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**Organic Semiconducting Agents for Deep-tissue Molecular Imaging:
Second Near-Infrared Fluorescence, Self-luminescence, and Photoacoustics**

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Abstract: Although optical imaging has played a pivotal role in biology and medicine, it faces challenges of relatively low tissue penetration and poor signal-to-background ratio (SBR) due to light scattering and tissue autofluorescence. To overcome these issues, second near-infrared fluorescence, self-luminescence, and photoacoustic imaging have recently emerged, which utilizes an optical region with reduced light-tissue interactions, eliminates real-time light excitation, and detects acoustic signal with negligible attenuation, respectively. Because there are only a few endogenous molecules absorbing or emitting above visible region, development of contrast agents is essential for those deep-tissue optical imaging modalities. Organic semiconducting agents with π -conjugated frameworks can be synthesized to meet different optical requirements due to their easy chemical modification and legible structure-property relation. In this progress report, the deep-tissue optical imaging applications of organic semiconducting agents including small-molecule agents and nanoparticle derivatives are summarized. In particular, the molecular engineering or nano-formulation approaches to further improve the tissue penetration and detection sensitivity of these optical imaging modalities are highlighted. At last, current challenges and potential opportunities in this emerging subfield of biomedical imaging are discussed.

Keywords: contrast agents, nanoparticles, photoacoustic imaging, second near-infrared fluorescence imaging, self-luminescence imaging

1. Introduction

Molecular imaging enables visualization, characterization, and quantification of physiological or pathological processes at the cellular and subcellular dimensions in living subjects.^[1, 2] In conjunction with contrast agents and specialized instruments, molecular imaging has played a pivotal role in biomedical research and clinical practice. Clinical imaging modalities include radionuclide imaging (positron emission tomography (PET) and single-photon emission computed tomography (SPECT)), computed tomography (CT), thermal imaging, ultrasound, and magnetic resonance imaging (MRI), which rely on their respective energy types (γ -rays, X-rays, infrared radiation, or radiowaves) to achieve deep penetration ability in clinical practice.^[3] Among them, MRI, SPECT, PET, and thermal imaging have quantification capability, PET has the highest sensitivity (PET, $10^{-11}\sim 10^{-12}$ mol/L), MRI has the highest spatial resolution (20–100 μm), and ultrasound has the highest temporal resolution (seconds).^[4, 5] However, they have their own disadvantages such as availability of only anatomical information for CT, ultrasound, and thermal imaging, relatively poor spatial resolution for PET and SPECT (1–2 mm), reconstruction-induced low temporal resolution for CT, PET, SPECT, and MRI (minutes to hours), hazardous ionizing radiation for CT, PET, and SPECT, and expensive imaging systems for PET and MRI.^[6, 7]

Different from those conventional imaging modalities, optical imaging as a non-ionizing radiation method can simultaneously possess noninvasiveness, high spatio-temporal resolution, and high sensitivity.^[8-11] Because optical signals can be sensitively perturbed by fluorescence resonance energy transfer (FRET), photoinduced electron transfer, or self-quenching, optical imaging probes can be developed to have the ability to change their signals only in response to the specific biomarker or molecular event.^[12-14] Such smart activatable probes allow for correlation of optical signals not only with the concentration of biomarkers but also with the activity of biological molecules, leading to the real-time specific imaging at

the molecular level.^[15] However, such a smart approach is not often available for those conventional imaging modalities, because their electromagnetic signals are relatively difficult to be designed to change in respond to the target of interest.^[16, 17]

As an optical modality that is commonly used for *in vivo* imaging, near-infrared (NIR) fluorescence imaging relies on the excitation/emission wavelengths in the NIR spectrum ranging from 650 to 950 nm.^[18, 19] This window is defined as biological transparency window and also known as the first NIR window (NIR-I), which can achieve a tissue penetration depth up to ~1 cm (**Table 1**). However, when NIR-I light passes through or interacts with the tissue, it still encounters significant light scattering, tissue absorption, and autofluorescence (**Figure 1a,e,f**).^[20] Thus, NIR-I fluorescence imaging has a relatively poor signal-to-background ratio (SBR) and is less ideal for deep-tissue imaging.

To enhance the optical imaging depth, second near-infrared (NIR-II, 1,000–1,700 nm) fluorescence, self-luminescence and photoacoustic (PA) imaging have been recently developed. NIR-II fluorescence relies on a more ideal optical region with less light-tissue interactions (**Figure 1a**); self-luminescence imaging eliminates concurrent-excitation-induced autofluorescence by detecting photons after cessation of light excitation (**Figure 1b**); and PA imaging detects acoustic signal with negligible attenuation (**Figure 1c**). In contrast to NIR-I fluorescence imaging, NIR-II fluorescence, self-luminescence and PA imaging thus have increased tissue-penetration depth (**Table 1**), enabling deep-tissue molecular imaging based on light.

Imaging probes are essential for generation of specific signal correlated with the concentration or activity of biomarkers. Optical agents such as organic dyes,^[21] quantum dots (QDs),^[22] up-conversion nanoparticles,^[23] metal nanoclusters,^[24] carbon-based,^[25] and silica-based nanomaterials^[26] have been widely employed for molecular imaging. However, inorganic nanoparticles sometimes suffer from critical safety concerns including heavy metal–

induced toxicity and the inability of fast clearance from body post-imaging. To facilitate the translation for clinical applications, organic systems are relatively preferred. Because traditional NIR organic dyes often suffer from poor photostability, organic semiconducting agents including semiconducting polymer nanoparticles (SPNs) and semiconducting molecule nanoparticles (SMNs) have emerged as alternative candidates. These agents are made from completely organic and biologically inert π -conjugated semiconducting polymers (SPs) or semiconducting molecules (SMs) that have optical and electrical activities.^[27-35] Moreover, organic semiconducting agents often have larger absorption coefficients, more tunable optical properties and controllable size relative to other inorganic optical agents.^[36-38] To date, organic semiconducting agents have been exploited for many biomedical imaging applications, such as cell imaging and tracking,^[39-43] tumor imaging,^[44, 45] acute edema imaging,^[36] cardiovascular imaging^[46] and real-time evaluation of drug-induced hepatotoxicity.^[47]

The publications and citations about organic semiconducting agents are increasing in number, and there are some reviews nicely summarizing their syntheses, optical properties, and general sensing applications.^[30-32, 48-63] This review instead focuses on the recent progress on the development of organic semiconducting agents for deep-tissue molecular imaging including NIR-II fluorescence, self-luminescence and PA imaging, and highlights the chemistry and imaging mechanisms towards biological applications (**Table 2** and **Table 3**). As follows, the molecular design and applications of NIR-II fluorescence and self-luminescence imaging agents are first discussed. Then, the methods to develop smart activatable probes, amplify brightness and enhance imaging depth for PA imaging are described. At last, summary and outlook is given to further stimulate the research interests in the development of such imaging agents to fully explore their potential in deep-tissue imaging.

Table 1. Comparison of tissue-penetration depth for NIR fluorescence, self-luminescence and PA imaging.

Imaging modality	Fluorescence		Bio-luminescence	Chemi-luminescence	Afterglow	Photoacoustics	
	NIR-I	NIR-II				NIR-I	NIR-II
Penetration depth (cm)	~1	~3–5	~2	~2.5 ^{a)}	4 ^{b)}	5.2	11.6
Depth-to-resolution ratio	~15	~400	~1 ^{c)}	N.A. ^{d)}	N.A.	~200	N.A.
Reference	[8, 37]	[64–66]	[67, 68]	[47]	[69]	[70–72]	[73]

^{a)} Data was obtained in tissue-mimic gel phantoms. ^{b)} Data was obtained in chicken breast tissue. ^{c)} Data was applied in natural bioluminescence with emission wavelength in visible range. ^{d)} N.A.: Not applicable.

Figure 1. Illustration of interactions between light and biological tissue in (a) fluorescence imaging, (b) self-luminescence imaging, and (c) PA imaging. (d) Absorption spectra of oxyhemoglobin (red) and deoxyhemoglobin (blue) in human blood through a 1-mm long path. (e) Scattering coefficients of various biological tissues and phantom as a function of the wavelength ranging from 400 to 1,700 nm (blue: Visible, green: NIR-I and red: NIR-II). (f) Ex vivo autofluorescence spectra of mouse liver (black), spleen (red) and heart (blue) excited at 808 nm. (d)–(f) Reprinted with permission.^[74] Copyright 2017, Nature Publishing Group.

Scheme 1. Chemical structures of representative SPs and SMs used for NIR-II fluorescence imaging.

a Organic molecules

b Amphiphilic polymers/oligomers

Scheme 2. Chemical structures of (a) small organic molecules, (b) amphiphilic polymers and oligomers used for coprecipitation with SPs or SMs for preparation of SPNs or SMNs.

2. NIR-II Fluorescence Imaging

NIR-II fluorescence imaging detects photons in the spectrum ranging from 1,000 to 1,700 nm, which has reduced photon scattering (inverse wavelength dependence: $\lambda^{-\alpha}$, $\alpha = 0.2-4$) and tissue autofluorescence (a nearly negligible autofluorescence region beyond $\sim 1,500$ nm) than those in NIR-I window (**Figure 1**).^[46, 75] Moreover, NIR-IIa (1,300–1,400 nm) and NIR-IIb (1,500–1,700 nm) sub-windows refrain from the interference of water overtone absorbance peak ($\sim 1,450$ nm, assigned to the combination of OH vibrations including symmetric and asymmetric stretches), further improving the imaging quality.^[76, 77]

Most NIR-II imaging agents are inorganic nanoparticles such as single-walled carbon nanotubes (SWNTs),^[78-80] rare-earth-doped nanocomposites,^[81, 82] and QDs,^[75, 83, 84] because their intrinsic emission wavelengths can be easily adjusted to NIR-II window through the control of nanostructures. However, their optical properties, kinetics and biodistribution are difficult to be optimized to fulfill diverse applications. Alternatively, organic semiconducting agents can be used for NIR-II fluorescence imaging (**Scheme 1** and **Table 2**).^[85] To reduce the bandgaps so as to generate NIR-II fluorescence, SPs/SMs are often synthesized to have donor-acceptor (D-A) units (**Table 2**). Many electron-withdrawing or electron-donating building blocks, and π -bridging moieties have been screened and used to adjust the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) levels of SPs/SMs.^[86, 87] Currently, the main focuses are to make organic semiconducting agents (SPNs/SMNs) possess a relatively high fluorescence quantum yield (QY), ideal pharmacokinetics and favorable renal clearance.

Table 2. Optical properties and imaging applications of NIR-II fluorescent SPs/SMs.

Agent	λ_{ab} [nm]	λ_{em} [nm]	Φ_f [%] ^{a)}	Molecular engineer	Imaging application	Ref. ^{c)}
SP1 (pDA)	654	1047	1.7	D–A	Mouse arterial blood flow	[46]
SM1 (IR–1061)	780	1064	1.8	N.A. ^{b)}	Hindlimb and abdomen	[85]
SM3 (CH1055–PEG)	~750	1055	0.3	D–A–D	Lymphatic vasculature and brain tumor; tumor surgery guidance	[64]
SM4 (CQS1000)	~880	1000	N.A.	D–A–D	Circulatory system and resection of tumor guidance	[86, 87]
SM5 (SCH1100)	~865	1100	N.A.	D–A–D	Targeted prostate cancer	[86]
SM6 (IR–E1)	830	1071	0.7	D ₂ –D ₁ –A–D ₁ –D ₂	Cerebrovascular injury in a mouse model of traumatic brain injury	[88]
SM7 (IR–FEP)	780	1047	2.0	S–D–A–D–S	Blood flow and tumor	[89]
SM8 (IR–FGP)	745	1050	1.9	S–D–A–D–S	3D	[90]
SM9 (IR–FTAP)	733	1048	5.3	S–D ₂ –D ₁ –A–D ₁ –D ₂ –S	Blood vessels	[91]
SM10 (CH–4T/FBS–HT)	~748	~1005	11	D–A–D	Hindlimb vasculature and lymph node	[65]

^{a)} Measured in aqueous solution. ^{b)} N.A.: Not applicable. ^{c)} Ref.: Reference.

2.1. Molecular Design towards Renal Clearance

The first trial of organic semiconducting agents for NIR-II fluorescence imaging was conducted by Dai's group on poly(benzo[1,2-*b*:3,4-*b'*] difuran-*alt*-fluorothieno-[3,4-*b*] thiophene (SP1, **Scheme 1**), consisting of electron donor (benzo[1,2-*b*:3,4-*b'*] difuran) and acceptor (fluorothieno-[3,4-*b*]thiophene) units. As SP1 was not water-soluble, it was coprecipitated with 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (poly (ethylene glycol))-5000] (DSPE-mPEG5kDa, **Scheme 2b**) to form water-soluble nanoparticles (SPN1).^[46] As shown in **Figure 2a**, SPN1 had a large Stokes shift of ~ 400 nm with the absorption and emission maxima at 654 and 1,047 nm, respectively. SPN1 had a QY

of 1.7%, which was 4-fold higher than that of SWNTs.^[37] The brightness of SPN1 allowed for a much shorter exposure time (~20 ms) than SWNTs (~100 ms),^[37] enabling the high imaging speed (25.6 frames/second) and thus a clear observation of mouse arterial blood flow by NIR-II fluorescence imaging (**Figure 2b–e**). The dynamic NIR-II fluorescence intensity at a region of interest of the femoral artery showed that the spaced humped signal increased with linearly increasing baseline (**Figure 2c**). The regular humps were caused by periodic systolic phases in cardiac cycles and the increasing baseline was attributed to continuous perfusion of SPN1 after blood circulation. Thus, the dynamic fluorescence intensities of the femoral artery after linear baseline deduction revealed that the blood flow variations were closely correlated with the two phases of each heartbeat and thus were used to trace the cardiac cycles. According to the NIR-II imaging data, one cardiac cycle was calculated to be 206.7 ms (**Figure 2f**), which agreed with the result from real-time microscopic imaging operated on a specialized set-up.^[92] Thus, SPN1 enabled NIR-II fluorescence imaging with high temporal and spatial resolution, providing an optical dynamic way to monitor blood flows and cardiac cycles *in vivo*.

Low toxicity and fast clearance of an imaging agent are necessary for its potential US Food and Drug Administration (FDA) approval and clinical translation. However, because SPN1 had a large particle size (~17 nm), it had slow excretion from body through hepatobiliary metabolism, resulting in long-term toxic concern. Thus, to achieve renal clearance, a small organic SM (SM1, **Scheme 1**) was used as an NIR-II fluorescent ingredient instead of a SP to form water-soluble SMN1, which had the size of 5.8 nm after co-precipitation with DSPE-mPEG5kDa.^[85] The smaller size of SMN1 relative to SPN1, SWNTs^[25] and rare-earth nanoparticles^[81] led to the ability of kidney excretion. However, because the size of SMN1 was still above the renal filtration threshold (~40 kDa, 5.5 nm),^[85, 93, 94] SMN1 did not have an optimal renal excretion kinetics.

Figure 2. NIR-II fluorescence imaging. (a) Absorption and fluorescence spectra of SPN1. (b) The NIR-II fluorescence image of the mouse femoral artery. Scale bar: 5 mm. Integration of the NIR-II fluorescence intensity before (c) or after (d) subtraction of a linear increasing baseline inside the ROI (red box) in b as a function of time. (e) NIR-II fluorescence images of the red box area shown in b as a function of time. The signals are subtracted by baseline in c. Scale bar: 1 mm. (f) Fitted calibration curve for intensity spikes corresponding to ventricular ejections versus the numbers of heart pulses. (g) Absorbance and fluorescence spectra of SMN3. (h) Representative NIR-II fluorescence images (1,200 nm long-pass filter, 100 ms) of a mouse at indicated time points after an intravenous injection of SM3 (upper panel) or SWCNTs (lower panel) ($n = 3$ mice). (i) Quantification of NIR-II fluorescence intensities for both the liver and bladder regions after administration of SM3. (j) Fluorescence image of mouse brain vasculatures ($n = 2$ mice) after treatment with ICG (850–900 nm, 100 ms, left) or SM3 (1,300 nm long-pass filter, 1 s, right). (k) NIR-II fluorescence image of a mouse before (left) or after (right) performing NIR-II fluorescence imaging-guided surgery to excise the tumor at $t = 24$ h post-injection of SM2-affibody (60 μg). (l) SBR for the NIR-II fluorescence images at different time points post-injection of SM2-affibody in targeting and

blocking experiments ($n = 3$, $p < 0.05$). (a)–(f) Reprinted with permission.^[46] Copyright 2014, Nature Publishing Group. (g)–(l) Reprinted with permission.^[64] Copyright 2015, Nature Publishing Group.

Fully water-soluble SM agents with rapidly renal excretion were developed by Dai's group and used for NIR-II fluorescence imaging guided surgery.^[64] The carboxylic acid groups of SM2 (**Scheme 1**) were PEGylated to increase its hydrophilicity, yielding the contrast agent SM3 (**Scheme 1**). The backbone of SM3 was designed to have a donor-acceptor-donor (D-A-D) architecture, wherein the strong electron acceptor unit (BBTD: benzo[1,2-*c*:4,5-*c'*] bis([1,2,5]thiadiazole) and the strong electron donor unit (triphenylamine) were connected by π -bridging moieties. Such D-A-D type reduced the bandgap, leading to the absorption and emission maxima at ~ 750 and $\sim 1,055$ nm, respectively (**Figure 2g**). Additionally, the emission of such agents could be further red-shifted through adjusting the bandgaps between HOMO and LUMO levels (SM3, SM4, and SM5, **Scheme 1**): for instance, the emission maximum of SM5 was $\sim 1,100$ nm.^[86, 87]

Because the molecular weight of SM3 (8.9 kDa) was below the size threshold for renal clearance, SM3 had a high renal excretion efficacy $> 90\%$ (**Figure 2h, i**), while other NIR-II nanoagents such as SWCNTs were excreted slowly through the reticuloendothelial system. Brain tumor and vasculature were clearly visualized with higher clarity and imaging quality by NIR-II fluorescence of SM3 relative to the NIR-I fluorescence of indocyanine green (ICG) due to reduced photon scattering and tissue autofluorescence in NIR-II window (**Figure 2j**). To endow the specificity for EGFR+ cancer cells, SM2 was linked to an anti-EGFR affibody (SM2-affibody). Quantification demonstrated that the SBR of in vivo tumor imaging reached ~ 15 within 6 h post-injection, which was 5-fold higher than that of NIR-I imaging with the same affibody (**Figure 2k-l**).^[95] The relatively lower SBR for the anti-EGFR affibody blocking group further proved the specificity of SM2-affibody (**Figure 2l**). Moreover, the first proof-of-concept NIR-II imaging-guided tumor surgery was performed (**Figure 2k-l**),

which provided a clearer differentiation between cancerous and healthy tissue than white light did.

2.2. Molecular Design towards Enhanced Brightness

Although the water-soluble SMs had the ability to be cleared through kidney excretion, they had the drawback of low fluorescence QYs due to strong interactions between the π -conjugated segments and other molecules in the environments such as water. Thus, the molecular structures of SMs were optimized. For instance, different from SM3, SM6 (**Scheme 1**) was designed to comprise a bulky donor, 3,4-ethylenedioxythiophene (EDOT) instead of triphenylamine. Such replacement reduced the inter- and intra-molecular interactions between the π -conjugated backbones, resulting in 2.3-fold enhancement in QY (0.7%) at ~1,071 nm in aqueous solution relative to that of SM3 (0.3%).^[88]

To improve the optical properties, the shielding units were introduced into SMs. For instance, SM7 and SM8 were constructed using a shielding unit-donor-acceptor-donor-shielding unit (S-D-A-D-S) structure with dialkyl-fluorene as the shielding unit, BBTD as the acceptor, EDOT (SM7) or tert(ethylene glycol) (TEG)-thiophene (SM8) as the donor (**Scheme 1**).^[89, 90] Compared with SM6, the introduction of shielding unit for SM7 further reduced the intermolecular interactions (e.g., aggregation) of π -conjugated backbone due to the presence of the bulky alkyl chains at the 9,9'-position of fluorene, leading to an increased QY of 2.0% in aqueous solution.^[89] In addition to the introduction of dialkyl-fluorene as the shielding part, the electron-donating TEG-thiophene was incorporated into SM8 to distort the conjugated backbone and increase the dihedral angle between the acceptor and the donor relative to SM3 and SM6.^[90] Such design also reduced molecular interactions/aggregation and led to the QY of 1.9% for SM8.

In addition, the donor units were elongated in SM9 so as to have a shielding unit-donor 2-donor 1-acceptor-donor 1-donor 2-shielding unit (S-D₂-D₁-A-D₁-D₂-S) structure (**Scheme**

1). The alkyl thiophene and the unsubstituted thiophene were employed as the first and second donor to connect the acceptor unit (BBTD) and the shielding unit, respectively.^[91] Such a structure further reduced intermolecular interactions as compared to the molecules with other donor units (e.g., EDOT and thiophene), resulting in an enhanced QY of 5.3% in aqueous solution.

In addition to the modification of molecular structures, supramolecular assembly represented a facile strategy to enhance the NIR-II fluorescence of SMs. To form supramolecular assemblies with serum protein (fetal bovine serum) through non-covalent interactions (e.g., hydrophobic and electrostatic interactions), SM2 was functionalized with sulfonic acid to yield a completely water-soluble SM10 (**Scheme 1**). Such a self-assembly avoided the intermolecular aggregation-induced self-quenching, resulting in a 110-fold enhancement in fluorescence intensity relative to that of SM3.^[65] Such a high NIR-II QY (11%) of SM10 not only enabled imaging of cardiac cycles with high temporal resolution, but also led to deep-tissue imaging of lymph nodes at the SBR (29.0) superior to that in NIR-I window (4.86).

3. Self-luminescence Imaging

Self-luminescence imaging detects photons without real-time light excitation and thus eliminates the tissue autofluorescence, permitting deep-tissue imaging at a higher SBR level than corresponding fluorescence imaging. There are three kinds of self-luminescence imaging approaches: bioluminescence, chemiluminescence, and afterglow imaging (**Figure 1b**). Bioluminescence relies on the enzymatic reaction to generate photons, and thus involves the enzyme and substrate, while chemiluminescence is produced by chemical reactions between substrates and often reactive species.^[96, 97] Different from bioluminescence and chemiluminescence triggered by addition of a substrate into the system, afterglow imaging

requires pre-irradiation of light to generate the chemical or energy defects, and detects photons released from those defects after cessation of light irradiation.^[69, 98, 99]

Table 3. Optical properties, self-luminescence and PA imaging applications of SPs/SMs.

Optical modality		Agent	Optical signal [nm]	Size [nm]	Biomarker	Imaging application	Ref.
Self-luminescence	Bioluminescence	SPN2–BF	780	27	--	Lymph nodes and targeted tumor	[68]
	Chemiluminescence	SPN3–CF	820	50	H ₂ O ₂	Drug–induced hepatotoxicity	[47]
		SPN7	780	~20	H ₂ O ₂	Peritonitis and neuroinflammation	[100]
	Afterglow	SMN11–T	~530	~65	--	Lymph nodes	[101]
SPN2–NCBS, –thiol		780	34	Biothiols	Lymph nodes, tumor and drug–induced hepatotoxicity	[69]	
Photoacoustics	Activatable PA	SPN8	700, 735, 820	41	ONOO [–] /ClO [–]	Lymph nodes and zymosan–induced acute edema	[36]
		SMN14	680, 750	20	ONOO [–]	Tumor	[102]
		SMN15	680, 780	34	ClO [–]	Tumor	[103]
		SMN16	680, 750	8.5	Acidic pH	Tumor	[28]
	Amplified PA	SPN9–11	712, 635, 748 (SPN9–11)	46	--	Tumor	[104]
		SPN12–14	665	40	--	Tumor	[105]
		SPN8–F0, –4, –10, –20, –40	650	54	--	Tumor	[27]
		SPN15, –SiO ₂	750, 740 (SPN15, –SiO ₂)	~20	--	Targeted tumor	[106]
		SM17	~700	20	H ₂ O ₂ /ONOO [–]	Tumor	[107]
		NIR-II PA	SPN16–II	1253	54	--	Penetration and brain cortex
	SPN17		1200	86	--	Tumor	[109]
	SM18		1042	--	--	Penetration and tumor	[73]

Scheme 3. Chemical structures of representative SPs and SMs used for self-luminescence imaging.

3.1. Bioluminescence Imaging

Naturally occurring bioluminescence is often in the visible region, and thus not ideal for in vivo imaging. Bioluminescence resonance energy transfer (BRET) is a general way to shift the visible luminescence to NIR for deep-tissue imaging. BRET was used to generate the NIR bioluminescence from the CdSe–ZnS core–shell QDs through covalent conjugating with a *Renilla reniformis* luciferase (Luc8).^[110] The emission of Luc8 in the presence of the substrate coelenterazine was at 480 nm, which was shifted to the QDs emission at 655 nm *via* BRET without light excitation. Such kinds of BRET-based self-exciting QDs with tunable wavelengths (from the visible to the short NIR–II) were applied for tumor and lymph node imaging^[111, 112] as well as for molecular imaging of enzyme activities.^[113]

To solve the potential toxicity issue of bioluminescent QDs, Rao's group developed self-luminescent nanoparticles based on SPNs for in vivo NIR bioluminescence imaging (**Figure 3a**).^[68] The NIR bioluminescence was induced by a BRET/FRET relay process: Luc8 served as a BRET donor (480 nm) and poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (SP2, MEHPPV, **Scheme 3**) acted as the BRET acceptor and the FRET donor (594 nm), while silicon 2,3-naphthalocyanine bis(trihexylsilyloxy) (NCBS, **Scheme**

2a) was used as the FRET acceptor to result in NIR emission (780 nm) (**Figure 3a**). The bioluminescence spectrum of SPN2–BF demonstrated the efficient energy transfer occurring from Luc8 to SP2 and then to NCBS (**Figure 3b**). Cyclic–RGD peptide was conjugated to this SPN to obtain SPN2–BF–cRGD, permitting targeted tumor imaging (**Figure 3c**). Due to the negligible background of bioluminescence, the tumor was clearly visualized with strong bioluminescence signal at $t = 5$ min post-injection; in comparison, the fluorescence signal failed to do so (**Figure 3c**). Moreover, NIR bioluminescence imaging permitted imaging tumors as small as 2–3 mm in diameter with the SBR (~ 100) that was ~ 30 –fold higher than that of fluorescence imaging (**Figure 3d**).

Figure 3. Bioluminescence imaging. (a) Schematic illustration of the self-luminescent BRET–FRET NIR SPNs (SPN2–BF–cRGD). An amphiphilic polymer, polystyrene–grafted poly(ethylene glycol) functionalized with carboxyl groups (PS–g–PEG–COOH, **Scheme 2b**) was used to coprecipitate with SP2 to obtain the water–soluble and biocompatible nanoparticles. Cyclic RGD peptide was conjugated to the nanoparticle surface for targeted *in vivo* tumor imaging. (b) Bioluminescent emission spectrum of SPN2–BF in PBS buffer. (c) Representative bioluminescence (upper panel) and fluorescence (lower panel) images of tumor in living mice at indicated time points after administration of SPN2–BF–cRGD (~ 50

μg). (d) SBR for bioluminescence and fluorescence imaging of tumor in c. Reprinted with permission.^[68] Copyright 2012, Nature Publishing Group.

3.2. Chemiluminescence Imaging

Although bioluminescence imaging has high imaging sensitivity and specificity, their signals are often affected by enzyme microenvironment and substrate biodistribution in living animals. In contrast, chemiluminescence imaging does not require the enzyme, but relies on the reaction of substrate with reactive oxygen species (ROS), and thus is simpler and more versatile. Chemiluminescence resonance energy transfer (CRET) for *in vivo* NIR imaging was first employed on an inorganic system, which was composed of luminol (a chemical that specifically reacted with hypochlorous acid, HOCl, to generate blue chemiluminescence) physically mixed with the NIR fluorescence-emitting QDs as the CRET acceptor. Such system was used to monitor myeloperoxidase activity through chemiluminescence imaging of HOCl generated by myeloperoxidase.^[114] However, this system was a physical mixture with relatively poor biodistribution and thus limited applications.

Organic semiconducting agents were exploited as the CRET acceptor for NIR chemiluminescence imaging of drug-induced hepatotoxicity in living mice.^[47] SPN3-CF was designed to have two optically independent channels that used CRET and FRET to detect hydrogen peroxide (H₂O₂) and peroxynitrite (ONOO⁻), respectively (**Figure 4a**). In this system, a chemiluminescent substrate bis-(2,4,5-trichloro-6-(pentylloxycarbonyl)phenyl)oxalate (CPPO, **Scheme 2a**) was encapsulated into the poly[2,7-(9,9'-dioctylfluorene)-*alt*-4,7-bis(thiophen-2-yl)benzo-2,1,3-thiadiazole] (SP3, PFODBT, **Scheme 3**) matrix, which acted as the chemiluminescent donor to react with H₂O₂, generating luminescence without external excitation. The core SP3 served as both the CRET acceptor and the FRET energy donor, while a cyanine dye 2-[4'-(β-carboxyethylthio)-7'-(1'',3'',3''-trimethylindolenine)-3',5'-trimethyleneheptatrien-1-yl]-1,3,3-trimethylindolenium (IR775S, **Scheme 2a**) was used as the FRET energy acceptor to induce NIR emission. Because reactive

nitrogen species (RNS) such as ONOO^- could decompose IR775S, the NIR fluorescence emission of IR775S was inhibited in the presence of ONOO^- , leading to the abolishment of FRET and thus ratiometric fluorescence signals. To target hepatocytes, a PEG-grafted poly(styrene) copolymer conjugated with galactose (PS-*g*-PEG-Gal, **Scheme 2b**) was co-encapsulated with those agents. *In vitro* study demonstrated that CRET occurred and chemiluminescence intensity of SPN3-CF responded linearly to H_2O_2 with a limit of detection (LOD) of 5 nM (**Figure 4b**). Upon treatment with ONOO^- stepwise, the fluorescence intensity of SPN3-CF decreased at 820 nm and concurrently increased at 680 nm, allowing for ratiometric (I_{680}/I_{820}) detection of ONOO^- with a LOD of 10 nM (**Figure 4c**). Thus, SPN3-CF could detect nanomolar concentration of both H_2O_2 and ONOO^-

Figure 4. Chemiluminescence and fluorescence imaging of drug-induced hepatotoxicity. (a) Illustration of simultaneous and differential detection of H_2O_2 and $ONOO^-/OCl$ respectively via chemiluminescent channel (upper) and fluorescent channel (lower) by SPN3–CF. (b) Chemiluminescent intensities of SPN3–CF (1 $\mu g/mL$) as a function of H_2O_2 concentration. Inset: the corresponding chemiluminescence images. (c) Fluorescence spectra of SPN3–CF (1 $\mu g/mL$) after incremental additions of NaONOO. (d) Representative chemiluminescence (upper) or fluorescence (lower) images of mice after treatment with saline, APAP (300 mg/kg, intraperitoneally), and APAP (300 mg/kg, intraperitoneally) with GSH (200 mg/kg, intravenously), 1–ABT (2 $\times$ 100 mg/kg intraperitoneally), or DCE (0.2 mg/kg intraperitoneally), followed by SPN3–CF (0.8 mg, intravenously). (e) Chemiluminescence or (f) fluorescence intensities of the liver after indicated treatment in d as a function of time. Reprinted with permission.^[47] Copyright 2014, Nature Publishing Group.

Drug-induced hepatotoxicity is a long-standing concern of modern medicine.^[115] It is one of the most common reasons that the FDA withholds drug approval.^[116] Evaluation of potential hepatotoxicity in advance of regulatory approval is challenging because current *in vitro* methods often have low predictive power.^[117] SPN3-CF was explored to detect drug-induced hepatotoxicity, and acetaminophen (APAP), an antipyretic analgesic drug, was used as a model drug. Dose-dependent ROS/RNS activity in the liver was detected by SPN3-CF within minutes, which obviously preceded the appearance of histological changes in drug-damaged tissue. This confirmed the effectiveness of SPN3-CF in the early detection of hepatotoxicity *in vivo*. The mechanism for APAP bioactivation involves CYP450-mediated oxidation to an iminoquinone, N-acetylparaquinonimine (NAPQI), which can bind directly to cellular proteins to induce mitochondrial dysfunction and the production of superoxide ($O_2^{\bullet-}$) and $ONOO^-$ or undergo reduction by molecular oxygen to directly produce $O_2^{\bullet-}$ and H_2O_2 . Upon treatment with the antioxidant (GSH) or the CYP450 inhibitors (1-ABT: 1-aminobenzotriazole and DCE: trans-1,2-dichloroethylene), both chemiluminescence intensities and fluorescence index were reduced (**Figure 4d-f**), indicating successful remediation of oxidative and nitrative stress, respectively. This study proved that SPNs could be developed into effective chemiluminescence probes to detect drug-induced toxicity and monitor remediation outcome, potentially contributing to improving drug development processes.

Figure 5. Amplified chemiluminescence imaging of neuroinflammation. (a) Schematic illustration for the mechanism of CRET from TCPO to SPNs. (b) The fluorescent and chemiluminescent QYs of SPNs as a function of the energy intervals between the LUMO of 1,2-dioxetanedione and the HOMO of SPs. (c) Chemiluminescence and fluorescence spectra of SPN7. (d) Representative chemiluminescence images of mice treated with saline, LPS (10 mg/kg, 2 μL) or LPS (8 mg/kg) with GSH (500 mg/mL, 1 μL), followed by an intracerebral injection of SPN7 (10 mg/mL, 2 μL) at $t = 4$ h later. Chemiluminescence image was acquired at $t = 1$ min after administration of SPN7. (e) Quantification of chemiluminescence intensities in (d). *Statistically significant difference between LPS treatment and control or GSH remediation group ($n = 3$, $P < 0.05$). Reprinted with permission.^[100] Copyright 2016, American Chemical Society.

To further improve the NIR chemiluminescence efficacy of SPNs, Pu's group screened five polyfluorene-based SPs (SP3–7, **Scheme 3**) with different molecular orbitals. Another hydrophobic chemiluminescent substrate, bis(2,4,6-trichlorophenyl) oxalate (TCPO, **Scheme 2a**), was encapsulated into the SPNs.^[100] To explore the underlying mechanism that governed the chemiluminescence process between peroxalate and the SP matrix, their chemiluminescence intensities were correlated with their energy levels. The TCPO-mediated chemiluminescence process is known as chemically initiated electron exchange luminescence (CIEEL) (**Figure 5a**). High energy intermediate—1,2-dioxetanedione was firstly generated spontaneously and instantaneously after oxidation of TCPO by H_2O_2 , followed by reduction

of a SP to generate the SP radical cation and the carbon dioxide radical anion. Then, the back-electron transfer occurred between the cation and the anion to produce the excited SP, ultimately leading to the luminescence from the SP. Thus, the intermolecular electron transfer from the SP to 1,2-dioxetanedione in CIEEL was the key step in determining the chemiluminescence efficiency of SPNs, which could be qualitatively evaluated according to the energy interval between the HOMO of a SP and the LUMO of 1,2-dioxetanedione. Among these SPNs, the energy interval between the HOMO of poly [(9,9'-dioctyl-2,7- of 11.6 cm. This is the highest penetration depth reported for NIR-II imaging so far. ADDIN SP7) and the LUMO of 1,2-dioxetanedione was the smallest, and thus the intermolecular electron transfer between them was most favored, leading to the highest chemiluminescence efficiency (**Figure 5b**). The QY of the optimized chemiluminescence was calculated to be 2.30×10^{-2} einsteins/mol, higher than the previously reported chemiluminescence systems including dye-doped peroxalate nanoparticles^[118] and luminol.^[119]

With the high brightness and the low LOD (5 nM) for detection of H₂O₂, SPN7 was doped with the NIR dye (NCBS) to obtain SPN7-NCBS. Such doping shifted the CRET emission from 452 nm (SP7) to 778 nm (NCBS) (**Figure 5c**), making it useful for deep-tissue imaging. Neuroinflammation is associated with neurodegenerative diseases and characterized by the activation of brain glial cells, primarily microglia and astrocytes that release various soluble factors that include ROS, cytokines and lipid metabolites. SPN7-NCBS was able to detect H₂O₂ *in vivo* in the mouse model of neuroinflammation induced by intracerebral injection of lipopolysaccharide (LPS) (**Figure 5d-e**).

3.3. Afterglow Imaging

Bioluminescence and chemiluminescence imaging modalities rely on additional enzymatic or chemical reaction to produce light emission, which makes the signals easily perturbed by enzyme microenvironment or substrate biodistribution. Furthermore, consumption of

substrates in bioluminescence and chemiluminescence imaging results in gradually decreased overall signal, compromising their ability for longitudinal imaging to monitor long-term biological processes. In contrast, afterglow imaging that detects the signal after cessation of light irradiation and can be repeatedly activated is a more promising approach for longitudinal imaging.

Figure 6. *In vivo* ultra-long phosphorescence imaging. (a) Proposed mechanism for ultra-long phosphorescence of SMNs enhanced by the formation of H-aggregates. (b) Normalized phosphorescence spectra of SMNs. (c) Schematic illustration for the detection of ultra-long phosphorescence after light irradiation. (d) Ultra-long phosphorescence images of SMNs *in vitro*. (e) Ultra-long phosphorescence and fluorescence images of lymph node at $t = 1$ h after intradermal injection of SMN11-T. (f) SBR for lymph node imaging in e. Reprinted with permission.^[101] Copyright 2017, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

One way to separate the light excitation and signal collection is to take advantage of ultra-long phosphorescence.^[101] Because the phosphorescence is often sensitive to oxygen and water, a top-down approach (from the solid crystals of SM11–13 to nanoparticles, **Scheme 3**) was used to synthesize the water-soluble SMNs–T (SMN11–T, SMN12–T, and SMN13–T). Such a formulation method greatly retained the H-aggregation in the resulted nanoparticles; in contrast, the nanoparticles made from the bottom-up approach (nanoprecipitation) (SMNs–B: SMN11–B, SMN12–B, and SMN13–B) did not possess such feature. Because the H-aggregation provided the lower energy levels and acted as additional energy traps to effectively stabilize the triplet excited states, SMNs–T had much longer phosphorescence life-time than that for SMNs–B (**Figure 6a–d**). Among these SMNs–T, SMN11–T had the brightest ultra-long phosphorescence and its longest life-time (861 ms). The ultra-long phosphorescence of SMN11–T could be repeatedly activated with nearly no loss in total intensity, leading to the feasibility for longitudinal *in vivo* imaging such as lymph node mapping. After intradermal injection of SMN11–T, the axillary lymph node was clearly delineated by afterglow imaging, showing an SBR of 40 (**Figure 6e**). In contrast, fluorescence imaging failed to do so due to the strong tissue autofluorescence at the visible wavelength (**Figure 6f**). This study not only revealed a universal methodology to extend the life-time of phosphorescent molecules but also demonstrated the first example of water-soluble SMNs for *in vivo* afterglow imaging.

Despite the effectiveness of ultra-long phosphorescence for afterglow imaging, the half-life was still relatively short (~milliseconds) and the emission was in the visible region. Recently, Pu's group discovered the long-lasting NIR afterglow behavior of poly(phenylenevinylene) (PPV)-based SPNs (half-life up to 6 min in aqueous solution).^[69] The mechanism of PPV-based afterglow luminescence was revealed to involve light-induced formation of unstable chemical defects that stored the light energy and released photons as downstream products over time (**Figure 7a**). Light irradiation of PPVs generated singlet

oxygen ($^1\text{O}_2$), which oxidized the vinylene bond via $\pi^2-\pi^2$ cycloaddition to form a PPV-dioxetane intermediate. This intermediate was unstable and spontaneously degraded into a PPV-aldehyde and generated photons. Because $^1\text{O}_2$ played a key role in determining the afterglow brightness, the afterglow of SPN2 was amplified and red-shifted into the NIR region (778 nm) by doping the $^1\text{O}_2$ photosensitizer (NCBS). The afterglow intensities after pre-irradiation at either 514 nm or 808 nm increased with increasing doping amount of NCBS due to enhanced generation of $^1\text{O}_2$ and was optimized at 5_{w/w}% doping amount (**Figure 7b**). Quantification indicated that the absolute afterglow intensity of the optimal SPN2-NCBS5 induced by pre-irradiation at 514 nm was increased by 6.8-fold relative to non-doped control SPN (SPN2). Moreover, the afterglow of SPN2-NCBS5 could be further enhanced by 11-fold if pre-irradiation was conducted at 808 nm. This was attributed to the stronger $^1\text{O}_2$ generation ability of NCBS than MEHPPV. The NIR-induced afterglow of SPN2-NCBS5 penetrated through a living mouse with an SBR (237 ± 22) that was 4.7 and 120 times higher than the visible-light-induced afterglow (50.7 ± 4.5) and the NIR fluorescence (1.98 ± 0.09), respectively (**Figure 7c-e**). SPN2-NCBS5 was then tested for passively targeted imaging of tumors in living mice. The tumor was clearly visualized at $t = 2$ h post-injection for afterglow imaging, but not possible for NIR fluorescence imaging at this time point (**Figure 7f-g**). The SBR of afterglow imaging was higher than that of NIR fluorescence at all time points due to a low background of afterglow (**Figure 7h**). In addition, SPN2-NCBS5 had the good biocompatibility and enzymatic biodegradability, showing its promise for clinical translation.

Figure 7. Molecular afterglow imaging. (a) Proposed mechanism for the afterglow luminescence of PPV-based SPNs. R_1 and R_2 represent alkyl chains. (b) Quantification of the afterglow luminescence and fluorescence intensities of SPNs at indicated doping amount of NCBS. PEG-*b*-PPG-*b*-PEG was used to coprecipitate with SP2 and NCBS at various doping amount (0%, 1%, 2.5%, and 5%) to obtain water-soluble SPN2-NCBS0, SPN2-NCBS1, SPN2-NCBS2.5, SPN2-NCBS5, and SPN2-NCBS10, respectively. (c) Schematic illustration of penetration study of SPN2-NCBS5. (d) Afterglow luminescence and fluorescence images of the SPN2-NCBS5 solution through a living mouse. (e) SBRs for indicated images in d. (f) Schematic illustration of tumor imaging. (g) Afterglow luminescence (left panel) and fluorescence (right panel) images of tumor in living mice at $t = 2$ h post-injection of SPN2-NCBS5. (h) SBRs for tumor imaging in living mice as a function of time. (i) Schematic illustration of the design of SPN2-thiol for biothiol-activatable afterglow imaging. An amphiphilic oligomer $C_{18}PEG_{12}$ -DNBS was used to coprecipitate with SP2 and NCBS to obtain water-soluble SPN2-thiol. (j) Representative afterglow luminescence images of mice with indicated treatment, followed by administration of SPN2-thiol *via* tail vein at $t = 20$ min

later. Intensities values are the mean $\pm$ s.d. for $n = 3$ mice. (k) SBRs for indicated imaging of liver as a function of time. Reprinted with permission.^[69] Copyright 2017, Nature Publishing Group.

Oxidative stress induced by drug challenge and thereafter depletion of antioxidants in the liver are concurrent early events in hepatotoxicity.^[120] Biothiols including GSH, cysteine (Cys) and homocysteine (Hcy) constitute a major portion of the total body antioxidants that defend against oxidative stress. Thus, real-time *in situ* imaging of biothiol levels can be a feasible way to evaluate drug-induced hepatotoxicity. To achieve this, Pu's group developed an activatable afterglow probe (SPN2-thiol) for biothiol imaging. SPN2-thiol had an electron-withdrawing quencher (2,4-dinitrophenylsulfonyl, DNBS) on its surface, and thus efficient electron transfer from core to DNBS occurred, leading to the quenched afterglow at its initial state. In the presence of biothiols (GSH, Cys and Hcy), the sulfonamide bond on the surface of SPN2-thiol was cleaved, DNBS was subsequently released from the nanoparticle surface, and the electron transfer was consequently prevented, triggering afterglow turn-on (**Figure 7i**). SPN2-thiol exhibited good selectivity towards biothiols, particularly Cys with a LOD of 0.60 μ M. The imaging ability of SPN2-thiol was further validated in mouse model to detect APAP-induced hepatotoxicity. APAP bioactivation induced oxidative and nitrosative stress and in turn consumption of biothiols. The afterglow signals for APAP-treated mice were 1.99-fold lower than that for saline-treated control mice at $t = 2$ h post-injection of SPN2-thiol (**Figure 7j, k**). By contrast, both inhibition of APAP bioactivation using (1-ABT and DCE) and scavenging of ROS using the antioxidant drug (*N*-acetyl-*L*-cysteine, NAC) led to the recovery of afterglow signals to the basal level (**Figure 7j, k**). Moreover, the SBRs of afterglow imaging were ~ 25 -fold higher than that of NIR fluorescence at all time points (**Figure 7k**), confirming the higher sensitivity of afterglow imaging.

4. Photoacoustic Imaging

PA imaging is an emerging hybrid modality that combines optical excitation with ultrasonic detection.^[18, 72, 121-128] Unlike fluorescence imaging that has the shallow penetration depth limited by photon–tissue interactions, the imaging depth of PA is mainly determined by the penetration capability of excitation photons because ultrasonic signals possess minimal attenuation due to negligible sound scattering in biological tissues. Thereby, PA imaging breaks through the imaging depth limit of photons, and achieves several centimeters of tissue penetration depth (**Figure 1c&Table 1**).^[129] Moreover, because of the availability of ultrasound modality, PA imaging can simultaneously provide anatomical and molecular imaging in real time.

Scheme 4. Chemical structures of representative SPs and SMs used for PA imaging.

4.1. Activatable PA Imaging

Endogenous (i.e., hemoglobin and melanin) and exogenous agents (i.e., ICG and MB: methylene blue) have been used for many PA imaging applications, such as brain and sentinel lymph nodes imaging.^[121, 130-132] Because the signal relies on the concentration difference between normal and diseased tissues, those agents generally provide a low SBR and are less capable of correlating the signal with complexed biological and pathological processes. Thus, activatable PA probes have been developed, which can respond to biomolecular targets or events of interest by sending out distinct PA signals.^[133-135]

To date, PA probes based on NIR dyes,^[136-138] metallic nanoparticles (e.g., gold nanorods),^[139-147] carbon nanotubes,^[148-150] two-dimensional (2D) nanomaterials,^[151-153] QDs,^[154] porphyrins,^[155-159] up-conversion nanoparticles,^[160, 161] genetically encoded fluorescent proteins,^[162-166] and organic semiconducting agents^[27, 36, 104] have been developed for imaging of diseased tissues, enzymes, chemical mediators, and physiological indexes. The activatable PA probes based on organic semiconducting agents are generally designed to contain biomarker-responsive sensing component and inert optical component (**Figure 8a** and **Table 3**). Up to now, these activatable PA probes have been developed for imaging of ROS^[36, 102, 103] and pH.^[28]

By virtue of the higher photostability and ROS resistance than other agents such as organic dyes, gold nanoparticles and QDs, poly(cyclopentadithiophene-*alt*-benzothiadiazole) (SP8, **Scheme 4**) was transformed into SPN8 as an activatable PA probe for ROS imaging by co-precipitation with the ROS-sensitive dye (IR775S) in the presence of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, **Scheme 2b**).^[36] The PA spectrum of SPN8 showed approximately equal intensities at 700, 735, and 820 nm. Upon treatment with ONOO⁻ or ClO⁻, the PA peak at 700 nm assigned to SP8 remained nearly the same, while the peaks of IR775S at 735 and 820 nm significantly decreased, leading to the ratiometric PA signal

(PA₇₀₀/PA₈₂₀) for ROS detection (**Figure 8b**). Such a spectral change was attributed to ROS-induced decomposition of IR775S. The probe (SPN8) was tested in murine model of zymosan-induced acute edema (**Figure 8c**). At 120 min post-injection of SPN8, PA₇₀₀/PA₈₂₀ for zymosan-treated mice (2.7 ± 0.31) was 1.9-fold higher than that for control mice (1.4 ± 0.22) (**Figure 8d**). Thus, this study confirmed that SPN8 could be applied for activatable PA imaging of ROS.

Figure 8. Activatable PA molecular imaging. (a) Schematic illustration of SPs-based activatable probes (SPNs) for PA imaging. RC: responsive component. (b) Representative PA spectra of SPN8 (5 $\mu\text{g/mL}$) before and after treatment with 5 μM ROS. (c) PA/Ultrasound overlaid images of regions in the thigh of living mice after intramuscular injection of SPN8 (3 μg , 50 μL). The regions of thighs were pretreated with saline (i) or zymosan (ii) for 20 min before SPN8 injection. (d) The ratiometric PA signals ($\text{PA}_{700}/\text{PA}_{820}$) as a function of time post-injection of SPN8. *Statistically significant difference in $\text{PA}_{700}/\text{PA}_{820}$ between two groups at all time points ($p < 0.05$). (e) Schematic illustration of the SMN-based activatable nanoprobe for pH sensing. (f) PA spectra of SMN at pH = 7.4, 6.4, or 5.5. (g) Ratiometric images ($\Delta\text{PA}_{680}/\Delta\text{PA}_{750}$) of xenograft HeLa tumor in mouse model before and 6 h post-injection of SMN16 (25 $\mu\text{g}/\text{mouse}$). (h) Quantification of ratiometric PA signals ($\Delta\text{PA}_{680}/\Delta\text{PA}_{750}$) as a function of time post-injection of SMN16. **No statistically significant difference in $\Delta\text{PA}_{680}/\Delta\text{PA}_{750}$ between 3 and 6 h ($p > 0.05$, $n = 3$). (b)–(d) Reprinted with permission.^[36] Copyright 2014, Nature Publishing Group. (f)–(h) Reprinted with permission. Copyright 2016,^[28] WILEY–VCH Verlag GmbH & Co. KGaA, Weinheim.

To increase the specificity of activatable PA probes towards a certain ROS, Pu's group designed an organic SM nanoprobe (SMN14) composed of a boronate-caged boron-dipyrromethene (BODIPY) dye (BBD, SM14, **Scheme 4**) and a bulky borane (TPB: triphenylborane, or TPBF: tris(pentafluorophenyl)borane, **Scheme 2a**).^[102] SM14 was a dye sensitive to both ONOO^- and H_2O_2 , while the bulky borane was the blocking agent that only could be consumed by ONOO^- . Thus, large excess of the bulky boranes in SMN14 protected SM14 from oxidation by H_2O_2 , leading to the high specificity towards ONOO^- . Upon treatment with ONOO^- , the PA signal of SMN14 at 750 nm increased greatly while the signal at 680 nm remained nearly unchanged, leading to the ratiometric PA signal ($\text{PA}_{750}/\text{PA}_{680}$) towards ONOO^- . The imaging capability of SMN14 was also validated for *in vivo* PA imaging of ONOO^- in the tumor of living mice.

Similarly, an activatable and degradable SM nanoprobe (SMN15) was developed for *in vivo* PA imaging of ClO^- .^[103] SMN15 was designed to contain SM15 (**Scheme 4**) as a water-soluble nanocarrier to encapsulate ROS-inert NCBS within it. In the presence of ClO^- , the

phenothiazine in the backbone of SM15 was oxidized and degraded, while NCBS remained intact, resulting in the decrease of PA intensity at 680 nm but nearly no change at 780 nm. With the high sensitivity and selectivity towards ClO^- , SMN15 was successfully used for ratiometric PA imaging of ClO^- in the xenografted tumors of living mice.

Apart from ROS detection, Pu's group developed an activatable PA nanoprobe (SMN16) for *in vivo* ratiometric imaging of pH.^[28] pH is an important physiological factor that plays a pivotal role in maintaining the homeostasis of living organism, and detection of aberrant pH is thus imperative to diagnosis, monitoring, and prognosis of various diseases ranging from inflammation,^[167] cancer,^[168] to Alzheimer's disease.^[169] The SMN16 was designed to consist of a SM (F-DTS, SM16, **Scheme 4**) to act as the inert PA matrix and a BODIPY dye (pH-BDP, **Scheme 2a**) to serve as both PA enhancer and pH sensor. The photoinduced electron transfer (PET) quenching of SMN16 and in turn the PA enhancement of SMN16 (**Figure 8e**). A hydroxyl group was designed on the backbone of pH-BDP, which could undergo protonation upon acidification. Thus, the PA intensity at 750 nm decreased significantly while the intensity at 680 nm barely changed with pH decreasing from 7.4 to 5.5 (**Figure 8f**). Because the PA signal of SMN16 was amplified by 3.1 folds relative to that of non-doped SM16 nanoparticles, the injection dosage was minimized to 25 $\mu\text{g}/\text{mouse}$, lower than that for carbon nanotube (~ 40 $\mu\text{g}/\text{mouse}$)^[148] and SPN8 (50 $\mu\text{g}/\text{mouse}$).^[36] The *in vivo* ratiometric PA signals of SMN16 for ratiometric imaging of pH. ADDIN EN.CITE <record><rec-number>224</rec-number><foreign-keys><key app="EN" type="record">224</key></foreign-keys></record> post injection (**Figure 8g,h**), allowing for pH estimation in the tumor of living mice.

4.2. Strategies for Amplified PA Imaging

Imaging sensitivity determines the dosage of imaging agents, and thus the dosage can be lowered down with increasing sensitivity, potentially mitigating the toxicity issue. To increase the PA brightness of SPNs, a series of copolymers (SP9–11, **Scheme 4**) were synthesized to

comprise the same electron deficient diketopyrrolopyrrole (DPP) and three different electron donating units to form the electron D–A alternating backbone structures.^[104] The polymers were coprecipitated with DSPE–mPEG2kDa (**Scheme 2b**) to form the SPNs (SPN9–11). Among SPN9–11 and SPN8, the PA signal of SPN11 was $\approx$ 5.40, 3.70, and 3.20 times brighter than that of SPN10, SPN9, and SPN8, respectively; while SPN8 had the strongest fluorescence relative to other SPNs, which was 10, 20, and 54 times higher than that of SPN9, SPN10, and SPN11, respectively. Such opposite tendencies of fluorescence and PA brightness complied with the underlying photophysical mechanisms. The excitation energy absorbed by the chromophore is generally dissipated through the following pathways: i) fluorescence emission, ii) generation of heat through vibrational relaxation (thermal deactivation), and iii) intersystem conversion to long-lived species (e.g., phosphorescence).^[170] PA property is based on the PA effect that measures the thermal deactivation (pathway (ii)) of the excited chromophore. As these SPNs are not phosphorescent, the pathway (ii) competes with pathway (i) to determine the final PA brightness. Thus, SPN11 with the strongest D–A backbone structure and narrowest bandgap favored nonradiative deactivation most and thus had the lowest fluorescence but the highest PA brightness among these nanoparticles.

The structure–property study implies that the fluorescence quenching is the way to enhance the PA intensities of SPNs.^[104] Thus, SPs with self–quenched fluorescence were developed by incorporating an electron–deficient unit, benzothiadiazole (BT), into the polymer backbone of poly{3–(5–(9–hexyl–9–octyl–9H–fluoren–2–yl)thiophen–2–yl)–2,5–bis(2–hexyldecyl)–6–(thiophen–2–yl)pyrrolo [3,4–c]pyrrole–1,4(2H,5H)–dione} (PDPPF, SP12, **Scheme 4**). As such, photoinduced electron transfer was promoted within the BT-containing PDPPFs, leading to an increased non–radiative decay and thus heat generation upon light irradiation.^[105] The SPs with different doping amount of BT in the polymer backbone including SP12 (0% BT), SP13 (5% BT), and SP14 (10% BT), were coprecipitated with an amphiphilic triblock copolymer (PEG–*b*–PPG–*b*–PEG, **Scheme 2b**) to obtain SPN12, SPN13,

and SPN14, respectively. As expected, SPN14 displayed 1.14 and 1.7-fold higher PA intensity relative to those of SPN13 and SPN12, respectively.

Figure 9. Doping-amplified PA imaging. (a) Schematic illustration of PA amplification for SPNs through photoinduced electron transfer. (b) Fluorescence (left) and PA (right) images of SPN solutions in tubes. (c) IR thermal images of SPN solutions after laser irradiation for 300 s. (d) PA images of tumor at $t = 6$ h after systemic administration of SPN8-F0 (left) or SPN8-F20 (right) (200 μ L, 100 μ g/mL). (e) Quantification of PA intensities at 750 nm as a function of post-injection time of SPN8-F0, SPN8-F20, or saline. Reprinted with permission,^[27] Copyright 2016, American Chemical Society.

To avoid the time-consuming synthesis, a facile intraparticle molecular orbital engineering approach was introduced to enhance the PA brightness of SPNs.^[27] SP8 was coprecipitated with (6,6)-phenyl-C71-butyric acid methyl ester (PC70BM, **Scheme 2a**) at the amount of 0, 4, 10, 20, and 40 w/w% to yield SPN8-F0, SPN8-F4, SPN8-F10, SPN8-F20, and SPN8-F40, respectively. The energy levels between SPN8 and PC70BM were matched to favor photoinduced electron transfer (**Figure 9a**), resulting in quenched fluorescence and thus

amplified PA brightness. With increased doping amount of PC70BM, the fluorescence intensities decreased rapidly, the solution temperature increased gradually, and the PA intensities increased obviously (**Figure 9b–c**). Among these nanoparticles, SPN8–F20 with the highest PA brightness was used for PA imaging of tumor in living mice. The result showed that SPN8–F20 had 1.8–fold higher PA brightness than that of SPN8–F0 at $t = 6$ h post-injection (**Figure 9d–e**).

Figure 10. Surface-engineering amplified PA imaging. (a) Schematic illustration of PA amplification for SPNs through surface coating. (b) Representative PA images of SPN15 and SPN15–SiO₂ at different concentrations. (c) Simulation of PA signal using the wave equation of PA and the Green's function method. (d) Schematic illustration of the COMSOL simulation for SPN15 (left) and SPN15–SiO₂ (right). (1 and 2 are the two test points for measuring temperature and PA signal). (e) Simulation of the temperature near the surface of the SPNs (point 1) after a temporal laser pulse. (f) Simulation of PA signals near the surface

of SPNs (point 1) after a temporal laser pulse. Reprinted with permission,^[106] Copyright 2017, Elsevier.

Many PA SPNs are non-fluorescent, and thus not eligible for signal amplification through photoinduced electron transfer. An alternative surface engineering approach was developed to amplify PA brightness based on the heat dissipation mechanism.^[106] SPN15 was developed into the silica-coated counterpart (SPN15-SiO₂) (**Figure 10a**). The PA signal of SPN15-SiO₂ was ~1.4-fold higher than that of SPN15 at various concentrations (**Figure 10b**). The theoretical simulation was carried out to reveal the underlying mechanism (**Figure 10c-f**). The interface between the silica layer and water in SPN15-SiO₂ had higher heat interfacial conductance than that of the SP core and water in SPN15. According to formula $\Delta T = RQ$ (where Q is the heat flux through the interface and R is the interfacial resistance),^[171] the higher interfacial conductance (lower R) favored the faster heat dissipation from nanoparticle to the surrounding water. Thus, at point 1 (7 nm away from the center of nanoparticle), SPN15-SiO₂ had a sharper increasing temperature and thus a lower peak temperature at the interface than SPN15 (**Figure 10d-e**). The final equation of PA intensity was performed through heat transfer simulation conducted by COMSOL multiphysics (COMSOL Inc, USA.), followed by coupling to acoustic module with justification by wave equation and the Green's function method (**Figure 10c**). The analytic equation indicated that the PA amplitude was positively correlated with the heating function (A(r) and H(t)). Thus, the higher interfacial heat conductance of SPN15-SiO₂ resulted in a sharper temperature increase, and consequently led to a larger PA signal relative to SPN15 (**Figure 10e,f**).

Size-dependent heat dissipation also allowed us to design a self-assembly approach to turn on the PA signals for *in vivo* imaging of ROS.^[107] A SM zinc-tetra(2-((1-(3-amino-3-oxopropyl)-1H-1,2,3-triazol-4-yl)-methoxy)-2-oxo-1-(4-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)oxy)phenyl)ethyl methoxy poly(ethylene glycol)succinate)

phthalocyanine (PCBP, SM17, **Scheme 4**) was designed and synthesized to have a hydrophobic phthalocyanine core and four hydrophilic PEG branches linked by ROS responsive phenylboronic acid pinacol ester groups. In the presence of ROS (i.e., H_2O_2 and ONOO^-), the aryl boronic ester group in PCBP was oxidized, leading to the release of boronic pinacol ester and subsequent cleavage of hydrophilic PEG *via* a self-immolative process. Because the residual part of phthalocyanine segment had high hydrophobicity, it spontaneously self-assembled and regrew into large nanoparticles (**Figure 11a**). Such ROS-induced nanoparticle regrowth eventually resulted in enhanced PA signals due to increased heat transfer (**Figure 11b**).^[158, 172] PCBP was used to detect elevated ROS levels in the tumor after treatment with *D,L*-buthionine-(*S,R*)-sulphoximine (BSO). The PA signal in the tumor for BSO-treated group was 2.0-fold higher than that for untreated group at 8 h post-injection (**Figure 11c, d**), proving the utility of PCBP for monitoring the biomarker concentration along with cancer chemotherapy.

Figure 11. Self-assembly enhanced PA imaging. (a) Schematic illustration of PA amplification for SM probe (SM17) through self-assembly. (b) PA amplitudes at 700 nm as a function of the average diameters of SM17. (c) Representative PA maximum intensity projection images of tumors for BSO-pretreated and untreated mice ($n = 3$) after intravenous

injection of SM17 (30 μg). (d) PA amplitude increment at 700 nm (ΔPA_{700}) as a function of post-injection time of SM17 in BSO-pretreated and untreated mice. The error bars represent the standard deviation of three separate measurements. Reprinted with permission.^[107] Copyright 2017, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

4.3. NIR-II PA Imaging

Although it had been well validated that fluorescence imaging in NIR-II window outperformed that in NIR-I window, it was unclear if shifting PA imaging from NIR-I to NIR-II window could further improve the imaging performance. Due to the lack of NIR-II absorbing contrast agents, PA imaging were often carried out in the NIR-I window mainly.^[109]

To compare PA imaging in NIR-I and NIR-II, poly(diketopyrrolopyrrole-*alt*-thiadiazoloquinoline) (SP16, **Scheme 4**) that absorbed at both NIR-I and NIR-II regions was synthesized.^[108] SP16 was designed to have a D-A₁-D-A₂ structure, which had smaller bandgap as compared with SP11 with a D-A alternating backbone structure. SP11 and SP16 were coprecipitated with PEG-*b*-PPG-*b*-PEG to yield water-soluble SPN11-I and SPN16-II, respectively. SPN16-II had a broadband absorption spectrum ranging from visible to NIR-II region with the maximum at 1253 nm due to the enhanced charge transfer within the backbone of SP16, while SPN11-I only had NIR-I absorption with the maximum at 744 nm (**Figure 12a**). In addition, SPN16-II had nearly identical PA amplitudes at 750 and 1064 nm (**Figure 12b**), allowing to directly compare PA imaging at both wavelengths. Due to the lower tissue auto-PA background in the NIR-II window, the SBR of PA imaging at 1064 nm was higher than that at 750 nm at all depths (**Figure 12c-e**), particularly 1.4-time higher at the imaging depth of 3 cm. With such a higher deep-tissue imaging ability, SPN16-II was further used for *in vivo* imaging of brain vasculatures in living rats (**Figure 12f**). The SBR of PA imaging at 1064 nm was 1.5-time higher than that at 750 nm. Thereby, this study not only showed the first broadband absorbing organic contrast agent applicable for PA imaging in

both NIR-I and NIR-II windows but also proved the advantages of NIR-II light over NIR-I light in PA imaging.

Figure 12. NIR-II PA imaging. (a) Absorption spectra of SPN11-I and SPN16-II (40 µg/mL). (b) PA spectra of SPN11-I and SPN16-II (200 µg/mL). (c) Photograph of the agar gel phantom containing SPN16-II at different concentrations (1–4: 0.05, 0.2, 0.5, and 1 mg/mL, respectively). (d) PA images of the agar gel phantom containing SPN16-II solutions acquired in both NIR windows at different depths. (e) SBR for PA images of [SPN16-II] = 1 mg/mL at 750 or 1064 nm as a function of the depth of chicken breast tissue. (f) Representative PA images of rat cortex at 70 min post-injection of SPN16-II at 750 and 1064 nm and the representative photograph after PA imaging and removal of skin. (g) SBR for images in f. Reprinted with permission.^[108] Copyright 2017, American Chemical Society.

Similarly, other NIR-II PA agents such as thienoisindigo (TII)-based SP17 (**Scheme 4**) were developed for PA imaging.^[109] The PA signal of the corresponding nanoparticle (SPN17) at 1064 nm could be detected through a 5.3 cm chicken breast tissue at a low concentration of

40 $\mu\text{g mL}^{-1}$. SPN17 was also used for tumor imaging and the PA signal of tumor at 1300 nm after the administration of nanoparticles was 13.3-fold higher than that of the control without nanoparticle administration. In addition, the phosphorus phthalocyanine (SM18, **Scheme 4**) was tested for NIR-II PA imaging, and its PA signal could be detected through the chicken breast tissue at the depth of 11.6 cm. This is the highest penetration depth reported for NIR-II imaging so far.^[73] Note that because the maximum permissible exposure (MPE) increases with the laser wavelength (the MPE for skin is 100 and 25 mJ/cm^2 for 1064 and 750 nm laser, respectively), the imaging depth and SBR of NIR-II PA imaging can be further improved.

5. Conclusions and Outlook

Deep-tissue molecular optical imaging including NIR-II fluorescence, self-luminescence, and PA imaging provides unprecedented advantages over traditional NIR fluorescence imaging, allowing for *in vivo* detection of biological and pathological processes at improved tissue penetration depth, depth-to-resolution ratio and SBR. Compared with traditional NIR fluorescence imaging, NIR-II fluorescence imaging has further reduced light scattering, self-luminescence imaging avoids tissue autofluorescence, and PA imaging detects phonons rather than photons upon light excitation. Because there are few endogenous molecules that can absorb or emit in NIR region, exogenous contrast agents are required to generate signals for these optical imaging acquisition. In particular, smart activatable probes are used to correlate signals with the concentration or activity of biomarkers. Among many optically active materials, organic semiconducting agents not only possess equal or even better optical properties, but also have the versatility to be developed into different structures for molecular targeting, sensing and even therapy in living systems. Moreover, there are few categories of materials that can be easily finetuned to fit the different optical requirements for NIR-II fluorescence, self-luminescence, and PA imaging. The intrinsic advantages of organic semiconducting agents coupled with deep-tissue optical imaging methods have permitted

ultrasensitive detection of tissues (tumors and lymph nodes et al.) and biomarkers (enzymes, reactive species and biothiols et al.), real-time accurate monitoring of physiological indexes and functions (pH and cardiac cycle et al.) in living mice.

Because the application of organic semiconducting agents in deep-tissue molecular imaging is in its infancy, there are many opportunities for optimization and exploitation in preclinical research and clinical translation. First, the highest QY of NIR-II fluorescent organic semiconducting agents is reported to be ~11%, and this can be increased through improved molecular engineering or new nano-formulation method. Second, new approaches towards self-luminescence in addition to ultra-long phosphorescence and bio/chemiluminescence are highly wanted to improve the performance of self-luminescence imaging including increased half-life time and red-shifted emission wavelength (from NIR-I to NIR-II). Third, there are very few NIR-II fluorescent/PA activatable probes,^[173] which is mainly caused by the difficulty in chemical modification of current organic semiconducting agents. With the emergence of new NIR-II emitting and absorbing dyes, it is envisioned that more and more NIR-II fluorescent/PA activatable probes will be developed to fully unlock the potential of NIR-II optical imaging in medicine, particularly early diagnosis.

Because of the hydrophobicity of π -conjugated components in organic semiconducting agents, they can be facilely transformed into water-soluble nanoparticles through nano-precipitation or nano-emulsion. Such straightforward preparation allows to incorporate other components into those nanoagents, leading to multi-functionalization. For instance, doping of other agents such as MRI agents can lead to multimodal complementary imaging, while co-precipitation with therapeutic agents can produce theranostic agents with chemotherapy, immunotherapy or gene therapy capability. Moreover, the intrinsic photodynamic and photothermal features of organic semiconducting agents provide the feasibilities for them to act as remote controller for imaging-guided photoactivation of specific biological functions such as thermosensitive protein ion channels and gene transcription.^[174-177]

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Keyword: contrast agents, nanoparticles, photoacoustic imaging, second near-infrared fluorescence imaging, self-luminescence imaging

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Second Near-Infrared Fluorescence, Self-luminescence, and Photoacoustics

ToC figure

Molecular optical imaging including second near-infrared fluorescence, self-luminescence, and photoacoustic imaging provides enhanced tissue penetration depth and detection sensitivity as compared with traditional optical imaging. This Progress Report summarizes the recent development of organic semiconducting agents for these deep-tissue optical imaging modalities, and highlights the molecular design guidelines to amplify the signal intensity, improve the sensing specificity and enhance the tissue penetration.