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Ratiometric Photoacoustic Nanoprobe for Bioimaging of Cu^{2+}

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ABSTRACT: Aberrant copper content implicates numerous diseases including Alzheimer's disease and Wilson's disease. Conventional copper detection technologies are difficult to offer non-invasive and accurate deep tissue detection of copper. Here we report a photoacoustic (PA) nanoprobe (NRh-IR-NMs) for ratiometric PA imaging of Cu^{2+}. The nanoprobe consists of a selective Cu^{2+}-responsive probe (NRh) as the indicator and a non-responsive dye (IR) as the internal reference. In the presence of Cu^{2+}, a selective Cu^{2+}-induced structure change of NRh would take place, resulting in the increase of PA signal intensity increment at 716 nm (ΔPA716). However, the ΔPA834 which attributes to IR shows negligible change. Therefore, the ratiometric PA signal (ΔPA716/ΔPA834) could be used as an indicator for Cu^{2+} detection. This ratiometric PA detection method offers a non-invasive technology with high selectivity and tissue penetration depth, which is a promising tool for deep tissue detection of Cu^{2+} in living organisms.

KEYWORDS: photoacoustic probe; copper ion detection; ratiometric imaging; biosensor; nanotechnology

1. Introduction

Photoacoustic (PA) imaging is a newly emerged non-invasive imaging technology.[1-3] By converting pulsed near-infrared (NIR) laser excitation into ultrasonic emission, PA imaging combines the advantages of both optical imaging (high selectivity) and ultrasonic imaging (improved tissue penetration depth).[4-6] Furthermore, ratiometric imaging, which is based on internal reference approach, can eliminate the environmental effects and thus give more reliable detection result.[7-11] Owing to the merits of ratiometric PA imaging, it has been used as a promising non-invasive technique in a wide range of *in vivo* bioapplications (*e.g.* tumor imaging, thrombus imaging, therapy monitoring, pH detection, enzyme detection).[12-22] However, the application of PA imaging in metal ions detection is still in its infancy.[23-27]

Divalent copper ion (Cu^{2+}) is an important metal ion in living organisms which plays a significant role in many biological processes.[28-29] The copper levels in the human liver and brain are as high as 5 μg per gram of tissue.[30] It has been demonstrated that aberrant Cu^{2+} level is implicated in numerous severe diseases.[31-33] For example, the increased concentration of Cu^{2+} ions in the brain would promote the aggregation of pathological protein, which is a distinct feature of Alzheimer's disease (AD).[34-36] Additionally, Wilson's disease (WD) is another copper ion-related disease in which copper builds up in various tissues (*e.g.* liver, brain, kidneys, and cornea) and thus damages these organs and nervous system.[37-39] The liver copper concentration of WD patients is 4.5−16.5-fold higher than that of healthy individuals.[30] Hence, reliable copper detection is of great significance. Conventional copper detection technologies are mainly based on inductively coupled plasma methods.[40-42] Although these technologies enable copper detection with high accuracy, they usually involve invasive procedures that need adequate tissue samples and require tedious procedures. Moreover, these methods can only

measure the average copper content of the sample, rather than the copper mapping of the whole tissue. For example, liver biopsy is the gold standard and the most accurate test for the detection of copper content in liver.[43] However, performing liver biopsy is extremely invasive and is contraindicated in some patients.[44] Furthermore, in the non-renewable organs (*e.g.* brain, kidneys) of living organisms, copper contents are difficult to be detected by these techniques. Although various fluorescent probes have enabled detection of metal ions,[45-48] most of them are suboptimal for *in vivo* applications due to tissue autofluorescence and limited tissue penetration.[49-50] Recently, Li *et al.* developed acoustogenic probes for *in vitro* chemoselective imaging of Cu^{2+}.[23] The PA probes show great potential due to their high selectivity and the distinct advantages of PAI. Therefore, the development of more PA probes for *in vivo* copper detection is highly desirable.

Herein, we report a ratiometric PA nanoprobe (NRh-IR-NMs) for *in vivo* deep tissue detection of Cu^{2+}. As shown in Figure 1a, two types of organic probes, selective Cu^{2+}-responsive NRh and non-responsive IR820 (IR), were encapsulated into nanomicelles (NMs) to form NRh-IR-NMs. In this system, NRh, which can realize selective Cu^{2+}-induced absorbance change, acts as an indicator; while IR, whose absorbance is inert to Cu^{2+}, serves as the internal reference. The NRh-IR-NMs has a single absorption peak at about 834 nm (belongs to IR) in the NIR region, resulting in an obvious PA signal at 834 nm (PA834). However, in the presence of Cu^{2+}, a selective Cu^{2+}-induced spirolactam ring-opening and further hydrolysis of NRh would take place, thus converting NRh into NRh1. The absorbance of NRh1-IR-NMs at 716 nm (belongs to NRh1) would be increased significantly (Figure 1b), resulting in an amplified PA signal intensity increment at 716 nm (ΔPA716). In contrast, the ΔPA834 of NRh1-IR-NMs shows virtually no change when compared to that of NRh-IR-NMs. Therefore, the ratiometric PA signal (ΔPA716/ΔPA834) could be used for Cu^{2+} detection.

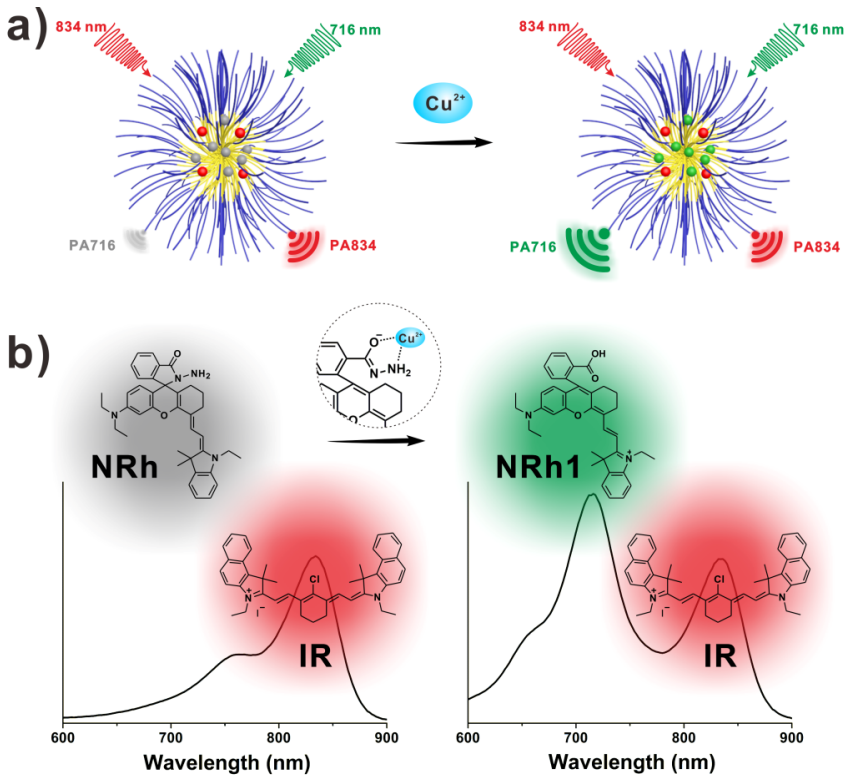

Figure 1. a) The scheme showing the detection of Cu^{2+} by PA nanoprobe. b) The scheme showing the structure and absorption spectra changes of the nanoprobe in the presence of Cu^{2+}.

2. Experimental section

2.1 Materials

Poly (ethylene glycol) methyl ether (mPEG, Mn 5,000), ε-caprolactone (CL) and tin(II) 2-ethylhexanoate ($Sn(Oct)_2$) were purchased from Sigma-Aldrich.

2.2 Synthetic Methods

Experimental procedures for the synthesis of NRh, IR and poly(ethylene glycol)-poly (ε-caprolactone) (PEG-PCL) can be found in the Supporting Information (Scheme S1-S3).

2.3 Cu^{2+}-induced Response of the NRh

To examine the Cu^{2+}-induced response of the NRh, $CuCl_2$ (10 eq.) aqueous solution was added to 10 μM NRh in the MeOH solution (1 eq.). The mixture was vigorously stirred for 15 min, and the

absorption spectra of the solution were measured.

2.4 Preparation and Characterization of the NRh-IR-NMs

NRh (0.470 mg, 0.8 μmol), IR (29.56 μg, 0.04 μmol) and PEG-PCL (4 mg) were dissolved in 500 μL of THF. Then 2 mL of pure water was added into the solution under sonication. The THF was then evaporated under reduced pressure at room temperature.

The effective particle diameter and colloidal stability of the NRh-IR-NMs were determined by dynamic light scattering (DLS) (SZ-100 nano particle analyzer, HORIBA Scientific, USA) at room temperature (n = 3). NIR absorption spectra of the samples were measured by Genesys 10S UV-Vis-NIR spectrophotometer (Thermo Scientific, Waltham, MA) (n = 3). *In vitro* PA imaging of the samples was measured on a Vevo 2100 LAZR system (VisualSonics, Inc., New York) (n = 3). PA signals were acquired within the range of 680-900 nm.

2.5 Cu^{2+}-induced Response of the NRh-IR-NMs

The aqueous solution of NRh-IR-NMs (NRh concentration: 0.2 mM) was incubated with Cu^{2+} (or other metal ions) aqueous solution (2 mM) at room temperature for different periods of time. Then the absorption spectra and PA signals of the solutions were measured as mentioned above (n = 3).

2.6 *In Vitro* Cell Experiments

The *in vitro* cell cytotoxicity of NRh-IR-NMs was assessed on HeLa cells, which was purchased from American type culture collection (ATCC). HeLa cells were seeded into 96-well plates and incubated at 37 °C for 24 h. Then the samples were added into each well for an additional 24 h of incubation. Afterwards, the relative cell viabilities were measured by the methyl thiazolyl tetrazolium (MTT) assay (n = 5).

2.7 *In Vivo* PA Imaging

All animal experiments were performed under a National Institutes of Health Animal Care and Use Committee (NIHACUC) approved protocol. A total of 50 μL (2 mg/mL) of NRh-IR-NMs aqueous solution and different Cu^{2+} solutions were subcutaneously injected into the thigh of living mice (Harlan, Indianapolis, IN). At 2 h postinjection, the *in vivo* PA imaging was performed on a Vevo 2100 LAZR system (VisualSonics Inc. New York, NY) equipped with a 40 MHz, 256-element linear array transducer (n = 3).

3. Results and Discussion

3.1 Characterization of NRh and IR

The NRh molecule was synthesized as shown in Scheme S1 (Supporting Information).[51-55] Nuclear magnetic resonance (NMR) and liquid chromatography-mass spectrometry (LC-MS) characterizations demonstrated the chemical structure of NRh (Figures S1-S3, Supporting Information). To examine the Cu^{2+}-induced response of the NRh (Scheme S4, Supporting Information), excessive Cu^{2+} aqueous solution was added to the NRh solution. The solution color turned to green rapidly, and the absorbance at 600-750 nm increased significantly, demonstrating the conversion from NRh into NRh1 (Figure S4, Supporting Information). The LC-MS spectra further confirmed the Cu^{2+}-induced structure change of NRh (Figure S5, Supporting Information). Then IR molecule was synthesized according to literature (Scheme S2, Supporting Information).[56] NMR spectra confirmed the successful synthesis of IR (Figures S6-S7, Supporting Information). As shown in the absorption spectra (Figure S8, Supporting Information), IR molecules have a single absorption peak at about 834 nm. In the presence of Cu^{2+}, the IR didn't show any absorbance change in NIR region, indicating its inertness to Cu^{2+}. Furthermore, the presence of IR would not affect the reaction between NRh and Cu^{2+} (Figure S9, Supporting Information); thus, IR can be used as the internal reference of the ratiometric

PA probe.

3.2 Preparation and Characterization of NRh-IR-NMs

Amphiphilic diblock copolymer, PEG-PCL, was synthesized by ring-opening polymerization of ε-caprolactone to prepare the nanomicelles (NMs) (Scheme S3 and Figure S10, Supporting Information). The two small molecular probes at the NRh:IR molar ratio of 20:1 were encapsulated into PEG-PCL based NMs to obtain NRh-IR-NMs. The hydrodynamic diameter of NRh-IR-NMs was measured to be 84.2 ± 16.5 nm by DLS (Figure 2a). Furthermore, the NRh-IR-NMs showed good colloidal stability for 7 days (Figure S11, Supporting Information). The nanosized diameter and good colloidal stability of the NRh-IR-NMs made them suitable for *in vivo* bioapplication. Then NIR absorption spectra of blank NMs and different probe-loaded NMs were measured. As shown in Figure 2b, both blank NMs and NRh-NMs showed no obvious absorbance in the NIR region; however, IR-NMs and NRh-IR-NMs showed a single characteristic absorption peak of IR at 834 nm.

3.3 Detecting Cu^{2+} *In Vitro*

To examine Cu^{2+} response of the NRh-IR-NMs, the absorption spectra of the NRh-IR-NMs aqueous solutions incubated with different concentrations of Cu^{2+} were measured. The absorption spectra of blank NMs aqueous solution showed little change upon addition of Cu^{2+}, indicating non-response of blank NMs (Figure S12, Supporting Information). However, upon the addition of Cu^{2+}, the absorbance at 716 nm (Abs716) of NRh-IR-NMs solution increased gradually, indicating the reaction between NRh molecules and Cu^{2+}. Different from NRh, IR molecules are inert to Cu^{2+}, thus the absorbance at 834 nm (Abs834) of NRh-IR-NMs showed no appreciable change (Figure 2c). As a result, the ratio of Abs716 to Abs834 (Abs716/Abs834) increased over time. As shown in Figure S13 (Supporting Information), after 120 min of incubation with Cu^{2+}, the value of Abs716/Abs834

increased to 1.407, which is about 7-fold higher than its initial value. Moreover, the Cu^{2+} concentration-dependent changes in absorption spectra of NRh-IR-NMs also gave a significant change of Abs716/Abs834 (Figure 2d and Figure S14, Supporting Information). Indeed, the sensing rate of NRh-IR-NMs in aqueous environment is relatively low due to the hydrophobicity of NRh. However, it has been reported that WD and AD patients suffer from persistent copper excess in major organs. Therefore, the slow sensing rate will not affect the application potential of the nanoprobe in these diseases. Next, the selectivity of NRh-IR-NMs was investigated. Various metal ions were selected as control groups. As shown in Figure 2e and 2f, only Cu^{2+} induced prominent changes in solution color, absorption spectra and Abs716/Abs834, while negligible changes were observed in all the control groups, demonstrating great selectivity of the NRh-IR-NMs.

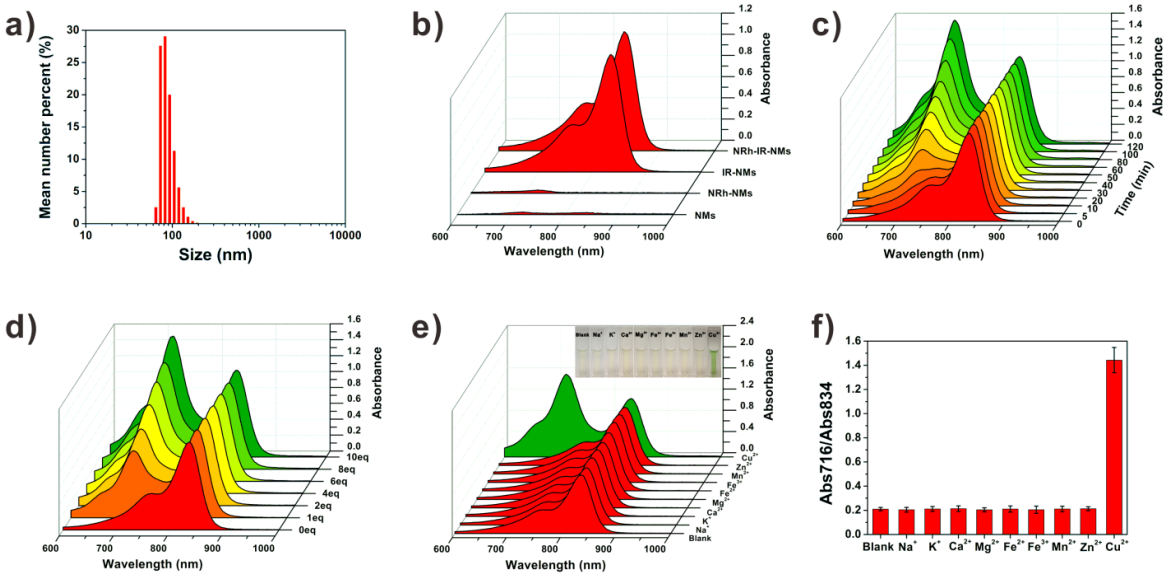

Figure 2. (a) Effective particle diameter of the NRh-IR-NMs. (b) Absorption spectra of NMs, NRh-NMs, IR-NMs and NRh-IR-NMs. (c) Time-dependent changes in absorption spectra of NRh-IR-NMs solution upon the addition of Cu^{2+} solution (10 eq). (d) Absorption spectra changes of the NRh-IR-NMs solutions upon addition of different amounts of Cu^{2+} solution. (e) Absorption spectra and color (inset) changes of the NRh-IR-NMs solutions upon addition of different metal ions. (f) Changes in absorbance ratio (Abs716/Abs834) of NRh-IR-NMs solutions upon addition of different metal ions (n = 3).

Considering the significant Cu^{2+}-induced absorption change in NIR region, we further measured the PA properties of NRh-IR-NMs *in vitro*. As shown in Figure 3a and 3b, compared to the background, the NRh-IR-NMs aqueous solution showed an obvious ΔPA834 and a relatively low ΔPA716, resulting in a very low ΔPA716/ΔPA834 (≈ 0.5). However, in the presence of Cu^{2+}, as expected, the ΔPA834 of the NRh1-IR-NMs showed no prominent change, while the ΔPA716 increased significantly, resulting in an increased ΔPA716/ΔPA834 (≈ 3.4), which is about 7-fold higher than that of NRh-IR-NMs (Figures S15-S16, Supporting Information). The calculated ΔPA716/ΔPA834 was higher than the Abs716/Abs834, which may be because the different photothermal conversion efficiencies between NRh and IR. Furthermore, the changes of ΔPA716/ΔPA834 showed Cu^{2+} concentration dependence in the range of 0.5-10.0 eq. (Figure 3c). In contrast, with the addition of other metal ions, the PA intensities and the ΔPA716/ΔPA834 of the solutions showed no changes (Figure 3 and Figures S15-S16, Supporting Information), which further demonstrated the selectivity of the PA nanoprobe.

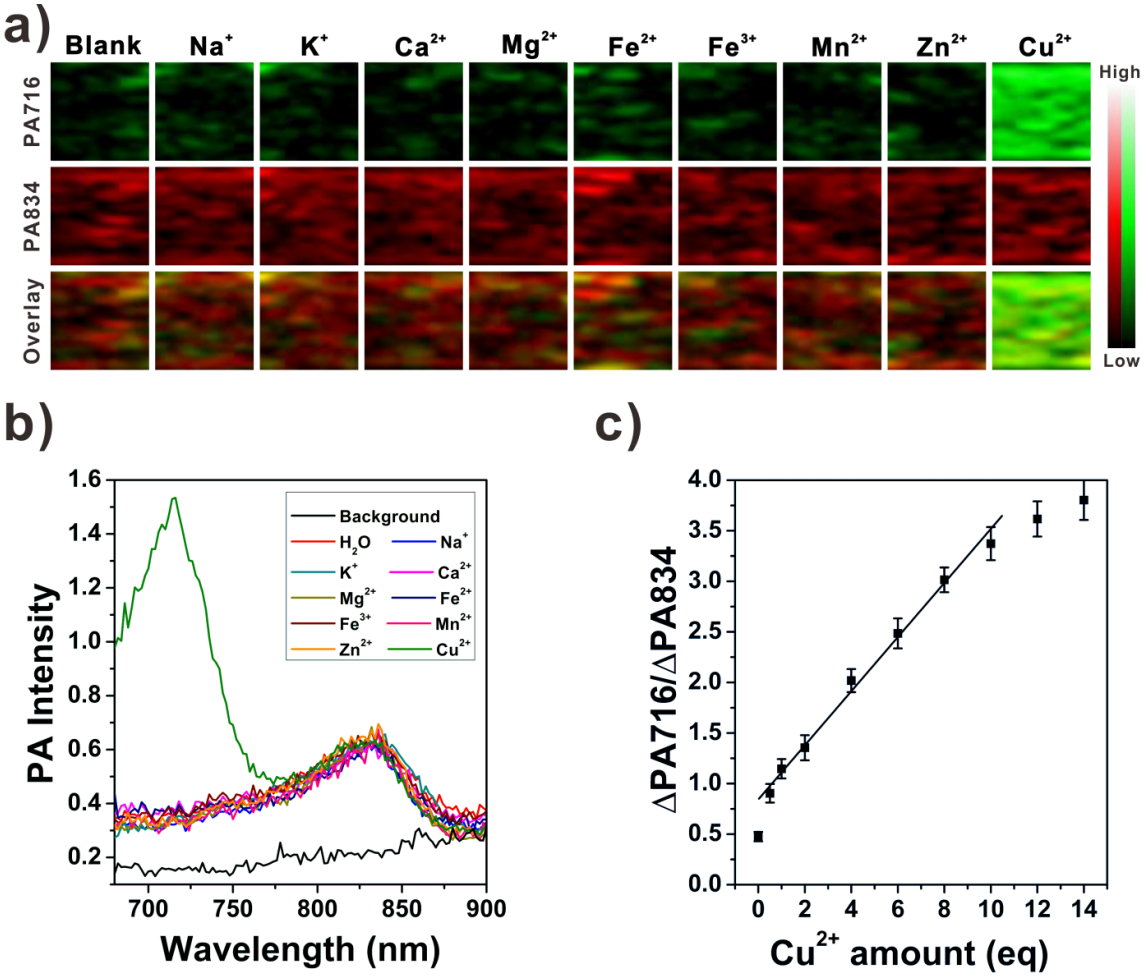

Figure 3. *In vitro* PA images (a) and PA spectra (b) of the NRh-IR-NMs aqueous solution with the addition of pure water or various metal ions solutions. (c) ΔPA716/ΔPA834 of the NRh-IR-NMs solution as a function of Cu^{2+} concentration (n = 3).

3.4 Cytotoxicity

To assess the cytotoxicity and biocompatibility of NRh-IR-NMs, HeLa cells were incubated with NRh-IR-NMs at different concentrations (0-400 mg L^{-1}). After 24 h of incubation, the cell viabilities were investigated by the MTT assay. As shown in Figure S17 (Supporting Information), even at a high concentration (400 mg L^{-1}) of NRh-IR-NMs, the cell viability was still more than 90%, indicating the negligible cytotoxicity of the NRh-IR-NMs.

3.5 In Vivo PA Imaging of Cu^{2+}

In vivo PA imaging of Cu^{2+} was tested by subcutaneous administration of Cu^{2+} solution and NRh-IR-NMs in the thigh of living mice. At 2 h post-injection, the PA spectra were monitored. For the mice treated with saline (control group), the background PA signals at both 716 nm and 834 nm were very low (Figure 4a and 4b). After the injection of NRh-IR-NMs, obvious PA834 was observed; while the PA716 showed negligible change (Figure S18, Supporting Information). The calculated ΔPA716/ΔPA834 thus has a relatively low value (about 0.3, Figure 4c). However, for the mice injected with Cu^{2+}, increased PA716 can be clearly detected, indicating the reaction between NRh molecules and Cu^{2+}. As a result, the ΔPA716/ΔPA834 values of the NRh-IR-NMs/Cu^{2+} groups were much higher than that of NRh-IR-NMs only group (Figure 4c). Furthermore, the ΔPA716/ΔPA834 value within the region is dependent on the dosage of the administrated Cu^{2+}. To investigate the potential of NRh-IR-NMs for deep-tissue application, as a proof of concept experiment, Cu^{2+} solution and NRh-IR-NMs were injected subcutaneously into the thigh of living mice. Then the thigh was covered by 2 mm pork tissue and observed by PA imaging. As shown in Figure 5, PA signals in deep region (depth at 2.0-4.0 mm) were clearly obesrved. All of these results manifest the potentiakl of NRh-IR-NMs for deep tissue detection of Cu^{2+} in living mice. Based on the size and surface modification of the nanoprobe, we believe that the NRh-IR-NMs are suitable for intravenous injection and the intravenously injected nanoprobe will be mainly distributed in liver. Considering high copper concentration in the liver of WD patients, we anticipate that the NRh-IR-NMs will be useful for liver copper detection in a WD model.

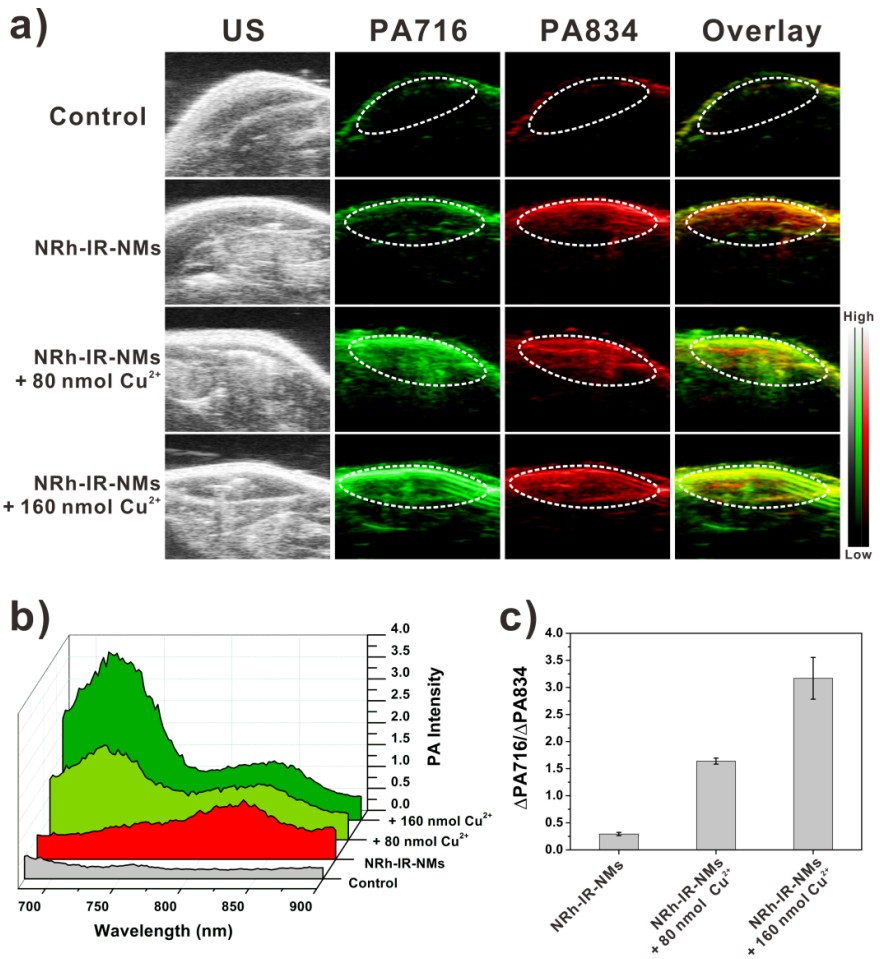

Figure 4. (a) PA images of living mice after subcutaneous administration of saline and NRh-IR-NMs solution with or without Cu^{2+}. (b) PA spectra of region of interest (ROI) in (a). (c) Ratiometric PA signals (ΔPA716/ΔPA834) based on data in (b).

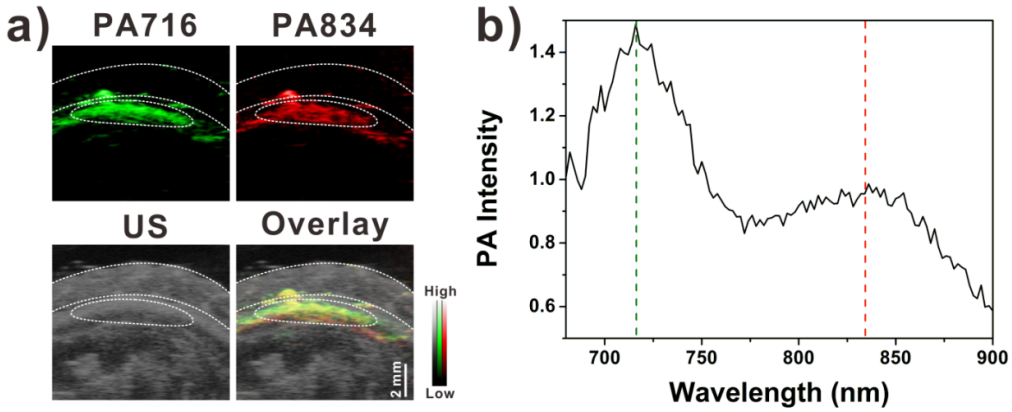

Figure 5. (a) PA images of living mice after subcutaneous administration of NRh-IR-NMs solution with Cu^{2+} (covered by 2 mm pork tissue). (b) PA spectrum of ROI in (a).

4. Conclusion

In summary, a non-invasive strategy based on ratiometric PA nanoprobe (NRh-IR-NMs) was developed for *in vivo* detection of Cu^{2+} in living mice. In this system, two small molecular NIR probes, NRh as a Cu^{2+}-responsive indicator and IR as a Cu^{2+}-inert internal reference, were encapsulated into NMs simultaneously. In the presence of Cu^{2+}, a selective Cu^{2+}-induced reaction would take place and thus lead to changes of PA intensities, and then ratiometric PA imaging can be used to detect the Cu^{2+} *in vivo*. This ratiometric PA detection method is a non-invasive technology with high selectivity and improved tissue penetration depth, which may be a great tool for Cu^{2+} detection in living organisms. Considering the critical role of Cu^{2+} for major neuronal functions, the ratiometric PA nanoprobe has great potential for detection of Cu^{2+} in numerous diseases such as AD and WD.

ASSOCIATED CONTENT

Supporting Information

Supporting Information Available: Synthesis process and NMR characterization of NRh, IR and PEG-PCL. UV-vis-NIR absorption spectra and PA spectra of different samples. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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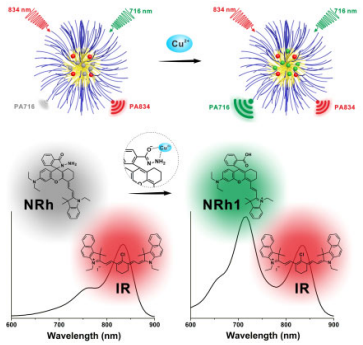

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