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In vivo target bio-imaging of Alzheimer’s disease by fluorescent zinc oxide nanoclusters†

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Alzheimer’s disease (AD) is an irreversible neurodegenerative disease which is difficult to cure. When Alzheimer’s disease occurs, the level of zinc ions in the brain changes, and the relevant amount of zinc ions continue decreasing in the cerebrospinal fluid and plasma of Alzheimer’s patients with disease exacerbation. In view of these considerations, we have explored a new strategy for the in vivo rapid fluorescence imaging of Alzheimer’s disease through target bio-labeling of zinc oxide nanoclusters which were biosynthesized in vivo in the Alzheimer’s brain via intravenous injection of zinc gluconate solution. By using three-month-old and six-month-old Alzheimer’s model mice as models, our observations demonstrate that bio compatible zinc ions could pass through the blood–brain barrier of the Alzheimer’s disease mice and generate fluorescent zinc oxide nanoclusters (ZnO NCs) through biosynthesis, and then the bio-synthesized ZnO NCs could readily accumulate in situ on the hippocampus specific region for the in vivo fluorescent labeling of the affected sites. This study provides a new way for the rapid diagnosis of Alzheimer’s disease and may have promising prospects in the effective diagnosis of Alzheimer’s disease.

Introduction

Alzheimer’s disease (AD), the most common cause of dementia, is a progressive and age-related irreversible neurodegenerative disease.1,2 It is reported that AD could affect one in nine people over the age of 65 years;3 with the acceleration of the aging population, this figure is sure to rise dramatically.4 Therefore, Alzheimer’s disease has become one of the major diseases to harm the health of the aged. It exerts great influence on the family and society as a global health problem.

In recent years, enormous resources have been used to understand the mechanism of AD, and much progress has been made in this field. However, the relevant mechanism still needs to be revealed that sets off the neurobiological events leading to severe cognitive and neuropsychiatric dysfunctions. Many studies have demonstrated that AD is a multi-factorial neurodegenerative disorder defined by both neuropathology and clinical symptoms. Pathologically, AD is characterized by two hallmark elements: numerous senile plaques (SPs) composed of β-amyloid (Aβ) peptides and abundant neurofibrillary tangles (NFTs) formed by filaments of highly phosphorylated tau proteins in the brain.5–7 When Alzheimer’s disease occurs, the relevant amount of zinc ions in the brain is also found to change when compared with the normal ones.8 Additionally, in the early stage of Alzheimer’s disease, there exists the apparent decrease of cell proliferation and neuronal survival in the hippocampus, while low oxygen metabolism and glucose metabolism in the cortex, temporal lobe and parietal lobe have been observed in the brain. For example, positron emission tomography (PET) images of 18-F marked deoxidization glucose showed that glucose metabolism was reduced significantly in the brain of Alzheimer’s patients.9,10 Meanwhile, T. Lu et al. have reported that the repressor element 1-silencing transcription factor (REST) is a universal feature of normal ageing in human cortical and hippocampal neurons, which can repress genes that promote cell death and Alzheimer’s disease pathology, inducing the expression of stress response genes and protecting neurons from oxidative stress and β-amyloid (Aβ) peptide toxicity.11,12 Furthermore, additional proposed mediators of AD progression include age, genetic factors, prion proteins, oxidative stress, inflammation and altered mitochondrial function,13,14 while nutrition, environment, physical activity and education may also potentiate AD-like pathology, making the disease difficult to elucidate.15,16
Clinically, AD is characterized by cognitive and memory dysfunctions, such as memory loss, language impairment, and personality or behavioral changes.\textsuperscript{17} Unfortunately, to date, there is no efficient cure for ADs. Thus, early diagnosis of patients with AD is an important key in improving decisions for medical treatment as early as possible. Although some intelligence and psychological tests as well as imaging technologies including computerized tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) etc., have been used in the relevant clinical diagnostics, yet Alzheimer’s disease still cannot be diagnosed in the early stage and consequently many patients lose precious time for precocious treatment.

Our recent research illustrates that synthesized fluorescent nanoclusters (NCs) like gold or silver NCs could be readily utilized as a great potential probe for the highly sensitive optical imaging of oxidative stress linked pathologies such as cancers.\textsuperscript{18–20} During these previous investigations, we also found that cancer cells could be detected by \textit{in vivo} fluorescent bio-imaging through the biosynthesis of the biocompatible zinc oxide nanoclusters, and it reduced the further development of tumors. Based on the above considerations, in this contribution we have explored a new strategy for the \textit{in vivo} bio-imaging of Alzheimer’s disease through fluorescent zinc oxide nanoclusters biosynthesized \textit{in vivo} in AD’s brain through intravenous injection of zinc gluconate, which is an ideal organic zinc supplement with good biological compatibility and high bioavailability. As illustrated in Fig. 1, by using three-month-old and six-month-old Alzheimer’s model mice as experimental models, our observations demonstrate that the biocompatible zinc ions could readily pass through the blood–brain barrier to lead to the biosynthesis of fluorescent zinc oxide nanoclusters that accumulate in a hippocampus specific region. This allows a direct \textit{in situ} labeling of the affected sites, thus providing a new way for the rapid and ultrasensitive diagnosis of Alzheimer’s disease.

**Results and discussion**

Zinc is one of the essential trace elements in the human body, which is closely related to a variety of physiological processes and has extremely important biological functions \textit{in vivo}, including nervous system, body immunity, growth and development, metabolic system, etc.\textsuperscript{21,22} In view of these considerations, we have explored the possibility of the \textit{in vivo} fluorescence imaging of Alzheimer’s disease through fluorescent zinc oxide nanoclusters which were biosynthesized \textit{in vivo} in the Alzheimer’s brain via intravenous injection of zinc gluconate solution. By using three-month-old and six-month-old Alzheimer’s model mice as models, our observations demonstrate that biocompatible zinc ions could pass through the blood–brain barrier of the relevant Alzheimer’s model mice and generate fluorescent zinc oxide nanoclusters, then accumulate \textit{in situ} on the hippocampus specific region for the \textit{in vivo} fluorescence labeling of the affected sites. As shown in Fig. 2 and 3, it is evident that the rapid-target fluorescent brain-bioimaging \textit{in vivo} could be readily performed under excitation wavelengths of 420 nm or 480 nm and emission wavelengths of 620 nm or 670 nm, spontaneously produced via tail-vein injection of zinc gluconate into AD’s model mice. In contrast, it is noteworthy that there is no fluorescence signal in the brain of the blank control group of mice (CON) (Fig. 2A) as well as the normal control group of mice (NOR) at different time points (1 h, 6 h, 18 h, 24 h) via tail-vein injection of 0.3 mL 5 mmol L\textsuperscript{−1} zinc gluconate solution (Fig. S2\textendash†), while the fluorescence signal could be observed in the three-month-old Alzheimer’s model mice (AD-1). 1 hour (1 h) after injection, the fluorescence intensity increased considerably with a relatively longer accumulation or circulation time after relevant injection of zinc gluconate solution, until the strongest fluorescence signal was detected in the brain of AD-1 model mice 6 hours (6 h) after injection. Afterwards, both the fluo-
rescence intensity and imaging area were observed to decrease significantly.

As illustrated in Fig. S3 and S2,† no fluorescence signal was observed in the normal control group besides noise whatever the time after injection, while there is a remarkable fluorescence signal in the three-month-old mice (AD-1) and the six-month-old mice (AD-2) after injection of zinc gluconate solution (Fig. 3). Besides, it is interesting to note the similar trend of the variations of the mean fluorescence intensity at various time points in AD-1 and AD-2’s fluorescence intensity. Moreover, fluorescence bio-marked areas and fluorescence intensities in AD-2 were displayed to be slightly larger than that in AD-1 after tail-vein injection of zinc gluconate solution from 6 to 18 hours. Additionally, in Fig. S3,† it can be seen that the variations of the mean fluorescence intensity of Alzheimer’s model mice (AD-1, AD-2) reached their peaks at 6 hours after injection of zinc gluconate solution, and then the mean fluorescence intensity of the Alzheimer’s model mice was many times than that of the normal control group of mice (NOR). These results show that the biosynthesized fluorescent zinc oxide nanoclusters may serve to bio-image the affected regions of AD mice brains.

It is reported that the main pathological characteristics of Alzheimer’s disease is senile plaque formation in the brain, and the key component of senile plaque is β-amyloid (Aβ) peptides, which is an important pathway of various factors of Alzheimer’s disease (AD) and thus plays a central role in the process of AD.23–26 The neurotoxicity of Aβ involves complicated molecular mechanisms pertaining to promoting the formation of free radicals, enhancing the inflammatory reaction caused by inflammatory cytokines and neuron apoptosis, etc.23 Aβ proteins have a strong complexing ability toward zinc ions. For instance, independent fluorescence competition titrations demonstrate that the Aβ protein can readily seize zinc ions from the zinc–porphyrin complex through competitive reactions to generate the zinc–Aβ complex, which can lead to the apparent changes of the relevant fluorescence intensity, from which the apparent binding constants of zinc gluconate molecules could readily cross the lesion area of the AD brain rapidly, as exemplified by the in situ formation of zinc oxide nanoclusters, may thus also facilitate their scavenging
function of reactive radicals. This is consistent with our previous studies, as shown in Fig. 2 and 3 as well as Fig. S4.†

Owing to the above observations, we further explored the detailed fluorescence imaging of dissected brains of NOR, AD-1 and AD-2 mice after their treatment by tail-vein injection of zinc gluconate solution. As illustrated in Fig. 4(A)–(G), no fluorescence signal could be observed in the dissected brain of the NOR. Conversely, strong fluorescence signals were detected in the dissected brain of AD-1 mice, and stronger ones in AD-2, as shown in Fig. 4 and S4.† It is evident that fluorescence signals were bigger in the hippocampus where more serious Alzheimer-induced damage results in diseased brains.

As shown in Fig. 4(H) and (I), typical TEM images illustrate that 96% of the resulting zinc oxide NCs biosynthesized in vivo in AD’s brain after tail-injection of zinc gluconate solution ranged from 1.9 to 2.9 nm in diameter, with a distribution peak at ca. 2.5 nm. Besides, they were almost spherical and had no noticeable trend to agglomerate; this fact suggests that the surface of these nanoclusters is stabilized by appropriate ligands. The HRTEM image (the inset) indicates that zinc oxide nanoclusters kept their interplanar spacing of ~0.18 nm. The mean zeta potential of the biosynthesized zinc oxide nanoclusters obtained from the brain of AD mice is ca. −57.5 mV (Fig. S7†), and the quantum yield of the as-prepared zinc oxide nanoclusters in solution is about 6% and the fluorescence lifetime of the zinc oxide nanoclusters in solution is about 4.37 ns. In Fig. 4(J), X-ray photoelectron spectroscopy (XPS) was used to investigate the valence of zinc in the biosynthesized zinc oxide nanoclusters, illustrating two peaks located at the binding energies of 1021.7 and 1045 eV, being consistent with the emission of 2p photoelectrons from Zn²⁺.

Meanwhile, as shown in the XPS-element mapping study of relevant AD’s brain tissues (from a random region of 50 µm × 50 µm) (Fig. 4(K)), the relatively bright points appeared in the slices of AD’s brains but not in that of CON (Fig. S6†), indicating that zinc oxide nanoclusters were only biosynthesized in vivo and distributed in AD’s brains.

![Fig. 4](image-url)

Fig. 4  (A–K) (A–F) Fluorescence imaging of the dissected brain of the NOR, AD-1 and AD-2 via tail-vein injection of 0.3 mL 5 mmol L⁻¹ zinc gluconate solution at 30 h post-injection. Each fluorescence image of the dissected brain was taken from two angles (dorsal view included A, B, C and ventral view included D, E, F). (G) The variations of mean fluorescence intensity of the dissected brain of the three groups of mice. (H) TEM image and HRTEM image (the inset) of biosynthesized zinc oxide nanoclusters. (I) Size distribution of the zinc oxide nanoclusters, ranging between 1.9 to 2.9 nm in diameter with a distribution peak at ca. 2.5 nm. (J) XPS spectra illustrate two peaks located at 1021.7 eV and 1045 eV, consistent with the emission of 2p photoelectrons from Zn²⁺. (K) XPS-element mapping of AD’s brain tissues, indicating special distribution of relevant nanoclusters biosynthesized in AD’s brain.
The excess iron accumulation and disrupted expression or function of iron metabolism proteins in the brain are closely related with neurodegenerative diseases such as Alzheimer’s disease (AD). In the AD brain, there exists excessive iron accumulation. However, no corresponding increase of ferritin for the regulation of iron metabolism was found in the AD brain, leading to an increase of the risk of producing oxidative stress damage via the Fenton reaction in the presence of H₂O₂, leading to cell damage and cell apoptosis for inducing AD. Besides, it can also regulate the expression of a variety of proteins including the β-amyloid precursor protein (APP) which has potential toxicity.

Based on these facts, we have tested if the Fenton cocktail consisting of Fe²⁺ and H₂O₂ could somewhat be responsible for the in situ formation of zinc oxide nanoclusters from zinc gluconate solution. For this purpose, in the in vitro experiments, we immediately added a 0.15 mmol L⁻¹ zinc gluconate solution to freshly mixed solutions of 0.3 mmol L⁻¹ FeCl₂ and 0.2% H₂O₂. The UV-Vis absorption spectrum of the resulting three-solution mixture readily displayed a new peak at about 350 nm, featuring the formation of zinc oxide (ZnO) nanoclusters as characterized afterwards by transmission electron microscopy (TEM) and high resolution transmission electron microscopy (HRTEM), as shown in Fig. S10. These ZnO nanoclusters had a size distribution ranging between 2.9 to 4.4 nm in diameter with a distribution peak at ca. 3.8 nm, with an interplanar spacing of ~0.19 nm (shown in Fig. S10 and S11†). Since the biological environment of Alzheimer’s brain is very complex, it is difficult to simulate it as a real biological process. Though the mean distribution of ZnO nanoclusters resulted in being a bit larger than those detected after in vitro biosynthesis (see above), the in vivo and in vitro morphologies of the two types of nanoclusters (size and interplanar spacing) were fully coherent. This supports the hypothesis that the in vivo biosynthesis of ZnO nanoclusters in the AD mice brains, where the presence of the OH⁻ species is crucial for the formation of ZnO nanoclusters which could readily attract the zinc cations to generate the key intermediate [Zn(OH)₄]²⁻, with subsequent dehydration to form ZnO nanoclusters. Thus, the in vivo biosynthesis of zinc oxide nanoclusters in AD mice is at least in part due to the presence of iron and hydrogen peroxide in the damaged zones of AD mice brains (Fig. 5). Besides, the Aβ protein may also have some impact on the generation of ZnO NCs, but the influence should be limited. Therefore, the relatively high level of the ROS (e.g. H₂O₂) in the disease sites of the AD mice will mainly account for the formation of the biosynthesized ZnO NCs when using tail-vein injection of zinc gluconate solution so that the relevant AD model mice could rapidly show fluorescence bioimaging in the disease sites.

To further explore the specific targeting ability of zinc gluconate as well as the possible biodistribution in different organs, the relevant fluorescence imaging of major organs (i.e., liver, spleen, kidney, lung and brain) of the NOR and AD-2 after injection of zinc gluconate has been obtained. As shown in Fig. 6, the results evidenced that none of the major organs of the NOR displayed a fluorescence signal, while in the organs of AD-2, a strong fluorescence signal appeared in the brain, though very weak or almost no fluorescence was detected in other organs. These results showed that the formation of zinc oxide nanoclusters in situ is restricted to hippocampus specific regions, which allows fluorescent labeling of the affected sites, thus providing early rapid imaging of Alzheimer’s disease.

![Fig. 5 Schematic illustration of the possible process for the relevant synthesis of ZnO nanoclusters. R refers to the reactive amino acid or protein, etc.](image-url)
As shown in Fig. 7, histopathological analysis was also performed on the tissues obtained from the harvested organs (i.e., liver, spleen, and kidney). As illustrated in these histopathological images of AD-1 and AD-2 treated by tail-injections of zinc gluconate solution, H&E-stained tissue sections showed that no histopathological abnormalities or lesions were observed in the organs (i.e., liver, spleen, and kidney). Furthermore, no changes were observed in eating, drinking, grooming, activity, weight, and neurological status in Alzheimer’s mice or normal mice injected with relevant zinc gluconate solution. These observations show that zinc gluconate has no obvious side effects on the major organs of AD mice at the injected doses, which is consistent with the literature reports that supplementing zinc properly can significantly improve the structure of the damaged neuron cells, and the structure and content of the cytoskeleton protein in the hippocampus.42

Conclusions

In summary, in this contribution we have explored the possibility of the in vivo fluorescence imaging of Alzheimer’s disease through rapid-target fluorescent bio-marking based on biosynthesized zinc oxide nanoclusters from the intravenous injection of zinc gluconate. These series of studies demonstrate that rapid and specific brain targeting fluorescence imaging through the real-time fluorescent labeling of the zinc oxide nanoclusters biosynthesized in AD mice’s brain could be readily realized by employing the peculiarities of the brain of Alzheimer without any additional agents, which could be utilized to efficiently and accurately bio-image the affected regions of AD mice brains in vivo and thus may have enormous potential to improve the precision of early AD diagnostics and treatments. Besides, the results of in vitro experiments establish a link between the formation of ZnO nanoclusters from Zn^{2+} and the production of Fenton conditions (i.e., to the simultaneous presence of Fe^{2+} and H_2O_2) in the brain of AD mice.

Competing financial interests

The authors declare no competing financial interests.

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