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Novel NIR-II organic fluorophores for bioimaging beyond 1550 nm[†]

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Near-infrared fluorescence imaging in the 1500–1700 nm sub-window (NIR-IIb) has shown a deeper penetration depth, higher resolution and zero auto-fluorescence for biomedical imaging. Till now, very few small molecule NIR-IIb fluorophores have been reported due to the extremely rare organic NIR-IIb skeleton and a notorious aggregation-caused quenching (ACQ) effect in aqueous solution. In this study, highly twisted NIR-II small molecule fluorophores such as HL3 (45.5° at the S₀ state) with the emission wavelength extending into the NIR-IIb region were designed and synthesized using an aggregation-induced emission (AIE) strategy. HL3 dots showed a remarkable increase in fluorescence intensity with a QY of 11.7% in the NIR-II window (>1000 nm) and 0.05% in the NIR-IIb region (>1550 nm) in water. High-resolution *in vivo* imaging of the whole body, cerebral vasculature, and lymphatic drainage beyond 1550 nm was achieved using NIR-II AIE HL3 dots for the first time. These attractive results may promote the development of small-molecule NIR-IIb fluorophores with the maximum emission wavelength beyond 1500 nm with a deeper penetration depth and higher resolution.

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Introduction

Optical fluorescence imaging is a promising modality for real-time monitoring of disease progression, drug delivery and image-guided surgery with high spatial and temporal resolution.¹ However, traditional fluorescence imaging techniques mostly focused on the visible and near-infrared region below 900 nm, where imaging resolution and penetration depths were largely limited due to the photo-scattering, auto-fluorescence and absorption of biological tissues. Hence, developing novel fluorophores with longer emission wavelengths to improve imaging resolution for *in vivo* deeper imaging is crucial and still a great challenge.²

During the past few years, molecular imaging in the second near-infrared region (NIR-II, 1000–1700 nm) has emerged as a powerful tool for the delineation and treatment of cancers.^{3–12} The first organic small-molecule dye CH1055 with 90% renal excretion for NIR-II bio-imaging was reported in 2015,⁵ and opened up a new era for small molecule imaging. Deeper imaging depths (up to 3 cm), excellent temporal and spatial resolutions (50 FPS and 1 μm), and a higher tumor-to-normal tissue (T/NT) ratio (up to 15) were achieved in the NIR-II region.^{5,12} Especially, the NIR-IIb sub-window (1500–1700 nm) showed tremendous advantages of near-zero auto-fluorescence, negligible scattering, and unparalleled tissue-imaging depths, and turned out to be a hot spot for *in vivo* fluorescence bio-imaging.¹³ Very few inorganic NIR-IIb fluorophores such as single-walled carbon nanotubes (SWNTs), rare earth doped nanoparticles, and quantum dots have been investigated for biosensing and bioimaging beyond 1500 nm.^{7g,8f,13–17} It is worth noting that organic FD-1080 J-aggregates were first accomplished with high resolution imaging of the cerebral and hindlimb vasculature with fluorescence emission tailing into 1500 nm with a quantum yield (QY) of 0.0545%.¹⁷ The signal-to-background ratio (SBR) was 3.3-fold higher than that of NIR-IIa (1300–1400 nm) imaging. However, small-molecule NIR-IIb fluorophores (beyond 1500 nm) are still in their infancy. Shifting small-molecule NIR-II fluorophore emissions, specifically into the NIR-IIb sub-window, is a great challenge but crucial for their expansion to *in vivo* biomedical applications. Thus, it is

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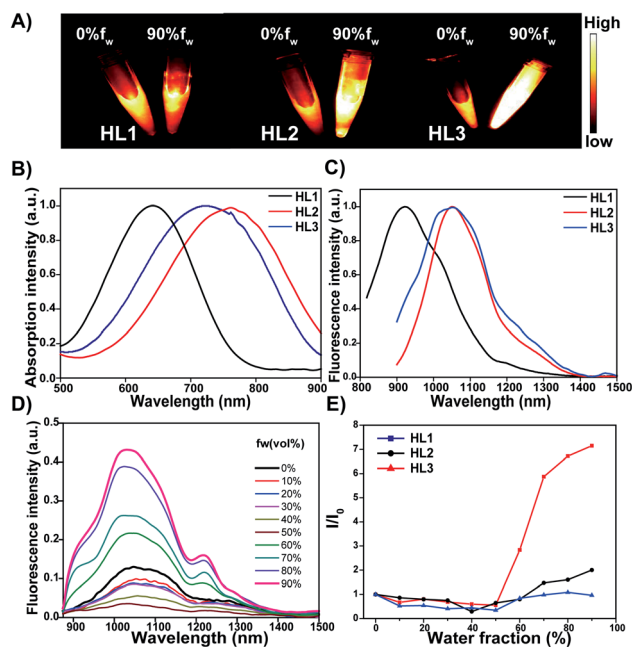


Fig. 3 (A) NIR-II fluorescence images (1000 LP, 808 nm excitation, 40 mW cm⁻², and 5 ms) of HL1–HL3 in THF and THF/water (f_w , 90%); (B) absorption and (C) fluorescence emission spectra of HL1–HL3 in THF under 808 nm excitation; (D) fluorescence emission spectra of HL3 obtained upon increasing f_w from 0 to 90% under 808 nm excitation; (E) fluorescence intensity ratios (I/I_0) of HL1–HL3 in different f_w s. I_0 is the fluorescence intensity of HL1–HL3 in THF.

(Fig. 4D). The quantum yields were calculated to be $\sim 11.7\%$ in the NIR-II region (1000–1700 nm) and $\sim 0.05\%$ in the NIR-Ib region (beyond 1550 nm). The molar extinction coefficient (ϵ) of HL3 dots in water was measured to be 9.3×10^3 L mol⁻¹ cm⁻¹. The reasonable quantum yields and NIR-Ib fluorescence emission of HL3 dots encouraged us to explore their NIR-Ib imaging capabilities *in vitro*. HL3 dots exhibited excellent fluorescence intensity beyond 1550 nm under 808 nm laser irradiation (1550 nm LP, Fig. 4D). As shown in Fig. 4E, the fluorescence intensity of the HL3 dots showed no obvious changes in different media (FBS, PBS and water) under continuous 808 nm laser irradiation for 1 h (90 mW cm⁻²). L929 mouse fibroblast cells were applied to evaluate the potential cytotoxicity of HL3 dots using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. High cell viability was observed even at a high concentration (100 μ g mL⁻¹, Fig. 4F). The pharmacokinetics of HL3 dots were also investigated through the measurement of the blood circulation half-life. The blood half-life of HL3 dots was 114 min (Fig. S9†). All these results have demonstrated that HL3 dots have superior stability and excellent biocompatibility, and are more applicable for NIR-Ib bioimaging (beyond 1550 nm) *in vivo*.

To explore the feasibility of HL3 dots as a novel AIE NIR-Ib probe for reliable imaging of the whole body and cerebral vasculature system in KM mice and C57BL/6 mice ($n = 3$ per group), HL3 dots (200 μ L, 1 mg mL⁻¹) were then injected into KM mice via the tail vein, and NIR-II and NIR-Ib images of blood vessels were recorded using an InGaAs camera with

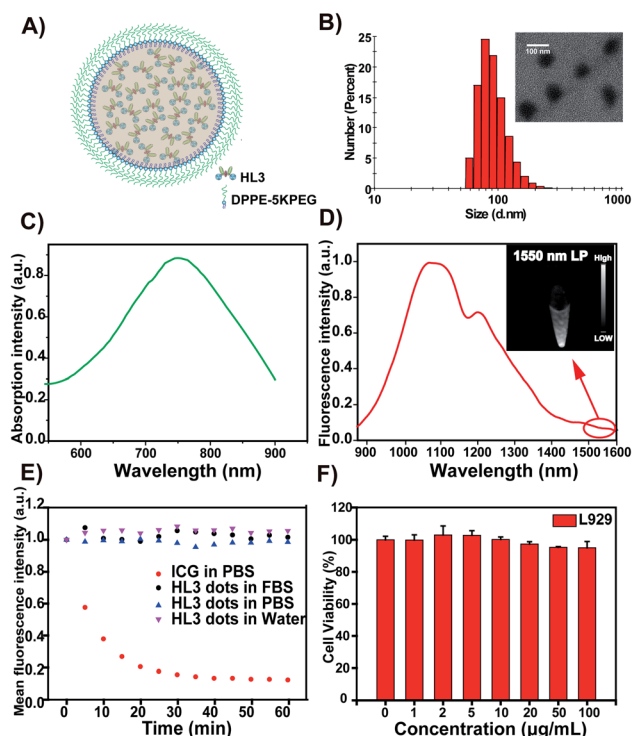


Fig. 4 (A) The formation of HL3 dots via nanoprecipitation; (B) the DLS and TEM images of the HL3 dots, scale bar: 100 nm; (C) the absorption spectra of HL3 dots in water; (D) the fluorescence spectra of HL3 dots, inset: NIR-Ib image of HL3 dots (1550 nm LP, 808 nm excitation, 90 mW cm⁻², and 500 ms exposure time) at a concentration of 0.3 mg mL⁻¹ in water; (E) the photo-stability of HL3 dots in water, FBS, PBS and ICG in PBS with continuous 808 nm laser irradiation for 60 min (90 mW cm⁻²); (F) the cytotoxicity of HL3 dots.

different LP filters (1000 nm, 1250 nm, and 1550 nm) and different exposure times under 808 nm laser irradiation (90 mW cm⁻²). After 5 min post-injection, whole blood vessels were clearly visualized, and the hind limb vasculature was chosen for analysis *via* the Gaussian-fitted full width at half maximum (FWHM) (Fig. 5). It was found that the imaging of HL3 dots in the NIR-II window (>1250 nm, 1250 nm LP) with a 50 ms exposure time exhibited highly superior resolution (Fig. 5B). An extended exposure time (500 ms) was needed for higher resolution NIR-Ib imaging beyond 1550 nm (>1550 nm, 1550 nm LP) (Fig. 5C). The FWHM values of the hind limb vasculature were 777 μ m (1000 nm LP), 768 μ m (1250 nm LP) and 719 μ m (1550 nm LP), respectively. The signal-to-background ratio (SBR) of NIR-Ib imaging (SBR = 2.5, 1550 nm LP) was much higher than that of NIR-II imaging (SBR = 1.4, 1000 nm LP and SBR = 1.8, 1250 nm LP) (Fig. 5). The cerebral vasculature system was also imaged using the NIR-Ib probe HL3 dots. The superior resolution of tiny vessels was obtained (Fig. 6). The corresponding SBR of NIR-II 1000 nm, NIR-II 1250 nm and NIR-Ib 1550 nm imaging was 1.4, 1.8 and 3.4, respectively (Fig. 6D and E). The SBR of NIR-Ib imaging (1550 nm LP) was 2.4-fold higher than that of NIR-II imaging (1000 nm LP). The FWHM values of the vessels at the same position (red dashed line) for different LP filters were calculated to be



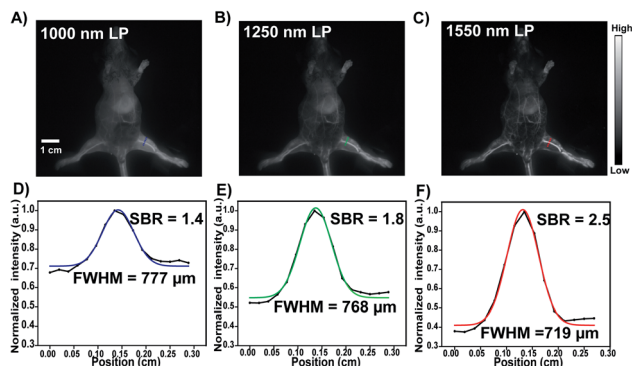


Fig. 5 NIR-II and NIR-IIb fluorescence images of whole mouse blood vessels after tail intravenous injection of HL3 dots ($200 \mu\text{L}$, 1 mg mL^{-1}) under 808 nm laser excitation (90 mW cm^{-2}) with different long-pass filters (left to right: 1000 nm, 1250 nm and 1550 nm). Scale bar (A–C): 1 cm. (A) 1000 nm LP, 5 ms exposure time, and 90 mW cm^{-2} ; (B) 1250 nm LP, 50 ms exposure time, and 90 mW cm^{-2} ; (C) 1550 nm LP, 500 ms exposure time, and 90 mW cm^{-2} ; (D–F) the fluorescence intensity profiles fitted using Gaussian, cross-section intensity (blue line), blue-dashed lines (1000 nm), green-dashed lines (1250 nm), red-dashed lines (1550 nm).

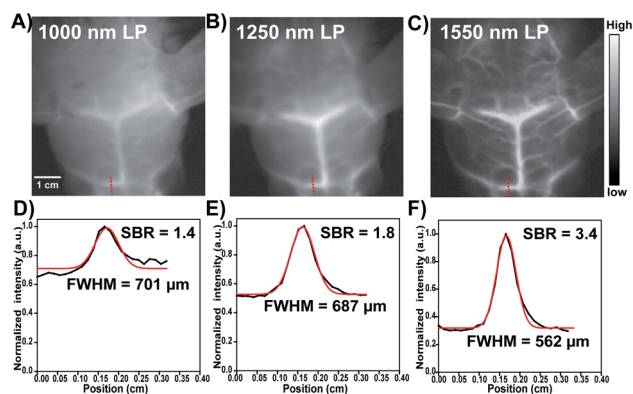


Fig. 6 NIR-II and NIR-IIb fluorescence images of cerebral vasculature with different LP filters in C57BL/6 mice ($n = 3$) after tail intravenous injection of HL3 dots ($200 \mu\text{L}$, 1.5 mg mL^{-1}). Scale bars (A–C): 1 cm. (A) 1000 nm LP, 4 ms exposure time, and 90 mW cm^{-2} ; (B) 1250 nm LP, 60 ms exposure time, and 90 mW cm^{-2} ; (C) 1550 nm LP, 500 ms exposure time, and 90 mW cm^{-2} ; (D–F) the fluorescence intensity profiles fitted using Gaussian, cross-section intensity (black lines), and the tiny vessel (red-dashed lines).

701 μm (1000 nm LP), 687 μm (1250 nm LP), and 562 μm (1550 nm LP), respectively. The NIR-II fluorescence images obtained using 1000 nm, 1250 nm and 1550 nm LP were also evaluated at the same exposure time (200 ms) at a concentration of 0.8 mg mL^{-1} . It was found that HL3 saturated the detector with 1000 nm and 1250 nm long-pass filters under imaging conditions suitable for HL3 with a 1550 nm long-pass filter (Fig. S10[†]). All these results indicated that HL3 dots have great potential for *in vivo* NIR-IIb imaging (beyond 1500 nm) at an extended exposure time.

The lymph node drainage plays a vital role in tumor metastasis. We next demonstrated the application of HL3 dots for

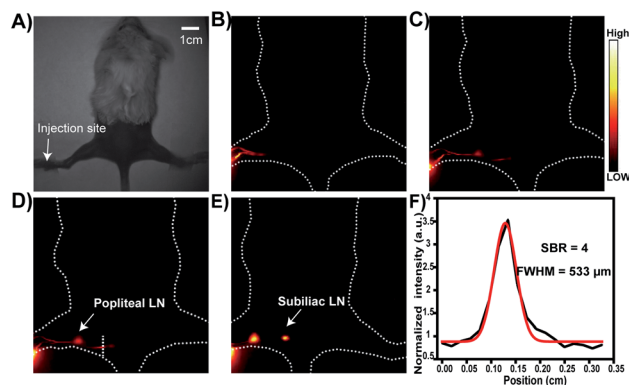


Fig. 7 Dynamic NIR-IIb imaging of lymphatic drainage in KM normal mice ($n = 3$) after injection of HL3 dots ($15 \mu\text{L}$, 1 mg mL^{-1}) under 808 nm laser excitation (1550 nm LP, 500 ms, and 90 mW cm^{-2}). (A) The digital photograph and *in vivo* NIR-II fluorescence imaging at 0 min; (B) 1 min; (C) 30 min; (D) 1 h; (E) 3 h after injection. (F) The fluorescence intensity profiles fitted using Gaussian in (D), cross-section intensity (black line) and the lymphatic vessel (white-dashed lines).

lymph node NIR-IIb imaging (beyond 1550 nm). HL3 dots ($15 \mu\text{L}$, 1 mg mL^{-1}) were injected intra-dermally at the left forefoot pad of KM mice ($n = 3$ per group) (Fig. 7). The process of lymphatic drainage was monitored under 808 nm laser irradiation (90 mW cm^{-2}) using 1550 nm LP. Lymphatic vessels were notably identified in 1 min after injection. The popliteal lymph node was gradually lighted up, and both the popliteal lymph node and the subiliac lymph node were clearly visualized in 2 h. The diameter of the lymphatic vessel between the popliteal lymph node and the subiliac lymph node was calculated to be 533 μm *via* FWHM, and the SBR reached 4 (Fig. 7F). The SBR values of the popliteal lymph node and the subiliac lymph node were 2.5 and 3.2 (1000 nm LP), 2.9 and 3.4 (1250 nm LP), and 5.1 and 4.8 (1550 nm LP), respectively (Fig. S11[†]). These results reveal that NIR-IIb imaging beyond 1550 nm of lymph node drainage can be achieved with a higher SBR.

Experimental section

All animal experiments were performed according to the Chinese Regulations for the Administration of Affairs Concerning Experimental Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of Wuhan University. And all the experimental details are provided in the ESI.[†]

Conclusions

In summary, we have successfully synthesized a series of small molecule fluorophores HL1–HL3 by introducing different donors and distortion groups. Among them, HL3 showed extremely stronger AIE characteristics and highly twisted donor–acceptor distortion. HL3 dots exhibited excellent water solubility, photo-stability and biocompatibility with a remarkable increase in NIR-II fluorescence intensity with QYs of 11.7%



and 0.05% in the NIR-II (>1000 nm) and NIR-IIb region (>1550 nm), respectively, in water. Superior quality NIR-IIb imaging beyond 1550 nm of the whole body, cerebral vasculature and the lymphatic drainage system was demonstrated for the first time with a higher SBR. It is hoped that this novel NIR-II fluorophore HL3 obtained using an integrated AIE and D-A distortion strategy may become a practical strategy to develop small-molecule NIR-IIb fluorophores with the maximum emission wavelength beyond 1500 nm with a deeper penetration depth and higher resolution.

Conflicts of interest

There are no conflicts to declare.

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