A tumor microenvironment–induced absorption red-shifted polymer nanoparticle for simultaneously activated photoacoustic imaging and photothermal therapy

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Tumor microenvironment–responsive therapy has enormous application potential in the diagnosis and treatment of cancer. The glutathione (GSH) level has been shown to be significantly increased in tumor tissues. Thus, GSH can be used as an effective endogenous molecule for diagnosis and tumor microenvironment–activated therapy. In this study, we prepared a tumor microenvironment–induced, absorption spectrum red-shifted, iron-copper co-doped polyaniline nanoparticle (Fe-Cu@PANI). The Cu(II) in this nanoparticle can undergo a redox reaction with GSH in tumors. The redox reaction induces a red shift in the absorption spectrum of the Fe-Cu@PANI nanoparticles from the visible to the near-infrared region accompanying with the etching of this nanoparticle, which simultaneously activates tumor photoacoustic imaging and photothermal therapy, thereby improving the accuracy of in vivo tumor imaging and the efficiency of photothermal therapy. The nanoparticle prepared in this study has broad application prospects in the diagnosis and treatment of cancer.

INTRODUCTION

Photoacoustic (PA) imaging is a novel biomedical imaging technique based on the combination of photoexcitation and ultrasonic detection (1–5). Because the attenuation of acoustic waves in biological tissues is two to three orders of magnitude lower than that of light waves, PA imaging of biological tissues provides excellent contrast, superspace resolution, high penetration, and high sensitivity (6–8). PA imaging can avoid strong light scattering in biological tissue and provide a PA signal capable of penetrating 7 cm with a spatial resolution of up to 100 μm (9), which surpasses the optical diffusion threshold and penetration depth of conventional optical imaging (10). Thus, it is a promising in vivo imaging technique with enormous potential in biomedical research and molecular diagnosis of diseases (11–13).

Because of its deeper tissue penetration and higher spatial resolution, the PA imaging technique has attracted considerable attention in recent years. Various materials, such as organic dyes (14), gold nanorods (15–18), carbon nanotubes (19, 20), two-dimensional graphene analogs (21, 22), sulfides (23, 24), and semiconductor polymer nanoparticles (25–27), have been used for PA imaging as in vivo bioactive molecular tracers, as well as probes for cancer diagnosis and therapy monitoring. However, most of these exogenous PA imaging contrast agents have “always-on” signal, and their signal-to-noise ratio is caused by the concentration gradient of PA agents between the disease site and normal tissue (28). PA agents that can be activated by specific biomolecules to produce a PA signal enable intrinsic signal evolution (from “OFF” to “ON” states) when recognizing a molecular target or event. This allows the visualization of pathological biomarkers at the molecular level in deep tissues in vivo, with high specificity and sensitivity for the detection of disease state and dynamically tracking processes associated with disease development. However, studies on the design and development of activatable PA probes are scarce to date (29). Considering their advantages, such as high signal-to-noise ratio, real-time detection ability between different probe states (i.e., activation and non-activation), and monitoring of pathological processes in deep tissue at the molecular level, developing activatable PA probes is of great importance for conducting biomedical research.

Photothermal therapy (PTT) is a cancer treatment regimen in which heat is generated from near-infrared (NIR) light irradiation using highly efficient photothermal agents. Compared with traditional cancer treatment methods, PTT has unique advantages, such as high tumor specificity, minimal damage to adjacent normal tissues, and spatiotemporal selectivity. Tumor microenvironment–responsive therapy using highly abundant endogenous biomolecules in tumor to activate the treatment can enhance the therapeutic efficiency. Furthermore, as a promising deep-tissue in vivo imaging technique, tumor microenvironment–activated PA imaging can be used to accurately locate the tumor site and guide PTT. Glutathione (GSH) is an important and most abundant small-molecule thiol antioxidant in cells. Compared to normal cells, the GSH concentration in cancer cells is about 1000 times higher (30, 31) and thus can serve as an important biomarker in the diagnosis of cancer (32, 33). Therefore, developing GSH-responsive nanomaterials with PA imaging and PTT features is an attractive option. The diagnostic and therapeutic functions of GSH-responsive nanoparticles are activated in tumor microenvironment and thus can be used for accurate in vivo PA imaging–guided photothermal tumor therapy.

In this study, we designed and prepared a tumor microenvironment–induced, absorption spectrum red-shifted, iron-copper co-doped polyaniline (PANI) nanoparticle (Fe-Cu@PANI). The Cu(II) ion in these nanoparticles underwent a redox reaction with GSH, which was highly abundant in tumor. The redox reaction produced protonated PANI and induced a red shift in the absorption spectrum of Fe-Cu@PANI nanoparticles from the visible to the NIR region, as well as accompanying with the etching of this nanoparticle to enhance...
GSH response. In addition, protonated PANI can generate heat under NIR light irradiation (34), thereby enabling tumor microenvironment–activated simultaneously PA imaging and PTT. The principle is illustrated in Fig. 1.

RESULTS AND DISCUSSION
Characterization of Fe-Cu@PANI nanoparticles and response to GSH
Fe-Cu@PANI nanoparticles were prepared by a one-pot method using bovine serum albumin (BSA) as dispersant and FeCl$_3$, CuCl$_2$, and aniline as raw materials. The preparation was performed by simply mixing the components, and the final nanoparticle product was collected after ultrafiltration. The loading amount of copper ions in the obtained Fe-Cu@PANI nanoparticles was 2.45%, which was determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Fe-Cu@PANI nanoparticles were highly dispersed and stable in pure water and phosphate-buffered saline (PBS), and the color of the aqueous solution was blue (fig. S1). The transmission electron microscopy (TEM) images showed that the average particle size of Fe-Cu@PANI nanoparticles was ~230 nm (Fig. 2A and fig. S2). The analysis of the composition of Fe-Cu@PANI nanoparticles by energy-dispersive x-ray spectroscopy (EDS) revealed that the nanoparticles were composed of Cu, Fe, N, and C elements (fig. S3A). The element mapping results further revealed the presence of elements Cu, Fe, N, and C in the as-prepared Fe-Cu@PANI nanoparticles (fig. S3B).

We then examined the response of GSH to Fe-Cu@PANI nanoparticles. The TEM images revealed a decrease in particle size after the reaction with GSH, and the particle size decreased gradually with the increase of the GSH concentration (Fig. 2, B and C, and fig. S4). We found that at GSH concentrations up to 1.0 mM, the particle size decreased to about 7 nm (Fig. 2C), which was much smaller at 5.0 mM GSH (fig. S4). The size change of the Fe-Cu@PANI nanoparticle in the presence of GSH was further confirmed by the dynamic light scattering (DLS) measurements (fig. S5), which produced results that were consistent with those obtained by TEM imaging. The change of nanoparticle size may be due to the redox reaction between the Cu(II) doped into the Fe-Cu@PANI nanoparticles and GSH, which resulted in the etching of the nanoparticles. To further verify the etching of nanoparticles, we determined whether there was a release of Cu and Fe from Fe-Cu@PANI nanoparticles after the reaction with GSH. Fe-Cu@PANI nanoparticles were incubated with GSH for 24 hours in PBS buffer, and then after centrifugation, the supernatant was analyzed by ICP-OES. The results showed that after incubation with GSH, a large amount of Cu and Fe ions was released compared to that without GSH, and the amounts increased with increasing GSH concentration (fig. S6). Thus, GSH could etch Fe-Cu@PANI nanoparticles. We also observed that the color of the solution of Fe-Cu@PANI nanoparticle changed from blue to yellow green with increasing concentration of added GSH (fig. S1), accompanied by a red shift in the absorption spectrum from the visible to the NIR region (Fig. 2D). The absorption spectra in Fig. 2D reveal that the maximum absorption wavelength ($\lambda_{max}$) red-shifted from ~615 to ~820 nm when the GSH concentration increased from 0 to 1.0 mM. Further increasing the concentration of GSH up to 5.0 mM resulted in no noticeable shift of $\lambda_{max}$ but there was an increase in the absorbance at 820 nm. The change of $\lambda_{max}$ ($\Delta \lambda$) was linearly correlated with GSH concentration in the range of 0 to 1.0 mM (fig. S7), while the absorbance at 820 nm of Fe-Cu@PANI solutions also showed a good linear relationship with the GSH concentrations from 0.4 to 5.0 mM (fig. S8). These results indicated that

Fig. 1. Schematic illustration of the preparation of Fe-Cu@PANI nanoparticles and the activation mechanism for PA imaging and tumor PTT. Fe-Cu@PANI nanoparticles can respond to endogenous GSH in tumor microenvironment, through the redox reaction between GSH and Cu(II) in this nanoparticle, which induces a red shift in the absorption spectrum of Fe-Cu@PANI nanoparticles from the visible to the NIR region, as well as accompanying with the etching of this nanoparticle to enhance GSH response. Thereby, tumor microenvironment activates simultaneously PA imaging and PTT.
Fe-Cu@PANI nanoparticles could be used for PA detection of GSH, due to the properties of the GSH-activated red shift in the $\lambda_{\text{max}}$ and GSH concentration-dependent change in absorbance. In addition, we also determined the absorption spectra of Fe-Cu@PANI solutions with and without 5.0 mM GSH at pH 6.5 to simulate the acid tumor microenvironment. The results shown in fig. S9 demonstrated that, under this simulated acid tumor microenvironment condition, the presence of 5.0 mM GSH also could induce a significant red shift of the $\lambda_{\text{max}}$ and an enhancement of the absorbance at 820 nm, indicating the potential application of Fe-Cu@PANI nanoparticles in tumor tissue. The response of Fe-Cu@PANI nanoparticles to other substances was also examined to evaluate their specificity for GSH. Amino acids, NaCl, NaI, NaNO₂, NaNO₃, H₂O₂, Na₂SO₃, Na₂SO₄, and NaHS at the same concentration of 5.0 mM did not cause the red shift of the absorption spectrum of the nanoparticle solution, and the absorbance of the solutions at 820 nm remained unchanged (Fig 2E), suggesting that Fe-Cu@PANI nanoparticles have excellent specificity for GSH.

**Response mechanism of Fe-Cu@PANI nanoparticles to GSH**

According to a previous report (35), the Fe(III) was functioned as an oxidant to catalyze the polymerization of aniline. We first prepared Fe-dopped polymer nanoparticles (Fe@PANI) without Cu doping to evaluate its response to GSH. The obtained Fe@PANI nanoparticle solution was blue in color (fig. S10A) with a $\lambda_{\text{max}}$ of ~620 nm (fig. S11A), but it could not be red-shifted to 820 nm even in the presence of GSH at up to 1.0 mM (fig. S11A). This implied that Cu doping in Fe-Cu@PANI nanoparticles was important for the GSH-induced NIR response. X-ray photoelectron spectroscopy (XPS) analysis was performed to determine the chemical states of Cu in the Fe-Cu@PANI nanoparticles before and after the reaction with GSH. The XPS spectra of Fe-Cu@PANI nanoparticles shown in Fig. 2F reveal peaks at 935.1 and 954.3 eV, which correspond to the electron energy peaks of Cu²⁺ 2P₃/₂ and 2P₁/₂, respectively (36, 37). After reacting with GSH (Fig. 2G and fig. S12), two new peaks for Cu⁺ 2P₃/₂ and 2P₁/₂ appeared at 932.9 and 952.4 eV, respectively (36, 37), and two valence states (Cu²⁺/Cu⁺) were simultaneously present.
present in the Fe-Cu@PANI nanoparticles after reacting with GSH. In
addition, it was found that, at higher GSH concentration, more Cu”
was produced (Fig. 2G and fig. S12). On the basis of these experi-
mental findings, we propose that the Fe-Cu@PANI nanoparticles
could respond specifically to GSH through the redox reaction be-
tween the Cu(II) in the Fe-Cu@PANI nanoparticles and GSH, ac-
cording to the following reaction equation: Cu^{2+} + GSH → Cu” +
GSSG (oxidized glutathione) + H^+ (38). It has been reported that
PANI exists in two forms including the emeraldine base (EB) and
emeraldine salt (ES). EB PANI can be converted into ES PANI by
protic doping with dopants (i.e., acids and transition metals), and
ES PANI has been shown to have strong NIR absorption (34, 39).
Thus, the proton generation in the redox reaction of GSH with Cu(II)
could facilitate the formation of ES PANI, thereby inducing the red
shift of the absorption spectrum. We also prepared Cu@PANI
nanoparticles without introducing the oxidant Fe(III) to evaluate
the GSH response. The Cu@PANI nanoparticle solution had a \( \lambda_{\text{max}} \)
of ~810 nm (fig. S11B) with light green color (fig. S10B), but adding
GSH only slightly increased the absorbance (fig. S11B). This implied
that most PANI in Cu@PANI nanoparticles was in the ES state, and
the reaction of GSH with Cu@PANI could not produce more ES. The
above results demonstrated that the GSH response to Fe-Cu@PANI
nanoparticles resulted from the Cu(II)/GSH redox reaction–induced
generation of ES PANI, which caused a red shift of the absorption
spectrum to NIR region. In addition, the etching of the Fe-Cu@PANI
nanoparticles after the addition of GSH could produce PANI
fragments with more exposed surface to undergo EB-to-ES conver-
sion during the redox reaction, thereby enhancing the GSH response.

GSH-activated PA properties of Fe-Cu@PANI nanoparticles
We also investigated the PA properties of Fe-Cu@PANI nanoparticles
after reaction with GSH by recording the PA signal at different con-
centrations of GSH. As shown in fig. S13, with the increase of the
GSH concentration, there was also a gradual red shift to about
820 nm in the PA spectrum of the nanoparticle solution. Under NIR
light irradiation at 820 nm, the PA intensity of the Fe-Cu@PANI
nanoparticle solution gradually increased with the increase of the
GSH concentration from 0 to 5.0 mM (Fig. 3A). The PA signal in-
tensity was found to be linearly correlated with the GSH concentra-
tion, with the following linear regression equation: \( I_{\text{PA}} = 1569.79 +
213.64C_{\text{GSH}}, R^2 = 0.9967 \) (Fig. 3, B and C). These results suggested
that Fe-Cu@PANI nanoparticles can be used as a PA probe for PA
detection of GSH.

Photothermal properties of GSH-activated
Fe-Cu@PANI nanoparticles
The photothermal properties of the Fe-Cu@PANI nanoparticle af-
after GSH activation were studied by monitoring the temperature of
the Fe-Cu@PANI solution containing GSH under 808-nm laser irra-
diation. The results shown in Fig. 3 (D and E) reveal that the tempera-
ture of all Fe-Cu@PANI nanoparticle solutions (60 \( \mu \)g/ml) increased
with the extension of irradiation time, while the temperature of the
aqueous solution remained unchanged. At the same time, when the
concentration of added GSH was increased, the temperature of
nanoparticle solutions was also increased, reaching to 39.0°, 46.5°,
57.3°, and 61.6°C after irradiation for 5 min at 0, 0.6, 2.0, and 5.0 mM
GSH, respectively. The above phenomenon demonstrated the GSH-
activated photothermal effect of Fe-Cu@PANI nanoparticles. The
redox reaction between GSH and Cu(II) produced protonated ES
PANI and caused the red shift of the absorption spectrum. Thus, at
a higher concentration of GSH, the NIR light absorption was stron-
ger, leading to more effective photothermal conversion (40). In ad-
dition, we found that the photothermal temperature was dependent
on the power density of the 808-nm laser (fig. S14). Moreover, the
photothermal conversion of the GSH-activated Fe-Cu@PANI nanopar-
icles still remained effective even after 6 cycles of laser irradiation
(Fig. 3F). The shape and intensity of the absorption spectrum of the
nanoparticle solution did not change significantly after 6 cycles of
laser irradiation (fig. S15). Together, the above results indicated
that GSH can activate and enhance the photothermal efficiency of
Fe-Cu@PANI nanoparticles. These findings demonstrate that Fe-Cu@
PANI nanoparticles can be used not only as PA probes for GSH
detection but also as an efficient activatable photothermal agent.

PTT assessment at the cellular level
Before bioapplication, we investigated the biocompatibility of Fe-
Cu@PANI nanoparticles by using the methyl thiazolyl tetrazolium
(MTT) assay to evaluate their cytotoxicity against the 4T1 (mouse
breast cancer) cell and HL-7702 (human normal hepatocytes) cell.
The results in Fig. 4A revealed that, after incubation with the Fe-Cu@
PANI nanoparticle solution (60 \( \mu \)g/ml) for 24 hours, the viability of
both cell lines was still higher than 90%, indicating that without la-
sker irradiation, the nanoparticle had minimal toxicity to normal
or tumor cells. On the other hand, after incubation with the Fe-Cu@
PANI nanoparticle solution (60 \( \mu \)g/ml) for 24 hours, followed by
irradiation with an 808-nm laser for 5 min, the viability of HL-7702
and 4T1 cells gradually decreased with the increase of the laser
power density. However, more 4T1 cells were killed than HL-7702
cells at each laser power density. When the laser power density was
1.5 W/cm², the viability of HL-7702 cells was higher than 70%,
whereas for 4T1 cells, it was about 10% (Fig. 4B). To further dem-
strate tumor cell–targeted phototherapy, we used confocal laser
scanning microscopy (CLSM) imaging to determine the viability of
HL-7702 and 4T1 cells after irradiation with an 808-nm laser and
costaining with Calcein-acetoxyethyl ester (Calcein-AM) and
propidium iodide. Calcein-AM stains living cells (cells with green
fluorescence), while propidium iodide stains dead cells (cells with red
fluorescence). In the images in Fig. 4C, no obvious dead cells are
observed in cells treated only with laser irradiation or Fe-Cu@PANI
nanoparticles, which was similar to that of the control group without
any treatment. The images of cells that were incubated with Fe-Cu@
PANI followed by irradiation with 808-nm laser reveal more wide-
spread and stronger red fluorescence staining for 4T1 than that for
HL-7702 cells, indicating selective killing of tumor cells. These re-
results show that Fe-Cu@PANI nanoparticles can be used as an effec-
tive photothermal agent for PTT of tumor cells.

Tumor microenvironment–activated PA imaging
To investigate whether the GSH in the tumor microenvironment

can activate the PA signal of Fe-Cu@PANI nanoparticles, we first
compared the PA signal from HL-7702 normal cells and 4T1 tumor
cells. Cells were first incubated with the Fe-Cu@PANI nanoparticle
solution followed by washing and centrifugation. The obtained cells
were used for PA imaging, and the results are shown in fig. S16. The
results showed that the PA intensity of 4T1 cell was much higher
than that of HL-7702 cells, indicating that the microenvironment of
tumor cells can activate PA signal. To demonstrate that the GSH in
the tumor cell microenvironment played an important role in the
activation of PA signal, we used N-ethyl-maleimide (NEM), a typical thiol-depleting agent to pretreat cells before staining with Fe-Cu@PANI nanoparticles. As anticipated, after depleting GSH in cells, the PA signals from tumor and normal cells both decreased (fig. S16). This indicated that the GSH in the tumor microenvironment could activate the PA signal of Fe-Cu@PANI nanoparticles.

After that, to evaluate the in vivo PA imaging performance, we injected Fe-Cu@PANI nanoparticles into the normal muscle tissues and the 4T1 cell-xenograft tumor site and then recorded and analyzed their PA signals (Fig. 5, A and B). Without injection of Fe-Cu@PANI nanoparticles, no obvious PA signal could be observed in both tumor and muscle tissues (control group). On the other hand, after injecting Fe-Cu@PANI nanoparticles, PA signals were clearly detected 0.5 hours later, and the intensity in the tumor site was significantly higher than that in muscle tissue, reaching its highest value within 1.0 hour. Then, with the prolongation of time, the PA signal both in tumor and muscle tissues gradually weakened, but in the tumor site, there was still a noticeable PA signal after 24 hours.
is due to the higher levels of GSH in tumor tissue than that in normal muscle tissue, and the highly abundant GSH in the tumor microenvironment can activate the PA signal of the injected Fe-Cu@PANI nanoparticles. A control experiment, in which exogenous 10 mM glutathione ethyl ester (GSH-EE) was first injected into muscle tissue followed by injecting Fe-Cu@PANI nanoparticles, was then performed to demonstrate the GSH-activated in vivo PA imaging. According to fig. S17, the PA signal intensity of muscle tissue pretreated with exogenous GSH-EE was significantly stronger than that of the muscle tissue without GSH-EE pretreatment. This result further indicated that the in vivo PA signal was correlated with the GSH level, and the stronger signal resulted from the higher concentration of GSH in the microenvironment.

On the basis of the above in vivo tumor microenvironment–activated PA imaging results, we used Fe-Cu@PANI nanoparticles as the PA probe for tracking the tumor growth during tumor development. We monitored the tumor growth at 4, 6, 8, 10, 12, and 14 days after injecting 4T1 cells in the root of the mouse hind legs. For each imaging, Fe-Cu@PANI nanoparticles were first injected into the tumor, and the PA signal was recorded 1.0 hour later. The results shown in Fig. 5C revealed that the PA signal gradually increased along with tumor growth. The PA intensity values measured at different xenograft days shown in Fig. 5D revealed that during the initial 6 days, the PA signal was relatively low, but from days 6 to 12, it was markedly enhanced and tended to stabilize after 12 days. This was due to the fluctuations in the GSH levels during tumor development. These findings indicated that the GSH level correlated with tumor growth rate, and the prepared Fe-Cu@PANI nanoparticles could be used as a tumor microenvironment–activated PA probe to monitor tumor growth.

The PA imaging of the tumor was also studied by intravenous injection of Fe-Cu@PANI nanoparticles. As shown in fig. S18, after intravenous injection, the PA signal in tumor sites was evident 1.0 hour later and achieved its highest intensity within 4.0 hours. Although the PA intensity gradually decreased with time, the PA signal in the tumor site was still noticeable even after 12 hours. This was due to the accumulation of nanoparticles in the tumor site through enhanced permeability and retention effect as well as the much higher GSH level in the tumor. The above results indicated that effective PA imaging of tumor could also be achieved through intravenous administration of Fe-Cu@PANI nanoparticles, which benefited real-time imaging-guided tumor therapy.

The distribution and metabolism of Fe-Cu@PANI nanoparticles were also investigated after intravenous administration. ICP-OES analysis was performed to determine accumulation of Cu and Fe in the main organs of nude mice at different time points after intravenous injection of Fe-Cu@PANI nanoparticles. Cu accumulated mainly in the liver and spleen after 24 hours (Fig. 5E), while Fe accumulated...
mainly in the spleen (fig. S19). With the prolongation of the in vivo retention time, the contents of Cu and Fe decreased rapidly within 7 days. These results demonstrated that Fe-Cu@PANI nanoparticles were biodegradable and could be scavenged in vivo.

**Tumor microenvironment–activated PTT**

The in vivo photothermal therapeutic efficacy of Fe-Cu@PANI nanoparticles was evaluated using 4T1 tumor–bearing mice, which were randomly divided into four groups and administered with different treatments (PBS, PBS + laser, Fe-Cu@PANI, and Fe-Cu@PANI + laser). In the laser-irradiated groups, we used 808-nm laser to irradiate the tumor site directly following intratumoral injection of PBS or Fe-Cu@PANI nanoparticles. The thermographic images shown in Fig. 6A reveal the enhancement of the photothermal signal in the Fe-Cu@PANI + laser group with increasing irradiation time, and it was much stronger than that in the PBS + laser group.
The tumor temperature achieved in the Fe-Cu@PANI + laser group increased rapidly and reached 55.6°C within 5 min (Fig. 6C). However, in the PBS + laser group, there was only a slight increase of the tumor temperature from 36.7°C to 40.3°C after 5-min irradiation. These indicated a good in vivo photothermal conversion of Fe-Cu@PANI, suggesting that it can be used for in vivo PTT of tumors. The images of tumor-bearing mice from four different groups at 0, 8, and 16 days after the first administration are shown in Fig. 6B. Without laser irradiation, the administration of PBS or Fe-Cu@PANI had no inhibitory effect on the growth of tumors. At the same time, without photothermal agent, even with laser irradiation, there was no obvious therapeutic effect on the tumor. Actually, only the developed PTT system combined with Fe-Cu@PANI nanoparticles and laser irradiation showed a remarkable inhibitory effect on tumor growth. The size of the tumors and body weight of mice were measured every 2 days during the treatment to evaluate the therapeutic efficiency.

Fig. 6. Fe-Cu@PANI nanoparticles for in vivo tumor PTT. (A) Thermographic images of tumor sites injected with Fe-Cu@PANI nanoparticles or PBS with an 808-nm laser irradiation. (B) Representative photographs of mice from the four groups with different treatments after 0, 8, and 16 days. (C) Corresponding temperature change versus irradiation time. (D) Tumor volume versus treatment time. (E) Mouse body weight versus treatment time. (F) Photograph of dissected tumors after 16 days. (G) H&E-stained tissues of heart, liver, spleen, lung, and kidney of mice from the PBS and Fe-Cu@PANI + laser groups. Photo credit: S.W., Guangxi Normal University.
and side effects. The results shown in Fig. 6D indicated that without laser irradiation, the tumors grew steadily, while the treatment with Fe-Cu@PANI nanoparticles followed by laser irradiation efficiently suppressed tumor growth and tended to disappear completely. The body weight of the mice treated with Fe-Cu@PANI nanoparticles followed by laser irradiation was slightly reduced in the initial stage of therapy but then increased steadily (Fig. 6E). In the other three control groups, there was no apparent loss of body weight during the therapy period. The tumors excised at the end of the various treatments also confirmed the above therapy result (Fig. 6F), showing that the tumor disappeared after PTT with Fe-Cu@PANI nanoparticles as the photothermal agent. Moreover, hematoxylin and eosin (H&E) costaining analysis and TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) assay showed that after PTT of the tumor for 16 days, no detectable lesions and damages were observed in the tissues from major organs including heart, liver, spleen, lung, and kidney (Fig. 6G and fig. S20). This confirmed that the proposed PTT using Fe-Cu@PANI nanoparticles and 808-nm laser irradiation did not cause noticeable side effects. Also, as for tumor treatment, normal muscle tissue was also injected with Fe-Cu@PANI nanoparticles and irradiated with laser irradiation. The temperature of the muscle tissue increased gradually but was much lower than that of the tumor site (fig. S21A), due to the lower GSH level in normal tissue compared with that in tumor tissue. The results of the histopathological analysis indicated that under the same photothermally treatment, the tumor tissue exhibited obvious damage, compared with normal muscle tissue in which damage was much less severe (fig. S21, B and C). This demonstrated that the PTT developed in this work is an effective tumor microenvironment–activated therapy with low side effect.

In summary, we have prepared an iron–copper co-doped PANI nanoparticle (Fe-Cu@PANI) and found that the Cu(II) in this nanoparticle could undergo redox reactions in the presence of GSH, which is highly abundant in tumor tissues. This induced a red shift in the absorption spectrum of Fe-Cu@PANI from the visible to the NIR region accompanying with the etching of this nanoparticle, thereby activating the PA signal for GSH and enhancing the therapeutic efficiency of tumor PTT. Fe-Cu@PANI nanoparticles here can be used not only as PA probes for in vivo GSH imaging in the diagnosis of tumor but also as an activatable photothermal agent for efficient tumor PTT. This study proposes a novel mechanism for the generation of PA signal by nanomaterials and provides new strategies to develop highly sensitive PA imaging nanoprobes. We have demonstrated that PA imaging of GSH can be used to monitor the tumor growth in vivo during the development of cancer, thereby providing a PA imaging–guided tumor therapy, which has great potential for the diagnosis and treatment of cancer.

**MATERIALS AND METHODS**

**Materials**

BSA was purchased from Amresco Inc. (Solon, OH, USA). Iron chloride hexahydrate (FeCl₃·6H₂O), copper dichloride dehydrate (CuCl₂·2H₂O), aniline, phenylalanine, histidine, threonine, tryptophan, lysine, sodium chloride (NaCl), sodium nitrate (NaNO₃) and sodium nitrite (NaNO₂), sodium sulfate (Na₂SO₄), sodium sulfite (Na₂SO₃), hydrogen peroxide (H₂O₂), sodium hydrosulfide (NaHS), NEM, and GSH-EE were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). GSH was purchased from Sigma-Aldrich (Saint Louis, MO, USA). All reagents were of analytical grade and used without further purification.

**Preparation of Fe-Cu@PANI nanoparticles**

The preparation method of BSA-PANI assemblies was as follows. The BSA-Fe⁴⁺/Cu²⁺ complex solution was first prepared by dissolving FeCl₃ (80 mg), CuCl₂ (20 mg), and BSA (70 mg) in 10 ml of deionized water with continuous stirring for 30 min at room temperature. Subsequently, the aniline/HCl solution, which was obtained by mixing hydrochloric acid (95 μl, 36% by mass) and aniline (95 μl) in 1.0 ml of deionized water and vigorous shaking for 5 min, was added to the above BSA-Fe⁴⁺/Cu²⁺ complex solution at 4°C upon the color change of the mixture to blue. The resulting solution was then placed in a refrigerator and kept at 4°C for 10 hours. Eventually, the Fe-Cu@PANI nanoparticles were obtained by ultrafiltration followed by lyophilization. For control experiments, Fe-@PANI nanoparticles and Cu@PANI nanoparticles were prepared using FeCl₃ (100 mg) and CuCl₂ (100 mg) as the raw materials, respectively.

**Cell culture and cytotoxicity assessment**

Murine breast cancer 4T1 cells and human normal liver HL-7702 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and all cultures were maintained in an incubator at 37°C with a humidified atmosphere containing 5% CO₂. Cells were seeded in a 96-well culture plate at a density of 10⁴ cells per well and then incubated for 24 hours at 37°C in a 5% CO₂ atmosphere. The corresponding culture medium solutions containing Fe-Cu@PANI nanoparticles at different concentrations (0, 20, 40, 60, 80, and 100 μg/ml) were added to the wells. The cells were then grown for 24 hours at 37°C in a 5% CO₂ atmosphere, and the cell viability was determined by MTT assay.

**In vitro and in vivo GSH-activated PA imaging**

PA imaging was performed on a multispectral optoacoustic tomography (MSOT) inVision 256-TF small animal scanner (iThera Medical GmbH, Munich, Germany). Commercial 6-mm transparent plastic tubes were each fully filled with either the control (PBS) solution or the sample solution and then fixed on the holder of the instrument. For in vitro PA detection, various concentrations of GSH (0, 0.2, 0.6, 1.0, 2.0, 3.0, 4.0, and 5.0 mM) were incubated with Fe-Cu@PANI solution for 0.5 hours and then inserted into the corresponding tube for PA detection. For in vivo PA imaging, the 4T1 tumor–bearing mice were anesthetized and set on the MSOT inVision 256-TF small animal scanner. The PA imaging was performed after injection of Fe-Cu@PANI (100 μl, 60 μg/ml) at different time points (0, 0.5, 1.0, 2.0, 12.0, and 24.0 hours). PA images of 4T1 tumor–bearing BALB/c mice determined at different days (4, 6, 8, 10, 12, and 14 days) were also acquired, in which Fe-Cu@PANI (100 μl, 60 μg/ml) was injected, and PA imaging was then performed 1.0 hour later. The whole mouse was scanned with a step size of 0.3 mm. After data acquisition, the PA images were reconstructed using a standard backprojection algorithm.

**Assessment of the photothermal therapeutic effect of Fe-Cu@PANI nanoparticles**

The 4T1 cells were seeded into 96-well culture plates at a density of 10⁴ per well and incubated at 37°C in a 5% CO₂ atmosphere for 24 hours. The solution of Fe-Cu@PANI nanoparticles at a concentration of 60 μg/ml in RPMI 1640 medium was added into the wells.
Then, the cells were irradiated for 5 min with an 808-nm laser at different laser power densities (0.5, 1.0, 1.5, 2.0, and 2.5 W/cm²). The cell viability was measured using a typical MTT assay. A CLSM imaging assay was also used to assess the dead and live cells after PTT. The Fe-Cu@PANI solution (60 μg/ml) was added to a cell culture plate containing 4T1 cells, and then the adherent cell layer was exposed to an 808-nm laser with a laser power of 1.5 W/cm² for 5 min. Sole addition of PBS, Fe-Cu@PANI (60 μg/ml), or NIR irradiation for 5 min only (1.5 W/cm²) was used as controls. After NIR laser irradiation, the live and dead 4T1 cells were detected by staining with calcein-AM and propidium iodide. The cells were then observed by CLSM (Carl Zeiss Microscopy GmbH, Jena, Germany).

For in vivo PTT, 20 tumor-bearing mice were divided into four groups (five mice per group). In the first group, mice were injected with 100 μl of the PBS solution. In the second group, mice were injected with 100 μl of the PBS solution and then irradiated with an 808-nm laser (1.5 W/cm²) for 5 min. In the third group, 100 μl of Fe-Cu@PANI (60 μg/ml) nanoparticles was injected into mice without laser irradiation. In the fourth group, mice were injected with 100 μl of Fe-Cu@PANI (60 μg/ml) and, 1 hour later, irradiated with an 808-nm laser (1.5 W/cm²) for 5 min. All groups were treated every other day for 16 days.

Tumor temperature and thermographic images were acquired using an Optris PI infrared camera (Optris Infrared Sensing LLC, Portsmouth, NH, USA). The tumor volumes after treatments were measured using a caliper, and the volume was calculated according to the formula \((V = \frac{a 	imes b^2}{2})\), where \(a\) and \(b\) are the long and short diameters of the tumor, respectively. The relative tumor volume \((V/V_0)\), where \(V_0\) denotes the initial tumor volume, i.e., day 0) was used to monitor tumor growth. The tumor volume and body weight were recorded every 2 days during the therapy period.

**Tumor-bearing mouse model**

The 8-week-old female Balb/c mice were obtained from Hunan SJA Laboratory Animal Co. Ltd. (Changsha, China). Animal handling procedures were approved by the Animal Ethics Committee of the Guangxi Normal University (no. 2019151-XC), and mice were kept under specific pathogen–free condition with ad libitum access to standard food and water. All animal experiments were performed in the Laboratory Animal Center of Guangxi Normal University, and the experiments were conducted under the protocols of the Care and Use of Laboratory Animals of the Guangxi Normal University. A 100 μl of suspension of 4T1 cells (10⁶ cells in PBS) was injected into subcutaneous tissue on the right hind side of the nude mice to develop the tumor.

**Material characterization**

TEM images were acquired on a FEI Tecnai G2 F-20 transmission electron microscope (FEI Company, Hillsboro, OR, USA) with an accelerating voltage of 200 kV. DLS measurements were performed on a Nano Zetasizer (Malvern Panalytical Ltd., Malvern, UK). The ultraviolet-visible spectra were recorded on an Agilent Cary 60 spectrophotometer (Agilent Technologies, Santa Clara, USA). XPS analysis was performed on an ESCALAB 250Xi x-ray photoelectron spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). EDS and element mapping images were acquired using a FEI Titan G2 F-30 instrument operated at 200 kV. The Fe and Cu were determined by ICP-OES analysis using a Flexar/NexION300X inductively coupled plasma optical emission spectrometer (PerkinElmer Inc., Waltham, MA, USA).

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**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/12/eabe3588/DC1

View/request a protocol for this paper from Bio-protocol.
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A tumor microenvironment–induced absorption red-shifted polymer nanoparticle for simultaneously activated photoacoustic imaging and photothermal therapy
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