

Photostable Small-Molecule NIR-II Fluorescent Scaffolds that Cross the Blood-Brain Barrier for Noninvasive Brain Imaging

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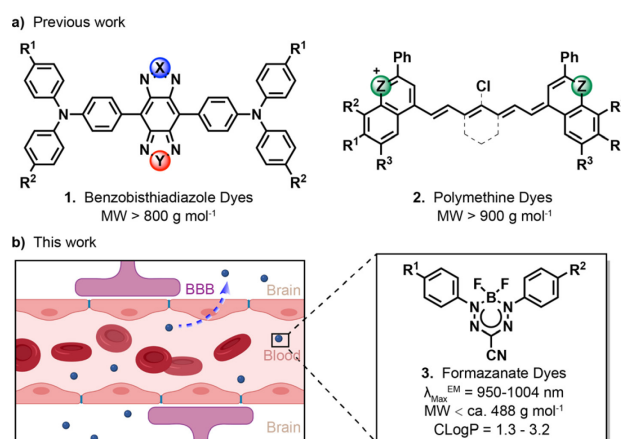
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ABSTRACT: The second near-infrared (NIR-II, 1000–1700 nm) fluorescent probes have significant advantages over visible or NIR-I (600–900 nm) imaging for both depth of penetration and level of resolution. Since the blood-brain barrier (BBB) prevents most molecules from entering the central nervous system, NIR-II dyes with large molecular frameworks have limited applications for brain imaging. In this work, we developed a series of boron difluoride (BF₂) formazanate NIR-II dyes which had tunable photophysical properties, ultrahigh photostability, excellent biological stability, and strong brightness. Modulation of the aniline moiety of BF₂ formazanate dyes significantly enhances their abilities to cross the BBB for noninvasive brain imaging. Furthermore, the intact mouse brain imaging and dynamic BBB diffusion were monitored using these BF₂ formazanate dyes in the NIR-II region. In murine glioblastoma models, these dyes can differentiate tumors from normal brain tissues. We anticipate that this new type of small molecule will find potential applications in creating probes and drugs relevant to theranostic for brain pathologies.

INTRODUCTION

The brain is a complex organ that represents the center of nervous system activity in mammals. Conventional imaging techniques for studying brain structure and activity include magnetic resonance imaging, positron emission tomography, computed tomography, and ultrasound imaging.^{1–6} Fluorescence brain imaging is particularly attractive because of its high temporal and spatial resolution coupled with its noninvasive and in situ real-time imaging capabilities. Despite these advantages, fluorescence imaging *in vivo* has been hampered for light microscopy in the visible spectrum (400–700 nm in wavelength) by poor penetration of photons into deep

tissues. Because of decreased photon scattering and reduced autofluorescence, optical bioimaging in the second near-infrared (NIR-II) channel (1000–1700 nm) is of special interest for deep-tissue imaging.^{7–13} Recent studies have indicated that NIR-II probes provide high-resolution bioimaging with deeper penetration (> 9 mm depth) than conventional visible or NIR-I probes (2 mm depth).^{14,15} Furthermore, the clinical utility of NIR-II biomedical imaging probes has been recently explored in human subjects.^{16,17} Both nanomaterials^{18–24} and small-molecule contrast agents^{25–28} have been developed for performing fluorescence imaging in the NIR-II window. In contrast to nanoparticles, small molecule NIR-II fluorophores have the advantage of providing well-defined structures, tunable emission wavelengths, adjustable photophysical properties, low toxicity, and rapid metabolism.²⁹ Indocyanine green (ICG) is



Scheme 1. Small-molecule scaffolds with NIR-II fluorescence. (a) Previous molecules which possess NIR-II fluorescence. (b) The schematic diagram of BF₂ formazanate dyes penetrating the BBB from the circulation.

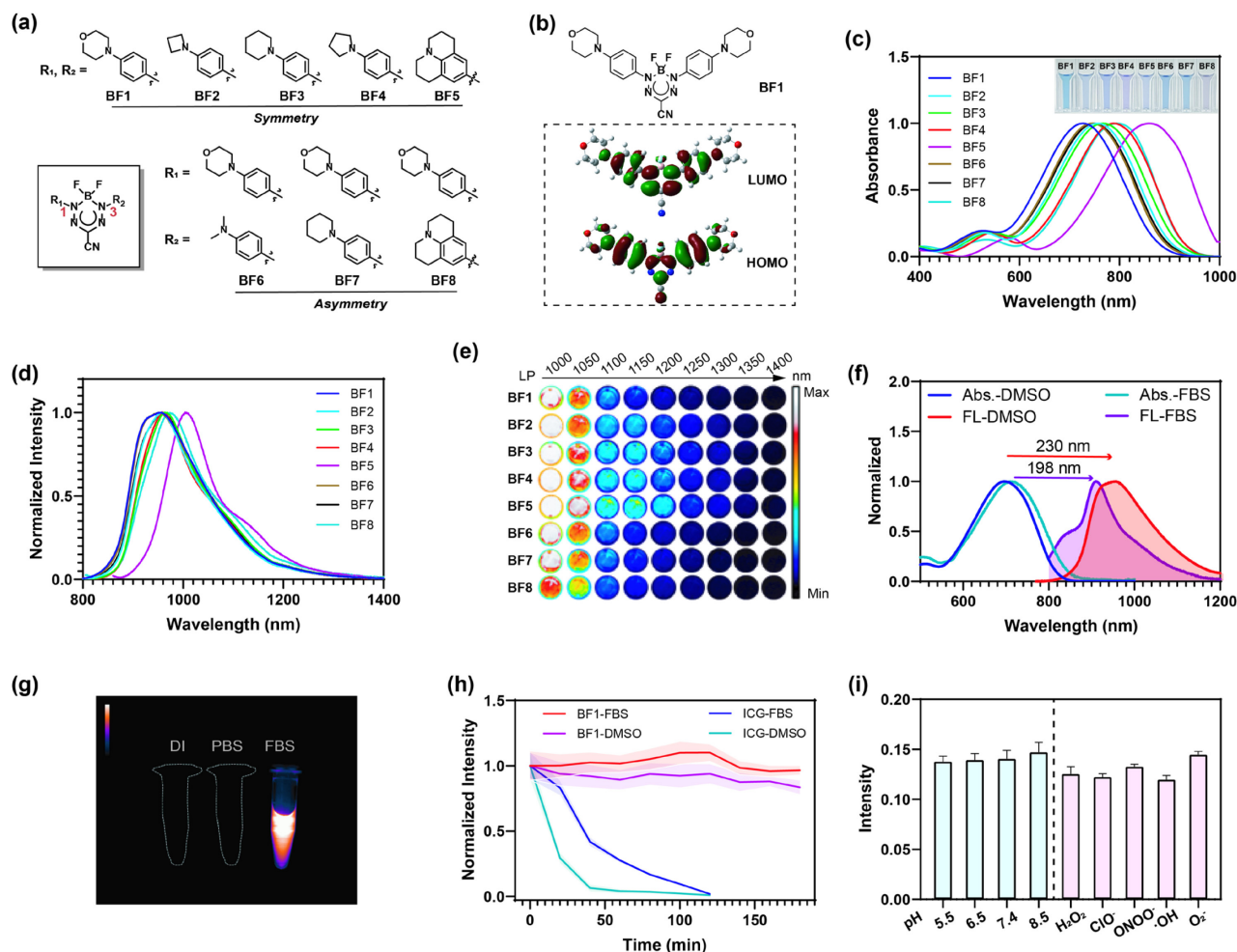


Figure 1. (a) Structures of BF₂ formazanate dyes **BF1–BF8** in this study. (b) HOMOs and LUMOs calculated for **BF1**. (c) The normalized absorbance spectra of **BF1–BF8** (20 μM) solution in DMSO. Inset shows photos of the solutions. (d) The normalized fluorescence spectra of **BF1–BF8** (20 μM) in DMSO. (e) False-colored images of **BF1–BF8** at 50 μM collected through long-pass (LP) filters with cut-on wavelengths of 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, and 1400 nm illumination was at 808 nm laser (45 mW cm⁻², 250 ms exposure). (f) Stokes shift of **BF1** with a concentration of 10 μM in DMSO and FBS. (g) NIR-II fluorescence images of **BF1** (10 μM) in deionized water (DI), PBS buffer, and FBS. (h) Stability of **BF1** and ICG (10 μM) under 808 nm light radiation within 180 minutes in FBS and DMSO. (i) Fluorescent stability of **BF1** (10 μM) in PBS (1% FBS) buffer with different pH and ROS species.

the first fluorophore in the near-infrared (NIR) region approved by the FDA for use as an intravenously administered contrast agent.^{30,31} Because of its tail emission in the NIR-II region, ICG was recently used for NIR-II fluorescence imaging *in vivo*.^{32–34} Furthermore, ICG-based NIR-II imaging has been recently explored in human cancer patients and shows that NIR-II imaging has significantly clinical advantages such as higher imaging contrast, resolution, and diseases detection sensitivity compared to NIR-I imaging.¹⁶ In addition to ICG, several other types of small molecule NIR-II dyes have been reported, including polymethine, benzobis-thiadiazole, and dipyrromethene boron difluoride NIR-II fluorophores (**Scheme 1a**).^{25,35–37} However, despite their success in NIR-II imaging, small-molecule NIR-II dyes capable of crossing the blood-brain barrier (BBB) are currently unavailable.

To construct an ideal small-molecule NIR-II fluorophore for brain imaging, several factors must be taken into consideration, including stability, fluorescence emission wavelength, signal strength, and molecular size. The latter factor is crucial, as evidenced by the fact that fewer than 2% of all US Food and Drug Agency (FDA)-approved small molecule drugs are able to cross the

intact BBB. Quantitative structure-activity relationship studies suggest that several key physicochemical parameters, including molecular weight, lipophilicity, polar surface area, hydrogen bonding, and charge, influence the BBB permeability of molecules.^{38–41} Boron difluoride (BF₂) complexes bearing *N*-bidentate ligands have been used as probes for cell imaging and analyte sensing.⁴² BF₂ complexes containing dipyrromethene ligands (BODIPYs) are most popular in this respect due to their high biocompatibility, tunable photophysical properties, and potentially high emission quantum yields. Recently, a new class of BF₂ formazanate complexes that possess tunable spectroscopic and electrochemical properties has been reported.^{42–45} Similar to BODIPY dyes, formazanate dyes are comprised of a BF₂ moiety bound to a stable six-membered *N*-bidentate ligand. Joe's group has made a great contribution to the modification of BF₂ formazanate substituent groups to tune their redox activities. They found that the modification of the aniline in the BF₂ formazanate framework can significantly affect its electrochemiluminescence and photoluminescence.^{46,47} Based on their work, aniline modification of BF₂ formazanates is likely to be a

promising strategy to modulate their molecular polarity, fluorescence emission, and BBB penetrability for noninvasive NIR-II imaging.

In this work, by modifying the aniline moiety of BF₂ formazanates, we developed a series of NIR-II fluorophores that possess relatively small molecular weights, log *P* values between 1 and 3, and low polar surfaces, fulfilling most of Lipinski's Rules for central nervous system drug design (Scheme 1b). After modification of BF₂ formazanate fluorophores with different electron-donating groups, the dyes exhibit desirable NIR-II emission spectra in fetal bovine serum (FBS) and dimethyl sulfoxide (DMSO). This type of dyes demonstrated ultrahigh photostability, large Stokes Shift, and strong brightness. Furthermore, *in vitro* and *in vivo* experiments establish that several of these dyes penetrate the BBB efficiently, allowing for their use in noninvasive NIR-II fluorescence-based brain imaging. The ability of these dyes to differentiate normal brain tissue from glioblastoma (GBM) tissue establishes their potential use in NIR-II imaging of brain tissue and abnormalities.

RESULTS AND DISCUSSION

Design and Characterization of NIR-II BF₂ Formazanate Dyes

BF₂ formazanate fluorophores possess rich photophysical properties owing to their highly delocalized π -systems and low-lying frontier orbitals that stabilize otherwise labile radicals.⁴² Most importantly, they have relatively small sizes, moderate lipophilicity, low polar surface area, and low charge, allowing for potentially high BBB permeability. In this work, a series of BF₂ formazanate fluorophores was designed with the aim to develop dyes with the emissions in the NIR-II region and the ability to cross the BBB (Figure 1a). Initially, the density-functional theory (DFT) calculations on these BF₂ formazanate fluorophores suggested that both the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) were mainly centered on the BF₂ formazanate backbones, with additional contributions from the π -conjugated aromatic *N*-1,3 position substituents (Figure 1b). We therefore altered the electron-rich π -conjugated system at the *N*-1,3 position substituents to optimize the photophysical profiles of these BF₂ formazanate dyes for NIR-II imaging. Aniline substituents are able to form classical quinoidal structures in BF₂ formazanate, thus dramatically extending the π -system to enhance electron delocalization.⁴⁶ The different-sized nitrogen-containing rings (from aziridine to julolidine) at the *N*-1,3 position were incorporated to obtain BF1–BF5 with symmetric structures. To adjust the molecular lipophilicity, the asymmetric BF₂ formazanate dyes with morpholine were also prepared at the

*N*1 position, and dimethylamine (BF6), piperidine (BF7), and julolidine (BF8) at the *N*3 position.

Next, the photophysical properties of these BF₂ formazanate fluorophores were studied in organic and biological relevant mediums. The BF1–BF8 dyes had NIR absorption peaks from 727 to 859 nm with large extinction coefficients of more than 44,000 M^{−1}cm^{−1} in DMSO. The dyes exhibited a red-shifting absorption trend in the order morpholine, azetidine, piperidine, pyrrolidine, and julolidine (Figure 1c). This trend was also observed in FBS (Figure S1). The fluorescence spectra for all dyes have broad emission bands ranging from 800 to 1400 nm in DMSO and FBS (Figure 1d, Figure S1). The julolidine substituent with enhanced electronic delocalization showed dramatically red-shifted absorption and fluorescence spectra, with an emission peak at 1004 nm in DMSO. TD-DFT calculations revealed strong intramolecular

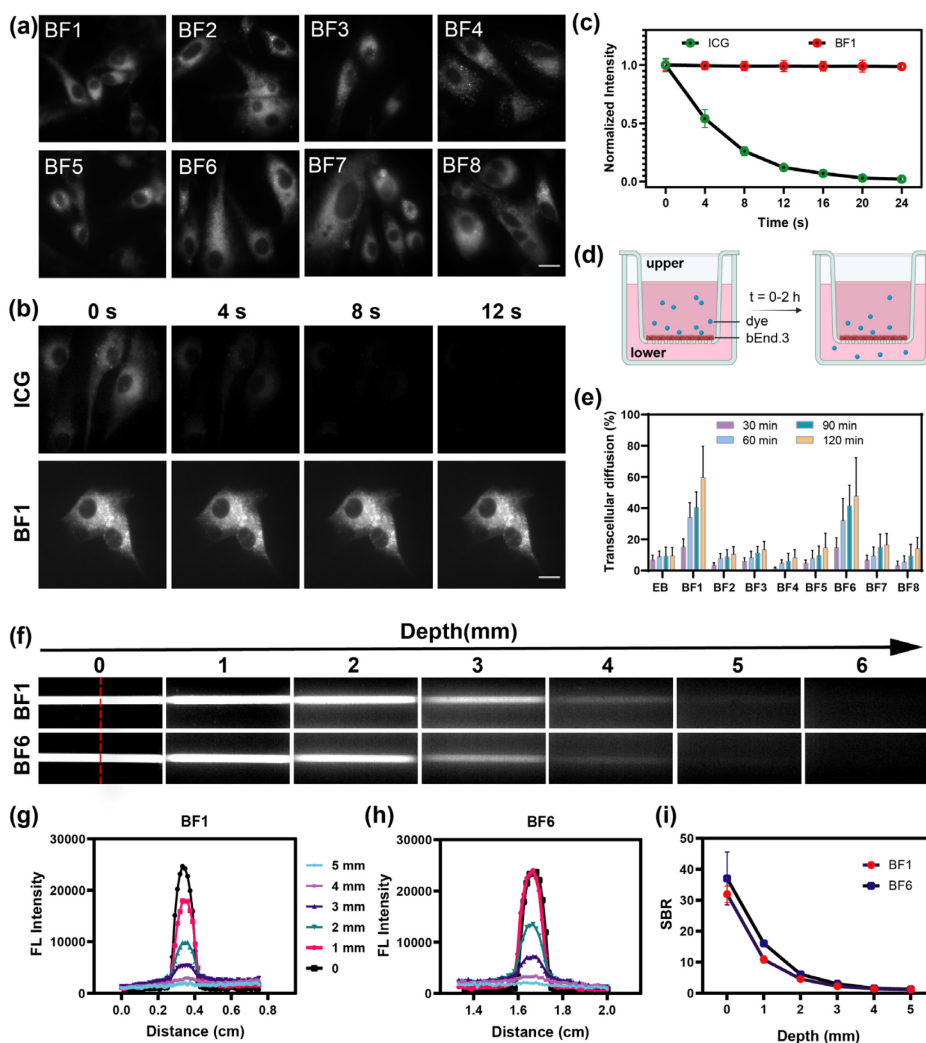


Figure 2. (a) Fluorescence imaging of BF1–BF8 in U-87 MG cells. All cell imaging experiments were performed using an NIR-II microscope with a 663 nm excitation laser (200 W cm^{−2}, exposure 10 ms) and 950 ± 50 nm emission filter. Scale bar is 10 μ m. (b) Fluorescence imaging of ICG and BF1 in U-87 MG cells at 0, 4, 8, 12 s under laser irradiation. Scale bar is 10 μ m. (c) Cellular photostability curves of ICG and BF1 within 25 seconds. (d) Schematic diagram of *in vitro* BBB model used in this study. (e) The relative transcellular diffusion amount of BF1–BF8 and EB *in vitro* within 2 hours. (f) Fluorescence images (excitation 808 nm, LP 1000 nm) of the capillary tube filled with BF1 and BF6 at depths of 0–6 mm in 1% Intralipid mimic tissue. (g, h) The cross-sectional fluorescence intensity profiles of BF1 and BF6 along the red dash line in (f) at different tissue depths. (i) The signal-background ratio (SBR) curves of BF1 and BF6 in 1% Intralipid®.

charge transfer (ICT) and small energy gaps (1.61–1.74 eV) between the HOMO and LUMO, which accounted for the NIR absorption of BF₂ formazanate dyes (**Figure S2**, **Table S1**). The HOMO-LUMO energy gaps become smaller from **BF1** to **BF5** and from **BF6** to **BF8**, suggesting electron-donating groups can significantly red-shift dye absorption and emission. In toluene, tetrahydrofuran (THF), and DMSO, the dyes showed the red-shift absorption and fluorescence. In FBS, the dyes showed a hypsochromic shift absorption and decreased extinction coefficient compared to those in DMSO (**Figure S3**, **S4**). The brightness of all dyes was dramatically enhanced in solvents with lower polarity (**Table S2**). To assess the capabilities of BF₂ formazanate dyes for NIR-II imaging, 50 μ M solutions of the dyes were imaged at different wavelengths. As shown in **Figure 1e**, NIR-II fluorescence properties of **BF1–BF8** were captured using a series of long-pass optical filters covering the NIR-II windows. To our delight, most of the dyes exhibited strong fluorescence beyond 1000 nm, with higher quantum yields comparable to other reported NIR-II fluorophores (**Table S2**).⁸ Notably, the **BF1–BF8** spectra exhibited large Stokes shifts (**Figure S5**, **S6**). In particular, the **BF1** spectrum shows a 230 and 198 nm Stokes shift in DMSO and FBS that simplifies filtering of excitation light (**Figure 1f**). The NIR-II fluorescence behavior of all dyes was studied in deionized water (DI), phosphate-buffered saline (PBS) buffer, and FBS. The result shows all dyes have a fluorescence quench in DI and PBS. But there are relatively strong fluorescence signals over 1000 nm in FBS (**Figure 1g**, **Figure S7**). Next, the photostability of these dyes and ICG was evaluated by monitoring fluorescence intensity in DMSO and FBS. Unlike the quick degradation of the ICG, all small-molecule BF₂ formazanate dyes exhibited ultrahigh emission stabilities in the different solvents for 3 hours long-term irradiations under high laser power irradiation (808 nm, 1.5 W cm⁻², **Figure 1h** and **S8a**). Photothermal and temperature-dependent fluorescence studies showed that most dyes have appreciable photothermal conversion and significant fluorescence increase from 25 to 65 °C in FBS (**Figure S8b**, **S8c**). **Figure 1i** showed that dye also had high pH and chemical stability in the presence of various reactive oxygen species (ROS). Thus, BF₂ formazanate dyes can be potentially used for *in vivo* imaging in the NIR-II region.

In vitro NIR-II imaging and evaluation of BBB permeability

Before testing the use of the BF₂ formazanate dyes for *in vivo* NIR-II imaging, we first applied them for NIR-II imaging of cells in culture. U-87 MG cells were incubated in 2 μ M **BF1–BF8** dyes for 20 minutes, followed by washing and imaging using an NIR-II inverted microscope.⁴⁸ Cell images showed that all dyes had good cell permeability and bright NIR-II fluorescence in cellular environment (**Figure 2a**). To study the subcellular localization of dyes in cell, we used commercial organelle-targeted trackers to analyze the colocalization with our dyes by confocal imaging. The result showed **BF1–BF4** and **BF6–BF8** have a better overlay with lipid droplet, which indicates BF₂ formazanate dyes can specifically target the lipid droplet in cells (**Figure S9**). Next, the cytotoxicity and photostability of these dyes were evaluated in brain vascular endothelial (bEnd.3) and U-87 MG cells, respectively. **BF1–BF8** exhibited negligible cytotoxicity even after incubation at 12 μ M for 24 hours (**Figure S10**). We further tested the intracellular photostability of **BF1–BF8** dyes. Gratifyingly, the BF₂ formazanate dyes exhibited excellent intracellular photostability. No significant fluorescence falls of BF₂ formazanate dyes were observed during a 10 min irradiation under high laser power density (663 nm, 200 W cm⁻²), while the intracellular fluorescence of ICG declined dramatically within 12 seconds under the same conditions (**Figure 2b**, **2c**, and **S11**, **supporting video 1**, **2**). These data confirmed that small-molecule **BF1–BF8** dyes are well-suited for *in vivo* imaging.

Physicochemical parameters, including molecular weight, lipophilicity (as calculated from log P), polar surface area, and hydrogen bonding, have been identified as key parameters for developing compounds with BBB permeability.^{39–41} In general, molecules with molecular weights less than 450 Da, log P less than 5, H-bond acceptors less than 7, and H-bond donors less than 3, exhibit possible BBB permeability.⁴⁹ We calculated these parameters for BF₂ formazanate fluorophores by experimental and computational studies. For the measurement of log P, we added dyes to octanol-saturated water. The log P values were calculated based on the ratio of the dye's concentration in the water-saturated octanol phase to its concentration in the octanol-saturated aqueous phase.⁵⁰ These preliminary analyses of **BF1–BF8** indicated that BF₂ formazanate dyes exhibited attractive physicochemical parameters for the potential BBB permeability (**Table S3**). To further evaluate the BBB permeability of BF₂ formazanate dyes, we used an *in vitro* BBB model prepared by seeding bEnd.3 cells onto gelatin-coated upper chambers of the transwell plates (**Figure 2d**). Successful establishment of the BBB layer was confirmed by the stable transendothelial electrical resistance (TEER) value (**Figure S12**). BF₂ formazanate dyes (**BF1–BF8**, 5 μ M) were then added to the upper chambers, and dye absorbance values in the lower chambers were measured at different time points (30, 60, 90, 120 min). Evans Blue (EB), which is incapable of passing through the BBB, was used as the negative control.^{51,52} By calculating the transcellular diffusion efficiency based on standard curves of **Figure S13**, **BF1** and **BF6** exhibited significantly higher BBB permeabilities among the BF₂ formazanate dyes tested, reaching an appreciable concentration in the lower chambers within 2 hours. In contrast, the slow absorbance increased in lower chambers for **BF2–BF5**, **BF7**, **BF8**, and EB, indicating a low rate of BBB penetration for these dyes (**Figure 2e**). To study the imaging performance of dyes, **BF1** and **BF6** were chosen to investigate the scattering and penetration in mimic tissue. Even up to 5 millimeters mimic tissue depth, the fluorescence signals of two dyes were clearly observed (**Figure 2f–i**).

In vivo brain imaging using NIR-II fluorescence

Encouraged by the NIR-II imaging properties and promising physicochemical parameters of BF₂ formazanate dyes and their ability to cross *in vitro* BBB monolayers, we explored the ability of these dyes to cross the BBB *in vivo*. To evaluate the use of the dyes for NIR-II imaging of cerebral tissue, we used tail vein injections to introduce **BF1–BF8** dyes into athymic nude mice, followed by *in vivo* NIR-II fluorescence imaging. ICG, a dye that has previously been used for *in vivo* NIR-II fluorescence imaging cerebrovascular, was included for comparison.⁵³ As shown in **Figure 3a** and **3c**, significant **BF1** and **BF6** fluorescence signals were observed in brain tissue after injection. The NIR-II fluorescence intensities of **BF1** and **BF6** reached maximum values 20 and 45 minutes after injection, respectively. In contrast to the blurred images obtained with visible and NIR-I light, sharp cerebral images with high signal-to-noise ratios (SNR) of 2.5–2.9 were obtained for the **BF1** and **BF6** dyes (**Figure 3b**, **3d**). In contrast, no significant NIR-II signals in cerebral tissue were observed after the injection of **BF2**, **BF3**, **BF4**, **BF5**, **BF7**, or **BF8** (**Figure S14**). As shown in **Figure 3e** and **3f**, ICG-treated mice exhibited fluorescence signals only in major brain blood vessels, rather than in the brain parenchyma. This vessel fluorescence gradually decreased after administration, and no fluorescence was observed from the brain tissue itself. This result is consistent with previous reports that ICG cannot penetrate the BBB.^{30,53} At 2 hours post-injection, multiple organs, including brain, heart, lung, kidney, spleen, liver, intestine, stomach, bone, muscle, and pancreas, were isolated and used for *ex vivo* NIR-II imaging. *Ex vivo* fluorescence images indicated that significant amounts of **BF1** and **BF6** accumulated in brain tissue (**Figure 3g**

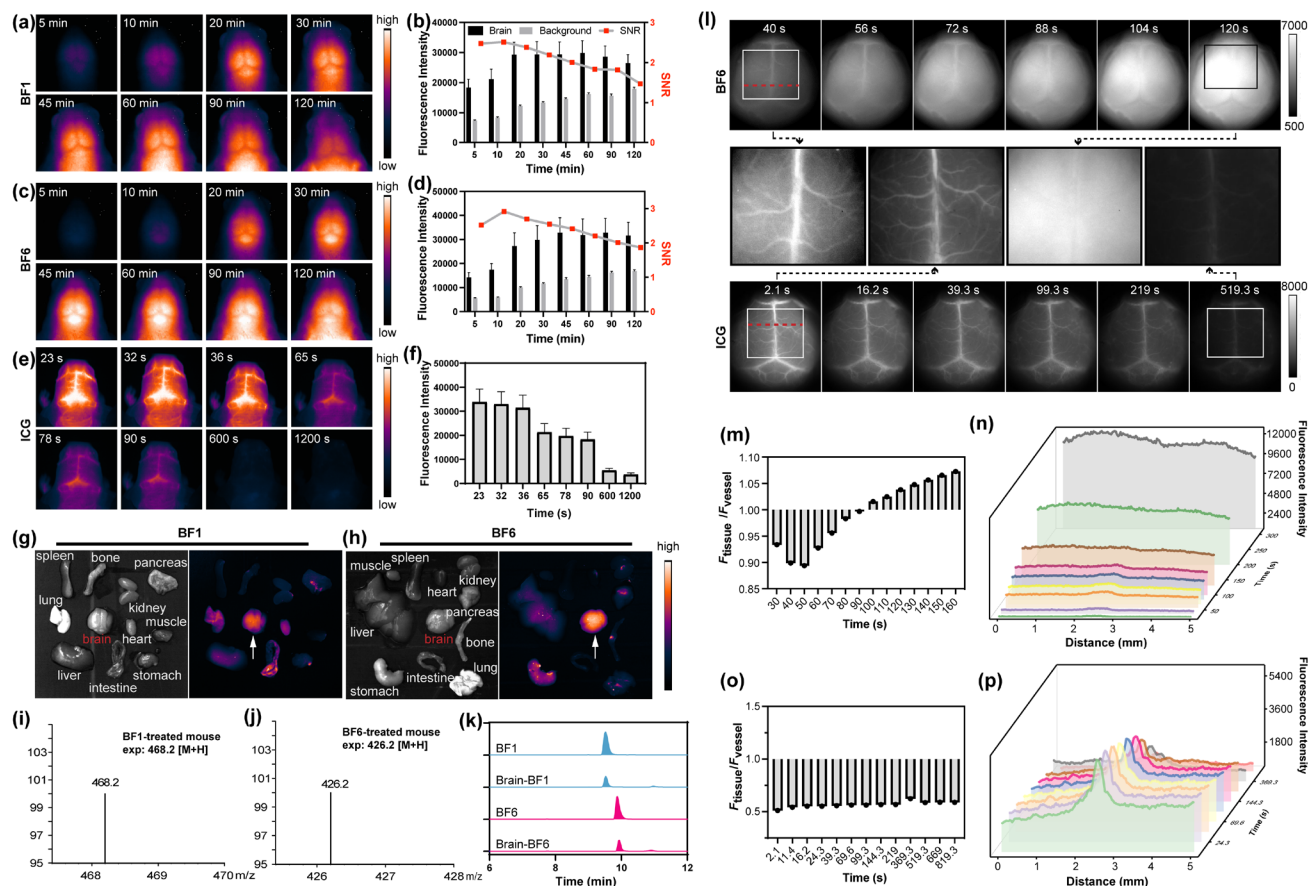


Figure 3. (a) *In vivo* NIR-II fluorescence imaging of cerebral tissue of mice injected with **BF1** (200 μ L, 200 μ M, tail vein injection) at time intervals from 5 to 120 min. (70 mW cm^{-2} , 808 nm laser, 1000 nm LP filter). (b) Time-dependent signal-to-noise ratio (SNR) changes determined by the NIR-II fluorescence imaging of **BF1**-treated mice. (c) *In vivo* NIR-II fluorescence imaging of cerebral tissue of mice injected with **BF6** as described in (a). (d) SNR changes of **BF6**-treated mice. (e) *In vivo* NIR-II fluorescence imaging of cerebral tissue from nude mice injected with ICG (200 μ L, 200 μ M) at certain time intervals from 5 s to 1200 s. (f) The fluorescence intensity changes of the brain at designated time points. (g, h) The fluorescence images of the heart, lung, kidney, spleen, liver, brain, intestine, stomach, bone, muscle, and pancreas at 2 hour after the injection of **BF1** and **BF6**. (i, j) Mass spectra from extracted brain tissue of mice after intravenous injection of **BF1** and **BF6** for 1 hour. (k) HPLC spectra of **BF1**, **BF6** standards and responding brain homogenate of mice treated with **BF1** and **BF6**. (l) *In vivo* NIR-II fluorescence imaging change of brain vessels and tissue at different time points after intravenous injection of **BF6** and ICG. (m) Fluorescence ratio changes of brain tissue and vessel after intravenous injection of **BF6** in (l) at different time points. (n) Cross sectional fluorescence intensity profile along the red dash line in (l) collected by a 1000 nm long-pass filter at designated time points after intravenous injection of **BF6**. (o) Fluorescence ratio changes of brain tissue and vessel after intravenous injection of ICG in (l) at different time points. (p) Cross sectional fluorescence intensity profile along the red line in (l) collected by a 1000 nm long-pass filter at designated time points after intravenous injection of ICG.

and **3h**). The biodistribution of **BF1** and **BF6** after 14 hours revealed that liver, kidneys, and bladder were successively illuminated (**Figure S15**). To further confirm BBB permeability of the **BF1** and **BF6**, brain homogenates from mice injected with these dyes were subjected to mass spectrometry analysis. Single ion monitoring (SIM) mass spectrometry revealed peaks of $[m/z + H] = 468.2$ and 426.2 , corresponding to the molecular weights of **BF1** and **BF6** (**Figure 3i** and **3j**). High-performance liquid chromatography (HPLC) analysis showed that **BF1** and **BF6** have accumulated in the brain tissue after injection compared to the standard curves (**Figure 3k**). This confirmed the high BBB permeability of **BF1** and **BF6**. **BF6** was chosen for further study due to its higher SNR.

We next studied the process of dye diffusion through the BBB. Prior to imaging, the mouse scalp was surgically opened under anesthesia to avoid interference from scalp vessels. Within the first 90s post injection, **BF6** brightly illuminated brain vessels more obvious than brain tissues upon excitation with the 808 nm laser

(**Figure 3l**). And with the diffusion of **BF6** from intracranial vessels to brain tissues, the entire brain was illuminated so that no clear boundary was seen between the blood vessels and brain tissues (**supporting video3**). Quantitative fluorescence data indicated that the NIR-II fluorescence ratio between tissue and vessels gradually increased within 160 s (**Figure 3m** and **3n**). The fluorescence intensity of brain tissue overtook that of intracranial vessels in 100 s, suggesting rapid BBB permeability of the **BF6** *in vivo*. **BF1**-treated mice exhibited a similarly rapid diffusion of the dye from vessels into brain tissue (**Figure S16**). By comparison, the mice treated with ICG, the NIR-II signal was restricted to blood vessels and additional diffusion into the brain tissue was not observed over time (**Figure 3l**, **3o**, and **3p**), which means the ICG dye is unable to cross the BBB. Based on the NIR-II fluorescent images of ICG, we found the diameter of brain vessels to be about 508 μ m, and it was consistent during the whole imaging process. While for the imaging with **BF6**, the calculated diameter of the intracranial vessels was

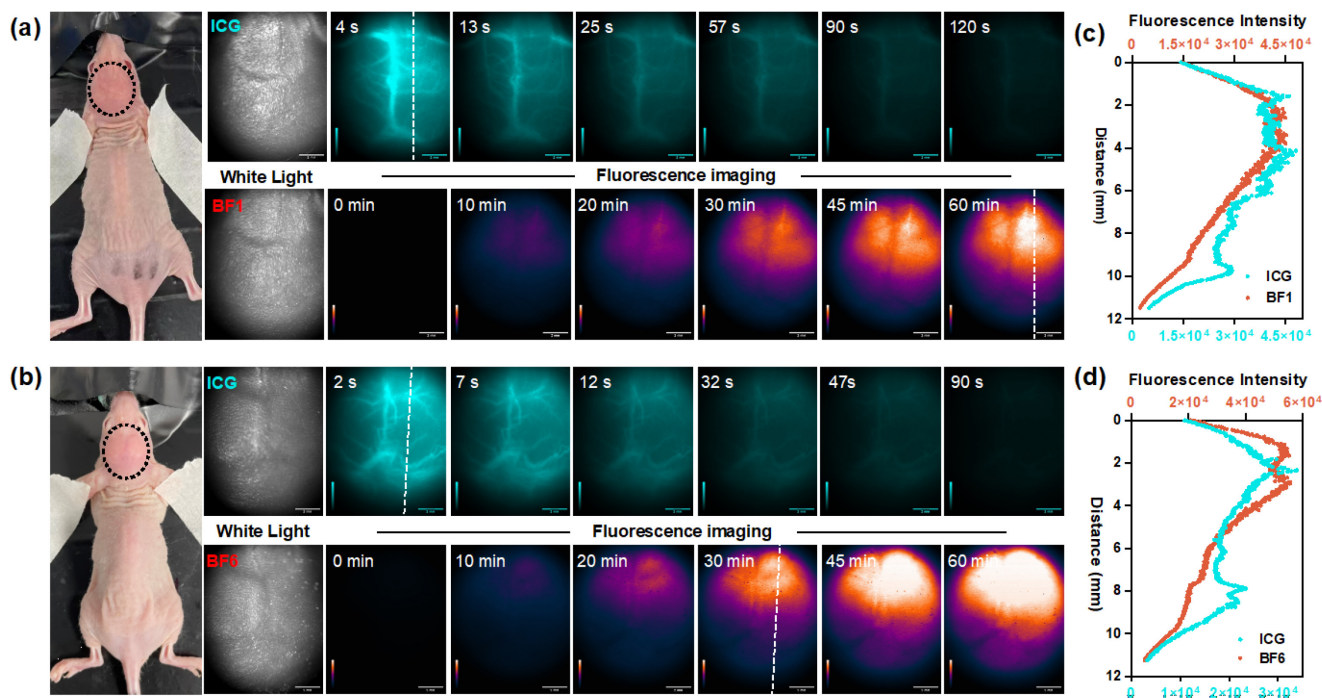


Figure 4. (a) White light and noninvasively *in vivo* NIR-II fluorescence imaging of mouse brains bearing primary glioma tumor after treating ICG and **BF1** (200 μ M, 200 μ L, tail vein injection) at six time points (4, 13, 25, 57, 90, 120 s for ICG; 0, 10, 20, 30, 45, 60 min for **BF1**). (b) White light and noninvasively *in vivo* NIR-II fluorescence imaging of mouse brains bearing primary glioma tumor after treating ICG and **BF6** (200 μ M, 200 μ L, tail vein injection) at six time points (2, 7, 12, 32, 47, 90 s for ICG; 0, 10, 20, 30, 45, 60 min for **BF6**), respectively. The laser power was 70 mW cm⁻², 808 nm laser, 1000 nm LP filter; (c, d) Cross-sectional fluorescence intensity profile along the white dash lines in (a) and (b) collected by 1000 nm long-pass filter.

about 663 μ m, which was larger than that of ICG because of BBB diffusion of **BF6** (Figure S17).

Importantly, no overt signs of toxicity were observed in mice treated with multi-doses **BF1** and **BF6** dyes. One month after the dye injection, mice were euthanized for collection of major organs, including heart, liver, spleen, lung, kidneys, and brain. Hematoxylin and eosin (H&E) staining of tissue sections suggested that **BF1** and **BF6** exhibited negligible toxicity in major organs compared with PBS-treated mice (Figure S18).

Biodistribution of BF₂ Formazanate Dyes in a Mouse Model of Glioblastoma

Glioblastoma multiforme is a uniformly fatal brain tumor characterized by infiltrative growth supported by angiogenesis. Glioblastoma-associated blood vessels are markedly abnormal, resulting in edema, hypoxia, and decreased delivery of chemotherapeutic agents.⁵⁴ Hoping that formazanate-based reagents might represent a novel approach to imaging glioblastoma, we explored the biodistribution of **BF1** and **BF6** in mice with GBM models. We introduced murine U87-MG GBM cells into the brain of nude mice based on a previously reported protocol.^{55,56} 3–4 weeks after tumor implantation, we injected ICG solution intravenously into the mice, followed by noninvasive imaging of the intact brains using NIR-II fluorescence. After 24 hours, **BF1** or **BF6** was used to treat the same mice with ICG treatment, followed by the noninvasive imaging again. Due to the fast metabolism of ICG in living mice, we collected fluorescent brain images at a six-time points within 120 seconds, while the images of **BF1** and **BF6**-treated mice were collected within 60 minutes. As shown in Figure 4a and 4b, irregular brain vessels were clearly illuminated by ICG, which indicates the glioma existence in the mouse brains. During 120 seconds, the fluorescence signals of ICG always were restricted in

the vessels and gradually disappeared. It confirmed that BBB prevents ICG from leaking into brain tissues in the presence of glioma. Surprisingly, we found the brains bearing tumors showed uneven fluorescence imaging, and fluorescence intensity of partial brain tissues increased from 0 to 60 minutes after **BF1** and **BF6** injection (Figure 4a and 4b). The cross-section fluorescence intensity profiles showed a significant difference between the ICG and **BF1** or **BF6** implying their different imaging behaviors (Figure 4c, 4d). Following *in vivo* imaging, mice were euthanized and brain tissues were collected for *ex vivo* studies. *Ex vivo* imaging showed a similar result with the *in vivo* imaging (Figure S19a, S19b). After the *ex vivo* imaging, brains were sliced and stained using Congo red and H&E for a detailed study. Isolated brains were characterized by obvious pathological features such as glioma-related edema (yellow arrows, Figure S19c, S19d) from three tangents along white dash lines in brain tissues. Most importantly, these results demonstrated that normal and brain tumor tissues were easily differentiated and delineated the margins using *in vivo* and *ex vivo* NIR-II fluorescence imaging. Time-dependent normal-to-glioma ratio (NGR) changes determined by the NIR-II fluorescence imaging of brain normal tissue and glioma after ICG, **BF1**, or **BF6** treatment (Figure S20a, S20b, S20d, S20e). Compared with ICG, brain images of **BF1**- and **BF6**-treated mice established a significantly larger NGR, suggesting **BF1** and **BF6** can better differentiate normal tissue from glioma than ICG (Figure S20c, S20f). Interestingly, we found that **BF1**- or **BF6**-treated mice exhibited higher fluorescence in normal tissue than in malignant tissue. To exclude the possibility that fluorescence differences were induced by the tumor microenvironment, the effect of pH and ROS stabilities on **BF1** and **BF6** fluorescence was tested. Like **BF1**, under low pH and oxidative environment, **BF6** showed only minor changes in fluorescence (Figure S21 and S22). Another possible reason for the

observed fluorescent differences in normal and malignant tissues may be the fluid pressure. Recent studies suggested that there is an increasing interstitial and intracranial fluid pressure in glioma regions, which may limit the diffusion of the dyes from blood vessels.^{57–59} The other potential reason can be the lack of transcellular and efflux transporters in the glioma niche. Overall, these results indicated that BF₂ formazanate dyes were potential tools for differentiating GBM tissue during different growth times, thus facilitating image-guided intervention in the clinic.

CONCLUSIONS

In summary, our work developed a series of NIR-II fluorophores that had ultrahigh photostability, high biological stability, large Stokes shift, relatively strong brightness, and capability to cross the BBB. Compared to other recently reported “small-molecule” NIR-II fluorophore scaffolds, BF₂ formazanate NIR-II dyes have key physicochemical parameters for the potential BBB permeability. We found that BF₂ formazanate fluorophores modified with morpholine can efficiently penetrate the BBB, which allows NIR-II brain imaging *in vivo* through the intact skin and skull. In a murine glioblastoma model, BF₂ formazanate fluorescence can be used to distinguish between tumor and normal tissue. Our work thus generates a new class of NIR-II fluorophores that are small in size and exhibit ideal lipophilicity, low polar surface areas, and adjustable photophysical properties, which provides a promising structural scaffold for the design of brain imaging and therapeutic probes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website: brief description (file type, i.e., PDF) brief description (file type, i.e., PDF)

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Notes

The authors declare no competing financial interests.

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