Aggregation-Induced Emission Luminogen with Near-Infrared-II Excitation and Near-Infrared-I Emission for Ultradeep Intravital Two-Photon Microscopy

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Supporting Information

ABSTRACT: Currently, a serious problem obstructing the large-scale clinical applications of fluorescence technique is the shallow penetration depth. Two-photon fluorescence microscopic imaging with excitation in the longer-wavelength near-infrared (NIR) region (>1100 nm) and emission in the NIR-I region (650−950 nm) is a good choice to realize deep-tissue and high-resolution imaging. Here, we report ultradeep two-photon fluorescence bioimaging with 1300 nm NIR-II excitation and NIR-I emission (peak ∼810 nm) based on a NIR aggregation-induced emission luminogen (AIEgen). The crab-shaped AIEgen possesses a planar core structure and several twisting phenyl/naphthyl rotators, affording both high fluorescence quantum yield and efficient two-photon activity. The organic AIE dots show high stability, good biocompatibility, and a large two-photon absorption cross section of 1.22 × 10⁵ GM. Under 1300 nm NIR-II excitation, in vivo two-photon fluorescence microscopic imaging helps to reconstruct the 3D vasculature with a high spatial resolution of sub-3.5 μm beyond the white matter (>840 μm) and even to the hippocampus (>960 μm) and visualize small vessels of ∼5 μm as deep as 1065 μm in mouse brain, which is among the largest penetration depths and best spatial resolution of in vivo two-photon imaging. Rational comparison with the AIE dots manifests that two-photon imaging outperforms the one-photon mode for high-resolution deep imaging. This work will inspire more sight and insight into the development of efficient NIR fluorophores for deep-tissue biomedical imaging.

KEYWORDS: near-infrared, aggregation-induced emission, two-photon fluorescence imaging, NIR-II excitation, brain imaging
sensitivity, real time, and easy accessibility.\textsuperscript{1,2} Currently, a serious problem obstructing the large-scale clinical applications of fluorescence technique is the shallow penetration depth.\textsuperscript{3,4} Near-infrared (NIR) (700–2500 nm) imaging is capable of affording good penetration capacity by virtue of the reduced light–tissue interactions.\textsuperscript{5–7} In general, the NIR biological region can be divided into four parts, \textit{i.e.}, NIR-I (650–950 nm), NIR-II (1100–1350 nm), NIR-III (1600–1870 nm), and NIR-IV (2100–2300 nm) windows.\textsuperscript{8–10} It is noted that there is a big water absorption peak at 1400–1500 nm.\textsuperscript{11} The NIR-I window shows weak tissue absorption, while other longer-wavelength NIR regions have more reduced light scattering.\textsuperscript{11–13} According to the previous work reported by Shi \textit{et al.}, NIR-II and NIR-III windows are the optimal wavelengths for bioimaging; in fact, NIR-III is called the “golden window”.\textsuperscript{5,10} However, the lack of high-performance emitters and suitable light source/detectors hinders the investigation of longer-wavelength NIR regions (\textit{e.g.}, NIR-III and NIR-IV).\textsuperscript{14} Recently, the modality of NIR-I excitation and NIR-II emission has attracted considerable attention for \textit{in vivo} imaging.\textsuperscript{14–16} Dai \textit{et al.} reported an NIR-membrane fluorescent photoluminescence with NIR-I excitation (808 nm) and NIR-II emission for image-guided tumor resection.\textsuperscript{14} Bawendi’s group reported the short-wave infrared imaging by using 808 nm NIR-I excitation based on commercially available NIR dyes in living mice.\textsuperscript{15} On the other hand, the protocol of employing NIR-II or even NIR-III excitation would be a better choice in terms of penetration depth, because the long-wavelength NIR light can penetrate into much deeper tissue.

Two-photon fluorescence (TPF) imaging is a promising method to realize deep-tissue and high-resolution bioimaging. TPF is a kind of nonlinear optical process in which two long-wavelength (low-energy) photons are simultaneously absorbed to generate the excited state followed by one short-wavelength (high-energy) photon-releasing process.\textsuperscript{17–19} Two-photon fluorescence microscopic (TPFM) technique has been widely used in helping the 3D reconstruction of main organs and monitoring biological processes/diseases.\textsuperscript{20–23} The conventional TPFM modality of NIR-I excitation and visible light emission has been investigated for more than a decade, but the imaging depth is limited due to the suboptimal excitation/emission wavelength.\textsuperscript{21} The NIR-I excitation and NIR-I emission TPFM protocol would be better than the previous one, but the overlap between the excitation and emission spectra is a serious obstacle. Recently, the emerging 1040 nm femtosecond (fs) laser based on ytterbium-doped photonic crystal fiber affords good imaging quality.\textsuperscript{22} Compared to the short-wavelength NIR light (700–1040 nm), the longer-wavelength NIR region is better regarding the attenuation depth because of the far more reduced light scattering in biological tissue.\textsuperscript{24–28} Alfano and co-workers studied the attenuation lengths of different NIR windows in chicken tissue, which suggested the good penetration capacity of 1200 and 1700 nm light.\textsuperscript{29} Xu \textit{et al.} reported the deep three-photon imaging of neuronal activity under 1300 and 1700 nm laser excitation.\textsuperscript{30,31} These results reveal the prominent advantage of long-wavelength NIR excitation for deep-tissue bioimaging. Except for the excitation light, the emission wavelength is also an important factor. Most of the previous two- and three-photon imaging exhibits emission in the visible range, which would impact the output ability of emitted light. The TPFM bioimaging protocol of employing NIR-II excitation with low tissue scattering and NIR-I emission with weak tissue absorption is promising but still undeveloped, since suitable probes are very rare.

At present, there are only two FDA-approved NIR fluorogens, \textit{i.e.}, methylene blue (MB) and indocyanine green (ICG), both of which are organic small molecules.\textsuperscript{12,29} This highlights that organic small-molecule emitters hold great potential for clinical translation, due to the notable characteristics of well-defined chemical structure, facile processability, and good biocompatibility.\textsuperscript{30,31} Nevertheless, conventional organic luminogens face several intrinsic drawbacks, including small Stokes shift (usually less than 50 nm), moderate photostability, and aggregation-caused quenching (ACQ) effect.\textsuperscript{32,33} The ACQ phenomenon severely hinders the applications of organic emitters, as the emission is intensively quenched at high concentrations or aggregate states. Moreover, to shift the excitation/emission wavelength to the NIR region, one needs to extend the conjugation length by introducing lots of aromatic blocks. This would result in a more planar and hydrophobic structure, and it has to be fabricated into nanoparticles (NPs) for bioimaging, making the ACQ effect even more pronounced.\textsuperscript{34,35} Aggregation-induced emission luminogens (AIEgens) are a kind of emitters that show weak emission in solution, but are strongly emissive in the solid/aggregation state due to the restriction of the intramolecular rotation (RIM) mechanism.\textsuperscript{36,37} The emission intensity of AIEgens intensifies when aggregated in a more condensed manner, which is particularly beneficial for NIR AIE materials because of their intrinsic hydrophobic nature. A lot of AIEgens have been reported, and AIE NPs exhibit good performance in biomedical applications including TPF imaging.\textsuperscript{38,39} Nevertheless, there is no report of TPF imaging with absolute NIR fluorescent AIEgens, \textit{i.e.}, the whole emission spectrum beyond 700 nm.

Recently, we have reported high-resolution bioimaging by employing the partial emission of AIE dots beyond 900 nm, suggesting that AIEgens could serve as efficient NIR contrast agents for \textit{in vivo} imaging.\textsuperscript{40} In this contribution, we develop ultradeep TPFM imaging with NIR-II excitation and NIR-I emission based on bright NIR AIE dots in living mouse (Scheme 1). Due to the crab-shaped AIEgen, high fluorescence quantum yield and two-photon excitation efficiency are simultaneously achieved. \textit{In vivo} TPFM imaging of mouse brain using 1300 nm NIR-II excitation helps to record 3D vascular information with superb spatial resolution (sub-3.5 μm) at white matter

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Scheme1.png}
\caption{Scheme 1. Schematic illustration of the AIE dots for NIR-II-excited \textit{in vivo} two-photon fluorescence imaging of mouse brain.}
\end{figure}
RESULTS AND DISCUSSION

For the TPF emitter, a large two-photon action cross section ($\delta\Phi$, where $\delta$ is the two-photon absorption cross section (TPACS) and $\Phi$ is the fluorescence quantum yield) is highly desirable.\textsuperscript{41-43} However, the $\delta$ and $\Phi$ values of organic fluorophores are usually opposite of or work against each other. To obtain good small-molecule TPF agents, the most effective strategies are boosting the intramolecular charge transfer (ICT) and conjugation length.\textsuperscript{44,45} The donor–acceptor (D–A) structure has been widely used to increase ICT and thus $\delta$, but it is detrimental for high $\Phi$. Alternatively, extending the conjugation length is also valid to improve $\delta$, yet the planar structure is prone to result in an ACQ effect. Therefore, it is a big challenge to simultaneously realize high $\delta$ and $\Phi$ values, especially for organic NIR emitters. Here, a crab-shaped D-A-type molecule ($N,N^\prime$-(5,6-diphenyl-1,2,5-thiadiazolo[3,4-g]quinoxaline-TQ) unit form a strong D–A interaction, which would facilitate ICT. Furthermore, the core unit (Ph-TQ-Ph, the red ellipse in Figure 1a) is a relatively planar architecture, which enables extended conjugation. Therefore, the planar D–A core endows the molecule with a large $\delta$ value. According to the RIM mechanism, the freely rotating phenyl and naphthyl rings around the planar core (Figure 1b) would inhibit intermolecular $\pi–\pi$ interaction, thus favoring an AIE signature (Figures S6, S7) and high $\Phi$ in the aggregate state. Density functional theory (DFT) calculation reveals that the electron density of the highest occupied molecular orbital (HOMO) is distributed along both the donor and acceptor moieties, whereas the lowest unoccupied molecular orbital (LUMO) is mostly localized on the electron-withdrawing TQ unit (Figure S8), indicative of a D–A-type chromophore with strong ICT.

To render the hydrophobic compound with good in vivo biocompatibility, we encapsulated TQ-BPN into AIE dots with the assistance of the FDA-approved surfactant pluronic F127, as schematically illustrated in Figure 1c (for details see the Experimental Section). As depicted in Figure 1d, the AIE dots exhibit maximal absorption and emission at $\sim630$ and $810$ nm, respectively, and the excitation profile (Figure S9) displays high intensity at $650$ nm, suggesting that they could be excited
by double-wavelength light near 1300 nm. Because of the AIE feature, the encapsulated AIE dots show a high fluorescence quantum yield of 13.9% (ICG in dimethyl sulfoxide with a quantum yield of 13% was used as the reference), which is higher than most organic NIR emitters in aqueous media.\textsuperscript{5,15} The morphology of the AIE dots is characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS) measurements, both of which reveal uniform spheres in shape with an average diameter of \(\sim 35\) nm (Figure 1e,f). Further scanning electron microscopy (SEM) characterization also manifests the sphere structure with similar size (Figure S10). It should be noted that the AIE dots exhibit good colloidal stability, as no precipitation is observed after storage at room temperature for 60 days, and the average diameter nearly does not change as well (Figure S11).

It is critically important for a fluorescent contrast agent to maintain a stable signal in different conditions. So we studied the fluorescence stability of the AIE dots with various treatments. As presented in Figure 1g, the photoluminescence (PL) intensity of the AIE dots proportionally increases with the concentration, indicating potential application for quantitative analysis. On the contrary, the emission of MB dye is slightly increased at very low concentrations, but significantly quenched at high concentrations, displaying an obvious ACQ phenomenon. Of note, the AIE dots exhibit good photostability, as only a slight decrease in the emission intensity (<5%) is observed after light irradiation (635 nm) for 1 h (Figure 1h). Reactive oxygen species (ROS) are a kind of important signaling molecules modulating physiological functions and widely existing in the living body.\textsuperscript{46,47} Thus, ROS-stable probes are essential to attain a stable imaging signal, but some agents (e.g., cyanine dyes) are easily destroyed by ROS. As depicted in Figure 1i, the AIE dots show superb stability, and no detectable bleaching is observed after treating with ROS. In contrast, ICG is not stable enough and undergoes severe bleaching in the presence of hydroxyl radical \((^\cdot OH)\), probably due to the oxidation of alternative single-double bonds.\textsuperscript{48} These results suggest that the AIE dots are suitable for intravital biomedical imaging.

To validate the penetration capability of NIR-II light for biological imaging, vectorial electric field Monte Carlo (EMC) simulation was employed.\textsuperscript{49,50} By simulating the tissue scattering and absorption coefficient (for details see the Experimental Section), the focal spots of light with various wavelengths (515, 635, 800, 1040, and 1300 nm) at different tissue depths are presented in Figure 2a, and the corresponding light intensity–tissue depth relationship is shown in Figure 2b. From visible to NIR-I, and further to the NIR-II spectral region, the light penetration ability considerably increases, especially at large depths. This can be attributed to the lower tissue scattering of longer-wavelength light and may explain why most current TPF imaging has a limited penetration depth of several hundred micrometers. It is interesting to note that the intensity of 1300 nm NIR-II light remains \(\sim 75\%\) of the original value at a depth of 1000 \(\mu\)m, suggesting a potent advantage for two-photon excitation.

The two-photon excitation process of the AIE dots is depicted in Figure 3a, in which two photons of 1300 nm (NIR-II) are simultaneously absorbed to generate an excited state; then a NIR-I photon is released via a radiative decay pathway. Under the excitation of a 1300 nm fs laser (1 MHz), the nonlinear optical property of the AIE dots was investigated. As shown in Figure 3b, the TPF spectrum is similar to the one-photon PL spectrum (Figure 1d), and the inset photograph...
shows bright two-photon emission from the AIE dots-filled capillary. Most of the emission from the AIE dots is located in the NIR-I range, being favorable for in vivo bioimaging because of the low tissue absorption and highly sensitive photodetector (peak response at ~800 nm) in this region. Noteworthy, the wavelength difference between the excitation and emission peaks of the TPF agent (a kind of "anti-Stokes shift") is as large as 500 nm, which is advantageous to avoid spectral overlap. To validate that the observed fluorescence signal was stemming from the two-photon-excited conversion process, we measured the emission intensities of the AIE dots under 1300 nm fs laser excitation with different power intensities. For a typical nonlinear two-photon optical process, the relationship between TPF intensity ($I_{flu}$) and the logarithm of incident laser power ($P_{inc}$) can be expressed as $\log I_{flu} = 2 \log P_{inc}$. Accordingly, we plot the logarithm of $I_{flu}$ of the AIE dots and the logarithm of $P_{inc}$, which shows a very good linear relationship (Figure 3c and Figure S12). The slope is calculated to be 2.2 (close to 2), indicative of TPF nonlinear optical process. To better understand the two-photon excitation process of the AIE dots, we measured the TPF lifetime ($\tau$), which was calculated to be 8.24 ns (Figure 3d). This would help to conduct high-contrast time-resolved imaging by eliminating the background signal.\(^{3,52}\) Interestingly, the $\delta$ value of the AIEgen at 1300 nm is calculated to be $1.22 \times 10^3$ GM (1 GM = $10^{30}$ cm$^2$ s$^{-1}$ photon$^{-1}$) using ICG as the reference (for details see the Experimental Section), which is one of the highest values for organic NIR two-photon fluorogens.\(^{53,54}\)

Considering the maximal absorption of the AIE dots was in the red-light region (~630 nm), we performed linear one-photon fluorescence confocal bioimaging under an excitation of 635 nm light. As shown in Figure S13, the microscopic system setup in our lab was equipped with a 635 nm CW excitation laser and a photomultiplier tube (PMT). The in vitro confocal imaging result indicates that the AIE dots are able to enter into HeLa cells and illuminate the cell region brightly (Figure S14). After confirming the good cellular imaging property, we performed the vascular imaging in living mice. The AIE dots were injected into female BALB/c mice through the tail vein, and the mice were imaged under the excitation of a low-power 635 nm laser (2 mW). Due to the bright NIR emission, one-photon fluorescence confocal imaging of mouse brain could detect light signal depth to 700 $\mu$m (Figure 4 and Video S1), representing the largest imaging depth with a one-photon imaging modality, to our knowledge; the current penetration depth of one-photon NIR-I fluorescence imaging of mouse brain in vivo is about 500 $\mu$m.\(^{3,55}\) It is noted that one-photon fluorescence confocal imaging offers a fine resolution of ~7.2 $\mu$m capacity at a depth of 600 $\mu$m (Figure S15). Moreover, we also examined the feasibility of our AIE dots for visualizing the vascular architecture of the mouse ear. As presented in Figure S16 and Video S2, the ear blood vessels could be clearly distinguished. These results indicate that one-photon fluorescence confocal imaging based on NIR-I-emitted AIE dots is capable of screening the intravital blood vasculature.

To obtain better penetration capability and spatial resolution, we further performed in vivo TPFM imaging to decipher the 3D vasculature information. A TPFM imaging system was built with a 1300 nm fs laser as the excitation light and a PMT with peak sensitivity at ~800 nm as the photosensor (Figure S17). We first performed the TPFM cellular imaging of HeLa cells by incubating them with AIE dots, which showed bright two-photon emission from the cells (Figure S18). The TPFM scanning was carried out with a 2D area of 700 $\mu$m × 900 $\mu$m on the mouse brain after injection of the AIE dots through the tail vein. The elegant brain vasculature at different depths is presented in Figure 5a. To gain a better evaluation of the imaging quality, lines were drawn across tiny capillaries at different depths (e.g., 600, 1000, and 1065 $\mu$m), and the pixel intensity was plotted as a function of position (Figure 5b and Figure S19). Gaussian fitting provides the profile of a capillary, which affords a high-resolution full width at half-maximum (fwhm) of ~3.4 $\mu$m at a
These results reveal that the TPFM imaging exhibits superb resolution of sub-3.5 μm beyond the white matter (>840 μm) and even to the hippocampus region (>960 μm),\textsuperscript{24} which is better than that of one-photon confocal imaging. Under the 1300 nm NIR-II excitation, TPFM imaging reaches as deep as 1065 μm, and a small blood vessel with a diameter of \(~5 μm\) can still be resolved (Figure 5a), being one of the largest penetration depths and best spatial resolutions of

Figure 5. (a) \textit{In vivo} TPFM images of mouse brain at different depths as indicated. (b) fwhm of a blood vessel at the depth of 1000 μm as indicated with the dotted white line in the 1000 μm image. (c, d) 3D reconstruction of brain vasculature. (e) Time-resolved decay profile of the TPF intensities and a TPFM lifetime image of the brain vessels \textit{in vivo} (depth = 100 μm). The scale bars in (a, e) and (c, d) indicate 100 and 150 μm, respectively.

Figure 6. Histological H&E staining for main organs (brain, heart, lung, liver, spleen, and kidneys) of the mice intravenously administrated with PBS and AIE dots for 2 h and 48 days. The scale bar indicates 100 μm.
the TPFM technique in vivo.\textsuperscript{36,57} Subsequently, the 3D reconstruction image of mouse brain vasculature was built (Figure 5c,d), in which the major blood vessels, capillaries, and junctions could all be vividly visualized. Moreover, TPFM imaging on the mouse ear also helps to delineate the vascular architecture below the ear dermis under 1300 nm fs laser excitation (Figure S20 and Video S3). The deep penetration and high-resolution of TPFM imaging is attributed to the good two-photon activity of the AIE dots and the excellent penetration capacity of NIR-II excitation in biological tissues. These results suggest that the crab-shaped AIEgen holds great promise for TPF applications, and the strategy of NIR-II excitation and NIR-I emission allows deep imaging in vivo.

Time-resolved fluorescence imaging is of great significance since it could provide superior resolution by resolving the excited-state lifetime differences of various fluorescent agents.\textsuperscript{58–60} On the basis of the TPF lifetime of the AIE dots, we conducted time-resolved intravital TPFM imaging of brain blood vessels. As depicted in Figure 5e, a uniform signal is observed in all the blood vessels, suggesting that the AIE dots are finely distributed in the vasculature. The AIE dots show a TPF lifetime of 8.86 ns in the blood vessels, in good accordance with the \textit{in vitro} measured lifetime (8.24 ns) in capillaries (Figure 3d). Our preliminary study illustrates potential applications of AIE dots for bioimaging in another dimension of the time scale. Biocompatibility and biosafety are very important for a contrast agent, so we carried out a histological study to evaluate the influence of the AIE dots on living mice. Hematoxylin and eosin (H&E) stain (Figure 6) reveals that no noticeable damage or inflammatory lesions are observed in the major organs (brain, heart, lung, liver, spleen, and kidney) of the mice after treating with AIE dots for a short time (2 h) and long time (48 days), suggesting good biocompatibility.

**CONCLUSIONS**

In summary, we report ultradeep \textit{in vivo} TPFM imaging with NIR-II excitation and NIR-I emission based on highly bright AIE dots. The crab-shaped AIEgen possesses a planar core structure and several twisting phenyl/naphthyl rings, affording both high brightness and efficient two-photon activity (\(\delta = 1.22 \times 10^3\) GM). In \textit{in vivo} TPFM imaging under 1300 nm NIR-II excitation enables a superb spatial resolution of sub-3.5 \(\mu\)m beyond the white matter (>840 \(\mu\)m) and even to the hippocampus region (>960 \(\mu\)m) and clear visualization of small vessels of ~5 \(\mu\)m at a depth of 1065 \(\mu\)m in mouse brain, suggesting that the AIEgens with NIR-II excitation and NIR-I emission hold great promise for deep-tissue and high-resolution \textit{in vivo} bioimaging. Future work will focus on the development of efficient organic NIR lumogens by finely tuning the molecular structure of AIEgens for longer-wavelength TPFM imaging, for example, in the NIR-III window.

**EXPERIMENTAL SECTION**

**Methods.** \(^1H\) NMR (400 MHz) and \(^13C\) NMR (100 MHz) spectra were measured on a Bruker AV 400 spectrometer. A high-resolution mass spectrum (HRMS) was obtained from a GCT Premier CAB048 mass spectrometer. High-performance liquid chromatography (HPLC) measurement was conducted on an Agilent 1260 Infinity. Absorption spectra were measured using a Shimadzu 2550 UV−vis spectrophotometer. PL measurement was conducted with a Horiba Fluorolog-3 spectrophluorometer. DLS measurements were performed on a Malvern Zetasizer Nano ZS-90. TEM images were acquired from a JEOL JEM-1200EX microscope. SEM images were captured by a Hitachi-SU8010 microscope. Molecular geometry optimization was calculated using the DFT method with the Gaussian 09 program package at the level of B3LYP/6-31G*.\textsuperscript{56,57} The Cartesian coordinates are presented in Table S1.

**Synthesis of TQ-BPN.** \(\text{NN}^-\{(5,6\text{-Dinitrobenzo[c][1,2,5}-\text{thiadiazole-4,7-diyl]bis(4,1-phenylene)}\)bis(N-phenylphenanthrene-1-amine) (0.81 g, 1 mmol) was dissolved in acetic acid (40 mL), followed by the addition of iron powder (1.68 g, 30 mmol), which was heated to 80 °C and stirred for 12 h. Afterward, water was added and the mixture was extracted with dichloromethane (100 mL × 3). After solvent evaporation, 4,7-bis(4-(naphthalen-1-yl)(phenyl)amino)-phenylbenzo[c][1,2,5]thiadiazole-5,6-diamine was obtained as a dark red solid, which was used directly. The crude diamine product and benzil (0.32 g, 1.5 mmol) were added into a 100 mL flask, and chloroform (15 mL) and acetic acid (15 mL) were then added, followed by heating to 65 °C and stirring overnight. Afterward, water was added, and the mixture was extracted with dichloromethane (100 mL × 3). The organic phase was combined and dried with MgSO\(_4\). The solvent was evaporated, followed by purification on a silica column with dichloromethane/hexane (v/v 1/1) as eluent. The collected product was recrystallized from hexane to give TQ-BPN as a dark solid (72% yield). \(^1H\) NMR (400 MHz, CDCl\(_3\)), 25 °C) \(\delta \) ppm: 8.07 (d, 2H), 7.96–7.89 (m, 6H), 7.83 (d, 2H), 7.61 (d, 4H), 7.56–7.46 (m, 6H), 7.43–7.34 (m, 4H), 7.30–7.24 (m, 12H), 7.21 (d, 4H), 7.06–6.99 (m, 2H), \(^{13}C\) NMR (400 MHz, CDCl\(_3\)), 25 °C) \(\delta \) ppm: 153.15, 152.57, 148.62, 147.89, 143.23, 138.58, 136.00, 135.34, 133.98, 131.40, 130.04, 129.45, 129.23, 128.50, 128.43, 128.15, 127.68, 127.34, 126.79, 126.57, 126.41, 126.22, 124.43, 123.12, 122.55, 119.52. HRMS (MALDI-TOF, m/z): [M]\(^+\) calcd for \(C_{36}H_{24}N_6S\), 926.3192; found, 926.3184. HPLC purity: >99.8%.

**Preparation of AIE Dots.** A 1 mg amount of TQ-BPN AIEgen and 12 mg of polyethylene-polypropylene glycol (pluronic F-127) were dissolved in 2 mL of CHCl\(_3\), followed by drying on a rotary evaporator. Afterward, 2 mL of phosphate-buffered saline (PBS) (\(pH = 7.4\)) was added, and the mixture was sonicated for 5 min to obtain a transparent blue solution of the AIE dots, which was used directly. Accordingly, the mass fraction of TQ-BPN in the AIE dots is estimated to be 7.7%.

**Monte Carlo Simulation.** The vectorial EMC simulation was used to simulate the absorption and scattering of light in biological tissue, in which the intensity distribution of a focal spot was simulated at certain depths. The biological sample was supposed as a tissue-like phantom composed of 1 \(\mu\)m scattering beads with a concentration of 0.1044 spheres/\(\mu\)m\(^3\). Water was considered as the main absorption matter. The scattering coefficient was calculated by Mie theory, in which the refractive index of the background and scattering beads was obtained from the Cauchy dispersion equation. \textsuperscript{48} Light of different colors (515, 635, 800, 1040, and 1300 nm) was chosen as the simulation wavelengths.

**Measurement of Two-Photon Absorption Cross Section.** TPACS (\(\delta \)) of the AIEgen was evaluated by the two-photon-induced fluorescence method. ICG in water was used as the reference. The \(\delta \) value at 1300 nm was measured with an uprigh TPF scanning microscope (Olympus, water-immersed objective: 25/1.00), which was connected to a 1300 nm fs laser (maximal output 200 mW, 1 MHz, 150–230 fs). A capillary filled with the AIEgen was irradiated with the laser beam. The objective was used to collect the TPF signal, in which a broad bandpass filter was equipped to select the light above 1000 nm.

\[
\delta_1 = \frac{F_1\Phi_2\varepsilon_3}{F_2\Phi_1\varepsilon_1}
\]

where \(F \) represents the TPF intensity, \(\delta \) is the TPACS, \(c \) is the molar concentration, \(\Phi \) is the fluorescence quantum yield, \(\varepsilon \) is the refractive index of the solvent, and subscripts 1 and 2 stand for the sample (AIEgen) and reference (ICG), respectively. According to the literature, the TPACS of ICG in water under 1552 nm excitation
We measured the TPF intensity of ICG in water ($c_1 = 0.129 \text{ mM}, n_1 = 1.332$) at 1552 and 1300 nm, which are 13.93 ($F_{1552}$) and 4.28 ($F_{1300}$), respectively. Thus, the TPACS of ICG ($\delta_2$) at 1300 nm is 64.5 GM. The TP intensity of AIEgen in toluene solution ($c_1 = 0.054 \text{ mM}, n_1 = 1.469$) was measured to be 406.7 ($F_{1300}$), and the fluorescence quantum yields of the solution of ICG ($\Phi_1$) and AIEgen ($\Phi_2$) above 1000 nm were measured to be 0.035% and 0.38%, respectively. As a consequence, the TPACS of the AIEgen ($\delta_2$) is calculated to be 1.22 $\times 10^3$ GM.

**Confocal Optical Imaging System.** One-photon confocal fluorescence imaging was conducted on a commercial microscope (Olympus, BX61W1-FV1000) with a 635 nm laser (2 mW) as the excitation light. The laser beam was focused onto the objective with an objective lens (XLPlan N, 25×, NA = 1.05, work distance = 2.0 mm), which was also used to detect the fluorescence signal from the emitters. The fluorescence signal was detected by a PMT (Hamamatsu, H7422-50) through descanned detection after a 700 nm long-pass filter.

**Two-Photon Fluorescence Microscopic Imaging System for Cells and Mouse Ear.** TPFM imaging of cells and mouse ear was performed on a commercial microscope (Olympus, BX61W1-FV1200) with a 1300 nm fs laser (maximal output power 200 mW, 1 MHz, 150–230 fs) as the excitation light. The 1300 nm light was generated from an optical parametric amplifier (Orpheus) pumped by a 1040 nm PHTAROS-10W fs laser (10 W), in which the emission wavelength can be tuned in the range of 1040–2600 nm. The 1300 nm fs laser beam was focused onto the objective with an objective lens (XLPlan N, 25×, NA = 1.05) with large NA, and the light reaching the objective must be 30 mW. The fluorescence signal was detected by a PMT (Hamamatsu, H7422-50) after a 700 nm long-pass filter.

**Two-Photon Fluorescence Microscopic Imaging System for Mouse Brain.** For the TPFM images of the mouse brain, we used a home-built scanning microscope and a 1300 nm fs laser (maximal output power 400 mW, 400 kHz, 35 fs) from a nonlinear optical parametric amplifier (Spectra Physics) pumped by a regenerative amplifier (Spectra, Spectra Physics) and included a custom-built dispersion compensation unit to maintain short pulse durations (50 fs) at the sample plane. The light reaching the objective was measured to be 30 mW. Detection included proper filters (920 LP and 712/90 BP) and PMT (H107750-PA, Hamamatsu).

**Confocal and TPFM Imaging of Cells.** Prior to the experiment, HeLa cells were cultured in cultivation dishes, and the AIE dots (100 μL, 1 mg/mL) were added into the cell chambers. After incubation at 37 °C for 2 h, the cells were washed twice with 1× PBS and imaged using the confocal or TPFM imaging system.

**Animal Experiments.** All the animal experiments were conducted under the guidance of the care and use of laboratory animals of Zhejiang University Animal Study Committee and EMBL’s Institutional Animal Care and Use Committee. Three mice were used for each kind of in vivo imaging (one-photon confocal, TPFM, and lifetime imaging).

**In Vivo One-Photon Confocal and Two-Photon Imaging.** Female BALB/c mice (8 weeks old) were used for all intravital imaging. The pretreatment of brain vasculature imaging is similar to that in a previous report.65 In general, the mice were anesthetized, and their skulls were removed to carefully offer a cranial window. For all in vivo one-photon confocal and TPFM imaging, the AIE dots in PBS (200 μL, 1 mg/mL) were injected into the mice through the tail vein before imaging. The images were taken every 5 μm, and the 3D reconstruction image was built using software (Imaris) subsequently. The scanning speeds of brain and ear imaging were 2.5 μm/μm (841 $\times$ 1024 pixels per frame) and 10 μs/μm (512 $\times$ 512 pixels per frame), respectively.

**TPFM Lifetime Imaging of Mouse Brain.** The pretreatment of the mice was similar to that for TPFM imaging. Under excitation of a 1300 nm fs laser, lifetime imaging based on the AIE dots was performed on a time-correlated single-photon counting system (Becker & Hickl GmbH, SPC-150, detection range 400–700 nm).

**Histological Study.** Healthy female BALB/c mice were divided into three groups ($n = 4$) at random, and different treatments were carried out prior to the measurement. Group 1 was used as the control and was injected with 200 μL of PBS through the tail vein. The mice in group 1 were sacrificed 2 h after the treatment. The other two groups were intravenously administrated with 200 μL of AIE dots in PBS (1 mM based on the AIEgen). Mice in group 2 and group 3 were respectively sacrificed 2 h and 48 days postinjection to study the acute and long-term toxicity. Main organs including the brain, heart, lung, liver, spleen, and kidneys were harvested, and the tissue samples were fixed in 4% paraformaldehyde. Afterward, the samples were embedded in paraffin, sectioned, stained with H&E, and then examined with an optical microscope.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.8b02452.

The structure characterizations, spectral data, calculation results, characterizations of the AIE dots, imaging setups, one-photon confocal and TPFM images (PDF)

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**Notes**

The authors declare no competing financial interest.

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