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Emerging Designs of Activatable Photoacoustic Probes for Molecular Imaging

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ABSTRACT: Photoacoustic (PA) imaging as a new hybrid imaging modality holds great promise for real-time *in vivo* monitoring of biological processes with deep tissue penetration and high spatial resolution. To endow PA imaging with the ability to provide real-time molecular information at disease sites, molecular probes that can change their PA signals responding to the target or event of interest have to be developed. This review focuses on the recent development of smart activatable PA probes for molecular imaging. A brief summary of PA imaging agents is given first, followed by the detailed discussion of the contemporary design approaches toward activatable PA probes for different imaging applications. At last, the current challenges are highlighted.

KEYWORDS: activatable probe, photoacoustic imaging, molecular imaging, nanoparticles, cancer

INTRODUCTION

Photoacoustic (PA) imaging is an emerging hybrid imaging modality that can provide three-dimensional images with real-time correlation, clinically relevant depths and relatively high spatial resolution using nonionizing radiation.¹⁻⁶ In the course of imaging, a nanosecond laser pulse is used to irradiate biological tissues of interest, which induces photons to propagate inside the tissue and subsequently leads to a localized temperature rise in a short time frame. The results are the transient thermoelastic expansion and in turn the generation of acoustic waves that can be captured by broadband ultrasonic transducers and converted into images.^{7, 8} Such an imaging process endows PA imaging with the combined merits of optical and acoustic methods including sensitive optical absorption contrast and minimized acoustic scattering. Thus, PA imaging not only has higher spatial resolution and deeper imaging depth than optical imaging but also exhibits better tissue contrast than ultrasound imaging. As most biological and pathological processes do not have detectable variations in PA contrast, development of PA imaging agents is critical, which partially defines its scope in medical applications and clinical usages.^{9, 10}

Smart activatable probes play a crucial role in furthering our understanding of biology and implementation of early diagnosis. As compared with “always on” conventional contrast agents, activatable probes are designed to send out specific signals in response to biomolecular targets or events of interest.¹¹⁻¹⁶ For instance, activatable probes are able to translate enzymatic activity into measurable and quantifiable signals both *in vitro* and *in vivo*. The advantages of activatable probes include low background noise, real-time correlation between signals and diseases status, and

concentration-independent contrast. Many optical (fluorescent or bioluminescent) and magnetic resonance imaging activatable probes have been developed and utilized for the detection of enzyme and biological related mediators in living animals.¹⁷⁻¹⁹ Moreover, imaging-guided surgery also benefited from the utilization of activatable probes due to their high signal-to-noise ratios.²⁰⁻

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Despite the important role of activatable probes in imaging, most current PA imaging agents rely on enhanced accumulation at diseases site *via* enhanced permeability and retention (EPR) effect or lock-and-key molecular recognition between the recognition groups and the overexpressed receptors to obtain a contrast for imaging.²⁴⁻²⁷ Thus, those accumulation probes generally provide low signal-to-noise ratios and are unable to report molecular activities. In contrast, activatable PA probes have been less developed so far, probably due to their relatively complex design and synthesis.

The publications and citations regarding PA imaging agents are increasing in number, and there are several reviews summarizing the development of PA imaging agents in a general way.^{3-9, 28-42} The focus of this review is instead to highlight the development of activatable PA probes for molecular imaging, pinpoint their contemporary molecular design strategies, and discuss their biological applications. As follows, the classification of PA imaging agents with an emphasize on the difference in their molecular constructs will be described first. Then, the chemistry and applications of activatable PA probes will be discussed in details. At last, summary and outlook will be given to further stimulate the research interests in the development of activatable PA probes so as to fully explore the potential of PA imaging in biology and medicine.

CLASSIFICATION OF PA IMAGING AGENTS

Because PA signals are mainly determined by the heat generation after light absorption, imaging agents with strong absorption in the near-infrared (NIR) spectral region are desired for PA imaging. Until now, many materials have been developed into PA imaging agents, which include small-molecule NIR dyes,⁴³⁻⁴⁵ metallic nanoparticles (e.g., gold nanorods),^{25, 46-53} carbon nanotubes,^{27, 54, 55} two-dimensional (2D) nanomaterials,⁵⁶⁻⁵⁸ quantum dots (QDs),⁵⁹ porphyrins,^{14, 60-63} up-conversion nanoparticles,^{64, 65} genetically encoded fluorescent proteins,⁶⁶⁻⁷⁰ and semiconducting polymer nanoparticles (SPNs).⁷¹⁻⁷³ According to the interactions between the probe and the molecular target, they can be categorized into three types: nontargeted, targeted and activatable imaging agents as shown in **Figure 1**.

Nontargeted PA imaging agents are endogenous or exogenous agents without any targeting moiety. Hemoglobin and melanin, which have much stronger absorption than normal tissue in both the visible and the NIR regions, are the two most important endogenous agents for enhanced PA imaging. Hemoglobin has been applied for PA imaging in a number of imaging applications such as mapping brain,^{1, 74} delineating tumor vasculature development and regression after therapy,⁷⁵⁻⁷⁷ monitoring hemodynamic activities,⁷⁸ and detecting low-speed blood flow in deep microcirculation.⁷⁹ Melanin, a natural pigment existing in skin, hair and eyes, is mainly used for the diagnosis, treatment planning and evaluation of skin melanoma by PA imaging.^{2, 80} Besides, its derivative has been applied for monitoring tumor vascular changes after therapy.⁸¹

Figure 1. Schematic illustration of design principles of (a) nontargeted, (b) targeted, and (c) activatable PA imaging agents. (a) Nontargeted PA imaging agents are NIR-absorption endogenous or exogenous agents without any targeting moiety. (b) Targeted PA imaging agents are composed of NIR-absorption components and targeting moieties such as antibodies and small-molecule ligand. (c) Activatable PA imaging agents usually contain a component that can response to a specific target or biological event by changing their molecular composition. The activatable PA imaging agents typically generate changed PA signals through i) aggregation of chromophore after enzymatic cleavage induced self-assembly; ii) retention of one chromophore and clearance of the other one after enzymatic cleavage; or iii) oxidation or protonation of the chromophore.

Despite using endogenous agents for imaging applications are safe and have the ability to reveal the true physiological conditions, it can only be applied for merely limited range of biological processes and is difficult to provide efficient signal to noise ratio for complexed biological

applications, such as cancer research. Thus, exogenous imaging agents are commonly used to enhance contrast. For instance, US Food and Drug Administration (FDA)-approved small molecular dyes indocyanine green (ICG) with the maximum absorption at 790 nm was used to image rat brains⁸² and map sentinel lymph nodes (SLNs).⁸³ Another FDA-approved small molecular dye, methylene blue (MB), was also used to detect the SLNs.⁸⁴ Free dyes without conjugating to other chemicals could potentially encounter the issues such as poor hydrophilicity, photoinstability, hard to functionalization and short circulation time.³⁹ Nanoparticles thus were used as the non-targeted PA imaging agents. For instance, gold nanocages and single-walled carbon nanotubes (SWNTs) were used for noninvasive PA mapping of SLNs.⁸⁵ The SLNs were successfully imaged *in vivo* with high contrast-to-noise ratio (>80) and good resolution (~500 μm). QDs were used as multifunctional PA agents for multimodal (PA and fluorescence) imaging and potentially for photothermal therapy.⁵⁹ 2D nanomaterials including graphene,^{86, 87} MoS₂,⁸⁸ WS₂,⁵⁸ and TiS₂,⁸⁹ are another class of nontargeted PA imaging agents.

Targeted PA imaging agents are composed of targeting moieties such as antibodies⁹⁰ and small-molecule ligand^{26, 91} in addition to the NIR absorption components. Thus, targeted PA imaging agents enable the readout of a specific biomarker or biological process at different stages of diseases. Many kinds of targeted PA imaging agents were developed, such as antibody conjugated gold nanoparticles,⁹² cyclic arginine–glycine–aspartic acid (RGD) or folate conjugated SWNTs,^{26, 27, 93, 94} and anti-HER-2 antibody conjugated ICG-doped nanoparticles.⁹⁵ In principle, all the aforementioned nontargeted PA imaging agents can be transformed into targeted PA imaging agents by conjugating with recognition groups.

Activatable PA imaging agents usually contain a component that can response to a specific

target or biological event by changing their molecular composition (**Figure 1c**), which consequently elicits the signal changes such as increased or ratiometric PA signals. As compared with targeted PA imaging agents with the signal “always on”, activatable PA imaging agents can only be activated by specific biomolecular recognition or interaction, leading to improved imaging sensitivity and specificity with higher signal-to-noise ratios. Upon activation, the PA probes typically alter their absorption states through retention, clearance, degradation or enhancement of nonradiative relaxation of the chromophore. To date, activatable PA probes have been developed to image cancer-related proteases such as matrix metalloproteinases (MMPs),⁴⁴ and furin,⁹⁶ as well as some small-molecule chemical mediators.^{71, 97}

DESIGN AND APPLICATIONS OF ACTIVATABLE PA PROBES

Enzymes

In vivo imaging of enzyme activity holds great promises for many applications in both basic and translational researches from deciphering the enzyme function in biology to better detecting diseases.^{15, 98} However, existing activatable probes generally rely on fluorescence as the signal readout, which has the shallow tissue penetration issue and thus limits *in vivo* applications.¹³ To address this issue, several activatable PA probes have been developed for imaging enzyme activity based on the specific enzymatic cleavage to the targeted substrate including MMPs,^{44, 99-104} furin⁹⁶ and hyaluronidase (HAase).¹⁰⁵ Such an enzymatic cleavage is used to result in the separation of chromophores or initiating further self-assembly so as to afford changes in PA signals with deep tissue penetration.

MMPs

MMPs are a family of zinc-dependent proteinases enabling the degradation of extracellular matrix (ECM) components and play key roles in both physiological and pathological processes,¹⁰⁶⁻¹⁰⁸ such as inflammation, wound healing, arthritis, cardiovascular disease, and cancer.¹⁰⁹⁻¹¹¹ Basement membranes of tumor cells are degraded by MMPs, which not only induce tumor invasion and migration,^{112, 113} but also provide space for growth of new blood vessels and release many angiogenic factors.^{106, 107} Elevated expression of MMPs, especially MMP-2 (gelatinase), are related to tumor stage,¹¹⁴ invasiveness,¹¹⁵ metastasis,¹¹⁶ and angiogenesis.¹¹⁷ Thus, molecular imaging of MMPs will favour early diagnosis of cancer.¹¹⁸

Gambhir's group reported an activatable PA probe targeted for proteolytic enzyme MMP-2.⁴⁴ A MMP-2 specific activatable cell-penetrating peptide (ACPP) CeeeeXPLGLAGrrrrK (X is a 6-aminohexanoyl acid) was labeled with a black hole quencher 3 (BHQ3) and Alexa750 on each side forming the activatable PA probe, named as Alexa750-APP-BHQ3 (B-APP-A) for short. This probe emitted PA signals at 675 and 750 nm, attributing to BHQ3 and Alexa750, respectively. *In vitro* cleavage of B-APP-A by MMP-2 induced the separation of the two chromophores and thereafter the change in the absorption spectrum (**Figure 2b**). After cleavage, the PA signal of B-APP-A at 675 nm was slightly higher than that at 750 nm (**Figure 2c**), caused by the different properties between the heterodimer and the monomeric chromophores that existed before and after the cleavage, respectively. Because the accumulation of BHQ3 and diffusion of Alexa750 after enzyme-mediated cleavage could not occur in solution, the *in vitro* PA signals responding to MMP-2 were limited. Gambhir's group further applied the MMP-activatable PA probe Alexa750-CXeeeeXPLGLAGrrrrXK-BHQ3 (B-APP'-A, X is a 6-aminohexanoyl acid) for the detection of follicular thyroid carcinoma.⁹⁹ For comparison, they also synthesized a noncleavable control

probe (control probe) with the same peptide sequence but consisting of D-amino acids. The subtraction PA signals for the noncleavable control probe did not change over time; in contrast, the signals for the activatable probe (B-APP'-A) increased steadily over time and the subtraction signals at t=140 min post injection were approximately 1.7-fold higher than the background (Figures 2d&2e). The imaging results confirmed that BHQ3 conjugated to the cell-penetrating part of the activatable probe (B-APP'-A) accumulated in tumor after MMP-2 cleavage, while the other dye Alexa750 diffused away, leading to enhanced subtraction PA signals ($PA_{680\text{ nm}}-PA_{750\text{ nm}}$).

Figure 2. (a) Schematic illustration of the probe design for MMP imaging. (b) Absorption spectra of the activatable probe (Alexa750-CeeeXPLGLAGrrrrK-BHQ3, B-APP-A) before (solid line) and after (dashed line) MMP-2 cleavage. (c) PA imaging of B-APP-A at 675 and 750 nm before and after MMP-2 cleavage. (d) PA imaging of thyroid tumor at t = 140 min after intravenous injection of the activatable probe (Alexa750-CXeeeXPLGLAGrrrrXK-BHQ3, B-APP'-A) and the noncleavable control probe. (e) The subtraction PA signals of the activatable probe (B-APP'-A) and the noncleavable control probe as a function of post-injection time. Subtraction PA signal

= $[(PA_{680\text{ nm}}-PA_{750\text{ nm}})_{\text{postinjection}} / (PA_{680\text{ nm}}-PA_{750\text{ nm}})_{\text{preinjection}}]$. (a), (b) and (c) are reprinted with permission from reference 44, Copyright 2010, American Chemical Society. (d) and (c) are reprinted with permission from reference 99, Copyright 2013, American Association for Cancer Research.

Similarly, Razansky's and Zeebregts's groups studied an activatable PA probe, MMPSense 680 (a commercial probe, MMP-sensitive activatable fluorescent probe), for MMPs imaging using the multispectral optoacoustic tomography (MSOT).¹⁰⁰ Upon treatment of MMPs, the absorption spectra of MMPSense 680 altered with the change of the peak intensities at 635 and 675 nm due to the characteristic spectral change of the extinction coefficient between the activated and non-activated states of MMPSense 680. Such changes of PA multiple wavelengths were resolved and reconstructed into three-dimensional images, permitting mapping of MMP activity deep in the vulnerable plaque of intact human carotid specimens.

In addition to the above small-molecule dye based probes, inorganic nanoparticles have also been developed into activatable PA probes for MMP imaging. Liu's and Chen's groups designed an activatable probe, CuS-peptide-BHQ3 (CPQ), based on copper sulfide (CuS) nanoparticles (absorption at 930 nm) by conjugating BHQ3 (absorption at 680 nm) with an MMP-specific peptide linker (**Figure 3a**).¹⁰¹ *In vitro* enzymatic cleavage showed that the absorption of the CPQ at 630 nm decreased obviously after incubation with MMP-13 (**Figure 3b**). In comparison, no obvious absorption change was observed after incubation with MMP-13 in the presence of MMP inhibitor III (MMPI-III) or without MMP-13, indicating that CPQ responded selectively towards MMPs and also was stable in the absence of MMPs. SCC7 breast cancer cell line was chosen as tumor model due to its overexpression of MMP-13. After intratumoral injection of CPQ, the PA signal at 680 nm decreased over time while at 930 nm

showed nearly no change. This demonstrated that MMP13 cleaved the peptide and released BHQ3, leading to the decrement of PA signal at 680 nm and thus decreased the ratiometric PA signals (PA_{680}/PA_{930}) (**Figures 3c&3d**). In contrast, the injection of CPQ together with MMPI-III inhibitor, the PA signals at both 680 and 930 nm showed no significant change even at 24 h post injection, proving that the inhibitor could prevent enzymatic cleavage of the MMP-specific peptide (**Figures 3c&3d**). In a similar way, Sun group developed a gold nanorod (GNR) based PA probe by conjugating a IRDye800 labelled MMP-cleavable peptide linker to the surface of GNR.¹⁰² Before enzyme cleavage, the probe exhibited strong PA signals both at 670 and 800 nm; after enzyme cleavage, the GNRs still retained in cells with signal at 670 nm, while IRDye800 was washed out, leading to the decreased the ratiometric PA signals (PA_{800}/PA_{670}).

Figure 3. (a) Schematic illustration of the mechanism of the nanoparticle-based PA probe (CuS-peptide-BHQ3, CPQ) for MMP detection. (b) Time dependent absorption spectra of CPQ in the presence of MMP-13. (c) *In vivo* PA imaging of mice tumors after intratumoral injection of CPQ, or CPQ with MMPI-III co-injection recorded at 680 nm and 930 nm. (d) Quantification of the ratiometric PA signals (PA_{680}/PA_{930}) of the CPQ group and the CPQ/MMPI-III group at

different post-injection time. Reprinted with permission from reference 101, Copyright 2014, Ivyspring International Publisher.

In addition to cleavage/retention design, self-assembly was used for the development of activatable PA probes for gelatinase (MMP-2 and MMP-9) imaging. For example, Wang's group synthesized a gelatinase responsive probe **1** consisting of purpurin 18 (P18) as the NIR absorbing dye, an enzyme-responsive peptide linker Pro-Leu-Gly-Val-Arg-Gly (PLGVRG), and Arg-Gly-Asp (RGD) as the targeting ligand (**Figure 4a**).¹⁰³ The authors also synthesized control probe 2 (P18-PMGMRGRGD) with scramble peptide sequence which was not recognized by the gelatinase and probe 3 (P18-PLGVRGRDG) without targeting ligand towards $\alpha v \beta 3$ integrin compared with probe 1. The PA signals of probe 1 at 730 ± 1 nm increased 3.5 folds after 1 h incubation with gelatinase compared with that after 5 min incubation in agar phantoms, while no PA signal enhancement of probe 2 was observed under the same conditions (**Figure 4b**). The enhancement of PA signals was due to the fact that after gelatinase-mediated cleavage of the peptide, the assembly of the remnant P18 motif was initiated to form nanofibers *via* intermolecular π - π stacking. Such a self-assembly enhanced photothermal conversion efficiency, causing increased PA signals. *In vivo* evaluation was performed on $\alpha v \beta 3$ integrin and gelatinase-overexpressed U87 cells xenografted mouse model. The PA signals of tumor after intravenous injection of probe 1 showed an increasing, reached its plateau at 6 h, and still remained strong after 24 h post-injection (**Figures 4c&4d**). However, the PA signals of tumor after intravenous injection of probe 2 showed a decreased signal after 2 h injection. The difference between probes 1 and 2 indicated that the enhanced PA signals of 2 overtime were due to the assembly induced retention effect and the assembly induced signal

amplification. In addition, the stronger PA signals of probe 1 relative to probe 3 at each time point (**Figures 4c&4d**) indicated that the RGD targeting moiety also contributed to the enhanced PA signals. In addition, the same group also used such design to enhance tumor therapeutic efficacy, which would pave the way to develop novel functional nanomaterials for cancer theranostics.

Wang's group further demonstrated the design concept for imaging of bacteria in living mice. The probe contained three parts, pyropheophorbide- α (Ppa) as a signalling molecule, Pro-Leu-Gly-Val-Arg-Gly (PLGVRG) as an enzyme-responsive peptide linker, and vancomycin (Van) as a targeting ligand to bind Gram-positive bacterial cell walls.¹⁰⁴ Once the probe reached bacteria, peptide linker was cleaved and then the hydrogelator was released. This triggered the self-assembly of the residual parts of the probe into the twisted fibers, leading to increased PA signals.

Figure 4. (a) Schematic illustration of the self-assembly design of activatable probe for

gelatinase imaging. Activatable probe 1 (Purpurin 18-PLGVRGRGD) was hydrolysed by gelatinase first, then the assembly of the remnant P18 motif was initiated to form nanofibers *via* intermolecular π - π stacking, causing increased PA signals. (b) PA signals of probe 1 (10 μ M) and control probe 2 (Purpurin 18-PMGMRGRGD, 10 μ M) after 5 min and 1 h incubation with gelatinase (15 ng/mL) at 730 ± 1 nm. (c) PA imaging in transverse sections of tumor up to 24 h after intravenous injection with probe 1, 2, and 3 (Purpurin 18-PLGVRGRDG) (200 μ M, 200 μ L, PBS: DMSO = 95:5, v/v) or PBS (200 μ L (PBS: DMSO = 95:5, v/v)). (d) Quantification of PA signals in tumor site as a function of time until 24 h post-injection. Reprinted with permission from reference 103, Copyright 2015, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Furin

Furin, a critical regulator of tumor progression and metastasis, has been proven to be an effective drug target.¹¹⁹ The ability to noninvasively visualize furin and furin-like activity in living animals is of importance for cancer biology and medicine.¹²⁰ By employing a biorthogonal reaction,¹²¹ Gambhir's and Rao's groups developed an activatable PA probe, Ac-RVRR(SET)K-(Atto740)-2-cyano-6-aminobenzothiazole (ESOR-PA01), for furin imaging.⁹⁶ The ESOR-PA01 probe consisted of a short peptide sequence (i.e., RVRR) specific towards furin, two pro-reactive moieties for initiating biorthogonal condensation reaction, and a NIR absorber PA reporter molecule, Atto740. The disulfide bond of cysteine was first reduced by the reductive environment (i.e., glutathione, GSH). Then, furin specifically cleaved the peptide RVRR substrate followed by releasing the amino group of cysteine. At last, a biorthogonal condensation occurred between 1,2-aminothiol and 2-cyano group of 2-cyano-6-aminobenzothiazole (CABT) for the probes, resulting in the formation of dimers and oligomers containing Atto740. The formed dimers and oligomers further underwent aggregation to form nanostructures (**Figure 5a**). Such activation triggered

accumulation of the probe, leading to enhanced PA signals. ESOR-PA01 was tested for imaging of furin activity in living cells (**Figure 5b**). The PA signals of ESOR-PA01 after incubation with furin overexpressing MDA-MB-231 cells produced 2.6-fold higher than that with furin-deficient LoVo cells. However, when the MDA-MB-231 cells were co-incubated with furin inhibitors, it caused 2.2-fold decrease in the PA signals as compared with untreated one (**Figure 5b**). ESOR-PA01 was further used to detect furin activity in living mice, generating 2.7-fold significant increase in the PA signals in the presence of furin relative to the negative control probe (ESOR-PA02), AcRRRVK(SET)K(Atto740)-CABT (**Figures 5c&5d**). Besides, a 7.1-fold increase in the PA signals was generated by ESOR-PA01 in furin-positive MDA-MB-231 tumors as compared with furin-deficient LoVo tumors at t = 60 min post injection (**Figure 5e**).

Figure 5. (a) Schematic illustration for the design of oligomerizable activatable PA probe (AcRVRRC(SET)K-(Atto740)-2-cyano-6-aminobenzothiazole, ESOR-PA01) for imaging of furin. (b) Quantification of the PA signals of LoVo and MDA-MB-231 cells (in the presence or absence of furin inhibitor I or II (200 μ M)) in phantom imaging after incubation with ESOR-PA01 (2 μ M) for 3 h recorded at 740 nm. (c) Schematic diagram of PA imaging of furin-like activity in tumor-bearing mice. (d) Representative PA images of LoVo and MDA-MB-231 tumors after $t = 0$ and 60 min intravenous injection of probe ESOR-PA01 (25 nmoles). (e) Quantification of the PA signals increase at $t = 60$ min post-injection of probe relative to $t = 0$ min either from the furin probe (ESOR-PA01) injected mice carrying LoVo ($n = 4$) and MDA-MB-231 ($n = 4$) tumors, or the furin negative probe (AcRRRVC(SET)K(Atto740)-2-cyano-6-aminobenzothiazole, ESOR-PA02) injected mice carrying MDA-MB-231 tumors. $**P = 0.01$, $*P = 0.03$, n.s. $P = 0.08$; error bars, SD. Reprinted with permission from reference 96, Copyright 2013, American Chemical Society.

HAase

HAase, as an endoglycosidase, can specifically degrade hyaluronic acid (HA) into small fragments which are highly angiogenic and allow facile cell adhesion and migration by activating focal adhesion kinase and mitogen-activated protein kinase pathways.¹²² Moreover, elevated expression of HAase is associated with the progression and metastasis of many types of cancers, such as prostate cancer, breast cancer, and ovarian cancer.¹²²⁻¹²⁵ Thus, development of HAase activatable probes allow for specific tumor imaging. Zhu's and Ma's groups developed a multifunctional Cy5.5-HANP/CuS (HANPC) nanocomposite by loading CuS into Cy5.5-conjugated hyaluronic acid nanoparticles (HANP) for activatable PA imaging of HAase.¹⁰⁵ The CuS nanoparticles in the HANPC nanocomposite with strong NIR absorbance appeared to be an excellent agent for PA imaging and also an effective photothermal therapy (PTT) agent, as they efficiently quenched the fluorescence of Cy5.5. Tested in the HAase overexpressing SCC7 tumor-

bearing mice model, strong PA signals (tumor-to-normal tissue ratio of 3.8) were observed at t=6 h post intravenous injection along with good tumor inhibition rate (89.74% on day 5) by PTT. The strategy thus is promising for PA imaging guided PTT.

Small Molecule Mediators

Small molecule mediators such as reactive oxygen species (ROS) and metal ions play an important role in the homeostasis of living organisms.^{126, 127} Aberrant changes in the concentration of small-molecule mediators are often associated with pathological conditions. Thus, real-time imaging at a high tissue penetration depth can provide mechanistic information on how they mediate the pathological processes, potentially leading to new methods for diseases diagnosis and drug development. In this respect, activatable PA probes have been developed for imaging of ROS⁷¹, pH^{97, 128-130} and metal ions.^{131, 132}

ROS

ROS is a hallmark of many pathological processes ranging from acute and chronic bacterial infections to chronic diseases such as cancer, cardiovascular disease, and arthritis.^{126, 133, 134} Imaging of ROS in living animals is challenging because many photonic imaging agents such as small-molecule dyes, QDs and gold nanoparticles are not stable in the presence of ROS. NIR-absorbing SPNs have emerged as a new class of photonic nanoagents for molecular imaging.^{73, 97, 135-142} By virtue of the high photostability and ROS resistance, SPNs were developed into activatable PA probes for imaging of ROS both *in vitro* and *in vivo*.⁷¹ Poly(cyclopentadithiophene-*alt*-benzothiadiazole) (SP1) was transformed into the nanoprobe by co-precipitation with a ROS-sensitive NIR dye (IR775S) and lipid (**Figure 6a**). The PA spectrum

of the SPN probe (RSPN) showed three PA peaks at 700, 735 and 820 nm with similar intensities. In the presence of ONOO^- or ClO^- , the PA peak at 700 nm assigned to SP1 remained nearly the same, while the peak at 735 nm decreased significantly and the peak at 820 nm almost disappeared due to the ROS-mediated rapid oxidative decomposition of IR775S (**Figure 6b**). The SPN probe thereby exhibited the ratiometric PA signals ($\text{PA}_{700}/\text{PA}_{820}$) upon detection of ONOO^- or ClO^- . The imaging capability was tested in a murine model of zymosan-induced acute edema (**Figure 6e**). $\text{PA}_{700}/\text{PA}_{820}$ gradually increased to 2.7 ± 0.31 at 120 min post-injection for zymosan-treated mice, which was significantly higher as compared with that for control mice (1.4 ± 0.22) (**Figure 6c**). Thus, the SPN probe was able to effectively monitor the production of ROS in living mice.

Figure 6. (a) Schematic illustration of the SPN-based activatable PA probe for ROS sensing. (b) Representative PA spectra of RSPN (5 $\mu\text{g/mL}$) in the absence and presence of ROS (5 μM). (c) PA/Ultrasound overlaid images of regions in the thigh of living mice after intramuscular injection of RSPN (3 μg , 50 μL) and the regions of thighs were pretreated with saline (i) or zymosan (ii) for 20 min before RSPN injection. (d) The ratiometric PA signals ($\text{PA}_{700}/\text{PA}_{820}$) as a function of time post-injection of RSPN. *Statistically significant difference in $\text{PA}_{700}/\text{PA}_{820}$ between zymosan-treated and saline-treated mice at all time points starting from 10 min ($p < 0.05$). Reprinted with permission from reference 71, Copyright 2014, Nature Publishing Group.

pH

pH is a critical physiological parameter that plays a critical role in cellular and tissue homeostasis and aberrant pH is associated with many diseases such as inflammation,¹⁴³ cancer,¹⁴⁴ cardiac ischemia,¹⁴⁵ and Alzheimer's disease.¹⁴⁶ In particular, as the extracellular pH of solid tumors is acidic, development of pH-sensitive probes is promising for cancer diagnoses, monitoring, and prognosis.^{144, 147}

A “turn-off” activatable PA probe was developed by Wang's and Duan's groups.¹²⁸ This probe was based on pH-sensitive micelle-like nanoparticles composed of a PEG grafted poly(β -amino ester). A fluorescence dye (squaraine, SQ) and an anticancer drug (doxorubicin, DOX) were loaded into the hydrophobic core of the nanoparticles. The polymer micelles were stable at physiological pH (7.4), while under the acidic biological environment (lysosomes), the tertiary amine groups on the alky chain of the copolymer underwent protonation, subsequently triggering the dissociation of micelles and then releasing the loaded DOX and SQ. After the dissociation of the micelles, the released SQ aggregated to form H-aggregates, which led to the decreased absorption and PA signals at 698 nm. The release of DOX triggered by pH was thus accompanied by the change of PA signals, allowing for the monitoring of drug release according to the decreased PA signals.

A “turn-on” activatable PA probe was developed by Han's group, which was based on sialic acid-capped polymeric nanovesicles comprising the NIR profluorophore (pNIR@P@SA).¹²⁹ The surface-anchored sialic acid was conjugated to the biocompatible shell of poly[styrene-*alt*-(maleic acid)] *via* covalent amide bond, allowing for tumor targeting. In acidic environment, the pNIR moiety underwent isomerization upon protonation, inducing a “turn-on” PA signal at 720

nm. The multifunctional pNIR@P@SA nanoparticles were used for PA “turn-on” imaging subcutaneous tumors and millimeter-sized liver tumor foci in mice.

Different from the above-mentioned PA probes, ratiometric PA probes were also developed for pH imaging. For example, Liu’s and Zeng’s groups synthesized a pH-responsive albumin-based nanoprobe (C-HSA-BPOx-IR825) for ratiometric PA imaging of pH.¹³⁰ Benzo[α]phenoxazine (BPOx) and heptamethine indocyanine dye (IR825) would initiate self-assembly of human serum albumin (HSA) to form albumin–dye nanocomplexes (HSA-BPOx-IR825) and the stability was greatly enhanced after using glutaraldehyde to induce covalent cross-linking of HSA, leading to stable nanoparticles, C-HSA-BPOx-IR825 (**Figure 7a**). The dye IR825 acted as an internal reference, whose absorption and fluorescence were inert to pH, while the pH-responsive dye BPOx served as a pH indicator for both ratiometric PA and fluorescence imaging. Along with the decrease of pH, BPOx was protonated and intramolecular charge-transfer occurred, which induced the increased PA signal of C-HSA-BPOx-IR825 at 680 nm with the unchanged PA signals at 825 nm contributed by IR825 (**Figures 7b&7c**). The ratiometric PA signals (PA_{680}/PA_{825}) showed an almost linear response to pH in the range of 5.0–7.0 (**Figure 7d**). C-HSA-BPOx-IR825 was applied for imaging the pH of mice bearing 4T1 tumor at different growth stages (6, 10, 14, and 18 d post-inoculation). Higher PA signals were observed in the tumor with the signals at 680 nm becoming particularly stronger than 825 nm (**Figure 7e**). Quantitative analysis showed that PA_{680}/PA_{825} increased with the tumor growth and the pH of tumors at $t = 6, 10, 14$ and 18-day post tumor inoculation were estimated to be $\sim 6.8, 6.6, 6.2$, and 6.0 , respectively (**Figure 7f**). Such results coordinated well with the fact that the pH of tumor became more acidic when the tumor grew larger due to the elevated production of lactate by cancer cells *via* the

anaerobic glycolytic pathway under hypoxic environment.¹⁴⁸ In a similar way, Liu's group also used the dye-albumin (croconine-HSA) self-assembly system to prepare nanoagents for pH-responsive ratiometric PA imaging and pH-responsive PTT.¹⁴⁹ Besides, the croconaine rotaxane dye itself was tested for ratiometric PA imaging of pH by Smith's group.¹⁵⁰

Figure 7. (a) Schematic illustration of the preparation of albumin-based nanoprobe (cross-linking-albumin-benzo[α]phenoxazine-heptamethine indocyanine dye, C-HSA-BPOx-IR825) and its ratiometric PA and fluorescence imaging of pH. (b) The scheme showing the protonation of BPOx moiety upon acidification. (c) Absorption spectra and (d) the ratiometric PA signals (PA_{680}/PA_{825}) of C-HSA-BPOx-IR825 measured at different pH. (e) PA imaging of tumors with different sizes after intravenous injection with C-HSA-BPOx-IR825 (3.5 mg kg⁻¹

BOPx, 1.6 mg kg⁻¹ IR825). Images were recorded at 680 nm and 825 nm excitations. (f) The ratiometric PA signals (PA₆₈₀/PA₈₂₅) of tumors with different sizes based on PA imaging data in (e) and their corresponding the pH estimation. Reprinted with permission from reference 130, Copyright 2015, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Our group developed an activatable PA semiconducting oligomer nanoprobe (SON) for *in vivo* ratiometric imaging of pH, which was composed of a semiconducting oligomer (SO, F-DTS) to act as the inert PA matrix and a boron-dipyrromethene (BODIPY) dye (pH-BDP) to serve as both PA enhancer and pH indicator (**Figure 8a**).⁹⁷ The photoinduced electron transfer (PET) effect existed between F-DTS and pH-BDP, resulting in quenched fluorescence of F-DTS and in turn enhanced PA brightness of the probe. Under acidic environment, the hydroxyl group on the backbone of pH-BDP underwent protonation generating the absorption changes of SONs. Using optimized 50_{w/w}% doping amount of pH-BDP (SON₅₀) as an example, with decreasing the pH, the absorption peak at 750 nm (Ab₇₅₀) assigned to pH-BDP decreased while the absorption peak at 680 nm (Ab₆₈₀) remained the same (**Figure 8b**). Consequently, the PA intensity at 680 nm remained negligible change, while the intensity at 750 nm decreased significantly upon pH decreased from 7.4 to 5.5 (**Figures 8c&8d**). Thus, such a pH response permitted ratiometric PA imaging of pH (**Figure 8e**). Because the PA signal of the probe was amplified by 3.1-fold as compared with the nondoped F-DTS nanoparticles, the injection dosage was minimized to 25 µg/mouse, lower than that for the carbon nanotube (~40 µg/mouse)²⁷ and the previous reported SPN (50 µg/mouse).⁷¹ The *in vivo* ratiometric PA signals of the SONs (ΔPA₆₈₀/ΔPA₇₅₀) reached 6.9 ± 0.6 at t=6 h post injection (**Figures 8f&8g**), allowing for estimating the pH value in the tumor of living mice to be ~6.3 according to the calibration curve in **Figure 8e**.

Figure 8. (a) Schematic illustration of the semiconducting oligomer nanoprobe (SON)-based activatable probe for pH sensing. (b) Absorption spectra of SON₅₀ ($1.2 \mu\text{g mL}^{-1}$) at different pH. (c) PA images of SON₅₀ solution at pH = 7.4, 6.4, or 5.5. A pulsed laser was turned to 680 or 750 nm for the ratiometric PA imaging. (d) PA spectra of SON₅₀ at different pH. (e) The ratiometric PA signals (PA_{680}/PA_{750}) of SON₅₀ at different pH. A linear calibration was obtained from pH = 7.4 to 5.5. (f) Ratiometric images ($\Delta PA_{680}/\Delta PA_{750}$) of subcutaneous HeLa tumor in nude mouse before and 6 h after intravenous administration of SON₅₀ ($25 \mu\text{g}/\text{mouse}$). (g) Quantification of the ratiometric PA signals ($\Delta PA_{680}/\Delta PA_{750}$) as a function of time post-injection of SON₅₀. To minimize the tissue interference, the PA intensity increments (PA intensity after injection of SON₅₀ subtracted by the tissue intensity before injection) at 680 and 750 nm (ΔPA_{680} nm and ΔPA_{750} nm) were used as *in vivo* PA signal. The pulsed laser was tuned to 680 or 750 nm for PA imaging. *No statistically significant difference in $\Delta PA_{680}/\Delta PA_{750}$ between 3 and 6 h ($p > 0.05$, $n = 3$). Reprinted with permission from reference 97, Copyright 2016, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Metal ions

Metal ions are essential cofactors in many biological processes such as energy metabolism and storage, signal transduction, and nucleic acid processing.¹²⁷ Development of reliable methods to detect and quantify metal ions is of importance for environmental monitoring, food safety, and medical diagnostics and imaging.^{127, 151-154}

Abnormal regulation of Cu(II) is related to numerous diseases including neurodegenerative diseases.¹⁵⁵ Chan's group designed and synthesized small-molecule-dye-based activatable PA probes (APC-1 and APC-2) for ratiometric imaging of Cu(II).¹³¹ Both APC-1 and APC-2 were based on an aza-BODIPY dye capped with 2-picolinic ester, but they had the different R substituting groups. Such difference resulted in the absorption red-shifted from 680 nm for APC-1 to 697 nm for APC-2 (**Figures 9b&9c**). In the presence of Cu(II), the probes were transformed to their corresponding uncapped phenoxide product, which had the maximum absorption at 755 nm for APC-1-H and 767 nm for APC-2-H. Thus, the ratiometric PA signals (PA_{755}/PA_{680} for APC-1 and PA_{767}/PA_{697} for APC-2) were generated in response to Cu(II) (**Figure 9a**). PA imaging of Cu(II) using APC-2 was demonstrated *in vitro* in fluorinated ethylene propylene copolymer (FEP) tubing covered by a 1 cm thick phantom (**Figures 9d&9e**). The PA signal at 697 nm showed almost no change while a dramatic enhancement at 767 nm was observed upon addition of Cu(II). The ratiometric PA signals (PA_{767}/PA_{697}) respectively increased 91.3- and 100.5-fold for treatment with 1 and 10 equivalent (equiv) of Cu(II) as compared with that for untreated one (**Figure 9f**), showing its effectiveness in PA imaging of Cu(II). A small-molecule dye based PA probes was also developed for calcium imaging as reported by Westmeyer's group.¹⁵⁶

Figure 9. (a) Schematic illustration of the aza-BODIPY-dye-based activatable PA probes for ratiometric PA imaging of Cu(II). Normalized absorption spectra of acoustogenic probe for copper (II)-1 (APC-1) (b) or acoustogenic probe for copper(II)-2 (APC-2) (c) (2 μ M) (dashed line) and their corresponding hydrolyzed product APC-1-H and APC-2-H (2 μ M, solid line) in PBS + 0.1% Cremophor® EL(CrEL) (pH 7.4). PA imaging of APC-2 (10 μ M, PBS + 0.1% CrEL, pH 7.4) in FEP tubing overlaid with a 1 cm thick phantom in the absence or presence of Cu(II) (100 μ M) with excitation at (d) 697 and (e) 767 nm. Scale bar represents 2 mm. (f) Quantification of the ratiometric PA signals (PA_{767}/PA_{697}) of APC-2 after treatment with 0, 1, or 10 equiv of Cu(II) for 90 min. The ratiometric PA values were normalized for control and Cu(II)-treated conditions. $n = 2$. Reprinted with permission from reference 131, Copyright 2015, American Chemical Society.

Lithium as a common drug for bipolar disorder has a narrow therapeutic window (0.6~1.2 mM) and also a low toxic dose (~2 mM), which makes it essential for real-time *in vivo* monitoring.^{157,}
¹⁵⁸ Clark's group designed a nanoprobe to continuously track lithium levels *in vivo* by ratiometric PA imaging.¹³² The nanoprobe was incorporated with an ionophore VI (L) in the core to extract lithium from the bulk solution. Such extraction of a cation into the core of the polymer caused a

shift in the internal pH of the nanoprobe, deprotonating the chromoionophore (CH^+) and thus changing its optical properties. With increased concentration of lithium, the PA signals at 515 nm increased along with the decreased PA signals at 660 nm, affording the ratiometric PA signals ($\text{PA}_{515}/\text{PA}_{660}$) responding linearly to the lithium concentration. The nanoprobe was applied for imaging lithium at a physiological concentration of 38 mg/kg in living mice, showing the increase of the ratiometric PA signals of 25%. In contrast, the fluorescence signal change was only 8% at the same lithium dose. The results suggested that the superior imaging depth of PA imaging could favor *in vivo* detection sensitivity.

Temperature

To monitor body temperature during PTT, reversible activatable probes that allow for multiple cycles of activation by input stimulus are highly demanded. Zheng's group reported a temperature-responsive PA nanoswitch based on the J-aggregates of porphyrin.¹⁵⁹ Light-absorbing porphyrin (bacteriopheophorbide α) was conjugated with host-lipid (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine) to form Bchl-lipid, which had the ordered J-aggregates of porphyrin within the amphipathic membranes (**Figure 10a**). The formed Bchl-lipid was mixed with dipalmitoylphosphatidylcholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamineN-[methoxy(polyethylene glycol)-2000] (mPEG2000-DPPE) to prepare the J-aggregating nanoparticles (JNP) *via* freeze-thaw extrusion of the lipid films (**Figure 10b**). Upon increasing the temperature, JNP16 with DPPC as the host lipid showed absorption decreased at 824 nm assigned to the J-aggregates of porphyrin, along with the increase at 750 nm attributed to the porphyrin monomer (**Figure 10c**). The spectral change was reversible and could be repeated with heating cooling cycles (**Figure 10d**). A range of temperature thresholds,

closely correlated with phase transition temperature of host lipid, could be achieved by using different host phospholipid with different length of acyl chain. The JNP based PA probes were successfully used for sensing the tissue temperature in tumor xenografts (**Figures 10e&10f**), demonstrating their potential for real-time monitoring of temperature during PTT.

Figure 10. (a) Molecular structure of bacteriopheophorbide-lipid (Bchl-lipid). (b) Schematic illustration of Bchl-lipid-based J-aggregating nanoparticles (JNP) prepared with 15% Bchl-lipid, 80% host phospholipid, and 5% mPEG2000-DPPE. Insets: (left) Representative transmission electron micrograph of JNP prepared with DPPC as host lipid (JNP16) and (right) photographs of JNP16 samples below and above phase transition temperature. (c) Representative NIR absorption spectra of JNP16 upon heating from 25 to 50 °C. (d) Reversibility cycles of PA signals of JNP16 at 824 and 750 nm during heating above and below JNP16's phase transition midpoint. (e) PA images of tumors at 680 and 824 nm after intratumoral injection of JNP16 (130 μM). (f) The PA signals of JNP16 at 824 nm response to temperature. (each colour represents data from 1 animal, n = 4). Reprinted with permission from reference 159, Copyright 2014, American Chemical Society.

SUMMARY AND OUTLOOK

Many applications of PA imaging have been identified ranging from oncology, to neuroscience and to cardiovascular diseases. With the help of activatable PA probes, PA imaging can be further advanced towards molecular level. Smart activatable PA probes summarized herein have been developed to image a variety of molecular targets of interest such as enzymes, small-molecule mediators, pH and heat, proving their capability to provide real-time molecular information on physiological and pathological processes in living animals.

The design approaches towards activatable PA probes are versatile, and not limited to particular system. Inorganic nanoparticles, organic nanostructures, macromolecules and small-molecule dyes can all be shaped into activatable PA probes for specific imaging task. Self-assembly/aggregation, enzyme cleavage and molecular structure variation are common ways to induced the changes of PA signals in responses to the target of interest. However, the level of signal difference before and after probe activation varies from system to system, and organic systems seem to have some advantages.

Despite the progress of activatable PA probes for molecular imaging in recent years, challenges need to be overcome to facilitate their utilization in basic research as well as in pre-clinical and clinical settings. The PA imaging has high tissue-penetration (up to 6 cm), but the PA signal of a material is mainly determined by the absorption-related heat generation. Thus, the sensitivity of activatable PA probes is limited, and usually lower than the fluorescent probes, which needs to be improved. Moreover, the dosage of activatable PA probes can be lowered down with increasing their sensitivity, potentially mitigating the toxicity issue. A feasible solution is to develop imaging agents with high photothermal conversion efficiency, which requires the deep understanding of

PA processes and materials photophysics. For the purpose of potential clinical translation, rapid clearance of imaging agents is always desirable. As compared with other designs summarized herein, the small-molecule design seems to be more promising, because they are more likely to be cleared out through urinary excretion, a process that occurs only when the size of imaging agents is smaller than the physiologic pore size of filtration slit in the glomerular capillary wall (~5 nm).

Although development of activatable PA probes is still in the early stage, they have proven their promise for understanding fundamental biology, early diagnosis and theranostics. Activatable PA probes with high sensitivity and rapid clearance are likely to emerge in the near future, which can probably allow imaging-guided surgery to perform in deep tissue in real time.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

PA, photoacoustic; EPR, enhanced permeability and retention; NIR, near-infrared; QDs, quantum dots; SPNs, semiconducting polymer nanoparticles; SON, semiconducting oligomer nanoprobe; 2D, two-dimensional; ICG, indocyanine green; SLNs, sentinel lymph nodes; SWNTs, single-walled carbon nanotubes; RGD, cyclic arginine–glycine–aspartic acid; MMPs, matrix metalloproteinases; HAase, hyaluronidase; ECM, extracellular matrix; BHQ3, black hole quencher 3; P18, purpurin 18; CABT, 2-cyano-6-aminobenzothiazole; GSH, glutathione; PTT, photothermal therapy; HA, hyaluronic acid; ROS, reactive oxygen species; SQ, squaraine; DOX, doxorubicin; HSA, human serum albumin; FEP, fluorinated ethylene propylene copolymer; DPPC, dipalmitoylphosphatidylcholine; JNP, J-aggregating nanoparticles

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