In Vivo Biosynthesized Zinc and Iron Oxide Nanoclusters for High Spatiotemporal Dual-Modality Bioimaging of Alzheimer’s Disease

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Supporting Information

ABSTRACT: Alzheimer’s disease is still incurable and neurodegenerative, and there is a lack of detection methods with high sensitivity and specificity. In this study, by taking different month old Alzheimer’s mice as models, we have explored the possibility of the target bioimaging of diseased sites through the initial injection of zinc gluconate solution into Alzheimer’s model mice post-tail vein and then the combination of another injection of ferrous chloride (FeCl2) solution into the same Alzheimer’s model mice post-stomach. Our observations indicate that both zinc gluconate solution and FeCl2 solution could cross the blood–brain barrier (BBB) to biosynthesize the fluorescent zinc oxide nanoclusters and magnetic iron oxide nanoclusters, respectively, in the lesion areas of the AD model mice, thus enabling high spatiotemporal dual-modality bioimaging (i.e., including fluorescence bioimaging (FL) and magnetic resonance imaging (MRI)) of Alzheimer’s disease for the first time. The result presents a novel promising strategy for the rapid and early diagnosis of Alzheimer’s disease.

INTRODUCTION

To date, Alzheimer’s disease (AD) is still fatal and neurodegenerative and lacks detection methods with high sensitivity and specificity. At present, the pathogenesis of Alzheimer’s disease is still unclear.1−5 Because of the long incubation period of Alzheimer’s disease, patients are already in late dementia when they are diagnosed. As the aging of the population is becoming increasingly serious in modern society, the incidence rate of AD has shown an upward trend every year. As we know, AD brings great pain to patients and their families and a heavy burden to our society.6−10 Therefore, the search for an early and rapid diagnosis method of Alzheimer’s disease is extremely urgent.

Presently, there are some imaging technologies and psychological or intelligence tests clinically used for the diagnosis of AD. Common imaging technologies mainly include magnetic resonance imaging (MRI), computed tomography (CT), and positron emission tomography (PET).11−14 With the well-known advantages including the high resolution of soft tissues, no radiation, and long-acting imaging, MRI is a kind of noninvasive method widely used in clinic diagnostics.15,16 However, MRI is relatively high-cost and consumes large amounts of time, so real-time high-spatiotemporal fluorescence imaging (FL) has attracted much attention. In our previous studies,17,18 we found that zinc gluconate solution could be readily utilized to realize fluorescence imaging of the lesion areas in the brains of Alzheimer’s model mice as a result of the obviously different redox environment and many metal ion

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distributions (e.g., zinc and iron) in AD brains compared to normal brains. To date, there has been little report on the combination of imaging for the diagnosis of Alzheimer’s disease. In this study, we have exploited the possibility of combining fluorescence and MRI for the rapidly dual-modality bioimaging of Alzheimer’s disease. It is observed that by taking different month old Alzheimer’s mice as models, the results demonstrate that both zinc gluconate solution and FeCl₂ solution could cross the blood−brain barrier and then were biosynthesized to fluorescent zinc oxide nanoclusters and magnetic iron oxide nanoclusters, respectively, thus enable targeting dual-modality bioimaging included magnetic resonance imaging (MRI) and fluorescence imaging (FL) of Alzheimer’s disease for the first time.

RESULTS AND DISCUSSION

Zinc and iron are essential trace elements for human and animal bodies. Both of them have many important physiological functions. Zinc closely pertains to the nervous system, immune system, growth and development, metabolic system, and so forth. Iron is the most abundant trace element in the human body. It participates in many life processes and is widely distributed in various tissues and organs. It is the necessary component not only for oxygen-transport proteins and electron-transfer proteins but also for many enzyme cofactors. Many iron nanoparticles (e.g., γ-Fe₂O₃, Fe₃O₄, and FePt), as the advanced contrast agents to shorten imaging time and increase image contrast and resolution, have been widely used in many fields, especially in biomedicine such as MRI, drug-targeting transport, and the diagnosis of cancer. In this study, we have demonstrated that both zinc gluconate solution and FeCl₂ solution could be readily biosynthesized to fluorescent zinc oxide nanoclusters and magnetic iron oxide nanoclusters, respectively, in the lesion areas of the AD model mice and thus enable fluorescence and MRI dual-modality bioimaging of Alzheimer’s disease.

Initially, we first injected 5 mmol/L zinc gluconate solution into the Alzheimer’s model mice via the tail vein and then...
injected 5 mmol/L ferrous chloride solution into the same Alzheimer’s model mice post stomach. As shown in Figure 2A−F, it is evident that no obvious fluorescence imaging signal appeared in the blank control group of the Alzheimer’s model mice (CON) without any medicine (given only with PBS) during the whole observation time (0−30 h). However, there was an obvious fluorescence signal in the same Alzheimer’s model mice (AD) with the given zinc gluconate solution and ferrous chloride solution. Moreover, the intensity of the fluorescence imaging gradually increased with time post injection and reached to the peak at 12 h. Then the intensity of relevant fluorescence was observed to decrease. This phenomenon was consistent with the fluorescence imaging of CON1 mice with only the injection of 5 mmol/L zinc gluconate solution.23 At the same time, it also showed in Figure 2 that the injection of ferrous chloride solution into the same AD model mice had no obvious effect on the fluorescence signal induced by the injection of zinc gluconate in vivo. However, as seen from Figure SSA−F, the results demonstrated that there was almost no fluorescence signal in the brains of the normal control group of mice (NOR) at different time points (0−30 h) via tail-vein injection of zinc gluconate solution and ferrous chloride solution post stomach.

Afterward, both the AD mice given 5 mmol/L zinc gluconate solution and 5 mmol/L ferrous chloride solution for 30 h and the normal control group of mice (NOR) without the injection of any medicine were dissected. Then their main organs such as brain, liver, spleen, kidneys, and lungs were harvested and imaged. Compared to the fluorescence imaging signal of the main organs of the AD mice with that of the NOR mice, as shown in Figure 3A−C, it is noted that there is almost no fluorescence signal in the organs of the NOR, whereas there appeared a strong fluorescence signal mainly in the brain of the AD mice. Additionally, a very weak fluorescence signal appeared in the liver of the AD model mice, which is probably related to metabolic residues, and no apparent fluorescence signal appeared in the relevant spleen, heart, kidneys, and lungs. These observations indicate that the adding of ferrous chloride solution had no obvious influence to the relevant targeting of zinc gluconate solution to the diseased sites for biosynthesizing the fluorescent zinc oxide nanoclusters to realize bioimaging of the Alzheimer’s brain.

Figure 3. (A) Fluorescence imaging of organs dissected from the normal control group of mice (NOR) without the injection of any medicine (given only with PBS). (B) Fluorescence imaging of the main organs dissected from the Alzheimer’s model mice (AD) with given 5 mmol/L zinc gluconate solution first and then 5 mmol/L ferrous chloride solution. (C) The contrast of fluorescence imaging the intensity of organs of the normal control group of mice (NOR) without the injection of any medicine (given only with PBS) and the Alzheimer’s model mice (AD) with 5 mmol/L zinc gluconate solution given first and then 5 mmol/L ferrous chloride solution given.

Comparing the fluorescence imaging of the cross section of the brain of the NOR and AD mice, as shown in Figure 4A,C, it can be seen that there is no fluorescence in the cross section of the brain of the NOR mice whereas a strong fluorescence signal appeared in the AD mice’s brain, especially in the hippocampus. It is already known that the damage to the hippocampus occurred regularly and seriously in Alzheimer’s disease. When 5 mmol/L zinc gluconate solution was given to AD mice, there was a strong fluorescence imaging signal in the AD’s brain,23 and the addition of ferrous chloride solution had no obvious influence on the target fluorescence imaging of zinc gluconate solution in the hippocampus of the Alzheimer’s brain.

After treatment for 24 h, both control groups of Alzheimer’s model mice (CON1) given with zinc gluconate solution alone and AD mice given with zinc gluconate and FeCl2 solution were in vivo imaged for T2-weighted MRI to evaluate the capability of the zinc and iron ions. As shown in Figure 4B,D, it was observed that an obvious darkening appeared in T2-weighted MRI in the hippocampus of the AD’s brain and no signal enhancement appeared in the hippocampus of CON1. The darkening signal (i.e., inside the red circle) should be iron oxide nanoclusters biosynthesized in the AD brain.
On the basis of these observations, the biosynthesized nanoclusters have been extracted from the relevant brain tissue of the AD brain for further characterization. As shown in Figure 5A, it can be seen that abnormal Zn and Fe could be readily detected in the EDS spectrum, and their levels are high at 0.05 and 0.07%, respectively. The typical transmission electron microscopy (TEM) and the size distribution curve (inset c) illustrate that 96% of the zinc oxide nanoclusters and iron oxide nanoclusters ranged between 4.5 to 5.3 nm in diameter with a distribution peak at ca. 4.9 nm. They were mainly spherical without an obvious trend toward agglomeration. To distinguish the zinc oxide nanoclusters and iron oxide nanoclusters clearly, it can be seen that their interplanar spacings are ca. 0.19 and 0.26 nm, respectively, from the HRTEM (insets a and b) in Figure 5B, which can be attributed to the (102) plane of ZnO and the (311) plane of iron oxide nanoclusters.23,27,28 In these experiments, only Fe$^{2+}$ was used as a precursor, thus the appearance of Fe$^{3+}$ should result from the partial oxidation of Fe$^{2+}$ in the AD’s brain tissues. Meanwhile, as illustrated in Figure 5C, X-ray photoelectron spectroscopy (XPS) was further used to demonstrate the valence of zinc atoms in the biosynthesized zinc oxide nanoclusters, shows two peaks located at the binding energies of 1025.0 and 1045.0 eV, corresponding to the emission of 2p photoelectrons from zinc(II), whereas for the Fe ions (Figure 5D), it was found that both Fe(II) and Fe(III) coexist in the AD’s brain tissue samples by deconvoluting the XPS band from 700 to 730 eV. From the integrated peak area, it was found that the +2 valence state of Fe accounts for 58.9% and the +3 valence state accounts for 41.1%, respectively, from total iron. Besides, by comparing the XPS mapping of the brain of the AD mice and that of the NOR mice (Figure S4), it can be seen that both Zn and Fe clearly increased in the AD’s brain to realize the high spatiotemporal fluorescence and MRI dual-modality imaging of the Alzheimer’s. Moreover, the fluorescence (FL) emission spectra of the biosynthesized ZnO NCs extracted from NOR, CON, and AD’s brain tissues demonstrate stronger fluorescence in AD than in NOR and CON (Figure S8).

In our previous study, we have successfully applied zinc gluconate solution to the AD model mice for the in vivo fluorescence bioimaging of the diseased sites, and the related mechanism has also been explored in detail.23 It is already known that the concentration levels of reactive oxygen species (ROS) such as H$_2$O$_2$ are much higher in the AD’s brain than in the normal brain.30−32 The existence of ROS will affect Fe$^{2+}$ and brings it to a higher valence state.33−36 The effect of H$_2$O$_2$ on Fe$^{2+}$ could readily lead to the appearance of Fe$^{3+}$. These further illustrate the relationship between Fe$^{3+}$ oxidation to Fe$^{2+}$ and hydroxyl radicals formed from H$_2$O$_2$ via the Fenton reaction.33 Besides, because there exists an inflammatory reaction, this allows reducing agents such as ascorbic acid to realize the body’s self-protection, which is present at a correspondingly high concentration in the brain.37−39 Then reducing agents such as ascorbic acid can reduce part of Fe$^{3+}$ to Fe$^{2+}$, maintaining redox balance in the microenvironment in the AD brain. On the basis of these observations, when the AD mice were treated with 5 mmol/L zinc gluconate solution via the tail vein first and then with 5 mmol/L ferrous chloride (FeCl$_2$) solution post stomach, ZnO NCs and Fe$_3$O$_4$ NCs could be biosynthesized in the following pathway.

$$Fe^{2+} + H_2O_2(ROH) \rightarrow Fe^{3+} + OH^- + \cdotOH(R^-) \quad (1)$$
\[
\text{Zn}^{2+} + 2\text{OH}^- \rightarrow \text{ZnO} + \text{H}_2\text{O} \\
\text{Fe}^{2+} + 2\text{Fe}^{3+} + 8\text{OH}^- \rightarrow \text{Fe}_3\text{O}_4 + 4\text{H}_2\text{O}
\]

Therefore, in the special biological microenvironment of the AD’s brain, the injection of zinc gluconate solution via tail vein first and then treatment with FeCl\(_2\) solution via stomach can readily generate ZnO nanoclusters for fluorescence bioimaging and Fe\(_3\)O\(_4\) nanoclusters for MRI imaging of the lesion area in the brain of the AD mice. Moreover, by collecting the fluorescence bioimaging signal at different time points after the injection of 5 mmol/L zinc gluconate solution and 5 mmol/L ferrous chloride solution to the AD model mice, the results showed that the relevant injection of FeCl\(_2\) solution into the AD mice had no obvious effect on the fluorescent imaging signal induced by the injection of zinc gluconate solution. Besides, treatment with FeCl\(_2\) solution for the AD model mice can readily realize MRI imaging of the lesion area of the AD’s brain, as shown in Figure 4B,D. Therefore, zinc gluconate solution can very well be in synergy with relevant FeCl\(_2\) solution for realizing the high spatiotemporal fluorescence and MRI dual-modality imaging of Alzheimer’s disease.

Moreover, on the basis of those described above, we have further explored the toxic effects on the related model mice under the given dosage. In our previous studies, the relevant results show that the injection of a given dosage of zinc gluconate solution will not produce obvious side effects.\(^{23}\) In this study, the model mice were given a 5 mmol/L zinc gluconate solution first and then a 5 mmol/L ferrous chloride solution a few minutes later. To test the toxic effects of zinc gluconate solution and ferrous chloride solution on the targeted mice, histopathological analysis of the dissected major organs (i.e., kidney, liver and spleen, etc.) of NOR mice (without any treatment) and AD mice (with injection of 5 mmol/L zinc gluconate solution and then 5 mmol/L ferrous chloride solution) was carried out, as showed in Figure 6A–F. The results show that no abnormal pathological phenomena could be observed in the kidney, liver, and spleen of the AD mice compared to those of the NOR mice. Besides, histopathologic analyses of H&E-stained brain sections from the gastrointestinal tract and brain of a blank normal control group of mice (NOR), blank control Alzheimer’s model mice (CON), a normal control group of mice (NOR’), and AD mice treated with zinc gluconate solution and ferrous chloride solution were carried out. These results showed that the structure of the gastrointestinal tract of this four-group model of mice is clear and morphologically intact, which indicated that both the structure and shape have no obvious abnormal change in either NOR’ mice or AD mice treated with zinc gluconate solution and ferrous chloride in Figures S6C,D and S7C,D compared to that of the blank NOR mice and CON mice in Figures S6A,B and S7A,B, respectively. Histopathologic analysis of H&E-stained brain sections of this four-group model of mice also showed that both zinc gluconate solution and ferrous chloride solution have good biocompatibility (Figure S9). Moreover, dosing the AD mice (injected with relevant zinc gluconate solution and 5 mmol/L ferrous chloride solution) for 3 to 4 weeks was found to have a very good effect on their diet, hair, behavior, and spirit. Therefore, both zinc gluconate solution and ferrous chloride solution have no obvious toxic effects on mice under the given dosage.

### SUMMARY AND CONCLUSIONS

In this study, we have exploited the possibility of the high spatiotemporal bioimaging of the lesion area of the target mice through the initial injection of zinc gluconate solution into Alzheimer’s model mice via the tail vein and then a combination of the further injection of ferrous chloride (FeCl\(_2\)) solution into the same Alzheimer’s model mice post stomach. Our observations demonstrate that in the special biological microenvironment of the AD’s brain both zinc gluconate solution and FeCl\(_2\) solution could readily cross the BBB and then accumulate on the disease sites of the AD’s brain to biosynthesize in vivo the fluorescent zinc oxide nanoclusters and magnetic iron oxide nanoclusters, respectively. These biosynthesized zinc oxide nanoclusters could precisely realize the in vivo fluorescence bioimaging of the lesion area in the brain of the AD mice, and iron oxide nanoclusters can well be in synergy for MRI imaging simultaneously. This raises the possibility to provide a new platform for the rapid and early diagnosis of Alzheimer’s disease through the biosynthesized functional nanoclusters as intelligent probes for fluorescence and MRI dual-modality imaging of Alzheimer’s disease.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.7b01516.

Materials and methods, fluorescence imaging of the CON without given any medicine and the normal control group of mice. TEM and HRTEM images and size distribution of the resulting ZnO NCs and Fe\(_3\)O\(_4\) NCs. XPS mapping of Zn and Fe in the dissected brain tissue of NOR mice and AD mice. Histopathologic analyses of H&E-stained tissue sections various mouse samples. Fluorescence emission spectra of the extracted biosynthesized ZnO NCs (PDF)

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### Notes

The authors declare no competing financial interest.
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