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A General Approach to Convert Hemicyanine Dyes into Highly Optimized Photoacoustic Scaffolds for Analyte Sensing

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Abstract: Most photoacoustic (PA) imaging agents are based on the repurposing of existing fluorescent dye platforms that exhibit non-optimal properties for PA applications. Herein, we introduce PA-HD, a new dye scaffold optimized for PA probe development which features an increase in sensitivity of 4.8-fold and a red-shift of the λ_{abs} from 690 nm to 745 nm to enable ratiometric imaging. Computational modelling was employed to elucidate the origin of these enhanced properties. Moreover, to demonstrate the generalizability of our remodeling efforts, we developed three probes for β -galactosidase activity (PA-HD-Gal), nitroreductase activity (PA-HD-NTR), and H_2O_2 (PA-HD- H_2O_2). We generated two cancer models to evaluate PA-HD-Gal and PA-HD-NTR. Additionally, we employed a murine model of Alzheimer's disease to test PA-HD- H_2O_2 . In this latter example, we observed a PA signal increase at 735 nm of 1.79 ± 0.20 -fold relative to background indicating the presence of oxidative stress. These results were confirmed via ratiometric calibration which was not possible using the parent HD platform.

cancer types and inflammatory conditions in human patients.^[2] However, as the demand for improved performance, better contrast, greater target specificity, and molecular information grew, efforts turned toward the design of targeted contrast agents^[3] and activatable photoacoustic probes (also known as acoustogenic probes)^[4] to reliably identify diseased tissue and to monitor biological analytes, respectively.

Owing to the intrinsic similarities between photoacoustic and fluorescence imaging (i.e., the requirement of light excitation), it is reasonable that most photoacoustic imaging agents to date have relied heavily on the repurposing of existing fluorophores. For example, indocyanine green (ICG), an FDA-approved cyanine-based fluorescent dye, was utilized extensively in early studies for contrast-enhanced photoacoustic imaging even though it is prone to photobleaching and oxidative decomposition. Likewise, the first small-molecule activatable photoacoustic probe (designed to detect Cu^{2+})^[5] was based on the aza-BODIPY dye, which is another NIR fluorescent platform.^[6] Subsequent studies have resulted in a palette of aza-BODIPY-based photoacoustic probes for hypoxia,^[7] nitric oxide,^[8] peroxynitrite,^[9] hydrogen peroxide,^[10] pH,^[11] redox status,^[12] and photodynamic therapy.^[13] In addition, our group put forth significant efforts to optimize this scaffold for photoacoustic imaging; however, this required the handling of unstable species such as reactive azirine intermediates, as well as challenging (and sometimes low yielding) purifications to separate the desired heteropyrrole coupling product from the unwanted symmetrical aza-BODIPYs.^[14] Due to these limitations, our design has not yet been widely adopted by other probe developers. Beyond aza-BODIPYs, other NIR fluorescent platforms such as xanthenes,^[15] cyanines^[16] and hemicyanine dyes (HD)^[17] have also been repurposed extensively (Scheme 1a).

Introduction

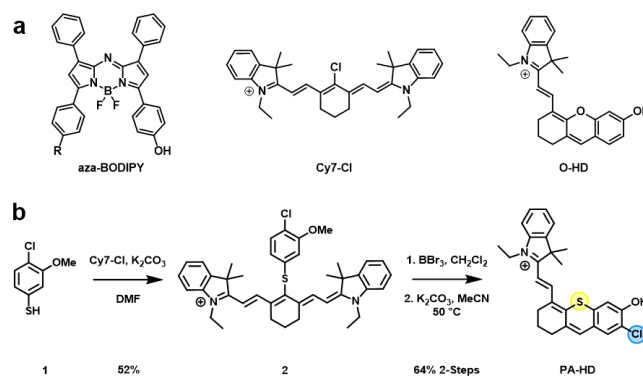
Photoacoustic imaging is a powerful *in vivo* approach that is characterized by the generation of ultrasound waves which result from the excitation of an optical absorber with light.^[1] In theory, any light-absorbing material can be induced to afford a photoacoustic readout; however, in practice, only molecules with a wavelength of maximum absorbance in the near-infrared (NIR) region ($\lambda_{\text{abs}} > 650$ nm), a high extinction coefficient ($\epsilon > 10^4 \text{ M}^{-1}\text{cm}^{-1}$) and a low quantum yield ($\Phi_{\text{FL}} < 5\%$) are desirable. Initially, when photoacoustic imaging emerged as an *in vivo* biomedical technique, various endogenous absorbers such as hemoglobin, oxyhemoglobin, and melanin were exploited to provide contrast. These studies set the stage for label-free imaging of various

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HDs have attracted considerable attention from the field owing to their ease of synthesis (one-step starting from a parent cyanine and resorcinol) and the presence of a convenient handle (i.e., -OH or -NH₂) to append analyte-responsive triggers.^[18] These features have made HD-based optical probes popular for detecting a diverse range of analytes.^[19] Unfortunately, HDs are far from ideal for photoacoustic analyte sensing. First, the λ_{abs} of a 'capped' probe (~600) typically falls below the NIR cutoff of 650 nm, as well as the lower wavelength limit of our commercial photoacoustic imaging systems (660 nm (MSOT iNvision, iThera) and 680 nm (Nexus 128+, Endra)). These constraints preclude ratiometric calibration which can account for imaging artifacts that result from photobleaching or differential probe clearance. Second, HDs are highly fluorescent molecules ($\Phi_{\text{FL}} = \sim 30\text{--}40\%$), meaning less of the excited state will relax via non-radiative decay to afford a photoacoustic signal. In this study, we introduce two highly effective modifications to transform existing HD designs into optimized scaffolds for photoacoustic imaging by substituting the endocyclic oxygen with a sulfur moiety and by tuning the phenolic pK_a value (herein referred to as PA-HDs). To demonstrate generalizability, we prepared three enhanced photoacoustic probes from PA-HD for β -galactosidase activity, nitroreductase activity, and hydrogen peroxide (H₂O₂). The β -galactosidase and nitroreductase probes were evaluated in ovarian and breast cancer models, respectively. Beyond subcutaneous tumors, we also employed our H₂O₂ probe to image oxidative stress in a murine model of Alzheimer's disease.

Results and Discussion

The design of PA-HD is based on the premise that sulfur-substitution can potentially red-shift the λ_{abs} value by up to 50 nm and thus, can facilitate deeper tissue imaging and ratiometric calibration. A similar approach was recently employed to red-shift chemiluminescent probes via sulfur-substitution.^[20] Moreover, we anticipate two additional benefits from this modification that favor photoacoustic imaging. First, we hypothesized the fluorescent quantum yield will be attenuated and second, we expect the extinction coefficient to be enhanced. It is noteworthy that although the apparent pK_a of regular HDs (herein referred to as O-HD) was reported to be ~5.6,^[18] this value appears to be inaccurate. In our hands we measured a value of 7.5 (Figure S1). This discrepancy likely resulted because the pH of each buffer system used to construct the corresponding pH-profile was not adjusted after the organic co-solvent component was added. A high pK_a value will decrease the photoacoustic sensitivity and lead to imaging artifacts since the protonated form of O-HD will have a similar absorbance profile to that of the unactivated probe. Therefore, in addition to performing the proposed O to S substitution, it is critical to install an ortho chloro group to lower the phenolic pK_a which ensures that upon unmasking of the trigger, the turned over PA-HD will exist predominantly in a deprotonated form. To this end, we synthesized PA-HD by first substituting the *meso*-chloro group of Cy7-Cl with thiophenol **1** to furnish cyanine **2** in 52% yield. This intermediate was then demethylated with BBr₃ and heated under basic conditions after solvent exchange to initiate the retro-Knoevenagel reaction to generate **PA-HD** in 64% yield over 2-steps (33% overall).



Scheme 1. a) Examples of NIR fluorescent dyes that have been repurposed for photoacoustic applications. b) Synthesis of PA-HD.

With PA-HD in hand, we first determined that the λ_{abs} was positioned at 745 nm which represents a bathochromic shift of 55 nm relative to O-HD (Figure 1a). Moreover, it is apparent that at pH 7.4, over half the population of O-HD is protonated and this results in a blue-shifted λ_{abs} and low ϵ . In contrast, since the measured pK_a value of PA-HD is 6.0, over 96% of the dye will be deprotonated at physiological pH (Figure S2). We also found that the ϵ had increased significantly by 62% from $8.2 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ to $13.3 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ and that the Φ_{FL} decreased by six-fold from 30% to 5%. As mentioned previously, meeting these two design criteria will translate into enhanced photoacoustic properties. To evaluate the impact of sulfur-substitution with respect to the photoacoustic signal intensity and potential ratiometric sensing capabilities, we conducted a series of head-to-head *in vitro* experiments. First, we embedded solutions of each dye (25 μM) in an agar-based tissue phantom (10% milk) and obtained photoacoustic spectra over a wavelength range of 660 to 980 nm

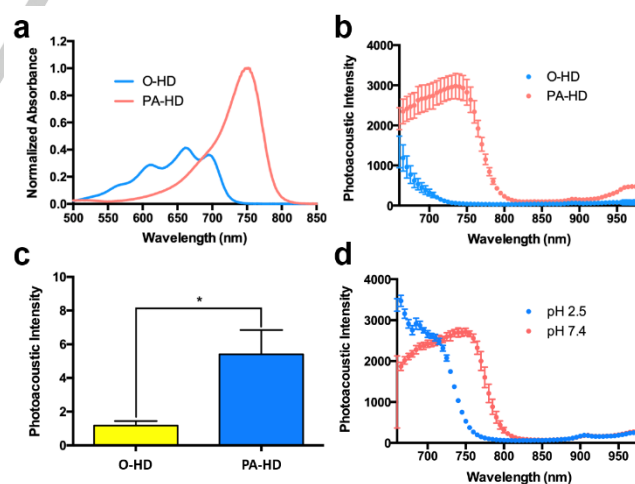


Figure 1. a) Absorbance and b) PA spectra of O-HD and PA-HD at pH 7.4 (1:1 v/v PBS:EtOH). Dye concentration was 25 μM for each dye (Each point represents Mean \pm SD, $n = 3$). c) Normalized photoacoustic intensity of O-HD and PA-HD in a tissue phantom containing 60% milk to mimic scattering effects in dense tissue. [PA-HD] = 0.5 μM (Mean \pm SD, $n = 3$). Statistical analysis was performed using two-tailed Student's t-test ($\alpha = 0.05$), *: $p < 0.05$. d) Photoacoustic spectra of PA-HD at pH 2.5 and 7.4 (Each point represents Mean \pm SD, $n = 3$).

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(Figure 1b). While the O-HD λ_{abs} clearly fell outside the NIR window, the highest intensity for PA-HD was centered at ~ 735 nm. This represents a 4.8-fold increase in sensitivity when comparing the highest signal for each dye. Additionally, we show a dose-dependent increase in the photoacoustic intensity after correcting for wavelength-dependent differences in fluence (Figure S3). Next, we reduced the dye concentration to $0.5 \mu\text{M}$ and changed the composition of milk in the phantom to 60% to mimic the scattering effects of dense tissue (e.g., brain). Even under these conditions that are designed to attenuate the photoacoustic signal, we could readily detect PA-HD but not O-HD (Figure S4). The corresponding normalized photoacoustic intensities (relative to background) were 5.4 ± 2.5 -fold and 1.18 ± 0.47 -fold, respectively (Figure 1c). To evaluate the potential of this new scaffold for ratiometric imaging, we subjected PA-HD to acidic conditions since protonation of the phenol is an effective proxy for the presence of a trigger at this position. Indeed, the protonated form of PA-HD exhibits a λ_{abs} at 650 nm (Figure S5). The ratios of the deprotonated and protonated forms at 650 nm and 745 nm based on absorbance were found to be 0.36 and 16.81, respectively (Figure 1d). This represents a theoretical turn-on response of 46.7 (defined as $\text{ratio}_{745}/\text{ratio}_{650}$). Similar results were obtained when the experiment was performed at 660 nm (lower wavelength limit) and 735 nm (maximum signal) via photoacoustic imaging to give a turn-on response of 3.7. Together these results demonstrate PA-HD can be employed for ratiometric imaging, while this is not possible with O-HD.

To better understand the origin of these enhanced properties, we modeled O-HD and PA-HD using state-of-the-art computational techniques. The resultant computed spectra at 0 K in acetonitrile shows excellent agreement with the experimental data (Figure S6). Analysis of the computed geometries (Figure 2a) reveal that the excited states of PA-HD is significantly different from the

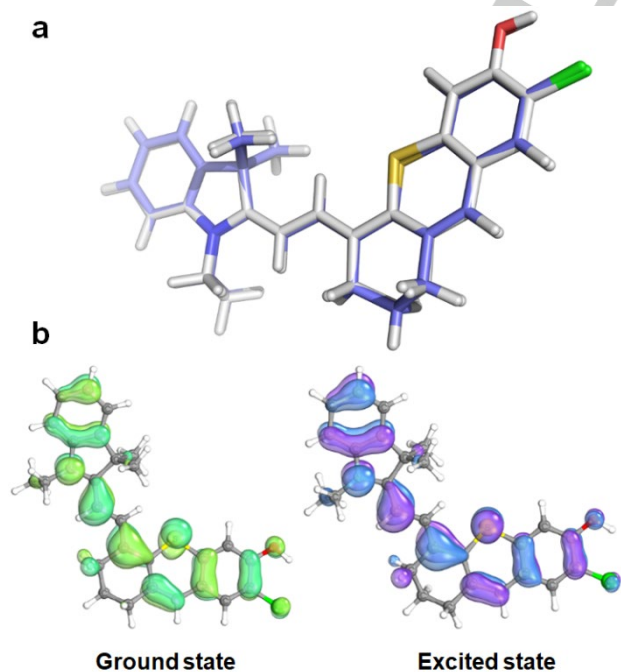


Figure 2. a) An overlay of the excited state geometry (white) with the ground state geometry (blue) for PA-HD. b) The HOMO of the ground state (green) and excited state (blue) structures of PA-HD.

ground state structures (RMSD 0.2) while the excited state geometry of O-HD remains similar to its ground state (RMSD 0.05). PA-HD is relatively planar in its excited state as evidenced by the dihedral angles involved in the extended π network ($\sim 9^\circ$). For comparison, the dihedral angle is $\sim 20^\circ$ for the ground state. However, O-HD is planar in both the ground and excited states. Furthermore, the HOMO for the excited state structure of PA-HD exhibits a much higher electron delocalization than the HOMO for the ground state (Figure 2b). We postulate that this increase in planarity for PA-HD, and thereby an increase in the conjugation, in the excited state compared to the ground state might be the origin of the better photoacoustic properties observed in PA-HD. Similar behavior is observed in S-HD (an analog lacking the ortho-chloro substituent), further supporting our hypothesis (see the supporting information for the S-HD results).

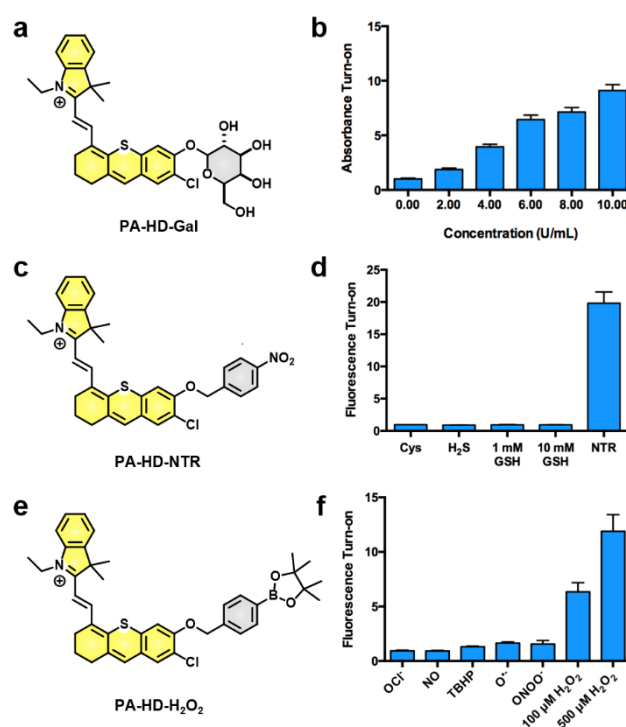


Figure 3. a) Chemical structure of PA-HD-Gal. b) Dose-dependent response of $5 \mu\text{M}$ PA-HD-Gal after 1 h to 0, 2, 4, 6, 8, or 10 U/mL of *E. Coli* beta-galactosidase at pH 7.4 (9:1 v/v PBS:MeCN) (Mean \pm SD, $n = 5$). c) Chemical structure of PA-HD-NTR. d) Response of $2 \mu\text{M}$ PA-HD-NTR to biologically relevant thiols (Cys and H_2S $100 \mu\text{M}$) and GSH at 1 mM and 10 mM for 15 min at pH 7.4 (9:1 v/v PBS:MeCN) (Mean \pm SD, $n = 3$). Complete activation mediated by 2 U/mL *E. coli* nitroreductase. e) Chemical structure of PA-HD- H_2O_2 . f) Response of $2 \mu\text{M}$ PA-HD- H_2O_2 to OCl^- , NO, TBHP, O_2^- , ONOO^- ($100 \mu\text{M}$) and H_2O_2 ($100 \mu\text{M}$ and $500 \mu\text{M}$) for 1 h at pH 7.4 (9:1 v/v PBS:MeCN) (Mean \pm SD, $n = 3$).

Next, we converted PA-HD into a series of three activatable photoacoustic probes to demonstrate that the enhanced properties afforded by the sulfur-substitution are generalizable. Specifically, PA-HD-Gal (Figure 3a), PA-HD-NTR (Figure 3c), and PA-HD- H_2O_2 (Figure 3e) were developed for β -galactosidase activity, nitroreductase activity, and H_2O_2 , respectively. We selected these three imaging targets because their corresponding triggers are commonly employed to validate new dye systems and

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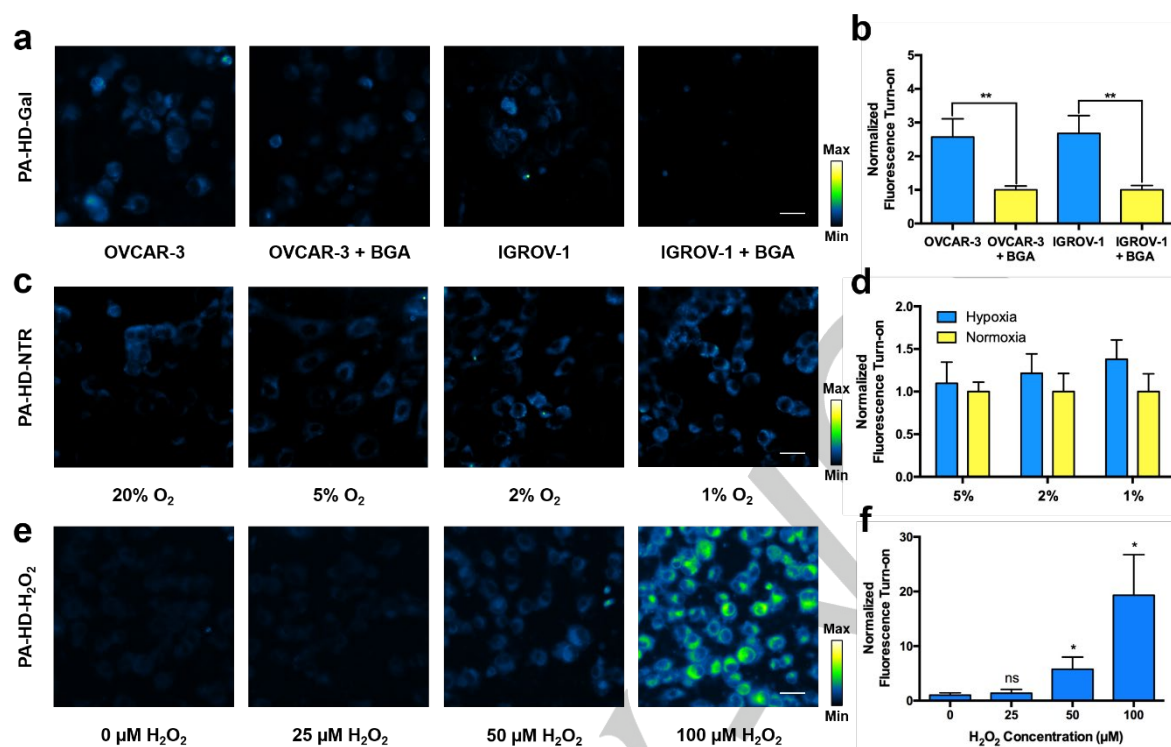


Figure 4. a) Fluorescent images of PA-HD-Gal (4 μ M) in ovarian cancer cell lines (OVCAR-3 and IGROV-1) pre-treated with a vehicle control or BGA, a beta-galactosidase inhibitor (1.5 mM). $n = 3$ for each condition. b) Quantified data from a) normalized to inhibitor condition (Mean \pm SD, $n = 3$). Statistical analysis was performed using two-tailed Student's t-test ($\alpha = 0.05$), **: $p < 0.01$. c) Fluorescent images of PA-HD-NTR (2 μ M) in ID8 cells cultured under the indicated oxygen atmosphere (20, 5, 2, or 1 % oxygen). $n = 3$ for each condition. d) Quantified data from c) normalized to a normoxic control (Mean \pm SD, $n = 3$). e) Fluorescent Images of PA-HD-H₂O₂ (10 μ M) in NeuroScreen-1 cells treated with various concentrations of H₂O₂ (0, 25, 50 100 μ M). $n = 3$ for each condition. f) Quantified data from e) normalized to the 0 μ M H₂O₂ condition (Mean \pm SD, $n = 3$). Statistical analysis was performed using two-tailed Student's t-test ($\alpha = 0.05$) compared to 0 μ M condition, *: $p < 0.05$. Scale bar represents 50 μ m.

they are important in a host of physiological and pathological processes. For instance, β -galactosidase expression has been used as a marker to identify senescent cells^[21] and ovarian cancer.^[22] Nitroreductase has been used as a target to detect gram positive and negative bacteria,^[23] as well as tumor hypoxia since it is overexpressed in many cancer types.^[24] Lastly, H₂O₂ is not only an important signaling molecule, at elevated levels it is a general indicator of oxidative stress in a variety of disease states.^[25]

After synthesizing the three probes, each was subjected to *in vitro* testing to assess target-responsiveness. We found that PA-HD-Gal was readily activated upon incubation with recombinant beta-galactosidase. Specifically, a dose-dependent (0 to 10 U/mL) change in the absorbance (shift of the λ_{abs} from 640 to 745 nm) was observed, representing a maximum signal enhancement of 9.1 ± 0.5 -fold based on absorbance (Figure 3b). We confirmed that this increase was due to the enzymatic activity because heat-inactivation of beta-galactosidase prior to incubation with PA-HD-Gal completely attenuated probe activation (Figure S7). Likewise, PA-HD-NTR was activated by recombinant nitroreductase from *E. coli* (19.8 ± 1.7 -fold change in fluorescence after 1 h). Moreover, the probe was demonstrated to be stable in the presence of biological thiols such as Cys, H₂S, and GSH which can potentially reduce the nitro group and lead to 1,4-elimination to give a false response (Figure 3d).^[26] Finally, we determined that PA-HD-H₂O₂ retained its responsiveness to H₂O₂. In particular, a 6.3 ± 0.8 -fold

and 11.9 ± 1.5 -fold change in fluorescence was noted after incubation of the probe for 1 h with 100 μ M and 500 μ M H₂O₂, respectively (Figure 3f). In contrast, when PA-HD-H₂O₂ was subjected to selectivity screening against a panel of reactive oxygen/nitrogen species (hypochlorite, nitric oxide, *tert*-butyl hydroperoxide, superoxide, peroxyxynitrite) there was no significant turn-on. Collectively, these results show that installation of established triggers onto PA-HD does not impact their performance. The photophysical properties of all dyes are summarized below in Table 1.

Table 1. Summary of photophysical properties

Compound	λ_{abs}^a (nm)	λ_{em}^a (nm)	ϵ^a ($10^4 \text{ M}^{-1} \text{cm}^{-1}$)	$\Phi_F^{a,b}$ (%)	PABF (10^4)	pK _a ^c
O-HD	690	712	8.2	30	5.7	7.5
PA-HD	745	765	13.3	5	12.6	6.0
PA-HD-Gal	640	728	3.8	0.7	3.8	N.D.
PA-HD-NTR	642	735	2.7	0.7	2.8	N.D.
PA-HD-H ₂ O ₂	645	738	2.8	0.7	2.7	N.D.

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a) Determined at pH 8.25 in 1:1 v/v PBS:MeOH. [b] vs. ICG in DMSO. [c] pKa values determined in 1:1 v/v Britton–Robinson buffers ranging from pH 2.6 to 11.3. N.D. = Not determinable.

Incubation of PA-HD for 6 h at concentrations up to 10 μM did not significantly impact cell viability (Figure S8). Likewise, the three probes were also not cytotoxic (Figure S9). With these results in hand, we focused our attention on evaluating the performance of each probe in cell-based studies. Beta-galactosidase is known to be expressed in ovarian cancer presumably because it is involved in the processing of glycans such as sialyl Lewis x.^[27] Upon incubation of two human ovarian cancer cell lines with PA-HD-Gal, OVCAR-3 and IGROV-1, we were able to observe a cytosolic staining pattern which suggests our probe was being activated by beta-galactosidase (Figure 4a). To confirm these results, we synthesized BGA, a potent beta-galactosidase inhibitor,^[28] to determine whether pretreatment would attenuate probe activation. Relative to OVCAR-3 and IGROV-1 cells that were not treated with the inhibitor, the signal intensity was 2.18-fold and 2.78-fold lower, respectively (Figure 4b). Next, the ability of PA-HD-NTR to distinguish between normoxic and hypoxic conditions via nitroreductase activity was tested. ID8 cancer cells were cultured in 20%, 5%, 2%, or 1% oxygen atmospheres for ~6 h before treatment with PA-HD-NTR. Under oxygen deficient conditions, nitroreductases present within cells can convert the aryl nitro group to the corresponding hydroxyl amine or amino moieties via multiple single electron transfer events. This reduction can facilitate unmasking of PA-HD via self-immolative chemistry. As anticipated, the signal was barely discernable from background when PA-HD-NTR was incubated under normoxic conditions (20% O_2) (Figure 4c). In contrast, the intensity was 1.38 ± 0.10 -fold higher at 1% oxygen (Figure 4d). Importantly, the low but statistically significant signal enhancement is a consequence of the trigger which requires chronic hypoxic conditions to upregulate nitroreductase expression. Finally, NeuroScreen-1 cells, a model system for neurons, was stained with PA-HD- H_2O_2 and treated with 0, 25, 50, or 100 μM H_2O_2 (Figure 4e). We selected this cell line because oxidative stress plays a central role in neurodegenerative diseases, as well as aging of the brain. Our results revealed a dose-dependent increase in the signal intensity where the highest concentration of H_2O_2 resulted in a 19.3 ± 7.4 -fold turn-on response after incubation (Figure 4f).

Next, we generated syngeneic models of ovarian cancer and breast cancer using ID8 and 4T1 cells, respectively. Intratumoral injection was employed to administer PA-HD-Gal to the ovarian tumors and PA-HD-NTR to the breast tumors. We selected this route of administration, as opposed to systemic injection, because we wanted to directly compare the signal change with the non-tumor bearing flank which can be treated with the same amount of probe via subcutaneous injection. Typically, we utilize tumors with a volume of at least 300–400 mm^3 for imaging purposes; however, to test PA-HD-Gal, the average volume was only ~30 mm^3 to showcase the enhanced sensitivity afforded by PA-HD. After 2.5 h, cross-sectional photoacoustic images were acquired at 735 nm using a MSOT imaging system to detect the turned over product. We observed a turn-on response (defined as the photoacoustic signal at 2.5 h over the initial scan) of 1.31 ± 0.21 -fold in the tumor. In contrast, there was no change (0.99 ± 0.10) for the PA-HD-Gal-treated flanks (Figure S10). These results were confirmed via *ex vivo* photoacoustic imaging because photoacoustic-active dark skin patches appeared directly over the

tumor site as a consequence of shaving and use of depilatory cream to prepare the C57BL/6 mice for ID8 cell implantation (a common occurrence with this strain). We obtained similar results when PA-HD-NTR was utilized to detect hypoxia in 4T1 tumors. 1.5 h after administration of the probe, the signal intensity at 735 nm increased by 1.40 ± 0.24 -fold relative to the non-tumor flank which changed by 1.09 ± 0.10 -fold (Figure S11). As mentioned previously, in our hands, the nitroreductase trigger only yields a modest change since there is a requirement on the overexpression of nitroreductase.

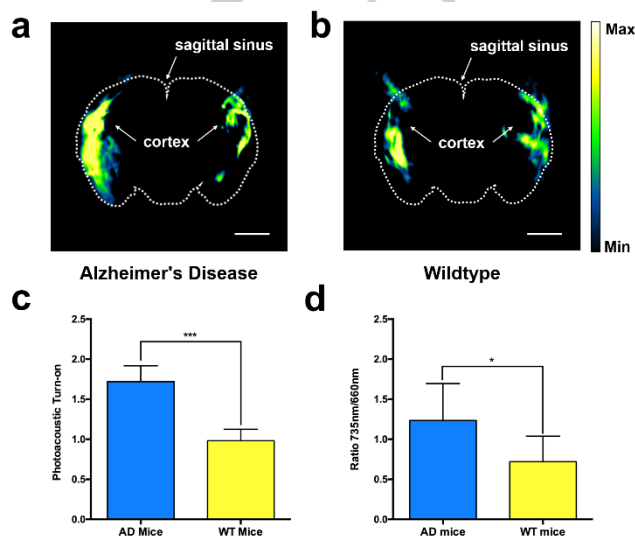


Figure 5. Representative photoacoustic images of the brain from a) an Alzheimer's disease mouse and b) a wildtype mouse captured 2.5 h after PA-HD- H_2O_2 was administered via retroorbital injection with excitation provided at 735 nm. Scale bar represents 2.5 mm. Dotted white outline of the brain is for visualization purposes only. Brain region cropped and overlaid onto a dark background. c) Photoacoustic turn-on response for Alzheimer's disease mice ($n = 5$) and wildtype mice ($n = 6$). Error bars = SD. Statistical analysis was performed using two-tailed Student's t-test ($\alpha = 0.05$), ***: $p < 0.001$. d) Photoacoustic ratio at 735 nm/660 nm of data from c) (Error bars = SD). Statistical analysis was performed using the Mann-Whitney U test ($\alpha = 0.05$), *: $p < 0.05$.

Finally, beyond subcutaneous tumor models, it is critical for us to evaluate whether the enhanced photoacoustic properties of PA-HD will effectively translate in a deep-tissue context. We sought to employ PA-HD- H_2O_2 to image oxidative stress (via H_2O_2 detection) in an Alzheimer's disease model. The brain consumes massive amounts of oxygen to fuel its stringent metabolic demands.^[29] However, when the natural antioxidant defense systems of the brain are compromised, a net increase in the generation of reactive oxygen species will result. Since oxidative stress is a hallmark of many neurological disorders including Alzheimer's disease,^[30] it is reasonable to assume that H_2O_2 will be elevated in the brain; however, there is limited direct evidence showing this to be true at the molecular level.^[31] In our study, we systemically administered PA-HD- H_2O_2 to 5xFAD mice which is a well-established transgenic model engineered to express the human amyloid beta protein precursor.^[32] We employed photoacoustic imaging at 660 nm to track probe uptake and observed a signal increase at this wavelength that plateaued after ~1 h. Of note, uptake of similar positively charged dyes through the blood-brain barrier have been reported in murine models of Alzheimer's

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disease.^[33] To ensure sufficient time for probe activation, we waited a total of 2.5 h before irradiating the brain at 735 nm (Figure 5a). A marked increase in the photoacoustic signal at this wavelength relative to the initial timepoint was observed (1.79 ± 0.20 -fold increase) indicating conversion of PA-HD-H₂O₂ to PA-HD (Figure 5b). In contrast, the turn-on response for healthy wildtype controls (B6SJLF1/J mice) was only 1.02 ± 0.14 -fold. To corroborate these results, we turned to ratiometric analysis (Figure 5b). We hypothesized the PA₇₃₅/PA₆₆₀ ratio will be higher in the 5xFAD mice compared to the B6SJLF1/J mice at the 2.5 h timepoint since this would represent probe activation and generation of PA-HD. Indeed, the ratio for the 5xFAD mice was 1.23 ± 0.46 , whereas the ratio for the B6SJLF1/J mice was 0.85 ± 0.07 . It is important to emphasize that ratiometric imaging would not have been possible with O-HD.

Conclusion

At the onset of this study, our goal was to establish a new dye platform that is optimized for the development of activatable photoacoustic probes. This approach represents a significant departure from the common practice of repurposing NIR fluorescent dyes that exhibit sub-optimal properties for photoacoustic imaging. One of the key criteria that we prioritized when developing PA-HD was accessibility, since we believe a high synthetic overhead (~ eight steps) would be less desirable to probe developers in the field. The O-HD dye developed by Lin and co-workers represents an attractive starting point for our remodelling efforts because it can be prepared from Cy7-Cl and resorcinol in a single step and has been shown to be a versatile platform for various biological applications. Beginning from 4-chloro-3-methoxybenzenethiol, PA-HD can be prepared in only three-steps with an overall yield of 33.3%. In this study, we demonstrated that substitution of the endocyclic oxygen atom with a sulfur group affords many desired properties. For instance, we observed a red-shifted λ_{abs} of 55 nm which facilitated access to deeper tissue and enabled ratiometric imaging. The ability to confirm an *in vivo* imaging result by tracking the signal change at two wavelengths clearly sets PA-HD apart from O-HD. While spectral unmixing is another strategy we could have potentially utilized to isolate the signal of PA-HD-H₂O₂ from that of background, this requires us to obtain a high-quality *in vivo* spectrum of the probe which we were unable to do in this instance. This highlights why ratiometric imaging is so critical. It is noteworthy that while we were evaluating the photoacoustic properties of PA-HD, a group prepared a NIR fluorescent probe based on a similar platform (lacking the *ortho*-chloro group and a different *N*-alkylation pattern) to image cysteine in live cells.^[34] However, as we have demonstrated in this work, sulfur-substitution results in a significant decrease in the fluorescent quantum yield which favors photoacoustic imaging while rendering the dye less effective for optical imaging. Indeed, to acquire the fluorescent cell images featured in Figure 3, it was necessary for us to irradiate with the light source set at 100% power in some instances. With this in mind, we envision PA-HD will facilitate the development of a diverse range of new activatable photoacoustic probes. We are optimistic that this work will result in a divergence between O-HD-based probes for fluorescent applications and PA-HD-based chemical tools for photoacoustic imaging.

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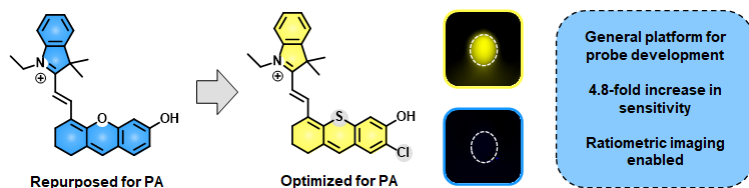
Keywords: Photoacoustic imaging • Acoustogenic Probe • Analyte Sensing • Hydrogen Peroxide • Alzheimer's Disease

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We have developed PA-HD, a new sulfur-substituted dye platform exhibiting enhanced properties for photoacoustic probe development. To demonstrate generalizability, we employed PA-HD to access probes for beta-galactosidase and nitroreductase activities, as well as H_2O_2 . Each probe was tested in live cells and *in vivo*. For instance, the H_2O_2 probe was employed to visualize oxidative stress in an Alzheimer's disease model.

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