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Molecular fluorescence and Photoacoustic Imaging in the Second Near-Infrared Optical Window Using Organic Contrast Agents

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Abstract: Non-invasive near-infrared (NIR) light ranging from 650 to 1000 nm (NIR-I) has been widely employed in fundamental research and clinical applications; however, a recently discovered second NIR (NIR-II) window from 1000 to 1700 nm exhibits even better deep-tissue imaging capability owing to the reduced photon scattering, minimized tissue autofluorescence, and increased applicable power at longer wavelengths. This review focuses on recent advances of organic contrast agents developed for in vivo fluorescence and photoacoustic imaging in NIR-II optical window. The superiority of NIR-II over NIR-I window for molecular imaging is first discussed in detail, followed by discussion of fluorescence and photoacoustic imaging of cancer, vasculature, and brain using organic contrast agents in the NIR-II window. At last, challenges and perspectives of organic contrast agents for NIR-II in vivo imaging are suggested.

1. Introduction

Near-infrared (NIR) light from 650 to 1000 nm, termed as the first NIR window (NIR-I) has been extensively investigated for optical imaging of various diseases.^[1] Compared with conventional visible region that undergoes significant attenuation by endogenous molecules, NIR-I light is able to achieve higher transmittance in biological tissues attributed to the reduced light-tissue interactions of photons at longer wavelengths (**Scheme 1b, c, and d**). As a consequence, NIR-I imaging can be used for deeper visualization of anatomical structures, pathological changes, and metabolic processes in vivo.^[2] However, NIR-I window remains suboptimal because of the intrinsic issues of high tissue autofluorescence and shallow tissue penetration depth.^[3]

Scheme 1. NIR-II vs NIR-I window for in vivo imaging. (a) Schematic illustration of light-tissue interaction. (b) Reduced scattering coefficients (μ_s') of various tissues plotted as a function of wavelength covering 400-1700 nm. (c) Vis-NIR absorption spectrum of water. OD represented optical density. NIR-IIa: 1300-1400 nm; NIR-IIb: 1500-1700 nm. (d) Effective attenuation coefficients of blood, skin, and fat plotted as a function of wavelength covering 200-1800 nm. (e) Ex vivo autofluorescence spectra of mouse liver, spleen, and heart tissue.

Excitation wavelength: 808 nm. (f) Quantum efficiency of cameras utilizing silicon (Si), mercury cadmium telluride (HgCdTe), or indium gallium arsenide (InGaAs) sensors as a function of wavelength (200-1800 nm). Figures from: (b, c, and e), Reproduced with permission.^[1] Copyright 2017, Nature Publishing Group. (d, f), Reproduced with permission.^[3] Copyright 2009, Nature Publishing Group.

Very recent studies have identified a new optical imaging region, the second NIR window (NIR-II, 1000-1700 nm), which has high potential to impart much superior imaging quality than NIR-I window.^[3] Advantages of NIR-II over NIR-I window could be well explained by the analysis of light-tissue interactions in the context of fluorescence and photoacoustic (PA) imaging.^[1] As shown in **Scheme 1a**, four typical processes are involved in light-tissue interactions when a laser beam penetrates through biological tissues: reflection, scattering, absorption that generates background PA signals, and autofluorescence emitted by endogenous fluorophores. Among these factors, scattering of light in biological tissues plays an explicitly crucial role in optical imaging since stray light usually contributes to background noise and also diminishes penetration depth. Note that light scattering follows an inversely proportional declining trend with the increase of wavelength in almost all kinds of tissues (**Scheme 1b**).^[4] Such reduced scattering pattern affords decreased background noise and thus contributes to the improved signal-to-background ratio (SBR) when going into tissues at longer wavelengths.

Autofluorescence caused by endogenous fluorophores significantly decays as a function of emission wavelength upon NIR excitation, reaching a nearly autofluorescence-free region above 1500 nm (**Scheme 1e**).^[5] The obvious decrease of autofluorescence at longer wavelengths contributes to the reduced background interference with respect to fluorescence imaging, largely improving the imaging performance of organs rich of endogenous fluorophores. Additionally, due to the lower energy of photons with longer wavelengths, much

higher maximum permissible exposure (MPE) is allowed for NIR-II than NIR-I lasers, giving rise to the enhanced applicable laser power especially for promoted PA imaging performance. Despite all these merits, the increased absorbance of water above 950 nm represents the pitfall for NIR-II window imaging (**Scheme 1c**). As an important endogenous absorber, water exhibits extremely high absorption centered at around 1450 nm with a surrounding range (1400-1500 nm), which has to be excluded from optical imaging.^[6]

The combination of reduced light scattering, minimized autofluorescence, and increased MPE readily outperforms the slightly higher photon absorbance in the NIR-II window. As a result, NIR-II light allows for even deeper tissue penetration than NIR-I light with improved SBRs and superior clarity.^[1] By virtue of NIR-II light, tissue depth unprecedented for NIR-I laser could be readily reached to detect biological processes happening deep inside living bodies. However, limited numbers of NIR-II contrast agents have been developed in the past few years. Thus far, the majority of NIR-II fluorophores are composed of inorganic nanomaterials such as quantum dots (QDs) and rare-earth based nanoparticles.^[7] However, issues of potential metal ion leakage and long-term safety concern need to be considered when it comes to the practical use of these inorganic agents. Carbon-based structures such as single-walled carbon nanotubes have also been utilized for NIR-II fluorescence imaging but suffer from drawbacks such as relatively low QY, limited optical tunability, and difficulty in in vivo clearance.^[5a, 6b, 8] Similar problems have also been confronted in NIR-II PA imaging, since most NIR-II PA agents are designed based on inorganic nanomaterials (copper sulfide nanoparticles, silver nanoplates, and gold nanostructures et al.) as well.^[9]

Recent development of organic contrast agents with the emission or absorption above 1000 nm opens new opportunities to in vivo imaging in NIR-II window.^[10] Fine-tuning of energy band gaps via molecular engineering allows for tunable emission or absorption peaks covering

the NIR-II region. Compared with inorganic nanomaterials, organic contrast agents eliminate the common concern of heavy metal ion-induced toxicity and are capable of in vivo biodegradation or fast excretion after careful design, reducing the risk of unknown long-term damage caused by residual contrast agents.^[11] More importantly, organic NIR-II agents are of similar chemical properties and pharmacokinetics with Food and Drug Administration (FDA)-approved NIR-I organic agents, highlighting their great potential towards clinical translation.

Scheme 2. Chemical structures of organic contrast agents that have been studied for (a) fluorescence and (b) PA imaging in NIR-II window.

In this review, we focus on organic contrast agents exploited for in vivo fluorescence and PA imaging in NIR-II window. Chemical structures of organic NIR-II fluorophores and PA absorbers are summarized in **Scheme 2**. In the following, we first introduce the recent development of organic NIR-II fluorophores utilized for fluorescence imaging of cancer, artery flow, and brain injury. Then, we survey the potential of organic NIR-II absorbers for PA imaging of brain vasculature and tumors. At last, challenges and perspectives of organic materials used for NIR-II fluorescence and PA imaging are discussed.

2. NIR-II fluorescence imaging

Over the past few decades, fluorescence imaging utilizing visible or NIR-I light has been widely-employed by researchers to probe biological or pathological processes in living organisms.^[12] In comparison with conventional tomographic imaging modalities such as positron emission tomography (PET), computed tomography (CT), and magnetic resonance imaging (MRI), fluorescence imaging provides benefits of non-invasiveness, simplified imaging system, easy handling, low cost, and excellent sensitivity etc.^[13] Notably, high spatial and temporal resolution of fluorescence imaging afford real-time wide-field imaging, making it an ideal approach for imaging-guided surgery.^[14]

Very recent development of NIR-II fluorophores allows for fluorescent imaging of biological processes in a superior window (**Table 1**). Thanks to the reduced light scattering and autofluorescence at progressively longer wavelengths, significant improvements of imaging performance could be achieved by extending emission to the NIR-II window, enabling micron-scale resolution at millimeter-scale depth in biological tissues. In addition, NIR-II fluorophores further facilitate molecular imaging guided surgery of tumors located in deep tissue, providing clinicians with more precision for efficient ablation of pathological sites.^[11b]

Table 1. Optical properties and applications of NIR-II organic fluorophores.

Fluorophore	λ_{abs} [nm]	λ_{em} [nm]	Stokes Shift [nm]	Φ_f [%]	Application
CH1055-PEG ^[11b]	~750	1055	~305	0.3	Lymphatic and brain tumor imaging; imaging guided tumor excision surgery
CH-4T/FBS-HT ^{a)} [11a]	~748	~1005	~257	11	Hindlimb vasculature and lymph node imaging
CQS1000 ^[15]	830	1000	170	--	Imaging of circulatory system
IR-EI ^[16]	830	1071	241	0.7	Traumatic brain injury
IR-1061 ^[17]	780	1064	--	1.8	Whole body imaging of nude mice; hindlimb and abdomen imaging
pDA ^[18]	654	1047	~400	1.7	Imaging of arterial blood flow
IR-FGP ^[19]	745	1050	~305	1.9	In vitro molecular imaging
IR-FEP ^[20]	780	1047	267	2.0	Tumor Imaging

^{a)}CH-4T after being heated in serum at 70 °C for 10 min

2.1. Tumor Imaging

Dai et al. reported the first NIR-II fluorophore CH1055 (**Scheme 2**) of rapid renal clearance and utilized it for fluorescence imaging of cancer diagnosis as well as image-guided surgery in NIR-II window.^[11b] CH1055 was PEGylated (CH1055-PEG, **Scheme 2**) to obtain excellent water solubility with an absorption peak at ~750 nm and a fluorescent emission peak at ~1055 nm (**Figure 1a**). Fast renal excretion of CH1055-PEG was endowed owing to the suitable molecular mass (~ 8.9 kDa) as well as small hydrodynamic radius (~3 nm) located well within the renal filtration threshold. **Figure 1b** demonstrated the excretion of CH1055-PEG monitored by NIR-II video-rate imaging. Within a few minutes after intravenous injection of CH1055-PEG in mice, strong fluorescent signal could be gradually observed in the bladder compared with very weak fluorescence in the liver. Such phenomenon was in sharp contrast with the high

hepatic uptake of single-walled carbon nanotubes (SWCNTs). Furthermore, in vivo excretion kinetics study validated the rapid urine excretion of CH1055-PEG with a short half-life (~ 1 h) in blood circulation and $\sim 90\%$ removal from mice within 24 h post-injection (**Figure 1c**). All of these results highlighted the first NIR-II fluorophore with comparable excretion ability to FDA-approved ones.

Figure 1. NIR-II fluorescence imaging for cancer diagnosis using CH1055 and its derivatives. (a) Absorption and fluorescence spectra of CH1055-PEG. Excitation wavelength: 808 nm. (b) Renal excretion of CH1055-PEG monitored by NIR-II imaging in the supine position of a mouse post-injection of CH1055-PEG ($n = 3$). (i-iv): $t = 15, 315, 520$, and 870 s, respectively. (c) Cumulative urine excretion curve (%ID, $n = 5$) and blood circulation (%ID/g) fitted with an exponential decay curve of CH1055-PEG injected mice for 24 h post-injection. Error bars indicate standard deviation. (d) NIR-II fluorescence image of lymphatic vasculature and inguinal lymph node of a living mouse post-injection of CH1055-PEG. The green asterisk indicates the location of xenograft U87MG tumor on the shoulder. The red arrow corresponds to the internodal collecting lymphatic vessel. The white arrow dictates the inguinal lymph node. (e) Brain vasculature and tumor imaging of C57BL/6 mice with shaved heads using either ICG (exposure time: 100 ms) (upper) or CH1055-PEG (exposure time: 1s) (down). (f) Schematic illustration of CH1055-anti-EGFR affibody (CH1055-affibody). (g) NIR-II molecular imaging

of tumor with CH1055-affibody. NIR-II fluorescence image was captured of a targeted mouse intravenously injected with CH1055-affibody (60 μ g) (upper) and a blocked mouse injected with CH1055-affibody together with free affibody (down) at 6 h post-injection. Inset demonstrates the SAS tumor after being excised from both targeted and blocked mouse, respectively. The white arrow indicates the tumor region and the red circle indicates the normal tissue region used to calculate the T/NT ratio. (h) NIR-II fluorescence imaging guided tumor surgery. The upper and down panels respectively demonstrate a mouse at 24 h post-injection of CH1055-affibody before and after fluorescence imaging guided surgery to resect the SAS tumor. Reproduced with permission.^[11b] Copyright 2016, Nature Publishing Group.

The superiority of fluorescence imaging performance in NIR-II relative to NIR-I window was further validated by lymphatic and tumor brain imaging using CH1055-PEG and ICG. After injection of CH1055-PEG or ICG near the base of the U87 tumor xenograft mouse tail, both internodal collecting lymphatic vasculature and inguinal lymph node could be clearly visualized (**Figure 1d**). Moreover, the signal-to-background ratio (SBR) of lymph node with CH1055-PEG was about 2-fold of that with ICG. The improved imaging quality of CH1055-PEG should be attributed to the lower tissue auto-fluorescence and background due to the reduced scattering of photons in NIR-II window. Such advantage was further demonstrated in brain vasculature imaging without skin removal. As shown in **Figure 1e**, an obviously higher degree of clarity of brain tumor and vasculature could be clearly observed with CH1055-PEG compared with ICG. More importantly, molecular imaging guided surgery was demonstrated on human squamous cell carcinoma (SAS) tumor-bearing mice using CH1055 conjugated with anti-epidermal growth factor receptor (EGFR) affibody for specific targeting (**Figure 1f**). Within 6 h post-injection of CH1055-affibody (**Figure 1g**), NIR-II imaging exhibited an increased tumor-to-normal tissue (T/NT) ratio of $\sim$ 5-fold higher than that from NIR-I imaging. Such enhanced imaging clarity allowed the first NIR-II imaging guided surgery to excise tumor after injection of CH1055-affibody (**Figure 1h**). All of these results demonstrated the outperformed

significance of CH1055 than FDA-approved NIR-I fluorophores for precision imaging of pathological sites and pinpointed its promising future in clinical trials to assist tumor excision during surgery.

2.2 Blood Flow imaging

Dai *et al.* reported a library of semiconducting polymers based on donor-acceptor structure with tunable emission wavelengths ranging from 1050 to 1350 nm.^[18] By virtue of the high QY (~1.7%), pDA was selected as the NIR-II fluorescent agent for artery blood flow imaging (**Scheme 2**). After functionalization by PEGylated surfactant, the resulted water-soluble pDA-PEG demonstrated an absorption peak at 654 nm and an emission peak at 1048 nm with a large Stokes shift (~400 nm) (**Figure 2a**). As a result of the bright fluorescence emitted from pDA-PEG, dynamic NIR-II imaging of arterial blood flow on living mice was achieved with short exposure time (~20 ms) and rapid frame rate (25.6 frames per second). Such ultrafast NIR-II imaging imparted real-time monitoring of the blood flow front in the hindlimb femoral artery with great clarity (**Figure 2b**). Moreover, a fast periodic oscillation in terms of blood velocity was clearly revealed attributed to the shorter time resolution (~20 ms) than a single cardiac cycle (~200 ms) (**Figure 2c**).

Figure 2. NIR-II artery blood flow imaging using pDA. (a) Absorption and fluorescence spectra of pDA-PEG. (b) NIR-II fluorescence images of arterial blood flow in a mouse hindlimb instantly after tail vein injection of pDA-PEG. The red arrow corresponds to the femoral artery. (c) Instantaneous velocity of arterial blood flow as a function of time. Velocity was calculated as the blood flow front travelled distance between two sequential frames divided by a time interval of 39 ms. (d) Integrated fluorescence intensity of ROI from the mouse femoral artery (Inset) as a function of time. ROI was indicated by a red box. Scale bar: 5 mm. (e) NIR-II fluorescence intensity of ROI as a function of time after subtraction of a baseline as shown in (d). (f) NIR-II fluorescence images of ROI after subtraction of a linear increasing baseline in (d). Scale bar: 1 mm. Reproduced with permission.^[18] Copyright 2014, Nature Publishing Group.

A region of interest analysis of NIR-II fluorescence signal from femoral artery was further performed in order to investigate the blood flow pattern. As shown in **Figure 2d**, evenly spaced intensity peaks, indicative of systolic phases, were detected with an increasing baseline referring to the perfusion of nanoparticles into artery vessel. After subtraction of the linear baseline in **Figure 2d**, sequential fluorescence spikes were clearly observed as a result of periodic ventricular ejections (**Figure 2e**). Moreover, blood flow variations over a complete heartbeat was demonstrated by the linear-baseline-subtracted NIR-II fluorescence imaging

(Figure 2f). Taken together, the high temporal and spatial resolutions of NIR-II imaging endowed by the high fluorescence QY of pDA were able to impart rich information about artery blood flow and cardiac cycles without the demand of cardiac gating. Such real-time hemodynamic imaging should be of great significance to the investigation of cardiovascular diseases in future clinical applications.

2.3 Brain Injury Imaging

Traumatic brain injury (TBI) has become a worldwide healthy problem and thousands of people suffer from it every year. It has been reported that vascular pathology could act as a biomarker for TBI and a variety of imaging modalities have been employed to study cerebral vasculature such as laser Doppler flowmetry, two-photon microscopy, and MRI. However, imaging performance using these techniques remains unsatisfactory due to invasive operation, limited resolution, or low sensitivity etc.

Figure 3. Traumatic brain injury (TBI) imaging in NIR-II window using IR-E1. (a) Absorption and fluorescence spectra of IR-E1 in aqueous solution. Excitation wavelength: 808 nm. (b) (i) and (iv): In vivo dynamic imaging of cerebral hypoperfusion. Dynamic NIR-II fluorescence images of TBI (i) and sham (iv) mice captured at 10 s after injection of IR-E1. (ii), (iii), and (v):

Leakage and accumulation of IR-E1 in brains of TBI and sham mice. Red box in (b, iii) indicates the ROI for the signal analysis in (d). (vi) PCA of image frames from dynamic NIR-II imaging of TBI mouse. Artery is depicted in red while vein in blue. Scale bar: 5 mm. (c) Cross-sectional fluorescence intensity along the dashed line in (b, ii) at 2, 8, 72, and 168 h after injection of IR-E1. (d) NIR-II fluorescence signal as a function of post-injection time in the healthy and injured ROI (in sham and TBI mice, respectively). Error bars indicate mean standard deviation ($n = 3$). (e) Dynamic imaging of hypoperfusion on a TBI mouse. Red arrows indicate the blood flow in a vessel. (f) Dynamic imaging of the leakage of IR-E1 into injured mouse brain. Red arrows indicate the appearing spotty leakage of IR-E1 into brain. The last image shows the PCA of image frames from dynamic imaging. Arterial and venous cerebral vessels are depicted in red and blue, respectively. While IR-E1 is depicted in green. (i) Superior sagittal sinus. (ii) Confluence of sinuses. (iii) Vein leakage. Scale bar: 2 mm. Reproduced with permission.^[16] Copyright 2016, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Dai, Luo, and Liang *et al.* reported an NIR-II fluorophore IR-E1 for fluorescence imaging of TBI in NIR-II window (**Scheme 2**).^[16] In order to impart a high QY (~0.7%), a bulky bridging group was introduced into donor-acceptor-donor structure to prevent intermolecular and intramolecular interactions in the conjugated backbone. The resulted IR-E1 was PEGylated to endow aqueous solubility with an absorption peak at 830 nm while a fluorescent emission peak at 1071 nm (**Figure 3a**). Because of the small molecular weight as well as hydrodynamic size, IR-E1 was capable of 83% clearance within 24 h post-injection via renal excretion. NIR-II fluorescence imaging of the mouse brain using IR-E1 was subsequently conducted on TBI mouse model without craniotomy (**Figure 3b, i-iii**). A reduced blood flow could be clearly observed in the injured region (right side) of the brain on TBI mouse (**Figure 3b, i; Figure 3c**). Nevertheless, such hypoperfusion was not seen in sham injury model (**Figure 3b, iv**). In addition, rapid clearance of IR-E1 from brain vasculature was observed in sham injury model (**Figure 3b, v**), in sharp comparison with the increased fluorescent signal in TBI model (**Figure 3b, ii**) attributed to the leakage of nanoparticles owing to the damaged blood brain barrier

(BBB) (**Figure 3d**). Furthermore, principal component analysis (PCA) of TBI mouse clearly depicted the hypoperfusion in arterial (red) and venous (blue) vessels of the injured region (**Figure 3b, vi**).

Dynamic NIR-II imaging of hypoperfusion in TBI mouse model was subsequently performed at single-vessel resolution (**Figure 3e**). The blood perfusion front was measured to move at an ultraslow speed of 0.002 mm s^{-1} in the injured region, so that blood perfusion into single vessels could be clearly observed. Moreover, a spotty leakage of NIR-II fluorophores into the injured region of TBI mouse was imaged with high resolution (**Figure 3f**), implying the venous subdural hematoma in the TBI mouse model. Such venous leakage of IR-E1 was also demonstrated by PCA of dynamic NIR-II frames. In conclusion, NIR-II fluorescence imaging using fluorophores of bright emission provides new opportunities to investigate the pathogenesis of TBI with unprecedented benefits, which should be of great importance to devise future therapeutic strategies.

3. NIR-II PA Imaging

Currently, PA imaging emerging as a promising diagnostic technique has been progressively studied worldwide, pushing forward related applications from bench to bedside.^[21] Different from traditional optical imaging, PA imaging relies on PA effect to transform photons absorbed by endogenous or exogenous chromophores into acoustic waves which experience much reduced scattering in biological tissues compared with photons.^[22] As a hybrid imaging modality, PA imaging combines the high contrast of optical imaging and high spatial resolution of ultrasound imaging, allowing for clear imaging at centimeter-scale depth in living bodies.^[23] Current PA imaging mainly capitalizes on NIR-I laser.^[24] Nevertheless, further investigations of PA imaging in a superior NIR-II window have been far less reported.

Increasing efforts have been made to develop PA agents that potently absorb light ranging from 1000 to 1350 nm within NIR-II window in recent years. Thanks to the largely increased MPE and reduced scattering of incident photons at longer wavelengths, NIR-II PA imaging is envisioned to endow further improved imaging depth as well as enhanced SBR relative to utilizing NIR-I laser, regardless of the slightly higher endogenous absorption in NIR-II window.^[9d, 25] In practice, a surprising depth of 11.6 cm in chicken breast tissue has been reached through PA imaging at 1064 nm using an organic contrast agent based on phosphorus phthalocyanine.^[26] Successive development of organic NIR-II absorbers is of vital importance to impart noninvasive PA imaging of physiological or pathological processes in centimeters deep in living organisms, contributing to effective diagnosis of various diseases at unprecedented depths.

Figure 4. NIR-II PA imaging of rat brain vasculature using SPN-II. (a) Schematic illustration of SPN-II. (b) UV-Vis-NIR absorption spectrum of SPN-II (40 $\mu\text{g mL}^{-1}$ in tetrahydrofuran). (c) PA spectra of SPN-II (200 $\mu\text{g mL}^{-1}$) and water. Error bars correspond to standard deviations of 10 separate measurements. (d) In vitro deep-tissue PA imaging in NIR-I and NIR-II windows. SPN-II (1 mg mL^{-1}) spots were embedded in an agar gel phantom placed under different depths

of chicken breast tissue. SNRs (dB) of SPN-II spots at 750 and 1064 nm were plotted as a function of the depth of chicken breast tissue, respectively. Energy density: 5.5 mJ cm^{-2} . $R^2 = 0.92549$ and 0.99172 for 750 and 1064 nm, respectively. (e) Two-dimensional NIR-II PA images of the agar gel phantom containing SPN-II spots at different depths of chicken breast tissue. Energy density: 20 mJ cm^{-2} at 1064 nm. Concentrations of SPN-II spots (from the white asterisk anti-clockwise): 1, 0.5, 0.2, and 0.05 mg mL^{-1} . (f) In vivo PA imaging of rat brain in NIR-II compared with NIR-I window. Representative PA images of rat cortex were captured at 70 min after tail vein injection of SPN-II (1.8 mg per rat) at 750 and 1064 nm, respectively. Energy density: 5.5 mJ cm^{-2} . (g) SNRs (dB) of brain cortex at 70 min post-injection of SPN-II at 750 and 1064 nm. Energy density, 5.5 mJ/cm^{-2} . *Statistically significant difference in SNR (dB) between NIR-I and NIR-II window ($p < 0.05$, $n = 3$). Reproduced with permission.^[25] Copyright 2017, American Chemical Society.

To substantially validate the advantage of NIR-II over NIR-I light for PA imaging, Pu *et al.* reported an organic PA agent SP2 with broadband absorption for the comparative study of PA performance in both NIR windows (**Figure 4a**).^[25] By virtue of the highly electron-deficient acceptor thiadiazoloquinoline and enhanced donor-acceptor interactions due to D-A₁-D-A₂ structure, SP2 exhibited lower band gap and subsequently strong absorption in NIR-II window (**Scheme 2**). After enveloping of SP2 by PEG-b-PPG-b-PEG, the resulted water-soluble SPN-II demonstrated a broadband absorbing property with an absorption peak at 1253 nm (**Figure 4b**). More importantly, SPN-II exhibited nearly identical PA signals at 750 and 1064 nm (**Figure 4c**), enabling itself an optimal contrast agent for the fair comparison of PA performance in both NIR windows.

In vitro deep-tissue PA imaging of SPN-II solutions embedded under different depths of chicken breast tissue was performed on a homemade PA system with switchable wavelengths between 750 and 1064 nm. As shown in **Figure 4d**, signal-to-noise ratio (SNR) in decibels of SPN-II solution imaged in NIR-II window was higher than that in NIR-I window at each depth of chicken breast tissue when compared at the same power density. In particular, the SNR of

SPN-II solution obtained at 1064 nm was 1.4-fold of that at 750 nm at a tissue depth of 3 cm. The maximum PA imaging depth was further enhanced to 5 cm in chicken breast tissue when increasing the energy density of 1064 nm laser to 20 mJ cm⁻² (**Figure 4e**).

Such superior PA imaging performance in NIR-II window was further validated by in vivo brain vasculature imaging on living rats. At 70 min post-injection of SPN-II, the PA image captured at 1064 nm exhibited improved clarity than that at 750 nm, as indicated by the higher contrast and clearer branch vessels (**Figure 4f**). Detailed ROI analysis indicated that the SNR gleaned at 1064 nm (~27.7 dB) was 1.5-times of that at 750 nm (~18.2 dB) (**Figure 4g**). Taken together, this work provided solid evidence of the superiority of PA imaging in NIR-II window compared with traditional NIR-I window, underlining the need for developing PA agents with strong absorption beyond 1000 nm.

Figure 5. NIR-II PA imaging of tumor. (a) UV-Vis-NIR absorption spectra of TII-PEG and TSPN. (b) In vivo PA (depicted in red and yellow) and US (depicted in grey) imaging of mouse tumors (dashed circles) with (upper) or without (lower) injection of matrigel inclusions of TSPNs (50 μ L, 40 μ g mL⁻¹). Energy density: 800 nm, 10-26 mJ cm⁻²; 1000 nm, 19-49 mJ cm⁻²; 1100 nm, 33-40 mJ cm⁻²; 1200 nm, 37-46 mJ cm⁻²; 1300 nm, 25-35 mJ cm⁻². (c) Vis-NIR

absorption spectrum of P1 NPs ($10 \mu\text{g mL}^{-1}$). (d) In vivo PA and US (depicted in grey) imaging of brain tumor post-injection of P1 NPs. Scale bar: 5 mm. (a-b): Reproduced with permission.^[27] Copyright 2017, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (c-d): Reproduced with permission.^[28] Copyright 2017, Royal Society of Chemistry.

Later, NIR-II PA imaging of cancer has also been carefully investigated. Mei and Cheng *et al.* developed a SP-based agent TSPN with strong NIR absorption and utilized it for multi-wavelength PA imaging of tumor in NIR-II window (**Figure 5a**).^[27] Thanks to the electron deficiency and high planarity of thienoisindigo (TII) unit, TII-TEG with triethylene glycol (TEG) side chains exhibited ultra-narrow bandgap and in turn strong absorption in NIR-II region (**Scheme 2**). Matching well with the absorption spectrum, the water-soluble TSPNs after formulation of TII-PEG with DSPE-PEG2000 generated strong PA signals in NIR-II region. As a result, the tube containing TSPNs with a low concentration of $40 \mu\text{g mL}^{-1}$ was clearly visualized by NIR-II PA imaging after embedding in chicken breast tissue at a depth up to 5.3 cm. Furthermore, TSPNs injected into the tumor could be clearly depicted by PA imaging conducted at multiple wavelengths ranging from 800 to 1300 nm (**Figure 5b**). In particular, thanks to the reduced interference from background tissue, the PA signal from tumor region was promoted by ~ 7 fold and ~ 13.3 fold at 1100 and 1300 nm after administration of TSPNs, respectively. To sum up, this study disclosed the optimal wavelengths (1100 and 1300 nm) in NIR-II window for deep tissue PA imaging with enhanced contrast. Further development of TSPN was expected to afford various functionalities for NIR-II theranostics.

Similar study of NIR-II PA imaging of cancer has also been reported on a brain tumor model.^[28] To endow a low bandgap and far-red absorption, a strongly electron-deficient acceptor benzobisthiadiazole (BBT) was utilized to copolymerize with a well-known electron donor benzodithiophene (BDT) to obtain P1 (**Scheme 2**). On account of the high planarity and intramolecular charge transfer (ICT) in the conjugated backbone, P1 exhibited red-shifted

absorption in the NIR-II region with a relatively high extinction coefficient ($\epsilon_{1064\text{ nm}} = 22.6\text{ mL mg}^{-1}\text{ cm}^{-1}$) after encapsulation by DSPE-PEG2000 to P1 NPs (**Figure 5c**). Consistent with the absorption spectrum, P1 NPs generated strong PA signals at 1064 nm so that a very low concentration of P1 NPs ($6\text{ }\mu\text{g mL}^{-1}$) could be detected by PA imaging at a limited power density (5.6 mJ cm^{-2}). More importantly, dual-modal PA/US imaging of orthotopic brain tumor was successfully performed at 1064 nm using P1 NPs on living mice. As shown in **Figure 5d**, the tumor was clearly delineated at 3 h post-injection of P1 NPs, as indicated by the strong PA signal detected under skin and skull. Notably, the tumor region at a tissue depth of $\sim 3.4\text{ mm}$ could be obviously visualized at 24 h post-injection with an unprecedented signal-to-background ratio of 59. Taken together, Liu and Zheng et al. reported the first organic contrast agent utilized for PA imaging of brain tumors in NIR-II window, highlighting the necessity to develop highly efficient NIR-II PA agents for potential applications in brain diseases.

4. Summary and Outlook

Progress of in vivo imaging in NIR-II window has been witnessed over the past few years. Taking advantage of the reduced photon scattering, minimal autofluorescence, and high MPE, both fluorescence and PA imaging demonstrate deeper tissue penetration and improved contrast (SBRs) when shifting the emission or excitation light from traditional NIR-I to NIR-II window. While most NIR-II fluorophores or PA contrast agents are composed of inorganic semiconductors, development of organic NIR-II nanoagents sharing similar chemical properties with FDA-approved NIR-I contrast agents provides new opportunities for the NIR-II in vivo imaging, which has enabled deep tissue imaging of cancer, circulatory system, and brain with high clarity.

While NIR-II window holds great promise for in vivo imaging, several concerns remain to be addressed before moving forward to clinical translations. First of all, despite several reports

about organic NIR-II fluorophores capable of fast renal clearance, the majority of organic NIR-II nanoagents tend to retain in organs and their long-term biosafety is unclear. To address this issue, new organic NIR-II nanoagent could be specifically devised either to endow biodegradability or to limit the overall diameter within the renal filtration threshold (~ 5 nm) for rapid clearance via urine excretion. The second problem lies in the overall low QY of existing organic NIR-II fluorophores which in general leads to poor temporal resolution. In particular, organic fluorophores with bright emission in the optimal autofluorescence-free region (>1500 nm) are scarcely reported. Thirdly, NIR-II fluorescence imaging requires InGaAs or HgCdTe cameras of high quantum efficiency above 1000 nm rather than silicon detectors commonly used in existing imaging systems (**Scheme 1f**). However, NIR-II fluorescence instrumentation using these new cameras is rather limited and demands further optimization. Similar dilemma has also been confronted in PA imaging, since thus far commercialized NIR-II PA system has yet to be fully developed. At last, very limited numbers of organic NIR-II absorbers have been reported, leaving NIR-II PA imaging largely remained to be explored.

In summary, development of ultra-small or biodegradable NIR-II organic contrast agents with bright emission or strong absorption above 1000 nm is of great interest to expedite NIR-II optical imaging towards clinical applications. Along with advancement in the imaging instrumentations, NIR-II optical imaging is envisioned to bring in significant enhancement in deep tissue imaging of anatomical structures, specific biomarkers, or physiological processes for improved diagnosis and theranostics of life-threatening diseases.

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TOC

The second near-infrared optical window (NIR-II, 1000-1700 nm) demonstrates the advantages of deeper tissue penetration and enhanced imaging clarity over the conventional first near-infrared window (NIR-I, 650-1000 nm). This review highlights the recent advances of organic contrast agents developed for fluorescence and photoacoustic imaging in the NIR-II window.