluorescence responsiveness (\(F_{Aβ}/F_0>25\)-fold) and a strong binding affinity (\(K_d=1.13 \, \text{μmol/L}\)). These results indicate that 6 binds more strongly to Aβ aggregates than to ThT (\(K_d=2.3 \, \text{μmol/L}, \, F_{Aβ}/F_0>111\)) and emits a higher fluorescence than BTA-1 (\(F_{Aβ}/F_0=4.1, \, K_d=0.30 \, \text{μmol/L}\)). Staining of AD transgenic mouse brains showed that this novel stilbene analog could be a useful imaging tool for AD studies\textsuperscript{[56]}.

As early as 2004, Li et al reported two styryl dyes, 2C40 (7)
and 2E10 (8), which showed a 27- and 30-fold increase, respectively, in fluorescence after exposure to Aβ1-40 fibrils, and a 20- and 16-fold increase, respectively, in fluorescence after exposure to Aβ1-42 fibrils. Moreover, these two probes co-stained Aβ plaques in AD mouse brain sections. In 2007, the same group synthesized novel agents derived from 7 and 8, removing the positive charge and increasing the hydrophobicity for better BBB permeability. One of these compounds, STB-8 (9), showed strong Aβ plaque staining in AD brain sections and a high binding affinity ($K_d$=3.2 μmol/L) to Aβ fibrils in solution. In ex vivo experiments, the Aβ plaques stained by 9 were confirmed by ThS co-staining and in vivo staining to overlap with Congo Red in AD transgenic mice. The results of in vivo and ex vivo imaging clearly demonstrated its BBB permeability and specific staining of Aβ plaques in AD mice.

Park et al. discovered compound 9n (10) and found that it exhibited a strong fluorescence response ($F_{Aβ}/F_{D}=40.84$) and binding affinity ($K_d$=1.84 μmol/L) to Aβ aggregates. 10 clearly stained both intracellular Aβ aggregates and Aβ plaques in transgenic AD model mice. Because the maximal emission wavelength of 10 when bound to Aβ aggregates is approximately 630 nm and the molecular weight is suitable for BBB penetration, 10 would be an excellent NIRF probe for Aβ detection with further in vivo tests.

Staderini et al. focused their attention on fluorescent compounds capable of staining Aβ fibrils while simultaneously blocking their aggregation. They developed a compound named 3 (11) that interacted with both Aβ and prion fibrils and stained them with high selectivity. 11 inhibits Aβ self-aggregation in a ThT assay (42.4% of fluorescence inhibition). Due to its low-toxicity and BBB penetrating ability, 11 is likely to become the first purposely designed therapeutic and diagnostic tool. However, the fluorescence spectrum of 11 makes it difficult to use in vivo for Aβ detection.

A recently reported fluorescence probe 1 (12) presented a novel fluorescence-enhancing mechanism. 12 is composed of three important components, an N,N-dimethylamino styrene as the binding unit, a triethylene glycol as the hydrophilic unit and fluorene as an “off-on type” fluorescence signaling unit. When bound to Aβ oligomers and insoluble aggregates, 12 showed disaggregation-induced fluorescence enhancement. Although 12 operates under a different mechanism than the other probes, the switch from aggregation to disaggregation depends on the polarity change in the surrounding environment. Therefore, the mechanism of action for 12 still belongs to the solvent-dependent mode.

Two derivatives of chalcone, probe 5 (13) and probe 6 (14), exhibited a significant increase in fluorescence intensity when bound to Aβ aggregates. The $K_d$ values of 13 and 14 were 1.59 and 2.30 μmol/L, respectively, which indicated a moderate affinity for Aβ aggregates. These probes specifically stained Aβ plaques in APP/PS1 transgenic mouse brain sections, which was confirmed by co-staining with an Aβ antibody. 13 exhibited a good performance in vitro and was selected for in vivo experimentation. 13 was confirmed to penetrate the BBB and to clearly stain Aβ plaques in 14-month-old APP/PS1 mice when compared with normal controls. However, the emission wavelength of 13 after binding to Aβ aggregates is 532 nm, thus it is still not qualified for in vivo experimentation.

Curcumin and its derivatives

Curcumin (Figure 3, 15), a diarylethannoid, is the principal curcuminoid of the popular South Asian spice turmeric. In AD research, 15 has been shown to have the following properties: anti-Aβ aggregation, anti-oxidation, inhibition of β-secretase and acetylcholinesterase, and Aβ-induced inflammation in vitro. Due to its fluorescent properties, 15 labels amyloid plaques, such as ThS in AD and transgenic mouse brain sections, and crosses the BBB to bind to plaques in vivo. In 2007, Garcia-Alloza et al. demonstrated that 15 could be applied in detecting Aβ plaques in vivo. However, it was only after 7 days of administration of 15 that Aβ plaques started to be labeled by 15, reflecting the low efficiency of 15 for in vivo imaging. Moreover, due to its short emission wavelength, practical applications of 15 as an NIRF probe are limited.

Using a two-step red-shift strategy, Ran et al. designed an
NIRF probe derived from curcumin, CRANAD-2 (16). 16 functions as a “smart” probe because of the 70-fold fluorescence intensity increase, an emission blue-shift (from 805 to 715 nm), a lifetime change, and quantum yield improvement upon binding to Aβ aggregates. This probe has a high affinity for Aβ aggregates (K_d=38.0 nmol/L), a reasonable Log P value (3), considerable stability in serum, and a weak albumin interaction. Meanwhile, high-contrast plaque staining in transgenic mouse tissue was observed, which colocalized with the signal from standard ThT-stained sections. Moreover, in vivo NIRF imaging indicated that 16 clearly differentiates between 19-month-old Tg2576 and wild-type mice at early time point (30 min)[38]. One factor limiting in vivo use is the slow washout from the brain.

Although 16 binds to Aβ aggregates/fibrils with high affinity, it lacks the capability to detect soluble Aβ species. To design a probe that is sensitive to soluble Aβ species, Zhang et al studied the structural stereohindrance compatibility of Aβ species and developed a compound called CRANAD-58 (17). 17 showed significant fluorescence property changes upon mixing with both soluble (monomers, dimers, and oligomers) and insoluble Aβ species in vitro. Histological staining of transgenic mice indicated that 17 could specifically highlight Aβ plaques that co-stained with ThS. In vivo NIRF imaging revealed that 17 was capable of differentiating transgenic and wild-type mice as young as 4 months old, an age that lacks visible Aβ plaques as Aβ is likely in its soluble forms[66]. The capability to detect soluble Aβ species makes 17 a promising probe for early AD diagnosis. Nevertheless, due to the neuroprotectivity of Aβ monomers[67], the higher affinity of 17 for Aβ monomers than other species might limit precise imaging when clinically applied.

Because curcumin is an effective inhibitor of Aβ aggregation, developing derivatives with both diagnostic and therapeutic functions seems possible. A communication published in 2014 reported a high quantum-yielding bifunctional curcumin analog, CRANAD-28 (18). 18 could penetrate the BBB to label plaques and cerebral amyloid angiopathies in a 9-month-old APP/PS1 mouse. Additionally, this probe inhibited copper- and naturally (phosphate buffered saline, PBS) induced Aβ crosslinking. Unexpectedly, the fluorescence intensity of 18 decreased upon mixing with the tested Aβ species, which differs from most other reported probes[68]. Further investigation is necessary before this probe could be a theranostic agent for AD.

Another group reported a bivalent ligand, BMAOI 14 (19), which is composed of curcumin and cholesterol. Experimentation demonstrated that 19 bound to Aβ monomers, oligomers, and fibrils with low, micromolar to submicromolar, binding affinities. Furthermore, 19 specifically bound to Aβ plaques in both AD human patients and Aβ precursor proteins from transgenic mouse brain tissues. Interestingly, although 19 has a molecular weight of more than 1000 Da, it could in fact rapidly cross the BBB, reach the brain tissue and become metabolized in a reasonable time window (~3 h), suggesting amenability in clinical applications[69]. This result suggests that cholesterol could be used as a promoting unit when a probe lacks BBB permeability.

Derivatives of the thiophenes
NIAD-4 (Figure 4A, 20) was designed with a classical push-pull structure, terminal donor (p-hydroxyphenyl group) moiety, acceptor (dicyanomethylene group) moiety and interconnected by a highly polarizable dithienylthienyl n-conjugated bridge. 20 strongly bound to Aβ fibrils (K_i=10 nmol/L) accompanied by an approximately 400-fold fluorescence enhancement.

Figure 4. Derivatives of thiophenes (A) and alkatrienes (B).
Additionally, it readily crossed the BBB after an intravenous injection and specifically labeled both the plaques and cerebral-vascular amyloid angiopathies in living brain tissue[5].

The maximal emission wavelength of 20 is 612 nm, outside the NIR range (650–950 nm). Based on the structure of 20, NIAD-11 (λ_{max}=690 nm) (21) and NIAD-16 (λ_{max}=720 nm) (22) were then synthesized. 21 exhibited a significant fluorescent increase and a 15-nm red-shift in the emission wavelength when bound to Aβ aggregates. 22 clearly stained Aβ plaques in AD mouse brain sections[50].

Derivatives of the alkarinienes

THK-265 (Figure 4B, 23) is an NIRF probe with an alkariniene chain bridging two pyrimidine rings. It has a maximal emission wavelength of more than 650 nm, a high quantum yield (38.5% in methanol solution), and a high affinity (K_d=97 nmol/L) for Aβ fibrils and a moderate Log P value (1.8). Ex vivo fluorescence imaging of brain samples in mice indicated a fast washout (>60% reduction of fluorescence within 60 min) of 23 from the brain. In vivo NIRF imaging demonstrated significantly higher fluorescence intensity in the brains of AD transgenic mice than in those of wild-type mice, even as early as 3 min in both 19- and 32-month-old APP/PS1 transgenic mice[71]. These results suggest that 23 could be a potential NIRF imaging probe for AD diagnosis.

Recently, Cui et al reported an NIRF probe, DANIR-2c (24), with a traditional donor-acceptor structure bridged by an alkariniene chain. 24 demonstrated a high affinity for Aβ aggregates in vitro (K_d=26.9 nmol/L, K_p=36.9 nmol/L) and stained Aβ plaques in brain sections from an AD patient and transgenic mice. It has a maximal emission wavelength of 665 nm, high in vitro stability in mouse serum, low cytotoxicity to human neuronal cells and ideal brain kinetics in normal nude mice. Additionally, in vivo NIRF imaging indicated a significant contrast in the fluorescent signal in brain regions of 22-month-old wild-type and APP/PS1 transgenic mice at 30 min post-injection of 24[72]. With a molecular weight of 249 Da, 24 may have the simplest structure of all of the Aβ NIRF probes. However, the blue-shift of 24 upon binding to Aβ means that further red-shifting of the maximal emission wavelength is required to make it a promising Aβ NIRF probe for AD diagnosis.

Fu et al introduced new acceptor moieties to the backbone of 24 and synthesized four novel NIRF probes with emission wavelengths ranging from 685 to 725 nm, longer than the 665 nm of 24. Among them, MCAAD-3 (25) displayed the highest fluorescence increase (26-fold) and affinity (K_d=106.0 nmol/L) when bound to Aβ aggregates. 25 readily penetrated the BBB, and as a result was selected for in vivo imaging in mice. The differences in the fluorescent signals between the transgenic and the wild-type mice could be seen after 30 min of injection. The in vivo results were confirmed by an ex vivo staining experiment. The authors conducted additional molecular docking simulations to explore the possible binding sites of their probes, which could provide insight into the discovery of novel Aβ probes[53].

Kim et al synthesized a novel class of two-photon absorbing dyes that are classified as n-extended acean analogs. Because limited autofluorescence is advantageous in tissue imaging and in vivo imaging of Aβ plaques, the authors synthesized compound 5 (26), an analog of 24. 26 exhibited a 41- to 61-fold increase in fluorescence upon binding to Aβ aggregates, with little interference from bovine serum albumin (BSA). It showed a high affinity for Aβ aggregates with a K_d of 44.6 nmol/L. In vivo imaging provided clear, bright red fluorescence images of Aβ plaques with little autofluorescence. It is believed that with proper instruments, two-photon probes would allow deeper tissue imaging in animals[74].

BODIPY-derived probes

Boron-dipyrromethene, BODIPY, is a classical fluorescent dye applied in a variety of fields[75]. Many notable features of BODIPY, such as the robustness against light and chemicals, the relatively high molar absorption coefficients and fluorescence quantum yields, the sharp emission bandwidths with high peak intensities, good solubility, excitation/emission wavelengths in the visible spectral region (≥500 nm), and fluorescence lifetimes in the nanosecond range, all contribute to the appeal of this structure as an important tool in a variety of imaging applications, as Boens et al reviewed[59].

Parhi et al reported the first exploration of synthesizing BODIPY-based fluorescent probes for detecting Aβ plaques[77]. However, they only characterized these probes chemically (the probes do not have off-on property) and no biological results were reported. Smith et al reported triazole-containing BODIPY dyes that showed different fluorescence increases in the presence of soluble Aβ_{1–42} oligomers with both unordered and ordered β-sheet-rich conformations[73]. Nonetheless, no further application of these dyes as Aβ fluorescent imaging probes has been reported.

A BODIPY-derived probe, BODIPY-7 (Figure 5, 27), was designed as a dual SPECT/fluorescent probe for imaging Aβ plaques in the brain. The radiolabeled 27 does not have characteristics for imaging Aβ plaques. Although 27 had a high fluorescent quantum yield (Φ=0.36), it exhibited shorter wavelengths of absorption/emission, at 606/613 nm, than those appropriate for optical imaging in vivo[70]. Another BODIPY-derived probe, BAP-1 (28), with a maximal emission wavelength of 648 nm, showed excellent efficacy both in vitro and in vivo. 28 bound to Aβ aggregates with a high affinity (K_d=44.1 nmol/L) and clearly stained Aβ plaques in the brains of transgenic mice. Moreover, 28 possesses ideal metabolic kinetics in vivo and could clearly differentiate between 25-month-old wild-type and Tg2576 mice at 1 h post-injection ex vivo[80]. The same group also developed derivatives of 28, BAP-2 (29), BAP-3 (30), BAP-4 (31), and BAP-5 (32), which contain most of the advantageous features of 28 but a better maximal emission wavelength, up to 700 nm. The estimated K_d values for 29, 30, 31, and 32 were 55, 149, 27, and 18 nmol/L, respectively, suggesting a high affinity for Aβ_{1–42} aggregates. These probes could clearly stain plaques in Tg2576 mice brain sections. One of these probes, 29, exhibited
ideal brain kinetics and was chosen for ex vivo experimentation, in which 29 showed selective binding of Aβ plaques with little nonspecific binding. However, 29 could not differentiate between transgenic and wild-type mice in vivo and showed higher accumulation in the scalp than in the brain in both groups. This result suggests that appropriate structural modifications to BODIPY derivatives are necessary for future in vivo applications.

Sozmen et al designed and synthesized a series of styryl-conjugated BODIPY dyes (EUA1-5) for fluorescence imaging of Aβ plaques. The emission wavelengths of these probes ranged from 654 to 763 nm. The dissociation constants of EUA1–5 were 320, 230, 320, 48.6 and 97 nmol/L, respectively. Among these probes, EUA-1 (33), EUA-2 (34) and EUA-4 (35) stained the Tg2576 mice brain sections efficiently and demonstrated good patterning. With the highest affinity and best fluorescence staining, 35 would be a promising diagnostic agent after further modification.

Other probes
In 2005, Novartis developed an oxazine-derivative NIRF probe, AOI-987 (Figure 6, 36), which could penetrate BBB and bind to Aβ plaques. However, the signal contrast between the wild-type and transgenic mice was not significant, probably due to the low fluorescence enhancement upon binding to Aβ aggregates observed in vitro. Structurally, 36 is categorized as a donor-acceptor but with a rigidified bridge, which may explain the non-optimal properties of 36.

Based on the donor–acceptor structure, Chang et al designed and synthesized a new family of fluorescent markers containing an amino naphthalenyl-2-cyano-acrylate (ANCA) motif. One of these compounds, ANCA-11 (37), showed increased fluorescence upon binding to Aβ aggregates (7.7-fold) and the highest binding affinity ($K_d=1.4 \mu$mol/L) among all compounds. In vitro tests indicated that all of the compounds could fluorescently stain amyloid deposits in human brain tissue from AD patients. However, further improvement of the binding affinity and maximal emission wavelengths of these probes is needed.

Gemma et al reported the identification and preliminary characterization of a new class of pyrrolo (imidazo) quinoxaline hydrazones (38) as fluorescent probes for Aβ fibrils. Except for the relatively high basic fluorescence intensities of the analyzed probes, the level of fluorescence changes when
mixed with Aβ fibrils is similar to that of ThT. Histological staining of AD mice suggested that these probes specifically recognize the β-pleated sheet structure of Aβ fibrils equivalently to the standard dyes such as ThS or Congo Red[88]. The advantage of these probes over traditional ThS or Congo Red is their BBB penetrating ability.

The solvent-independent mode as a new mode of Aβ labeling

Fluorescence self-quenching is not a new concept. In 1990, Munkholm et al. reported that fluoresceinamine, which has markedly quenched fluorescence, could be turned on by the conversion of its amine to an amide[89]. Therefore, fluoresceinamine could be used as a highly sensitive pH indicator. This compound has the very attractive feature of reducing background fluorescence when performing in vitro imaging, potentially allowing real-time imaging without a washing or clearance step[89]. In regard to biomolecule sensing (insulin, Aβ aggregates, etc), the above-mentioned mechanism lacks scope for its abilities. Kim et al.[85] systematically studied the fluorescent off-on mechanism for probes in which the fluorophore and the quencher are connected by a spacer. Changes in the distance between the fluorophore and the quencher, induced by solvent alteration, underlie the off-on mechanism.

Due to the high quantum yield, resistance to solvent polarity and pH, BODIPY-based fluorophores with off-on mechanisms for small-molecule sensing have already been developed[86, 88, 89]. However, no such probes have been developed for detecting macromolecules, such as Aβ aggregates.

Based on the off-on mechanism and features of BODIPYs, we synthesized different scaffolds and discovered molecule probe 1 (Figure 7, 39), which has an aniline group as the quencher and a nonpolar BODIPY as the fluorophore. 39 showed a high affinity for Aβ aggregates with a $K_d$ of 3.5 nmol/L. It clearly stained brain sections of APP/PS1 transgenic mice without a washing process. The plaques stained by 39 were co-stained by an Aβ specific antibody, further verifying its ability to label Aβ plaques with an off-on mechanism. Unlike other reported probes, this probe has almost no fluorescence in both polar and nonpolar solvents. After binding to Aβ aggregates, 39 exhibited a significant increase in fluorescence. We propose that the distance between the quenching group and BODIPY core would increase when the BODIPY motif bound to Aβ fibrils, and then the fluorescence of the probe would turn on[80].

The decreased environmental sensitivity and lack of washing procedure during high contrast brain tissue staining are advantages to 39 that make probes with this type of mode applicable to future in vivo imaging. We are now focusing on bathochromic shifting the emission wavelength of 39 to the NIR region on the premise of retaining its high Aβ binding affinity.

Conclusions and future prospectives

Over the past decade, a variety of fluorescent probes have been developed for labeling Aβ in vitro and in vivo. Most of these probes adopt a solvent-dependent mode with traditional donor-acceptor structures. Upon binding to the Aβ species, these probes exhibit enhanced fluorescence emission, a phenomenon often described as fluorescence “turn on”. Theoretically, this property allows these probes to be used as contrast agents for Aβ detection in vitro and in vivo, especially when compared to those fluorophores with Aβ binding affinity but no fluorescence change.

In reality, a large number of the reported probes showed efficacy in vitro but lack in vivo results. One of the probable reasons is a maximal emission below the NIR range (650−900 nm). NIRF probes are suited for in vivo detection due to their higher tissue penetration and lower background when compared to non-NIR probes. However, reported probes are often derived from already existent dyes with maximal emission wavelengths less than 600 nm. Only after further red-shifting of the probes is there potential for use in animals, presuming unaltered fluorescence properties.

Another probable reason for the lack of in vivo data is low BBB permeability. Traditional dyes, such as ThT and Congo Red, lack BBB penetration due to their charge or large molecular weight. Their improved derivatives are often neutral or have small molecular weights, which are suitable properties for the non-polarity needed to cross the BBB. However, the larger the non-polarity the lower the labeling specificity. Therefore, a balance between BBB penetration and labeling specificity should be maintained, which is a limiting factor in vivo. Of all the currently available Aβ NIRF probes that have been administered in vivo, only probes 17, 24 and 25 showed fast BBB permeability. After further modifications, these probes hold promise for early AD diagnosis.

Although much progress has been made during the last decade, there is still a long way to go before Aβ fluorescent probes truly have a foothold in the field of AD diagnosis. There is much room for improvement with the existing probes. (1) NIRF probes only account for a small number of probes, so red-shifting the existing probes is a current focus. (2) Most probes can only detect insoluble Aβ fibrils or plaques, and probes capable of targeting the soluble species, such as oligomers, are scarce. (3) A large number of recently discovered probes originate from traditional scaffolds with unsuitable properties for in vivo testing, so finding new scaffolds is an important task. (4) Although the traditional solvent-dependent mode is the leading mechanism of testing current probes (Table 1), introducing new mechanisms would help in discovering more efficient probes for in vivo imaging.

This review introduced for the first time an emerging mode
Table 1. Characteristics of representative probes with solvent-dependent mode.

| Probes | $\lambda_{ex}/\lambda_{em}$ (nm)* | Finished stage | $\alpha$ species detected | $K_d/K_i/IC_{50}$ | Fold increase | Ref |
|--------|----------------------------------|----------------|---------------------------|--------------------|--------------|-----|
| ThT & its derivatives | | | | | | |
| 1 | 450/482 | in vitro | $\beta$-fibrils | $K_d=890$ nmol/L | - | [91] |
| 2 | - | in vitro | $\beta$ fibrils | $K_d=4.38$ nmol/L | - | [52] |
| 3 | 340/410 | in vitro | $\beta$-fibrils | $K_d=3.27$ μmol/L | 36.1 | [53] |
| 4 | 540/634 | in vitro | $\beta$-fibrils | $IC_{50}=0.12$ μmol/L | - | [54] |
| 5 | 410/529 | in vitro | $\beta$-fibrils | $IC_{50}=0.11$ μmol/L | - | [54] |
| Derivatives of stilbene | | | | | | |
| 6 | 350/440 | in vitro | $\beta$-fibrils | $K_d=1.13$ μmol/L | 25 | [56] |
| 7 | 505/590 | in vitro | $\alpha$-fibrils | - | 27 | [57] |
| 8 | 464/503 | in vitro | $\beta$-fibrils | - | 30 | [57] |
| 9 | 373/407 | in vitro | $\beta$-fibrils | - | 16 | [58] |
| 10 | 430/636 | in vitro | $\beta$-fibrils | $K_d=1.84$ μmol/L | 40.84 | [59] |
| 11 | 540/636 | in vitro | $\beta$-fibrils | - | 60 | [60] |
| 12 | 412/462 | in vitro | $\beta$-fibrils | $K_d=2.25$ μmol/L | 6.5 | [61] |
| 13 | 400/570 | in vitro/ex vivo | $\beta$-fibrils | $K_d=1.59$ μmol/L | 49.9 | [62] |
| 14 | 438/582 | in vitro | $\beta$-fibrils | $K_d=2.30$ μmol/L | 58.9 | [62] |
| Derivatives of curcumin | | | | | | |
| 16 | 640/715 | in vivo | $\beta$-fibrils | $K_d=38.0$ nmol/L | 70 | [38] |
| 17 | 630/672 | in vivo | $\beta$-fibrils | $K_d=105.8$ nmol/L | 91.9 | [66] |
| | | | $\beta$-fibrils | $K_d=45.8$ nmol/L | 113.6 |
| | | | $\beta$-fibrils | - | 60 |
| | | | $\beta$-fibrils | - | |
| 18 | 498/578 | in vivo | $\beta$-fibrils | $K_d=68.8$ nmol/L | - | [68] |
| | | | $\beta$-fibrils | $K_d=159.7$ nmol/L | - | |
| | | | $\beta$-fibrils | $K_d=162.9$ nmol/L | - | |
| | | | $\beta$-fibrils | $K_d=85.7$ nmol/L | - | |
| | | | $\beta$-fibrils | $K_d=52.4$ nmol/L | - | |
| 19 | 445/535 | in vitro | $\beta$-fibrils | $K_d=2.03$ μmol/L | - | [69] |
| | | | $\beta$-fibrils | $K_d=2.17$ μmol/L | - | |
| | | | $\beta$-fibrils | $K_d=0.83$ μmol/L | - | |
| Derivatives of thiophenes | | | | | | |
| 20 | 475/612 | in vivo | $\beta$-fibrils | $K_d=10$ nmol/L | 400 | [37] |
| 21 | 545/690 | in vivo | $\beta$-fibrils | - | - | [70] |
| 22 | 470/720 | in vivo | $\beta$-fibrils | - | - | [70] |
| Derivatives of alkatrienes | | | | | | |
| 23 | 650/680 | in vivo | $\beta$-fibrils | $K_d=97$ nmol/L | - | [71] |
| 24 | 597/625 | in vivo | $\beta$-fibrils | $K_d=26.9$ nmol/L | 12 | [72] |
| 25 | 596/685 | in vivo | $\beta$-fibrils | $K_d=106.0$ nmol/L | 26 | [73] |
| 26 | 500/670 | in vivo | $\beta$-fibrils | $K_d=44.6$ nmol/L | 60 | [74] |
| BODIPY-derived probes | | | | | | |
| 27 | 606/613 | in vitro | $\beta$-fibrils | $K_d=108$ nmol/L | - | [79] |
| 28 | 614/648 | in vitro/ex vivo | $\beta$-fibrils | $K_d=44.1$ nmol/L | - | [80] |
| 29 | 650/708 | in vitro/ex vivo | $\beta$-fibrils | $K_d=55$ nmol/L | - | [81] |
| 30 | 663/705 | in vitro | $\beta$-fibrils | $K_d=149$ nmol/L | - | [81] |
| 31 | 636/704 | in vitro | $\beta$-fibrils | $K_d=27$ nmol/L | - | [81] |
| 32 | 649/723 | in vitro | $\beta$-fibrils | $K_d=18$ nmol/L | - | [81] |
| 33 | 518/654 | in vitro | $\beta$-fibrils | $K_d=322.8$ nmol/L | - | [82] |
| 34 | 597/667 | in vitro | $\beta$-fibrils | $K_d=226.2$ nmol/L | - | [82] |
| 35 | 624/673 | in vitro | $\beta$-fibrils | $K_d=48.6$ nmol/L | - | [82] |
| Other probes | | | | | | |
| 36 | 650/705 | in vivo | $\beta$-fibrils | $K_d=0.22$ μmol/L | - | [83] |
| 37 | 415/590 | in vitro | $\beta$-fibrils | $K_d=1.40$ μmol/L | 7.7 | [35] |
| 38 | 390/481 | in vitro | $\beta$-fibrils | - | - | [84] |

* Wavelengths measured without binding to $\alpha$ fibrils/aggregates.
for developing Aβ fluorescent probes, the solvent-independent mode that has a different mechanism of fluorescence enhancement than other existing probes. Our results from staining the brains of APP/PS1 mice suggested that traditional probes, such as ThS, and recent NIRF probes, such as 17, could not differentiate between wild-type and transgenic mice without a washing process. A probable explanation for this phenomenon is the interaction between residual probes and a nonpolar environment, overwhelming the Aβ signals. By contrast, our probe 39 clearly stained Aβ plaques even without a washing process. The further consolidated off-on mode of 39 holds possibility for future in vivo imaging as the high signal-to-background ratio can compensate for the low spatial resolution of NIRF imaging.

Identifying new biomarkers for early AD diagnosis meet difficult challenges. As we mentioned, Aβ oligomers, which have attracted increasing attention in understanding Aβ toxicity mechanisms, show great potential in early AD diagnosis. Aβ accumulation is known to start one to two decades before evident syndromes occur, and aggregated Aβ oligomers are believed to form early in this process. Therefore, examining the role of Aβ oligomers as a predictive marker requires a long period of longitudinal research. The results from probe 17 suggest that at 4-months, when Aβ is believed to be in an oligomeric form, a significant signal was detected in transgenic mice, suggesting that Aβ oligomers could be used as a potential marker for early diagnosis. Nevertheless, there are few probes capable of detecting Aβ oligomers, and fewer are NIRF probes that have been examined in vivo.

Currently, NIRF imaging technology has been widely used in different disease animal models and a non-specific contrast agent, ICG, has been used in off-label human clinical studies. However, the NIRF imaging devices and probes are in vivo holds, could not.

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