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论文题目：　　“针”知“拙”见——荧光探针制备及阿尔茨海默症早期诊断功能评价

Smart Probe Imaging Dementia

—— Synthesis of fluorescent probe and evaluation of its function for early diagnosis of AD

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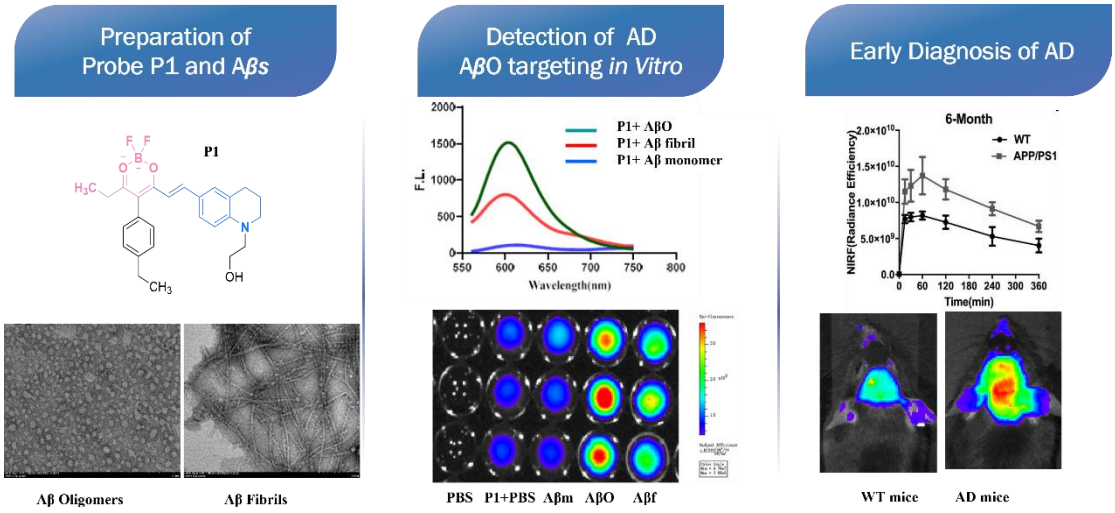
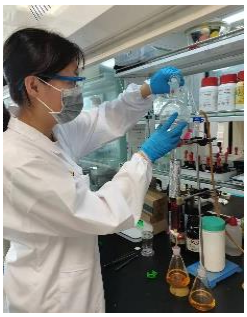
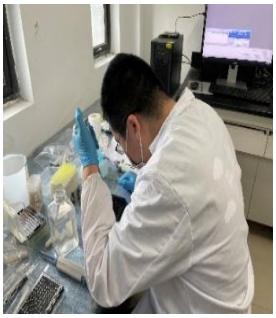
Graphical Abstract







Abstract

With the aging of society, Alzheimer's disease (AD) patients in China have increased year by year, with the number of patients exceeding 10 million. Currently, the effect of therapies for AD is very limited. One of the main reasons is that most of patients are already in the middle or late stages of the disease when they are diagnosed. Because the atrophy of the cerebral cortex and hippocampus can hardly recover, the best treatment time for the patients has already been missed. Sensitive and specific diagnostic methods are urgently needed to detect AD in an early stage, to gain opportunity and time for treatment of patients.

Near-infrared (NIR) fluorescence imaging has the advantages of high sensitivity, simple operation and fast imaging. Furthermore, the development of NIR small-molecule probes targeting AD biomarkers for real-time imaging of the disease is an important strategy for early diagnosis of AD. Amyloid beta oligomer (AβO) is a neurotoxic substance that begins to trigger nerve tissue damage in the brain more than 10 years before the onset of AD symptoms. Lencanumab, a drug successfully marketed this year, is the first to slow down the process of AD and works by binding AβO. This also proves that AβO is a reasonable target for disease diagnosis and treatment.

In this work, we synthetized a near-infrared fluorescent probe P1, which was shown to bind AβO strongly (Kd = 43.11 nM) and selectively. Probe P1 has a targeting function in in vitro experiments. The probe was able to image the brains of transgenic AD mice in the early stages and diagnose 6-month-old AD mice. Moreover, the probe P1 can easily cross the blood-brain barrier and has low cytotoxicity, so it has the potential to be developed as an imaging agent for early AD diagnosis.

Key words： Alzheimer’s Disease, early diagnosis, Amyloidβ oligomer （AβO）, Near-infrared fluorescence imaging, Fluorescent probes

“针”知“拙”见——荧光探针的制备及阿尔茨海默症早期诊断功能评价

摘 要

随着老龄化社会的到来，阿尔茨海默症(Alzheimer’s Disease, AD)患者逐年增多，患病人数超过了 5000 万，我国的患者有 1000 多万。目前，AD 的治疗效果不佳，主要的原因之一是大多患者被发现时已经处于疾病的中晚期，大脑皮质、海马等神经组织萎缩，难以恢复，错失了最佳治疗时间。临床亟需灵敏和特异性的诊断方法在疾病的早期发现患者，为治疗争取时间窗。

近红外荧光成像技术具有灵敏度高、操作简单和成像快的优势，研发靶向 AD 生物标志物的近红外小分子探针，用于疾病的实时成像，是 AD 早期诊断的重要策略。淀粉样蛋白寡聚体（Amyloidβ oligomer, AβO）是一种神经毒性物质，在 AD 症状出现的 10 多年前，触发大脑神经的损伤。今年成功上市的仑卡奈单抗是第一个减慢 AD 病程的药物，通过结合 AβO 发挥作用，也证明了 AβO 是疾病诊疗的合理靶点。本课题合成了近红外荧光探针 P1，在体外实验中显示，P1 对 AβO 的结合力强 (Kd =43.11nM)，选择性好具有靶向功能，在近红外荧光成像下，使 AD 小鼠大脑发光成像，诊断出疾病早期阶段的小鼠。并且，探针 P1 易于透过血脑屏障，细胞毒性低，具有成为 AD 早期诊断成像剂的潜力。

关键词：阿尔茨海默症, 早期诊断, 淀粉样蛋白寡聚体（AβO）, 近红外荧光成像, 探针

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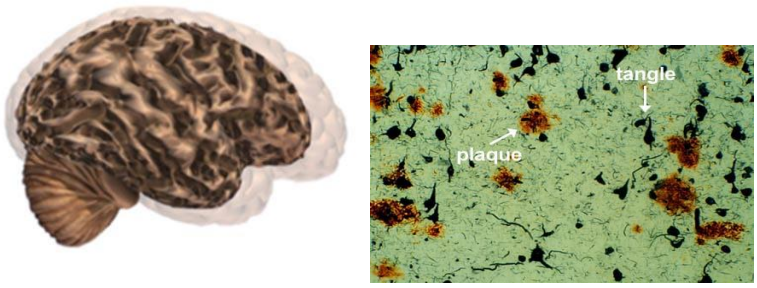
1. Introduction

Alzheimer's disease (AD) is the fifth leading cause of human deaths, accounting for 60-80% of dementia. The population of many countries is aging and the number of new cases of dementia is growing rapidly. Currently, more than 55 million people worldwide are suffering from dementia, with more than 10 million patients in China [1,2]。AD is characterized by a slow progression of the disease and a continuous degeneration of the nervous tissue. Initially, the patient suffers from loss of short-term memory, followed by motor disorders, speech disorders, emotional instability, and finally loss of physical function which leads to death. The average duration of the disease is about 3-9 years. [3] The large number of patients and the long duration of the disease impose a heavy economic and emotional burden on the world.

1.1 Hypothesis of amyloid protein cascade

The mechanisms underlying the pathogenesis of AD are complex, and they have not been fully understood now. In the late-time stage of disease, AD patients experience severe atrophy of the cerebral cortex and hippocampus (Fig.1a), with large numbers of beta-amyloid(Aβ) protein plaques and Tau protein tangles in the brain (Fig.1b). In 1992, John Hardy observed patients of familial AD and found that a missense mutation in the gene encoding an Aβ precursor protein (APP). The gene leads to an increased expression of Aβ protein and a higher number of plaques. As a result, Hardy proposed the well-known amyloid cascade hypothesis and stated that the core pathogenic factor in AD is the Aβ plaque. [4]



A B

Fig.1 A brain with advanced Alzheimer's and a normal brain(A); Plaques and tangles in the Alzheimer's brain(B).

How are amyloid plaques produced? In the brain of AD patients, the APP undergoes a two-step shearing process by β-secretase and γ-secretase to form Aβ monomers, which mainly include Aβ40 and Aβ42. Due to the presence of hydrophobic amino acids, the β-sheet structures of Aβ monomers aggregate with each other and gradually form soluble oligomers (AβO). AβO consists of small oligomers (2 and 3 oligomers), medium-sized oligomers (9 and 12 oligomers) and high-molecular-weight oligomers (protofibrils). AβO further aggregated to form insoluble fibrils, and the fibrils stacked together to form plaques (Fig.2) [5,6].

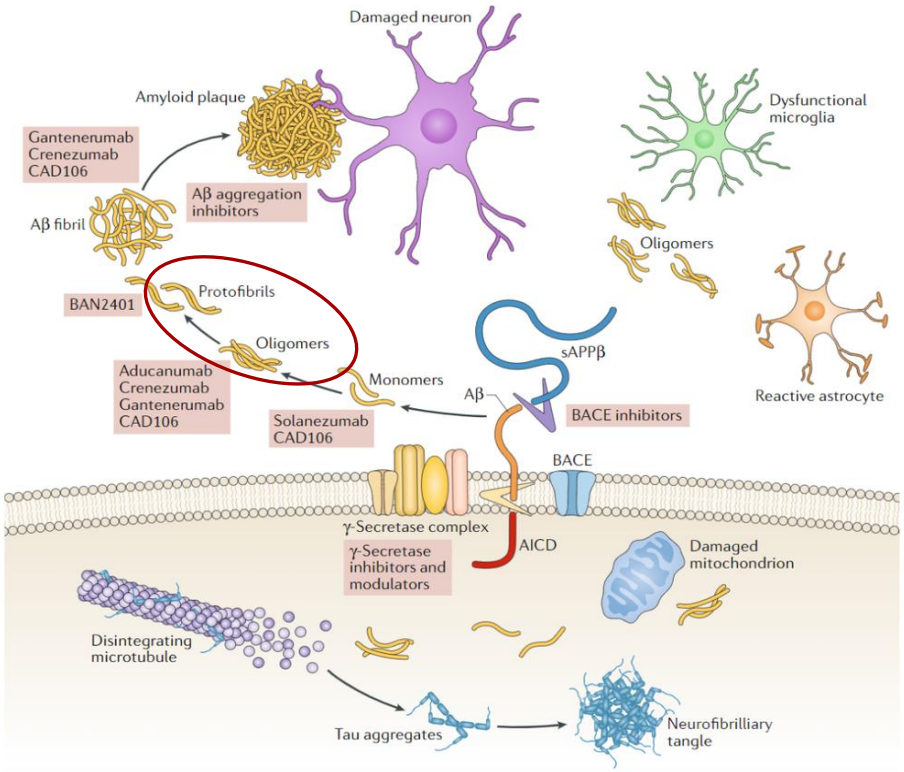


Fig.2 Forms of β-Amyloid and Neurotoxicity of oligomers. Aβs soluble species include monomers and oligomers，the Abs insoluble species include fibril and plaques. AβO could induced microtubule and mitochondrion dysfunction, and apoptosis of microglia before observable symptoms occur. AβO contribute significantly to the neurotoxicity in AD.

In recent years, the Aβ protein cascade theory has shifted from the ‘plaque hypothesis’ to the ‘oligomer hypothesis’. Different types of Aβ protein have different toxicity to nerve cells. Aβ monomer has no neurotoxicity and AβO has the strongest neurotoxicity. Insoluble fibers and plaques are deposited in the brain, so toxicity is reduced. AβO already exists in the brains of AD patients 20 years before the onset of clinical symptoms. It was found that AβO triggered mitochondrial and microtubule damage, neuronal apoptosis, impairment of microglia and astrocyte function, all of

which ultimately led to neuronal apoptosis and brain atrophy **(**Fig.2).[7] Amount of Aβ plaques does not correlate with severity of AD condition, In contrast, soluble AβO is not only more neurotoxic but also better correlated with disease progression. [8,9,10] Furthermore, in January 2023, Lencanumab was approved by the FDA to treat patients in the early stages of AD. It is the first drug that can slow the progression of AD. It reduces plaque in the brain by targeting high molecular weight AβO (protofibrils). [11] Pathogenesis and pharmaceutical research showed that AβO plays a key role in the development of the disease and is an effective target for treatment and diagnosis of the disease.

1.2 Near-infrared fluorescence imaging in disease diagnosis

Near-infrared fluorescence (NIRF) imaging is highly sensitive because of the low background and high intensity of the biofluorescent signal. NIRF probe is a "smart probe", which it significantly enhances the intensity of fluorescence when bounded to target proteins, enabling the diagnosis of disease. NIRF probes have already been used in the diagnosis of tumors in clinical practice. During tumor resection surgery, Pafolacianine and Indocyanine green (ICG) are able to "light up" small tumors that are difficult for the surgeon to see(Fig.3). As tracers for surgical navigation, they significantly improved tumor resection rates and prolonged patient survival in ovarian, lung, and liver cancer surgeries in ovarian, lung, and liver cancer. [12, 13] Similarly, NIRF probes can target AβO to "light up" the brains of AD patients, which has the potential to become an early diagnostic drug for AD. In addition, the advantages of NIRF probe imaging include: (1) non-invasive diagnosis and good safety; (2) rapid data acquisition and real-time imaging; (3) moderate cost, which does not require expensive equipment and personnel training costs. [14]

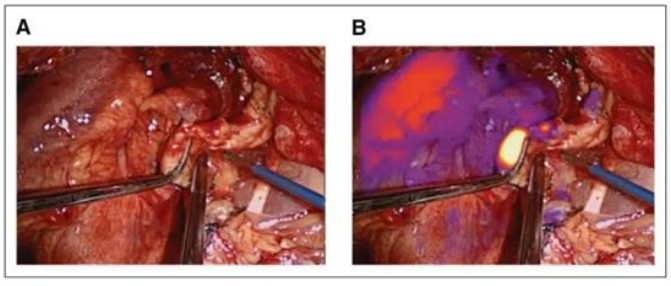


Fig.3 Visualization of ovarian cancer lesions in the right paracolic gutter using (A) normal white light compare with (B) NIRF imaging following Pafolacianine injection

1.3 Current status and progress in the diagnosis of AD

Currently, the diagnosis of an AD patient is based on the elevation of Aβ40/Aβ42 monomer in the cerebrospinal fluid and the presence of Aβ plaques in the brain. In other words, Aβ monomer and plaques are used as diagnostic targets. However, these methods have the following limitations: (1) The extraction of cerebrospinal fluid is invasive and carries the risk of complications; (2) Because the number of Aβ plaques does not correlate with disease progression, and Aβ plaque deposition is present in some normal older adults, a positive result does not confirm the diagnosis of AD or assess the severity of AD； (3) Clinical tracers have a high binding rate to insoluble Aβ plaques. However, there isn’t a diagnostic agent for targeting soluble AβO. These drawbacks will also promote the development of new testing technologies that better reflect the AD pathology process.

1.4 Research content and ideas

We are finding more and more Alzheimer's patients around us. One of my friend's grandmother was a warm and capable entrepreneur before she retired. She was diagnosed with AD three years ago and her memory became increasingly poor. When we were over at the friend's house, she repeatedly asked me "What's your name?" Although I just told her my name 10 minutes ago. Another AD patient, Ms. Wang is my neighbor and is 72 years old. The disease changed her from a gentle and polite person to a cranky and temperamental person. She sometimes can't find her way home, and her family must always be with her and take care of her patiently. These patients and their families have suffered tremendously, and we were eager to do something for them, so we became interested in AD research.

Since our first year of high school, Mr. Xu Liangliang has guided our research and we have read a lot of literature about AD. We realized that the disease is very difficult to treat, and medicine can only relieve the symptoms of the disease instead of curing it. In January 2003, Lencanumab was approved and is able to slow the progression of the disease, giving patients hope. However, it can only treat patients in the early stages of disease and is ineffective in patients in the middle and last stage of AD. Therefore, an effective method that can diagnose early patients with AD will play a key role in

improving treatment outcomes. Mr. Xu gave us professional and wise guidance during the research. We chose near-infrared fluorescent probe P1 to illuminate the toxic AβO to explore new ways to diagnose early AD. The study, named "Smart Probe Imaging Dementia" is composed of the following three parts (Fig. 4).

(1) Preparation of NIRF probe P1 and three types of A, and detection of their properties;

(2) Testing the ability of probe P1 to target AβO *in vitro*;

(3) Detection of the early diagnostic function of probe P1 in transgenic AD mice.

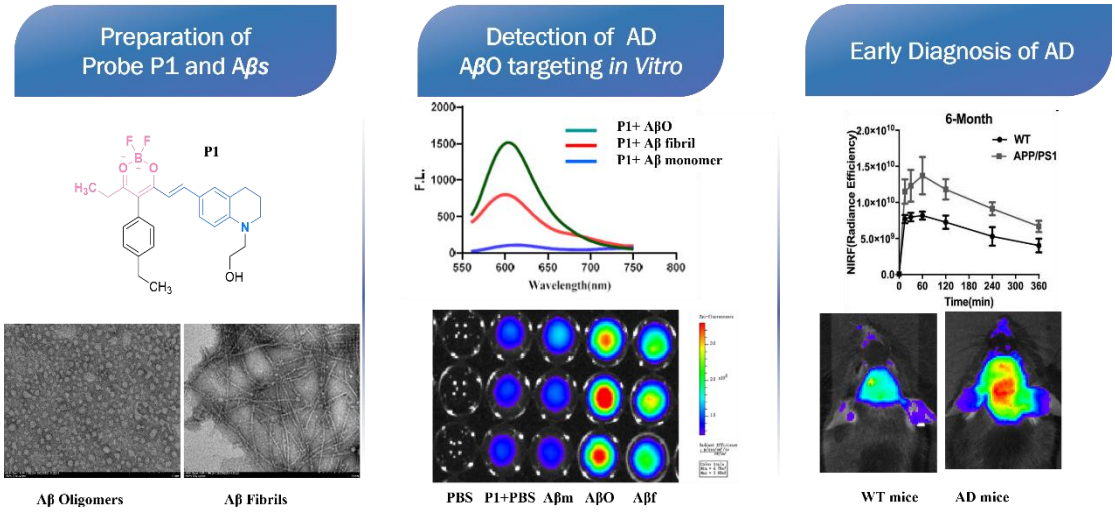
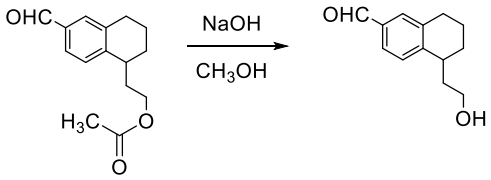


Fig.4 Reach contents of 'Smart Probe Imaging Dementia'

2、Experiments and Methods

2.1 Synthesis of Probe P1

2.1.1 5-(2-hydroxyethyl)-5,6,7,8-tetrahydronaphthalene-2-carbaldehyde



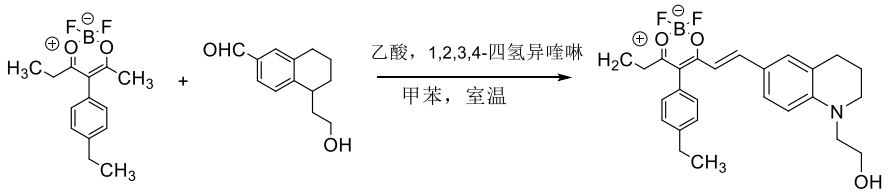
1 2

2-(6-formyl-1,2,3,4-tetrahydronaphthalen-1-yl)ethyl acetate, 1 (190 mg, 0.77 mol) was dissolved in methanol(8 ml), and then NaOH solution(1ml, 1.25mmol) was added dropwise. The reaction mixture was stirred at r.t. for 30min.

Dichloromethane(10ml) was added to the flask, then transferred to a dispensing funnel and washed with water (5 mlx3), saturated saline (5 mlx2). The resulting solution was dried using anhydrous Na_2SO_4, concentrated under reduced pressure and purified by column chromatography (PE:EA = 3:1) to give light yellow oily liquid 2 (120mg, 92%).

^{1}H NMR (300 MHz, Chloroform-d) δ 9.69 (s, 1H), 7.57 (dd, J = 8.6, 2.1 Hz, 1H), 7.50 (s, 1H), 6.75 (s, 1H), 3.94 (t, J = 5.8 Hz, 2H), 3.61 (t, J = 5.8 Hz, 2H), 3.57 – 3.48 (m, 2H), 2.85 (t, J = 6.3 Hz, 2H), 2.05 – 1.98 (m, 2H).

2.1.2 Probe P1



3 2 P1

4-ethyl-5-(4-ethylphenyl)-2,2-difluoro-6-methyl-2H-1 dioxaborinine, 3(260 mg, 0.97 mmol) and 2 (200 mg, 0.97 mmol) were dissolved in toluene (3 ml), followed by the additions of acetic acid (33.0 μL, 0.58 mmoL), tetrahydroisoquinoline (24 μL, 0.19 mmoL).The result solution was stirred at room temperature overnight. The mixture was extracted with CH_2Cl_2 (3 × 40 mL), and the organic phase was dried over Na_2SO_4. After the solvent was removed in vacuum, the residue was purified by

column chromatography to give P1 as a dark red solid. (77mg,17%), mp. 186-188 ℃. (Fig. 5)

^{1}H NMR (300 MHz, Chloroform-d) δ 8.05 (d, J = 15.1 Hz, 1H), 7.35 (s, 2H), 7.23 – 7.15 (m, 3H), 7.07 (s, 1H), 6.66 (d, J = 8.8 Hz, 1H), 6.15 (d, J = 15.1 Hz, 1H), 3.91 (t, J = 5.6 Hz, 2H), 3.58 (t, J = 5.7 Hz, 2H), 3.52 (t, J = 5.7 Hz, 2H), 2.78 (dt, J = 12.1, 6.6 Hz, 4H), 2.37 (q, J = 7.5 Hz, 2H), 1.99 (t, J = 5.9 Hz, 2H), 1.37 (t, J = 7.6 Hz, 3H), 1.18 (t, J = 7.5 Hz, 3H).HRMS(ESI) $C_{26}H_{30}BF_2NO_3$, $[M+Na]^+$ calculated = 476.2185; found = 476.2175.

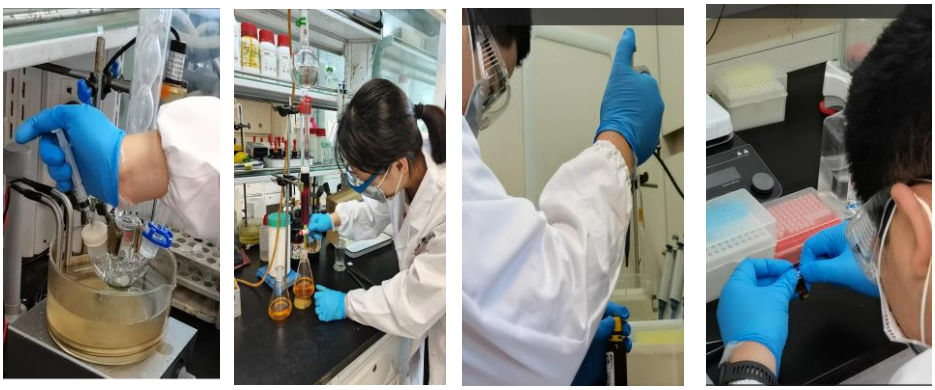
2.2 Preparation of the Aβ monomer, AβO and Aβ fibril

The preparations were performed according to previous reports. [15,16]。

Preparation of Aβ monomer：Aβ42 peptide (1.0 mg) was dissolved in HFIP(1.0 mL) as monomer state solution, and store at -20 ℃.

Preparation of AβO：The above solution(100μL) was added to DD H_2O(886μL) in an Eppendorf tube. After 20 min incubation at room temperature, the samples were centrifuged for 15 min at 14,000×G and the supernatant fraction was transferred to a new tube and subjected to a gentle stream of N_2 for 15 min to evaporate the HFIP. The samples were stirred for 3 hr. at 22℃.

Preparation of Aβ fibril： Aβ42 peptide (0.5 mg) was resuspended in 1% hydroxylamine solution (0.5 mL). The resulting solution was diluted 10-fold with PBS buffer (pH 7.4) and stirred a room temperature for 3 days. (Fig.5)



A B C D

Fig. 5 Some pictures in the process of the research: Synthesis of the probe P1(A, B); Preparation of AβO and Aβ fibrils(C,D).

2.3 Wavelength detection of probe P1

To a cuvette was added 980 μL PBS and 20 μL probe P1(25 μM). The solution was mixed well. The solution was scanned by a UV spectrophotometer in the range of 300-900 nm of wavelength. First, the maximum excitation wavelength of the probe was determined, followed by the maximum emission wavelength.

2.4 Stability studies of the probe P1 in mouse serum

The in vitro stability of P1 in mouse serum was determined by incubating P1 (10 μL, 10% acetonitrile solution, 10 μM), with 300 μL of mouse serum at 37 ℃ for 0, 15, 30, 60 min. Proteins were precipitated by adding 500 μL of acetonitrile after centrifugation at 5000 rpm for 15 min at 4 ℃. The supernatant was collected. Approximately 0.1 mL of the supernatant solution was analyzed using HPLC (UV detector, λ = 254 nm).

2.5 Blood−Brain Barrier Penetration Determination

Standard Curve: P1 solutions were prepared at concentrations of 100, 50, 25, 12.5, 5, 1, 0.5ug/mL in ethyl acetate respectively. The fluorescence intensity (F.I.) of the solutions was measured under the light of 540nm, and then concentration- F.I. standard curve was plotted by Prism (Fig.6).

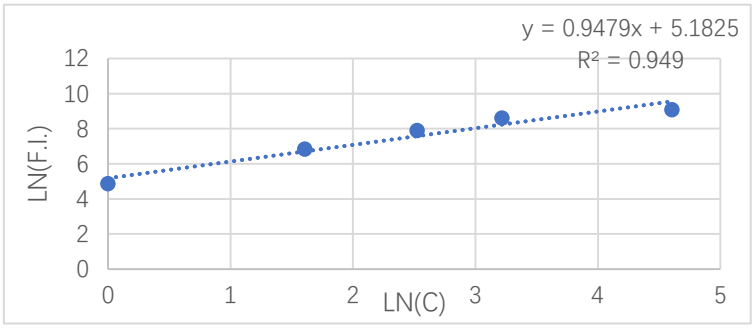


Fig.6 Concentration-fluorescence Intensity Standard Curve of Probe P1

P1 (0.4 mg/mL) solution was injected through the tail vein into 6 ICR mice divided into 2 groups. Three mice in each group were subjected to cardiac perfusion at 2min, 60min respectively, and then the whole brain was removed. The brains were made into a homogenate, and ethyl acetate (2 ml) was added and mixed on a shaker for 2 hours, then the supernatant was taken after centrifugation. The resulting solution

was centrifuged and measured the fluorescence intensity of supernatant. Based on the above standard curve, the concentration of P1 in the brain of ICR mice was obtained and the P1 amount in the brain of ICR mice is calculated: %ID/g = (concentration of the probe in the brain homogenate C/concentration of the administered drug C0)/weight of the brain of the mouse*100%.

2.6 MTT cytotoxicity of P1

SH-SY5Y cells were planted in 96-well plates at a concentration of 5000 per well. After around 12 h, the cells were treated with different concentrations of P1 for 24 h. Then the cells were incubated with 20 μL 5 mg/ml MTT solution for 4 h. After incubation, the media were discarded and 100 μL of DMSO was added into each well. Subsequently, the plates were read at 570 nm by using a Microplate reader. The experiments were repeated three times independently.

2.7 Near-infrared fluorescence spectral testing of probe P1 *in vitro*

2.7.1 Near-infrared fluorescence spectral testing of probe P1 with Aβs

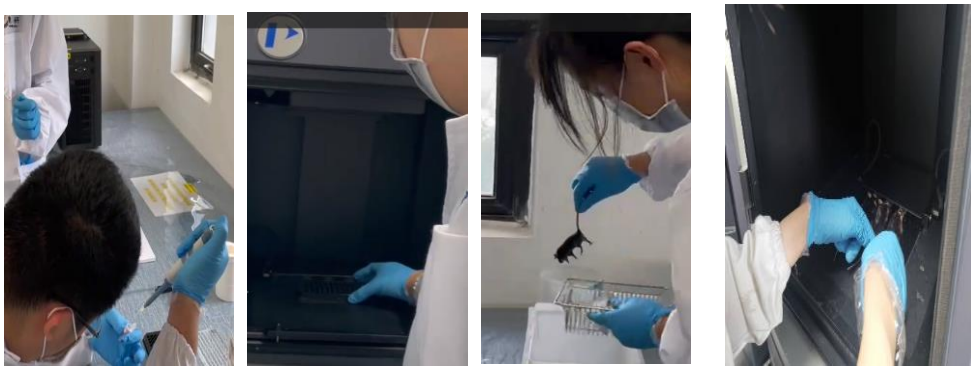
PBS buffer(0.96mL) was added to a quartz cuvette as a blank control and its fluorescence intensity was recorded. P1(10 μL, 25 μM) was added to the cuvette and the fluorescence spectrum of the solution was recorded with excitation at 540 nm and emission from 590 nm to 800 nm. To the above P1 solution, Aβs stock solution(30 μL 25 μM in HFIP for the monomers and in PBS buffer or double distilled water for the oligomers and Aβ42 fibril) was added to make a final Aβ concentration of 700 nM. The fluorescence spectra of this solution were recorded. The final near-infrared fluorescence spectra of P1 with Aβs were corrected using the fluorescence spectrum of blank control.

AβO solutions were prepared at concentrations of 100, 250, 500, 750, 1000, 1500, 2000, and 2500 nM, respectively. The fluorescence spectra tests were conducted following the above procedures. The data were processed with GraphPad Prism software to obtain the binding constants of probe P1 and AβO.

2.7.2 Brain phantom imaging

A 6-month old wild-type (C57BL/6) mouse was sacrificed and its brain was dissected to making a homogenization. PBS(2.0 mL) and the homogenate(0.1 mL) was added to 15 wells of a 96-well plate, and then added the P1(3.3μL, 250nM)and

the Aβ monomers, oligomers, and fibrils(9.9μL, 250nM), respectively. The resulting brain homogenates were imaged with Ex/Em = 540/610 nm using an IVIS® Spectrum imaging system (Fig.7).



A B C D

Fig. 7 Some pictures in the process of the research: Brain phantom imaging (A, B); Near-infrared fluorescence imaging in vivo (C, D).

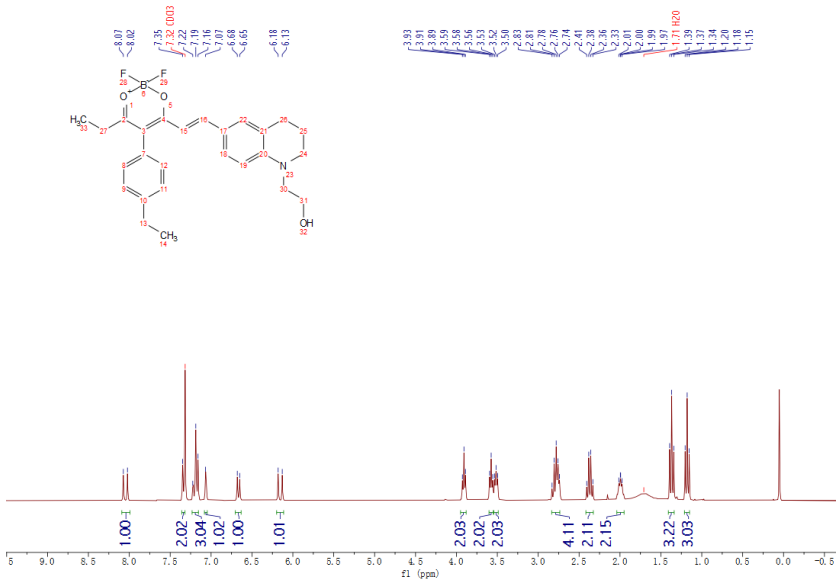
2.8 Near-infrared fluorescence imaging *in vivo*

An injection solution of P1 (4 mg/kg) was freshly prepared in 15% DMSO, 15% cremorphor, and 70% PBS. The solution was stabilized for 20 min before injection. 6-month old mice (female transgenic APP/PS1, n = 3 – 4 and age matched female wild type control mice, n = 3 – 4) were shaved before imaging. Each mouse was injected intravenously with 100 μL solution of P1. Fluorescence signals from the brain were recorded at 0, 30, 60, 120, 240 and 360 min after tail vein injection of the probe. In vivo NIR imaging was performed using an IVIS®Spectrum animal imaging system (Caliper LifeSciences, Perkin Elmer, Hopkinton, MA). The images were acquired with a 540 nm excitation filter and a 610 nm emission filter. An ROI was drawn in the brain region and data analysis was performed using LivingImage® 4.2.1 software (Fig.7).

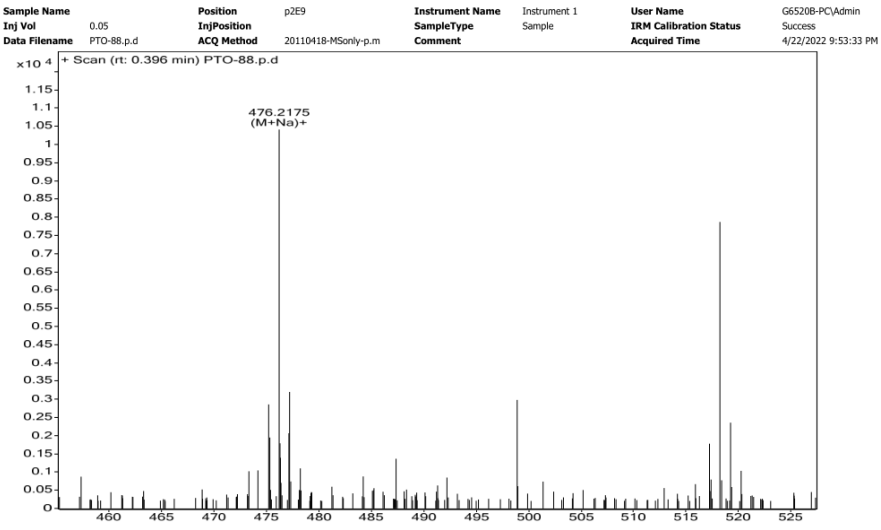
3、Results and Discussion

3.1 Structure identification of probe P1

Probe P1 was synthesized by two reactions including hydrolysis and condensation. It was confirmed by ^{1}H NMR and HRMS(Fig.8).



a. 1H NMR spectrum of probe P1: ^{1}H NMR (300 MHz, Chloroform-d) δ 8.05 (d, J = 15.1 Hz, 1H), 7.35 (s, 2H), 7.23 – 7.15 (m, 3H), 7.07 (s, 1H), 6.66 (d, J = 8.8 Hz, 1H), 6.15 (d, J = 15.1 Hz, 1H), 3.91 (t, J = 5.6 Hz, 2H), 3.58 (t, J = 5.7 Hz, 2H), 3.52 (t, J = 5.7 Hz, 2H), 2.78 (dt, J = 12.1, 6.6 Hz, 4H), 2.37 (q, J = 7.5 Hz, 2H), 1.99 (t, J = 5.9 Hz, 2H), 1.37 (t, J = 7.6 Hz, 3H), 1.18 (t, J = 7.5 Hz, 3H).



b. HRM:S spectrum of probe P1: HRMS(ESI) C26H30BF2NO3, [M+Na]+ calculated = 476.2185; found = 476.2175.

Fig.8 1H NMR(a) and HRMS(b) spectrum of probe P1

3.2 Characterization of the Aβ monomer, AβO and Aβ fibril

3.2.1 Thioredoxin T (ThT) Test

ThT is used as a standard compound to identify the type of Aβ protein. It fluoresces weakly on its own, but the more Aβ fibers, the higher the fluorescence intensity(FI) of ThT. ThT binds to Aβ monomer with weak FI and binds to Aβ fibril with the highest FI, which is about 3~4 times as strong as that of AβO[17]. It was confirmed that the FI of the Aβ fiber bound to ThT was about 1500, the FI of AβO was about 500, and the FI of monomer was less than 100, and the prepared Aβs were qualified (Fig.9).

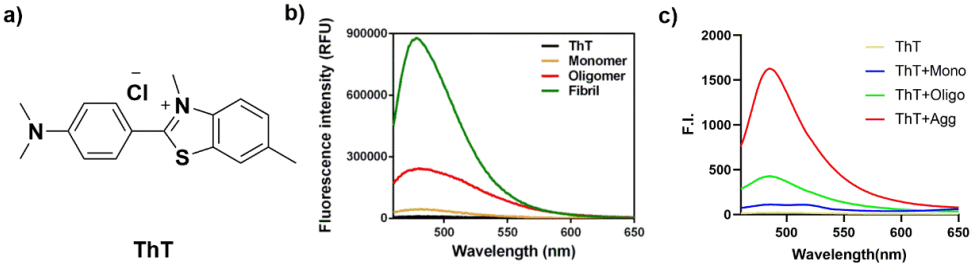


Fig.9 a. Structure of ThT； b. Fluorescence intensity of ThT upon interaction with Aβs repoted by literature； c. Fluorescence intensity of ThT upon interaction with Aβs in the PBS

3.2.2 Transmission Electron Microscope(TEM) imaging

TEM imaging could show the structure of protein at the nanoscale and is used to corroborate the morphology of proteins. TEM imaging shows that AβO consists of globular particles of various sizes，which is consistent with AβO having multiple forms, including dimer, hexamer and protofibril. Aβ fibril look like slender fibers (Fig.10).

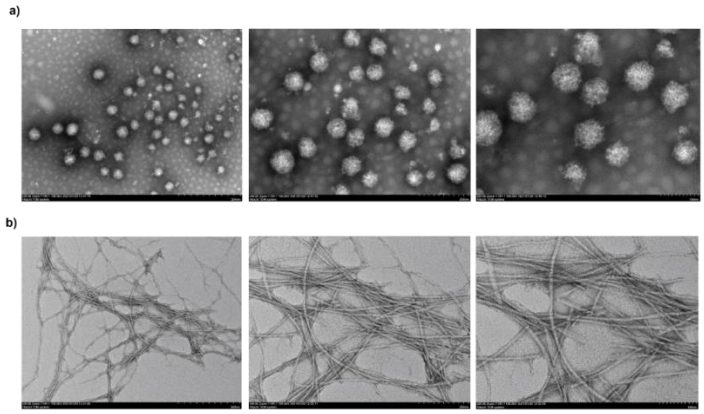


Fig.10 a. TEM image of Aβ42 oligomers； b. TEM image of Aβ42 fibrilsr

3.3 Excellent blood–brain barrier penetration of probe P1

Efficient blood–brain barrier (BBB) penetration is important for a brain-imaging

probe. To validate whether probe P1 can cross the BBB, P1was injected into wild type mice. After that, the mice were sacrificed and their brains were dissected at 2min or 60min, respectively. P1was extracted by ethyl acetate from the brain homogenates and the fluorescence spectra confirmed the existence of P1 in the extraction. It had a high initial BBB penetration, reaching 19.1% ID/g in the brain at 2 min and 10.7% ID/g after 60 minutes (Tab.1). The experimental results showed that P1 could cross the BBB and thus could be an excellent brain imaging probe.

Tab.1 Probe P1 BBB penetration

Tim(min)	the Brain Uptakes (ug/ml)				%ID/g
	1	2	3	AVG	
2	30.5	30.8	30.9	30.7	19.1
60	20.2	14.5	16.9	17.2	10.7

Fluorescent probe is transported through the bloodstream to reach the brain, so reasonable stability of the probe in serum is also important for imaging the brain. Before in vivo imaging, we also investigated the stability of P1 in serum, which can provide an indication of the potential stability of the probe in blood. In this regard, we incubated P1 in mouse serum at 37 ° C for 0, 30, and 60 min and measured its stability using HPLC. The experimental results showed that more than half of the P1 was not metabolized in 30 min (Fig.11), suggesting that P1 had decent stability in blood.

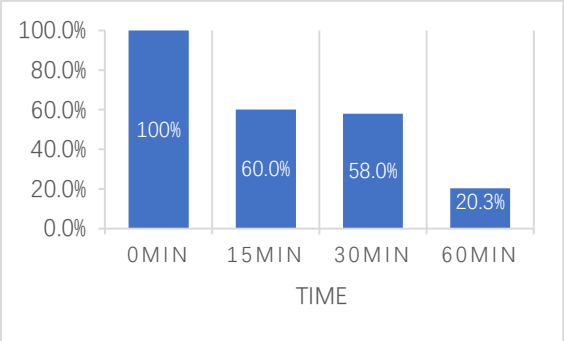


Fig.11 In vitro stability of P1 in mouse serum at 37℃. Quantification of HPLC peaks, and nearly 50% of P1 was remained after incubating in mouse serum for 30 min.

3.4 Low cytotoxicity of probe P1

An ideal imaging probe is characterized by low cytotoxicity. Consequently, cytotoxicity of P1 was evaluated in CCK-8 assay on SH-SY5Y cells at different

concentrations (Fig.12). As a result, Probe P1 was incubated with cells at a concentration up to 10 μM for 24 hours, and the cell survival rate was more than 85%. It shows that P1 is safe with no obvious toxicity to cells.

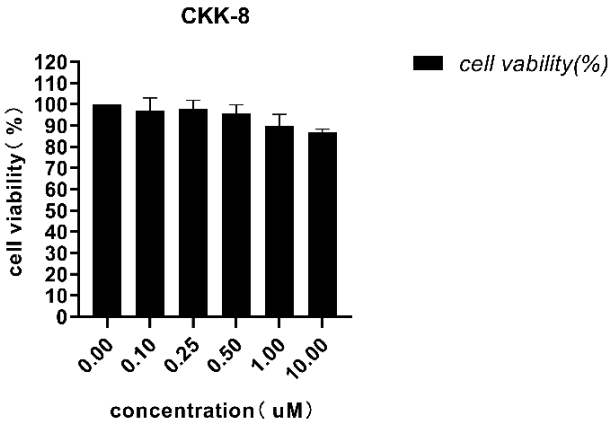


Fig.12 Cytotoxicity of P1 in CCK-8 assay on SH-SY5Y cells at different concentrations.Probe P1 has low cytotoxicity at a concentration up to 10 μM.

3.5 Probe P1 with specific detection for AβO, but not for Aβ monomer and Aβ fibril

AβO plays a key role in the development of AD and has received increasing attention as a target for detection and treatment of the disease. [18, 19] Mounting evidence indicates that AβO is highly neurotoxic, causing apoptosis and functional impairment in the brain 20 years before patients develop clinical symptoms. [20, 21, 22]Therefore, probes that specifically detect AβO will be used to detect early-stage patients and enable timely treatment.

We investigated the F.I. change of the probe upon binding to three types of Aβ proteins. P1 was hardly bound to Aβ monomer, however, it exhibited a significant increase in fluorescence intensity when incubated with AβO and Aβ fibers. In addition, the increase in F.I was especially pronounced when P1 was bound to AβO. The F.I. of AβO is about 12 times that of Aβ monomer and 1.8 times that of Aβ fiber, confirming the specific detection of probe P1 AβO(Fig.9).

The affinity of probe P1 to the protein indicates the strength of the interaction between the protein and the molecule, which is expressed in terms of the equilibrium dissociation constant Kd. The smaller the value, the stronger the affinity between the protein and the P1. Probe P1displayed impressive binding affinity for oligomers (Kd= 43.11nM), moreover, the binding showed a quantitative effect relationship (Fig.13).

Tab.2. Fluorescence intensity of PTO-41 upon interaction with Aβs in PBS

Probe	λex/λem (nm)	[a]I	[b]IM	[c]IA	[d]IO	[e]ΔIO/ΔIM	[f]ΔIO/ΔIA
P1	540/610	66.12	114.4	795.7	1485	12.98	1.87

[a] I stands for the f.I. of P1 itself; [b]IM stands for the f.I. of P1 bound to Aβ monomer; [c]IA stands for the f.I. of P1 bound to the Aβ fibrils; [d]IO stands for the f.I. of P1 bound to the AβO; [e]ΔIO/ΔIM stands for the ratio of the f.I. of P1 bound to the AβO and the monomer; [f]ΔIO/ΔIA stands for the ratio of the f.I. of P1 bound to the AβO and the fibrils.

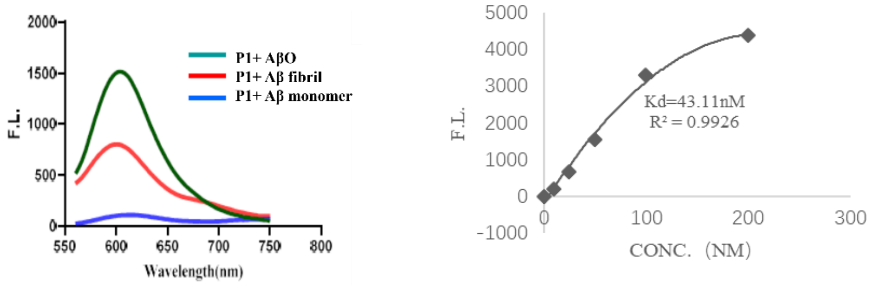
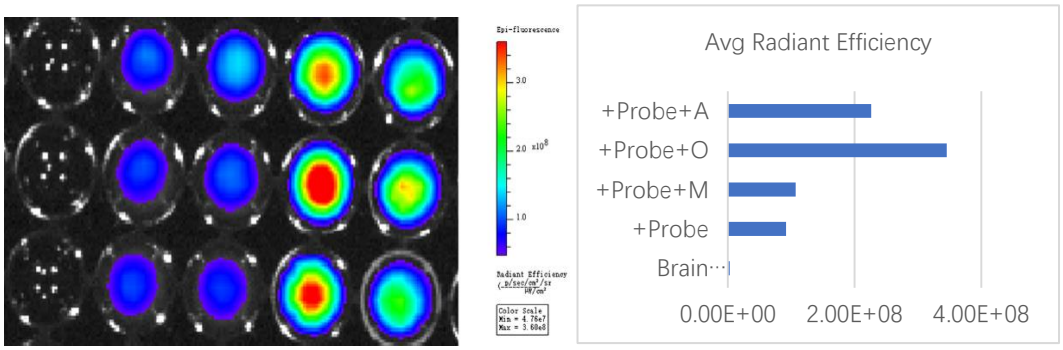


Fig.13 a. Fluorescence intensity of P1 upon interaction with Aβs in PBS; b. Kd value of probe P1with AβO

To verify whether the probe P1 is capable of targeting AβO in the brain, we investigated the interaction between probe P and Aβs in brain tissue homogenates. P1 showed significant selectivity for AβO, with fluorescence intensities 3.2 and 1.5 times higher than those of Aβ monomer and Aβ fiber, respectively (Fig.14). This demonstrates the potential of probe P1 to image Aβ oligomers in the brain.



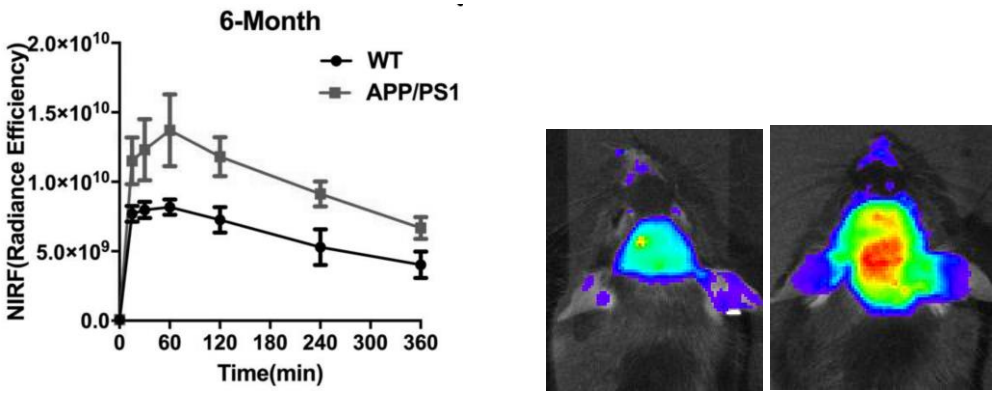
a b

Fig.14 a. Imaging picture of P1 upon incubation with Aβ monomers, Aβ oligomers, and Aβ aggregates in a biological environment (Ex = 540 nm and Em = 610 nm); b. quantitative analysis of the fluorescence intensity in the IVIS system.

3.6 Early diagnosis of transgenic AD mice by the probe P1

A broad range of data identifies AβO as the most neurotoxic species of Alzheimer's amyloid peptide. In the early stage of AD, though patients do not show clinical symptoms, AβO has already been present in the brain, causing neuronal apoptosis and impairment of brain function. Moreover, Lencanumab, a drug which was just approved by the FDA, is the first drug to slow cognitive decline of AD patients in clinical, and it works by inhibiting the AβO. Therefore, AβO is widely recognized as a reliable biomarker for early diagnosis of AD.

AβO is predominantly present in the brains of 6-month-old AD mice, while Aβ fibers and plaques are rare. Therefore, 6-month-old female mice (APP/PS1) and age-matched wild-type mice were used to assess the potential of P1 for specific AβO imaging in vivo using NIRF imaging. As shown in Fig.13, the fluorescence intensity in the brain regions of the AD mice were significantly higher than in the control mice. In particular, the fluorescence intensity of probe P1 in the brain of AD mice was 1.56 times higher than that of WT mice 60 min after iv injection of P1, and the higher fluorescence signal in the brain of AD mouse may result from the binding of P1 with AβO (Fig.15). Probe P1 is able to detect early AD mice by infrared fluorescence imaging and has the potential to be an early diagnostic drug.



a. b. WT mice AD mice

Fig. 15. In vivo NIRF imaging with P1 in 6th month mice. a. Time-course curves of NIRF from P1 in APP/PS1 and WT mice. b. Representative images of APP/PS1 and WT mice after I.V. injection with P1 at 60min.

4、Conclusions

We prepared a near-infrared fluorescent probe P1, investigated the specific affinity of probe P1 to AβO, and then assess the potential of P1 for AD early diagnosis in vivo using NIRF imaging.

① The synthesis process of probe P1 is mild, simple and inexpensive.

② Probe P1 has a strong affinity for AβO at the nanomolar level (Kd = 43.11 nM) . AβO is highly neurotoxic and appears in the brain 20 years before the onset of AD symptoms. It has significant advantages as a biomarker for early diagnostic agents.

③ Probe P1 has significantly higher fluorescence intensity in the brains of AD mice than that of normal wild-type mice, and has good prospects for the development of early diagnostic reagents.

Probe P1 specifically binds to AβO, and NIRF imaging "lights up" the brains of AD mice, providing a new strategy for the development of early diagnostic agents for AD. The discovery of such diagnostic agents will provide more opportunities for early intervention and treatment of AD patients.

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论文的选题来源、研究背景

在身边，有越来越多的人罹患阿尔茨海默病，看到患者和他们的家人因为疾病遭受巨大痛苦的时候，让我们非常希望能做点事情，所以萌发了研究阿尔茨海默症的想法。在许亮亮老师的指导下，我们调研了阿尔茨海默的治疗现状，发现这种疾病基本没有有效的治疗药物，更激发了我们的研究决心。

考虑到阿尔茨海默病到了晚期，脑细胞凋亡后，患者的治疗非常困难。在老师们的指导下，我们最终确定了研究的方案：合成化学探针，探索疾病的早期诊断方法，期望在疾病对健康造成不可挽回的伤害之前，为患者争取治疗的机会。回首一年以来的研究，我们经历了成功的喜悦，也遭遇了失败的忧伤。在研究工作告一段落的时候，对关爱、帮助、鼓励过我们的老师和学长致以最诚挚的谢意。

团队成员在论文撰写中承担的工作以及贡献

本课题的研究中，徐菡月同学完成了阿尔茨海默症探针的合成，转基因小鼠的近红外荧光成像，毛今泽同学完成了探针基本性质检测和探针对淀粉样蛋白寡聚体特异性结合的评价，我们共同完成了论文的撰写。

指导老师作用，及指导是否有偿

许亮亮老师是我们的化学老师，在得知我们的研究目标后，利用工作之余的时间，在调研文献，项目的设计、实验实施中无私无偿的给与指导。为了实验的顺利开展，帮助联系中国药科大学的科研资源。许老师的言传身教，使我们获益匪浅。

他人协助完成的研究成果

非常感谢中国药科大学的石磊教授提供实验的部分研究设备和场所，感谢中国药科大学的刘冰学长细致地指导仪器的使用方法和细致耐心的讲解。

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