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Dual Functional Small Molecule Probes as Fluorophore and Ligand for Misfolding Proteins

Xueli Zhang^{1,2} and Chongzhao Ran^{1,*}

¹Molecular Imaging Laboratory, MGH/MIT/HMS Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital/Harvard Medical School, Room 2301, Building 149, Charlestown, Boston, Massachusetts 02129

²Center for Drug Discovery, School of Pharmacy, China Pharmaceutical University, Nanjing, China

Abstract

Misfolding of a protein is a destructive process for variety of diseases that include neurodegenerative diseases such as Alzheimer's disease, Parkinson disease, Huntington disease, mad cow disease, amyotrophic lateral sclerosis (ALS), and frontal temporal dementia (FTD), and other non-CNS diseases such as diabetes, cystic fibrosis, and lysosomal storage diseases. Formation of various dysfunctional large assemblies of the misfolded protein is the primary consequence. To detect the formation of the aggregated species is very important for not only basic mechanism research but also very crucial for diagnosis of the diseases. In this review, we updated references related to the new development of the dual functional fluorescent small molecule probes for detecting the aggregated proteins *in vitro* and *in vivo*.

Keywords

Dual functional probes; fluorescent probe; misfolding proteins; neurodegenerative disease; *in vivo* imaging; fluorescent imaging; near infrared imaging; Alzheimer's disease; neurodegenerative diseases

1. INTRODUCTION

1.1. Protein Misfolding and Protein Misfolding Diseases

Protein is one of the most essential components of a biological system, and the folding/unfolding of proteins is a pivotal process of biological activities [1, 2]. To execute a normal function, the related proteins need to be folded and unfolded properly. However, if the folding/unfolding proceeds irregularly, the associated normal functions will be interrupted, which will result in diseases [3]. In the past decades, mounting evidence suggests that misfolding of protein is a fundamental problem during aging and metabolism [3–6]. The causes of the misfolding include inherited genetic factors and epigenetic factors [7–15]. Individuals with certain mutated genes are at high risk of developing certain diseases.

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*Address correspondence to this author at the Molecular Imaging Laboratory, MGH/MIT/HMS Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital/Harvard Medical School, Room 2301, Building 149, Charlestown, Boston, Massachusetts 02129.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

However, around 90% of patients with misfolding protein diseases are non-mutation carriers, and it is very difficult to pinpoint the exact causes for this population. Nonetheless, improper epigenetic regulations are believed to be the major contributors for the non-familial misfolding protein diseases [15–18].

Misfolding protein diseases could be divided into two large categories: central nerve system (CNS) related and non-CNS related diseases. Neurodegenerative diseases are CNS-related and aging-related, while non-CNS related diseases are more likely associated with metabolism abnormalities. Neurodegenerative diseases are the most studied misfolding protein diseases, including Alzheimer's disease (AD) [3, 19], Parkinson's disease (PD) [20], Huntington disease (HD), prion disease, ALS (Amyotrophic lateral sclerosis), and FTD (frontal temporal dementia) [3]. Each disease has one or more proteins that are prone to misfold and thus to aggregate (Table 1) Diabetes, cystic fibrosis, and lysosomal storage diseases are the most studied non-CNS related diseases [21, 22].

For the misfolding process, two features of proteins play very crucial roles. One feature is the structural change from alpha-helix configuration of native status to form a cross-beta structure. Normally, one or two hydrophobic core fragments are presented in the proteins, and they are the second necessary element for misfolding. During the formation of misfolded proteins, the hydrophobic interaction is the driving force and the cross-beta structure provides the platform for assembling. Compared to the native status of the protein, the misfolded assembly provides a changed microenvironment, which is normally more hydrophobic and is a very important feature for developing sensitive fluorescent probes [23, 24, Stefani, 2004 #2050].

1.2. Dual Functional Probe as Fluorophore/Ligand

To develop a molecular probe for a given target, two approaches are always used. The most used strategy is to design a molecular probe through a conjugation of a sensing moiety with a signal readout moiety. The sensing part is coming from a ligand of a specific target of a disease, which could be an inhibitor, activator, agonist, or antagonist of a given receptor or antibody of a targeted protein. Meanwhile, the signal readout part is normally consisting of a fluorescent/luminescent, radioactive, and magnetic (MRI) moiety [25]. The advantages of this strategy include its universal applications for very different targets, and its feasibility of multimodal detection through conjugation of different readout groups. On the other hand, the disadvantages include disruption of binding behavior, off-targeting, and tedious conjugation chemistry [26–29].

The second approach is to seek probes of dual functions of sensing and signal readout with a same scaffold. For such kinds of probes, the molecule serves as both ligand and sensor for binding and readout [30]. Apparently, the advantages include no binding disturbance and no tedious chemistry needed. However, the disadvantages are not suitable for a multi-modal readout, limited to small molecules, and narrow applications. The readout of the dual functional probes is always limited to fluorescent, luminescent and radioactive signal. It is very difficult to develop a probe that could be used as an MRI sensor while it itself is the ligand for the target. However, a recently developed MRI sensor from Jasanoff's group is worth noting which makes it possible to develop a probe of dual functions of MRI sensing and target binding [31]. Additionally, fluorescent probe with one or multiple ^{19}F had been used as dual functional probe for *in vivo* imaging A β plaques with ^{19}F MRI [32, 33].

Radioactive probes, which are derivatized from a ligand with replacement of one atom with a radionuclide, are widely used as dual functional probes for radioactive assays and nuclear medical imaging that includes *in vivo* PET and SPECT imaging [30]. Of note, radioactive dual probes that emit charged particles (β^+ and β^-) could be used as dual optical imaging

probes as well due to the newly discovered Cerenkov Luminescence Imaging (CLI) [1, 34–39].

Similar to dual radioactive probe, fluorescent (or luminescent) dual functional probes are also often used for various targets. In particular, these kind of dual functional probes are specially suitable for detecting small targets such as proteins of neurodegenerative diseases. In addition, the dual functional probes could be translated into clinical research using minor structural modification to make them as PET or SPECT probes [30]. Of note, compared to traditional PET probe development, it could be a cheaper and safer approach to develop a PET or SPECT probe based on first validation with fluorescent imaging.

In this review, we focused on small molecules with dual functions of fluorophores and ligands for misfolded Amyloid- β , tau, alpha-synuclein, insulin and prions.

2. AMYLOID- β

Amyloid- β (A β) deposits and Tau tangles are the most important pathological hallmark of Alzheimer's disease (AD). The formation of A β deposits arises from the aggregation of peptides A β 40 and A β 42, which are generated from amyloid peptide precursor (APP) by cleavage with β - and γ -secretases [40]. It is well known that A β species, including soluble monomers, dimers, oligomers, and insoluble fibrils/aggregates and plaques, play a central role in the neuropathology of AD. Initially it was thought that insoluble deposits/plaques formed by the A β peptide in AD brain cause neurodegeneration. However, it was later shown that amyloid plaque burden has poor correlation with Alzheimer's disease severity [41–43]. More recent evidence has been mounting that soluble dimeric and oligomeric A β species are more neurotoxic than insoluble deposits and may, in fact, be the agents that mediate neurodegeneration in AD [44–46].

Regardless of the nature of the primary contributors to AD pathology, it is broadly believed that the initial stage of the full course of amyloidosis is represented by the excessive accumulation of A β monomers caused by imbalanced A β clearance [47, 48]. The accumulated monomers gradually aggregate/polymerize/cross-link into neurotoxic soluble dimers and oligomers, the likely biomarkers of the presymptomatic stage and insoluble fibrils and plaques, the likely biomarkers of the symptomatic stage [47]. In the course of AD progression, all A β species are present, but the predominance of the subspecies changes gradually from soluble species, to insoluble fibrils and plaques [44] (Fig. 1).

Currently, probe development for imaging insoluble species has been very successful, and a few imaging agents have been approved for clinical diagnosis. [49, 50] Nonetheless, we still fall short of imaging probes capable of detecting soluble species. We believe such probes will be potentially beneficial for early/presymptomatic diagnosis.

2.1. Thioflavin and its Analogues

Thioflavin S and Thioflavin T are the most used fluorescent compounds for *in vitro* detection of A β aggregates in solutions and for visualizing A β plaques in brain slices during histological examination. Thioflavin T is a dual functional probe because it is fluorescent and binds to A β . Moreover, Thioflavin T could be considered as a “smart” fluorescent probe for A β species, because it changes its fluorescent properties dramatically upon binding. These changes include excitation and emission wavelength, red-shift, and significant fluorescent intensity increase once it binds to A β aggregates/fibrils. Thioflavins are charged benzothiazole molecules; therefore they are not BBB (brain blood barrier) penetrable. Researchers from different groups have worked on designing and synthesizing analogs of Thioflavin to render them BBB penetrable, and to investigate its application for *in vitro*

testing and *in vivo* imaging. An excellent review article by Mathis and Klunk summarized the progress of thioflavin for detecting A β species by fluorescent imaging and PET imaging [30, 51]. In this review, we summarized the most recent progress related to Thioflavin T for A β detection.

Kung *et al.* reported that IMPY, an analogue of Thioflavin T, displayed improved binding properties for amyloid imaging. In this compound, the benzothiazole of Thioflavin T was replaced by imidazo[1,2-a]pyridine. With this replacement, IMPY displayed a good binding affinity for preformed synthetic Ab40 aggregates in solution (K_i was about 15 nM) and showed selective plaque labeling on postmortem AD brain sections. Remarkably, a biodistribution study in normal mice with [125 I]IMPY displayed excellent brain uptake (2.9% at 2min) and fast washout (0.2% at 60 min). It is known that slow brain washout will result in high background-to-noise ratio and fast washout will result in low unspecific staining. Therefore, the high binding affinity and fast washout are highly desirable for amyloid plaque imaging agent development [52]. In 2009, Ono *et al.* reported a serial push-pull of benzothiazole, in which benzothiazole or styrylbenzothiazole was considered as the highly polarized bridge, and a dimethylamino group was designed as the donor, and a 2-cyano acrylate moiety as the acceptor. These compounds showed excellent binding toward A β 42 aggregates and high contrast for A β plaque staining in brain slices [53].

Recent mechanism studies suggested that Thioflavin T might bind in channels running parallel to the long axis of the fibril. In the channels, Thioflavin T probably binds in either a monomeric or dimeric form, and under this binding status the molecular conformation is likely to be planar [54]. Using molecular dynamics simulations, Wu provided strong support for the channel model. Their results suggested that linear dyes such as Thioflavin, bind parallel to the long axis of the fibril surface in grooves formed by side-chain ladders [55, 56]. Moreover, Biancalana used peptide self-assembly mimics (PSAMs) experimentally to define a minimalist Thioflavin T binding site, and found that the minimal discrete binding site for Thioflavin T is comprised from a channel across five or more betastrands of a flat beta-sheet surface [57, 58]. Most of the above mechanism studies indicated that Thioflavin binds to A β fibrils. However, Maezawa used surface plasmon resonance spectroscopy to investigate the binding sites of Thioflavin with A β species, and their results indicated that Thioflavin could also bind to A β oligomers, which are believed to be more toxic than fibrils for neurons [59].

In addition, recent studies indicated that Thioflavin T has certain therapeutic effect when it was tested with *C. elegans*. It was found that thioflavin could extend the lifespan of *C. elegans* [60].

Although Thioflavin and its analogues have been widely used for *in vitro* binding assay, screening of aggregating inhibitors and histological staining, none of these fluorescent probes could be used for *in vivo* imaging, due to their short emission wavelengths.

2.2. Congo Red, Chrysamine-G, stilbene and their Analogues

Like Thioflavin, Congo red is one of the most used compounds for staining A β plaques in brain slices. Much effort has been made to design and synthesize its analogues and several intensive review articles have been published [30, 61]. In this review, we summarized the new development regarding its synthesis and applications. Meanwhile, since Chrysamine-G is a very close analogue of Congo red, in this section, recent work related to Chrysamine-G was also included. In addition, because the structure of stilbene is half of Chrysamine G, literatures related to stilbene are included in this section as well. Stilbene derivatives are a large family for detecting A β plaques *in vitro* and *in vivo*, and several compounds have been successfully developed as PET imaging probes for clinical trials, and recently ^{18}F -AV-45

has been approved for clinical diagnosis of AD. An excellent review article about stilbene derivatives has been published in 2010 [62].

Yang group reported a series of fluorescent dyes based on a molecular rotor structure, in which an electron-donor unit was conjugated with an electron acceptor. In their series, π -conjugation of a dialkyl amino group was used as the electron donor and a 2-cyano acrylate unit was taken as the electron acceptor. Upon binding to A β fibrils, the fluorescent probes produced sensitive property changes due to the formation of twisted intramolecular charge-transfer (TICT) complexes. Their data showed Stilbene-based compound **1** produced 20nm blue-shift and 2.3-fold fluorescent intensity increasing after mixing with A β aggregates [63].

Based on a promising PET imaging probe SB-13, Hong *et al.* synthesized 34 stilbene analogues with various electron withdrawing and donating groups, and found four compounds exhibited more than a 6-fold fluorescence intensity increase. Remarkably, compound **2** displayed high fluorescence responsiveness (>25-fold increase) and strong binding affinity, and clearly labeled the peripheral region of A β plaques in brain slices [64].

In 2012, Ono *et al.* designed and synthesized a BODIPY-based stilbene analogue (BAP-1) for the imaging of β -amyloid plaques in the brain. Upon mixing synthetic A β aggregates, BAP-1 displayed a significant fluorescent intensity increase. Although BAP-1 failed to show the difference of transgenic mice and wild type mice from *in vivo* whole body imaging, it exhibited excellent fluorescent signal difference between transgenic and WT mice in the *ex vivo* imaging. The failure of *in vivo* imaging is probably due to two facts: 1) unspecific accumulation in scalp and 2) emission is still short (648 nm) for *in vivo* imaging. Histological staining indicated that β -Amyloid plaques in Tg2576 mouse brain were clearly visualized with BAP-1. [65]. Additionally, Li *et al.* synthesized and screened a stilbene-based compound library, and found dye 2C40 could be “turned on” upon incubation with A β aggregates, but the compounds may have little chance of penetrating the blood brain barrier (BBB) because of their high polarity [66].

2.3. NIAD-4 and its Analogues

Nesterov *et al.* designed an A β specific fluorescent probe NIAD-4 by employing a classical push-pull architecture with terminal donor and acceptor moieties that are bridged with a polarizable π system. In this compound, phenol moiety was used as the donor and dicyanomethane group was used as the acceptor, and two connected thiophene rings were used as the bridge. The unique properties of this compound include its small energy gap between HOMO-LUMO to enable it to emit at NIR range, and certain conformational freedom to make it as a “smart” probe through planarization of aromatic rings upon interacting with A β fibrils. The planarization is a rigidification process to reduce the vibrational-rotational levels, and thus leads to less non-radiation decay, thereby increasing the fluorescence quantum yield upon binding to A β aggregates [67]. NIAD-4 showed its *in vitro* smartnesses which include significant excitation and emission wavelength red-shift and remarkable fluorescent intensity increase upon mixing with A β aggregates. *In vivo* two-photon imaging indicated that NIAD-4 was capable of labeling A β plaques in brain and lighting up CAA (cerebral amyloid angiopathy) on the blood vessels. In addition, a series of NIAD-4 analogues were synthesized and tested by the same groups. They found NIAD-16 could be used for imaging A β plaques in tissue slices by lifetime imaging and spectral unmixing imaging [68].

2.4. Benzophenoxazine and its Analogues

Benzophenoxazine skeleton is a very common core structure for several natural dyes such as nile red, nile blue, and lacmoid, and these dyes are widely used for various biological

sample staining and cell imaging [69, 70]. For an example, Nile blue could be used to differentiate neutral lipids that are stained pink and acids that are stained blue [69, 70].

Hintersteiner *et al.* reported that benzophenoxazine derivatives could be used as a near infrared fluorescent (NIRF) imaging probe for transgenic AD mice. In their report, four benzophenoxazines were synthesized, and were tested *in vitro* and *in vivo*. It was found that AOI 987 was able to bind to A β aggregates *in vitro*. Histological staining indicated that AOI 987 could highlight A β plaques in brain tissue sections. Moreover, they demonstrated that AOI987 readily penetrated the intact blood-brain barrier. Remarkably, their report was the first to demonstrate the feasibility of *in vivo* NIRF imaging of transgenic AD mice, and practicability of monitoring the progression of amyloid plaques increase in APP23 transgenic mice during their aging process. However, AOI 987 could not be considered as a “smart” probe, because it was found that no significant fluorescent intensity changes occurred upon mixing with A β aggregates. Unlike “smart” NIRF probes, AOI 987 could not be used to differentiate the transgenic mice and wild type at early time points. Significant differences between the two groups could only be observed after certain washout time [71].

Abelein *et al.* reported lacmoid, a weak fluorescent compound of benzophenoxazine family, could bind A β 40 in a surfactant-like manner and inhibit the formation of all types of A β 40 and A β 42 aggregates. Their results indicated that lacmoid might stabilize a random coil conformation through interaction with monomeric A β , and thereby further counteract the formation of amyloid-like structures [72, 73].

Nile red was also successfully employed in detecting A β 42. Mishra *et al.* reported that Nile red bound very strongly to A β 42 fibrils (emission at 620 nm). However, it did not show significant fluorescent intensity change with monomeric A β 42, even though it showed an apparent blue shift. In addition, Nile red was proven to be efficient in discriminating between conformational isoforms of A β 42 amyloid fibrils grown under agitated and quiescent conditions [74].

2.5. Curcumin and its Analogues

Curcumin, a brightly colored powder, is the major curcuminoid of the Indian curry, and has been consumed daily for thousands of years in India and other regions. Curcumin is known for its antitumor, antioxidant, antiarthritic and anti-inflammatory properties [75–78]. Yang *et al.* reported that curcumin could be used as a histological staining reagent for senile plaques and demonstrated that curcumin could decrease amyloid deposits *in vivo* [79]. Further, Garcia-Alloza *et al.* demonstrated by two-photon imaging that curcumin could be visualized *in vivo* and could prevent the progress of amyloid plaque formation in the APP-tau transgenic mouse model [80]. Yanagisawa *et al.* showed that curcuminoids bind to soluble A β species [81]. Additionally, Ryu suggested that curcumin derivatives were potential amyloid PET imaging probes [82]. All of these studies indicate that curcumin has some specificity for amyloid plaques and displays high-affinity binding for A β aggregates (K_d = 0.20 nM). Our *in vitro* study indicated that curcumin is a “smart” fluorescent probe for various A β species, including monomeric, dimeric and aggregated A β species (Fig. 2A). Compared to dyes from other families, the unique properties of curcumin include its excellent response towards monomeric and dimeric A β species, indicating its ability to detect soluble A β species. So far, few fluorescent probes are capable of detecting soluble species. Moreover, we also reported that two non-conjugated curcumin analogues could be paired as FRET partners for detecting higher molecular weight A β species, including dimers, oligomers and aggregates [83]. Meanwhile, we proposed that, combining with Thioflavin T test, this FRET technique could be used to differentiate different A β species [83], which is a very difficult task.

Although curcumin is suitable for certain *in vitro* fluorescent tests, it is not a practical probe for *in vivo* NIR imaging due to its short emission wavelength, limited access across blood-brain barrier, and rapid metabolism [82]. Despite these limitations, we believed curcumin is a very good starting point for designing a series of fluorescent probes because of its excellent binding affinity towards various A β species. First, we designed a “smart” fluorescent probe, CRANAD-2, using a two-step red-shift strategy to enable it as a NIRF probe and suitable for *in vivo* studies. In this study, we first introduced difluorobron to block the diketone moiety of curcumin to make it red-shift 100 nm, and then 80 nm red-shift was achieved by replacing the hydroxyl group of curcumin with N,N’-dimethylamino group. The “smartness” of CRANAD-2 include a 70-fold fluorescence intensity increase, a 90 nm blue-shift (from 805 nm to 715 nm), a significant lifetime change, and a large increase in quantum yield upon interacting with A β aggregates (Fig. 2B). Moreover, this probe also shows a high affinity for A β aggregates (K_d = 38.0 nM), a reasonable Log P value (Log P = 3), considerable stability in serum and a weak interaction with albumin. Results of *in vivo* NIRF imaging showed that 24-month old Tg2576 mice exhibited a significantly higher relative signal than that of the control mice over the same period of time after intravenous injection of this probe. Remarkably, the fluorescent signal differences between transgenic and wild type mice could be seen at very early time points (10 min) due to its *in vivo* “smartness”(Fig. 2C) [84]. On the contrary, other NIRF probes such as AOI 987 were not able to show a difference of the two groups at early time points [71].

Recently, we designed and synthesized a series of curcumin-based fluorescent probes for *in vitro* and *in vivo* detecting A β species. We found that CRANAD-3 displayed significant fluorescent property changes not only for A β aggregates but also for soluble A β monomers and dimers [85]. These changes include emission intensity increase, emission wavelength shift, and lifetime increase. Our data suggested that these property changes could be used for different fluorescent imaging methods. The intensity increasing could be used for intensity-based NIRF imaging, and the wavelength change could be used for wavelength-based spectral unmixing imaging. Using CRANAD-3, we were able to differentiate transgenic APP/PS1 mice from wild type mice at an age of 2-months old (unpublished data). By utilizing the emission wavelength change, we demonstrated that spectral unmixing imaging with CRANAD-3 could differentiate bound and free probes *in vitro* and *in vivo*, and the signal of the bound CRANAD-3 was tightly associated with the concentration of A β species. *Ex vivo* whole brain and *in vivo* whole body imaging revealed that bound CRANAD-3 was primarily presented in the cortex area, and free probe was largely presented in the blood system. We showed that the signal of bound CRANAD-3 closely reflected the concentration A β species if A β species were saturated by the probe. We believe the unmixed bound signal could be used for a real-time report of concentration of A β (Fig. 2D, E, F) [86].

Other research groups also have reported the modifications of curcumin for fluorescent study and PET imaging. Ono *et al.* synthesized a series of curcumin analogue dibenzylideneacetones as potential PET imaging probes for A β plaques. However, no fluorescent data was reported for these compounds [87]. Liu reported a curcumin derivative, BMAOI 14, could be used to fluorescently label and detect A β aggregates in solution, and plaques in AD brain slices. In this probe, curcumin was used as the A β recognition moiety and cholesterol as an anchor to the neuronal cell membrane–lipid rafts. Their results indicated that BMAOI 14 bound to the A β monomers, oligomers, and fibrils with low micromolar to submicromolar binding affinities [88].

2.6. Other Fluorescent Dyes for Amyloid- β

Okamura reported a new scaffold of near infrared fluorescent (NIRF) probe, THK-265, for *in vitro* binding tests and *in vivo* NIRF imaging with transgenic mice. The structure of

THK-265, discovered from screening, does not belong to the families of the most studied fluorescent dyes (such as Congo red, Thioflavin, chrysamine, stilbene, curcumin, benzophenoxazine). The fluorescence intensity of THK-265 increased 3.6-fold with A β 40 fibrils and 6-fold higher with A β 42 fibrils compared to that without mixing with A β fibrils. However, unlike other “smart” probes, its emission was not significantly changed before and after incubation with A β species. Since it showed significant intensity increasing, it could be considered as a “smart” probe. *In vivo* imaging indicated THK-265 could be used to differentiate transgenic mice (APP/PS1) and wild type mice at ages of 19-, and 32-months old, and could be used to monitor the progress of A β formation during ages of 19-months to 32-months old. When a head to head comparison was made with AOI 987, THK-265 showed better contrast and higher sensitivity for plaque detecting *in vivo* [89].

Recently, Cook *et al.* reported that dipyrrophenazine Ruthenium(II) complex, a fluorescent/luminescent probe for DNA detection, was capable of detecting A β aggregates. It was found that the complex was not photoluminescent in aqueous solution nor in the presence of monomeric A β , but it displayed a strong photoluminescence in the presence of A β aggregates. It is believed that the light-switch is probably due to the microenvironment change upon interacting with A β fibrils. The microenvironment change probably leads to the complex switching from a low-lying dark state to a luminescent state. In addition, this ruthenium complex displayed a long-lived photoluminescence lifetime, which allows its application for the detection of A β aggregates in the presence of short-lived fluorescent backgrounds. It is known that thioflavin could not be used for monitoring the aggregation of A β if the fluorescence of the tested compound overlaps with fluorescent spectrum of Thioflavin. However, using time-gating technology, dipyrrophenazine ruthenium(II) complexes could be used for monitoring fibrillization in highly fluorescent media [90].

Ono reported chalcone could be considered as a new pharmacophore for binding of A β species. This pharmacophore is different from classical fluorescent compounds such as Thioflavin, congo red, and stilbene. *In vitro* tests showed the binding affinity of compound **3** for A β aggregate is about 3 nM. Biodistribution studies in normal mice with radioiodinated chalcones displayed high brain uptake (2.0–4.7% ID/g at 2 min.) and rapid clearance from the brain (0.2–0.6% ID/g at 30 min.), suggesting that the radioiodinated chalcones may be useful amyloid imaging agents for detecting A β -amyloid plaques in the brain of AD [91]. However, the author did not provide any fluorescent data for these chalcone compounds.

3. TAU TANGLES

Tau tangles in neuron cells are one of the most important pathological hallmarks for Alzheimer's disease. Under normal conditions, Tau proteins stabilize microtubules, which are essential for maintaining cell structure, providing platforms for intracellular transport, forming the spindle during mitosis, and other cellular processes as well. Tau proteins are abundant in neurons of the central nervous system [92]. Under abnormal conditions, tau no longer stabilizes microtubules properly, and this abnormality can result in Alzheimer's disease and other dementia such as Lewy body dementia [92]. Hyperphosphorylation of the tau protein (more than 30 sites) is a crucial step for self-assembly of tangles of paired helical filaments (PHFs) and straight filaments [93]. Recent studies indicated the concentration of p-Tau in CSF (cerebrospinal fluid) probably could be used as an important criterion for clinical AD diagnosis [94]. The aggregated tau are involved in the pathogenesis of Alzheimer's disease and other tauopathies such as Lewy body with dementia. In the past, it was widely believed that tau was primarily presented in the neuron cells, and the insoluble tau tangles are the cause of the neurotoxicity. However, more recent evidence indicated that soluble tau proteins are present in extracellular fluid as well, and could be toxic as well [92].

Because most of tau tangles are present inside of the neurons, it is more difficult to develop a fluorescent probe to detect it. Compared to A β , tau has been less accessible and fewer fluorescent probes have been discovered. Currently, no NIRF probe was reported for *in vivo* imaging tau tangles in transgenic mice.

3.1. Fluorescent BODIPY-Based Zn(II) Complex

Ojida *et al.* reported a well-designed fluorescent probe (compound **4**) for selectively detecting hyperphosphorylated tau proteins (p-Tau). Previously, binuclear zinc complexes of 2,2'-dipicolylamine (Dpa) were reported as artificial receptors for phosphorylated peptides.

In compound **4**, the two Dpa-Zn complex moieties were used as the sensors for multiple phosphorylates of tau proteins, and Bodipy dye was incorporated as the fluorescent readout and interactions with the β -sheet-rich structure of the NFTs, and triethylene glycol chains were introduced to improve the water solubility of the probe. In this designed probe, Bodipy is not only functioning as the fluorophore, but also as a ligand moiety to interact with the β -sheet of NFTs. *In vitro* test results indicated that compound **4** binds preferentially to peptides with phosphorylated groups at i and i+4 positions. Fluorescence titration study suggested that compound **4** binds strongly to p-Tau, whose concentration in CSF is believed to be a potential clinical diagnosis criterion [94]. Of note, compound **4** showed weaker interaction with artificial aggregates of the nonphosphorylated tau protein (n-Tau). Moreover, this probe also showed a weaker response toward A β 1–42 fibrils. In histological imaging studies with slices of an AD patient, compound **4** clearly highlighted deposits of NFTs, but not A β plaques (Fig. 3A). Although it is an excellent fluorescent probe for *in vitro* studies, it could not be used for *in vivo* studies due to its difficulty of BBB penetration [95].

3.2. Quinoline and Benzimidazole Derivatives

Okamura *et al.* reported that simple quinolone and benzimidazole derivatives could be used for staining neurofibrillary tangles (NFTs), neuropil threads of brain slices of AD patients. The fluorescent images with compounds BF-126, BF-158 and BF-170 clearly indicated that the compounds selectively labeled the tau tangles over A β plaques. It was found that the corresponding radiolabeled compounds exhibited excellent brain uptake and rapid clearance from normal brain tissue. Autoradiography using ¹¹C-labeled BF-158 also displayed clear labeling of NFTs in AD brain sections [96]. More recently, Villemagne reported that THK-523, a PET imaging probe and analogue of BF-170, displayed significantly higher retention of 18F-THK523 (48%; $P < 0.007$) in tau transgenic mice brains compared with their wild-type littermates or APP/PS1 mice using Micro-positron emission tomography (Fig. 3B) [97]. Although both fluorescent data and PET imaging data indicated that quinoline derivatives have excellent selectivity over A β plaques, and these compounds are structurally similar to thioflavin derivatives, it is not clear what is the unique interaction mechanism that results in the selectivity.

4. ALPHA-SYNUCLEIN

Alpha-synuclein (α -synuclein) protein, a subtype of synucleins and a 140 amino acids-long protein, is extensively localized in the nucleus of mammalian brain neurons [98]. It is predominantly expressed in the neocortex, hippocampus, substantia nigra, thalamus, and cerebellum. Although its normal physiological functions are unknown, its soluble form could be converted into insoluble neurotoxic fibrils, which could be found in pathological conditions characterized by Lewy bodies, such as Parkinson's disease, frontal temporal dementia (FTD) [99], and multiple system atrophy [100]. Three point mutations (A53T, A30P and E46K) have been identified in a rare familial Parkinson's disease. Compared to the wild-type (WT) protein, the mutants aggregate faster *in vitro*, and the formation of fibrils of mutated α -synuclein *in vivo* was considered as an important contributor for pathology of PD [101].

4.1. Mono- and Trimethine Cyanine Dyes

Volkova *et al.* studied a series of mono- and trimethine cyanine dyes for detecting α -synuclein, particularly for fibrils of α -synuclein. They found that T-284 and SH-516 displayed a significant fluorescent intensity increase upon mixing with α -synuclein fibrils. However, no significant intensity change was observed with monomeric α -synuclein. Furthermore, data of fluorescent anisotropy, lifetime and AFM experiments indicated that dye T-284 was specific towards fibrils of α -synuclein. Moreover, they suggested that dyes

of certain molecular dimensions (3 Å along z -axis and up to 6.5 Å along y-axis) could insert into “binding channels”. In addition, it was found that the dyes have a comparable binding constant to Thioflavin T with aggregated synuclein [102, 103]. However, it is not clear whether these compounds are selective for α -synuclein over other amyloid fibrils such as A β fibrils.

4.2. Styryl Benzazoles K114

The most used dyes for studying α -synuclein are Thioflavin and congo red (CR), and their analogues. Recently, K114, an analogue of CR, was reported as a fluorescent probe for staining α -synuclein in brain sections [104].

4.3. Hydroxyflavone (FE) and Other Dyes

Celej *et al.* reported that a derivative of 3-hydroxyflavone (FE) could be used for detecting various fibrils of α -synuclein. The unique fluorescent properties of FE include its triple emission peaks, and its sensitive responses toward microenvironment changes. They demonstrated unique and distinct spectroscopic signatures for the amyloid fibrils formed by the WT and mutant α -synuclein with 4-(diethylamino)-3-hydroxyflavone. These signature changes could probably be due to subtle differences in the tautomerized structures. Remarkably, the FE probe could be used to differentiate fibrils of WT, a mutated α -synuclein, particularly for A53T mutation. The remarkable difference of fluorescence spectra of the FE probe bound to the four α -synuclein variants probably is due to the tautomeric equilibria in the excited state and the existence of at least two different binding sites in the fibrils for the dye [105]. Moreover, they reported that AS140-MFC, a covalent adduct of 3-FE, could be used for continuous monitoring of fibrillation of α -synuclein [106]. In addition, the same group also reported that N-arylaminoanthralene sulfonate (NAS) derivatives could be used as fluorescent probes for monitoring α -synuclein fibrillation [107].

5. OTHER PROTEINS

Human insulin, a peptide hormone of 51 amino acids, plays a central role for regulating carbohydrate and fat metabolism, particularly for blood glucose levels in the body. External injection of insulin is one of the most important therapeutic treatments for type I diabetic patients. However, insulin is prone to aggregate and form fibrils, which could lead to serious problems, both in the production of the protein and its storage and delivery. Meanwhile, aggregated insulin could be found at repeat injection sites, and the aggregates could result in infection, bleeding, bruising, irritation, and inflammation. [108–112]. Therefore, monitoring its aggregation is very important for a high quality of insulin and avoiding side effects of insulin treatment. Monitoring the fluorescent intensity change of Thioflavin T is the most used method for detecting insulin aggregation. However, like monitoring other aggregating-prone proteins, this method is not very sensitive for the early aggregating stage [113, 114]. Recently, Hong *et al.* reported that a Tetraphenylethene based water-soluble fluorescent probe, BSPOTPE, could be used for detecting insulin aggregation. BSPOTPE is nonemissive when it is dissolved with native insulin. Remarkably, it starts to fluoresce when it is mixed with preformed insulin fibril. Of note, they also found that BSPOTPE could inhibit the nucleation process and impedes the protofibril formation of insulin. Simulation results indicated that BSPOTPE binds to partially unfolded insulin due to the hydrophobic interaction between the phenyl rings of BSPOTPE and hydrophobic residues of insulin [115]. Kitts *et al.* reported that Michler's hydrol blue (MHB), acting like a molecular rotor, could be used for detection of insulin fibrils. Once it binds to the fibrils, it displays a characteristic red-shift of excitation and an increase of fluorescent intensity. It was found that MHB is more sensitive to microenvironmental changes than Thioflavin T (ThT). When MHB was incubated with different fibrils, it exhibited different emission red-shifts,

suggesting it could be used to differentiate different fibrils [116]. In addition, Mishra reported that Nile red displayed a significant fluorescent intensity increase upon interacting with fibrils of insulin at pH 1.6 [74].

Prion protein, an infectious protein in its misfolded form, propagates by transmitting a misfolded protein state to properly folded proteins to form aggregated fibrils. Prions play a central role for the transmissible spongiform encephalopathies in a variety of mammals. Bovine spongiform encephalopathy (BSE, also known as "mad cow disease") in cattle and Creutzfeldt–Jakob disease (CJD) in humans are the most studied prion diseases [117].

In *ex vivo* experiments, Ishikawa showed that BTA-1 (a thioflavin derivative) and BSB (a Congo red derivative) selectively bound to compact plaques of prion protein (PrP) both in the brain specimens of human TSE and TSE-infected mice. After intravenous injection, prion plaques were clearly labeled by both fluorescent probes, and the staining could be observed for more than 42 h postinjection. In addition, they found these two probes inhibited new abnormal PrP formation, and *in vivo* data indicated that they could reduce the plaque loading in an experimental mouse model [118]. Recently, Stefansson *et al.* reported that the fluorescent intensity of benzothiazole and benzoxazole dimers could increase several folds when the dyes were incubated with Hamster prion proteins [119]. Bresjanac *et al.* reported that FDDNP could be used to label prion plaques (PPs) in brain tissue sections from patients with transmissible spongiform encephalopathies [120].

AOI 987

Lacmoid

Curcumin

CRANAD-2

CRANAD-3

dibenzylideneacetone

THK-265

[Ru(bpy)₂dppz]²⁺

Michler's hydrol blue

BTA-1

FDDNP

3

4

For other misfolding-prone proteins, references related to their dual functional probes are very limited. In this review, we didn't discuss these references.

6. CONCLUSION

Substantial progress has been achieved for the development of dual functional probes for misfolding proteins in the past decades. However, we believe much more efforts are needed to face the following challenges: 1) *More new scaffolds*: current available scaffolds for dual functional fluorescent probes are primarily from families of thioflavin, congo red, stilbene, benzophoxazine, curcumin, chalcone, FDDNP, and flavone. More new skeletons will enable

us to expand our arsenal. 2) *Species selectivity*: although current dual probes are very useful for detecting the misfolded proteins *in vitro* solution testing, they always have poor species selectivity, which is very important for detecting/diagnosing of disease status with samples from patients and for *in vivo* imaging. Table 1 shows that one misfolding protein is involved in multiple diseases and several misfolded proteins could be found in one clinical disease. For an example, misfolded A β s are related to AD, DLB, and FTD, and in DLB patients A β plaques, tau tangles, and alpha-synuclein fibrils could be found. In addition the overlap of distribution areas of multiple misfolding proteins in one disease is a very challenging issue for developing imaging probes and diagnosis tools. Currently, it is very difficult to use available dual probes to differentiate them in brain slices, extraction solutions and *in vivo* images. Development of species-specific dual probes is highly desirable for *in vivo* NIRF imaging and PET/SPECT imaging. 3) *Capability for detecting soluble species*: one of the primary causes of misfolding protein diseases is the over accumulation of soluble species that include monomers, dimers and oligomers. These species could probably be biomarkers for early/presymptomatic stages of disease. Therefore, a dual functional probe capable of detecting these soluble species is very necessary for early diagnosis and detection. Unfortunately, few agents are available for this purpose. Most of the current probes are primarily suitable for detecting insoluble large assemblies. 4) *More NIRF probes for misfolding protein beyond A β species*: NIRF imaging is a very useful tool for basic and preclinical research. Currently, all of the reported NIRF probes are developed for *in vivo* imaging of A β species. We still fall short of NIRF probes for other misfolding proteins.

Although limited SAR (structural activity relationship) has been discussed in several review references, and some compounds have been designed and tested [30]. However, their species-selectivity is not significantly improved. To design a new generation of dual functional fluorescent probes, we believe the following approaches are worth exploring. 1) Rational design of compounds based on the results of NMR studies. Because of the difficulty of preparing crystal of these misfolding proteins, X-ray crystallography could not be widely applied. Instead, structural NMR studies could be used for exploring the 3D structures of soluble (solution NMR) and insoluble aggregates (solid-state NMR). Particularly, investigation on the specific interaction sites of the protein with ligand could provide basis for further structural modifications of the lead ligand. 2) Design probes that could parallel insert into the beta-sheet of the target misfolding proteins. All of the misfolding-prone proteins contain certain length of hydrophobic core fragments, and the aggregating behaviors of these cores are probably alike. However, the amino acid residues nearby the cores of these misfolded proteins are very different across species, and we believe the differences could be utilized to design species-selective probe. 3) Design probes that have different ground state or excitation state upon binding. Very recently, Yang *et al.* demonstrated that 2-cyanoacrylate probes could fluorescently differentiate A β and prion protein in tissue slice due to their different ground states once the probe binds to A β and prion protein, and the ground state disparity is probably due to the different polarity of binding microenvironment [121]. We believe the different residues nearby the hydrophobic core fragment could provide varied microenvironments that could lead to different ground or excitation states upon interaction. 4) Seeking species-selective probes via high throughput screening. Some probes such as BF-126 and BF-170 were found through screening [96]; however, establishing more sensitive and efficient methods would definitely be beneficial for seeking species-selective imaging probes.

We believe dual functional probes are very essential for *in vitro* biological assays, drug development, mechanism studies, preclinical and clinical diagnosis, and *in vivo* imaging. To fulfill the above listed challenges, collaborations from interdisciplinary fields of medicinal chemistry, organic chemistry, biochemistry, structural biology, neurobiology, and biophysics are urgently needed to produce more reliable, selective, and practicable probes.

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ABBREVIATIONS

ALS	amyotrophic lateral sclerosis
AD	Alzheimer's disease
AFM	atomic force microscopy
APP	amyloid peptide precursor
APP/PS1	transgenic mice
A β	amyloid- β
BBB	brain blood barrier
BSE	bovine spongiform encephalopathy
CAA	cerebral amyloid angiopathy
CJD	Creutzfeldt-Jakob disease
CLI	Cerenkov Luminescence Imaging
CNS	central nervous system
CR	Congo red
CSF	cerebrospinal fluid
FE	hydroxyflavone
FRET	Förster (Fluorescence) resonance energy transfer
FTD	frontotemporal dementia
HD	Huntington's disease
HOMO	highest occupied molecular orbital
ID	injection dosage
LUMO	lowest unoccupied molecular orbital
MHB	Michler's hydrol blue
MRI	magnetic resonance imaging
n-Tau	nonphosphorylated tau
NAS	N-arylamino-naphthalene sulfonate
NFTs	neurofibrillary tangles
NIR	near infrared
NIRF	near infrared fluorescent
p-Tau	hyperphosphorylated tau
PD	Parkinson's disease
PET	positron emission tomography
PHFs	paired helical filaments

PPs	prion plaques
PrP	prion protein
PSAMs	peptide self-assembly mimics
SPECT	single-photon emission computed tomography
ThT	Thioflavin T
TICT	twisted intramolecular charge-transfer
TSE	transmissible spongiform encephalo-pathy
WT	wild type
2,2'-Dpa	2,2'-dipicolylamine

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Progression of Alzheimer's disease

Fig. (1).

The changes of the predominance of A β species during the progression of Alzheimer's disease, which starts from over-accumulation of monomers and ends up with the predominance of plaques. In the course of the development, all of the species are co-existed at most of the disease stages.

Fig. (2).
(A) Binding model of ThT to the KLVFFAE protofibril using molecular dynamics simulations, (left) the side-chain conformations of the PSAM ladders implicated in ThT-binding; (right) binding orientation for ThT (Permission from ref. 57). (B) ThT molecules binds to the protofibril of KLVFF (left), and a representative complex structure of the primary binding mode (Permission from ref.56).

Fig. (3).

Curcumin and its analogue for in vitro tests and NIRF imaging of AD mice. (A) Fluorescence spectra of curcumin with A β 40 monomers (left), A β 42 monomers (middle) and A β 40 aggregates (right); (B) Fluorescence spectrum of CRANAD-2 with A β 40 aggregates (left), emission wavelength change (middle) and lifetime change (right) in the present of A β 40 aggregates; (C) NIRF imaging with CRANAD-2 at different time points after i.v. injection (left: APP/PS1 mouse; right: wild type mouse). The signal difference between APP/PS1 and WT mice could be seen at very early time point (10 Min) after injection (Adapted and permission from ref. 84); (D) Ex vivo unmixed spectra of autofluorescence and bound and free CRANAD-3 with spectral unmixing imaging for an APP/PS1 mouse

brain; (E) Ex vivo unmixed images for autofluorescence (#1), bound CRANAD-3 (#2), free CRANAD-3 (#3) and merged image of #1,2,3 (#4). The images indicated that free CRANAD-3 was primarily existed in the blood system and bound CRANAD-3 was in the cortex area. (F) In vivo unmixed images for autofluorescence (#1), bound CRANAD-3 (#2), free CRANAD-3 (#3) and merged image of #1,2,3 (#4) (Adapted and permission from ref. 86).

Fig. (4).

(A) Histological images of compound **4** (BODIPY-Zn complex) for p-Tau detection. Upper panel: Various tau tangles stained with thioflavin T; Lower: the same tau species in upper panel stained with compound **4** (Permission from ACS publisher, ref.95); (B) In vivo PET imaging with THK-523. Upper left: Tau transgenic mouse rTg4510; Upper right: APP transgenic mice APP/PS1; Lower panel: aged-matched WT mice of the upper panel; (C) Quantitative analysis of brain uptake of THK-523 in transgenic Tau mice, transgenic APP/PS1 mice and aged-matched WT mice (Permission from ref.97).

Table 1

A Brief Summary of Misfolding Proteins Discussed in this Review and their Related Common Clinical Diseases and Primary Distribution Areas

Misfolding Proteins	Common Clinical Diseases	CNS/Non-CNS	Primary Distribution Areas
Amyloid beta	AD, FTD, DLB,	CNS	cortex, hippocampus, striatum, cerebral vessel, CSF
Tau proteins	AD, DLB, FTD, Pick's disease,	CNS	cortex, hippocampus, striatum, CSF
Alpha-synuclein	PD, DLB, multiple system atrophy	CNS	neocortex, hippocampus, substantia nigra, thalamus, cerebellum
Prion proteins	TSE, BSE, CJD	CNS	Infected and transmitted brain areas
Insulin	Type I/II diabetes	Non-CNS	Injection sites