Synthesis of silicon-substituted hemicyanines for multimodal SWIR imaging

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ABSTRACT: SWIR dyes offer many advantages over their more common NIR congeners; however, the available options are limited. New SWIR imaging agents can be accessed by remodeling existing NIR molecules (i.e., hemicyanines (HD)). In this study, we synthesized SWIR-HD, a modified HD featuring silicon-dimethyl and benzo[cd]indolium groups that are designed to red-shift the absorbance and emission to 988 and 1126 nm, respectively. SWIR-HD was tested in three cell lines and shown to exhibit excellent biocompatibility.

Introduction

Molecular imaging can enable real-time visualization of complex biological processes when augmented with robust optical dyes. Two of the most promising modalities for biomedical applications are fluorescence (FL) and photoacoustic (PA) imaging. Both techniques involve optical excitation of a chromophore to yield an excited state but differ through their ensuing relaxation pathways. FL involves radiative decay which releases a photon of light at a longer wavelength; whereas PA undergoes non-radiative relaxation to afford an ultrasonic signal via the PA effect. Until recently, the NIR range (650-900 nm) was the preferred spectral window. However, recent advances in instrumentation have facilitated a move into the shortwave infrared (SWIR) region (900-1700 nm) where interference from endogenous pigments are further minimized. It is noteworthy, a FL molecule does not need to absorb incident light in this window to generate a SWIR signal. For example, the λabs for indocyanine green (ICG) is 780 nm but features an emission tail beyond 900 nm that can be used for SWIR FL imaging. Similarly, most conventional PA imaging agents maximally absorb NIR light. However, in this spectral range, dyes must contend with PA-active pigments such as haemoglobin, resulting in higher background signals. In both instances, the application of SWIR excitation can lead to enhanced tissue penetration, higher signal-to-background ratios, and greater sensitivity.

A growing suite of SWIR imaging agents have recently been developed, with notable examples coming from the cyanines, xanthenes, and BODIPY families. However, these examples are hydrophobic and intrinsically prone to aggregation. While nanoparticle formulations can potentially offset these drawbacks, it is preferable to develop complementary SWIR dyes that do not require encapsulation for imaging. In this study, we strategically remodeled the hemicyanine dye (HD) (a common NIR platform) for multimodal SWIR FL and PA imaging. Of note, complementary remodeling efforts have yielded PA and NIR-II analogs. However, in the present study, we selected this platform owing to its relatively small size and ease of conversion into activity-based sensing probes. Unfortunately, these favourable properties come at a cost because HDs are essentially truncated cyanines which are blue shifted by ~100 nm. To compensate for the loss of π-conjugation, we introduced two key structural modifications using a modular synthetic route. Importantly, the best dye (SWIR-HD) we prepared,
absorbs and emits in the SWIR window. Moreover, SWIR-HD was tested in three cell lines and shown to exhibit excellent biocompatibility.

**Design of Silicon-substituted Hemicyanines**

The first modification made to the HD involves replacing the endocyclic oxygen atom with an electron-deficient functional group. Heteroatom substitution is a versatile strategy employed to shift the absorbance and emission profile of various dye platforms to longer wavelengths, with fluorescein and related molecules being the most notable examples. Substitutions include oxygen to boron, carbon, silicon, phosphorus, sulfur, as well as several other group 14 elements. From this list, we identified silicon as the most optimal heteroatom for our dye remodeling campaign. First, it has been shown that regardless of the scaffold being modified, replacing the endocyclic oxygen with a silicon group (i.e., SiMe$_2$) reliably red shifts the $\lambda_{\text{max}}$ by $\sim$90 nm. Second, unlike elements such as boron which can decompose in the presence of ROS, the SiMe$_2$ group is remarkably stable under oxidative conditions. Third, on the contrary to heavy-atoms such as selenium, silicon-based dyes are not known to generate singlet oxygen upon irradiation, which is important to limit unnecessary phototoxicity. Moreover, the inability to generate substantial levels of singlet oxygen likely contributes to the outstanding photosensitivity exhibited by silicon-substituted dyes.

Unfortunately, the canonical synthetic route utilized to access the HD core is incompatible with silicon incorporation (**Scheme 1** (top)). Specifically, the crucial carbon-oxygen bond that is formed between the cyanine (e.g., Cy7-Cl) and resorcinol (or 3-(diethylamino)phenol) precursors involve a conjugated substitution reaction where the latter acts as a nucleophilic species. However, silicon typically behaves as an electrophile, although strategies have been conceived to reverse this reactivity. We hypothesized it would be possible to employ organolithium chemistry to incorporate the requisite SiMe$_2$ group (**Scheme 1** (bottom)). Once attached, we would employ a non-templated vinylogous aldol condensation reaction to yield the dye backbone, which could be further extended using a Knoevenagel condensation reaction. The absorbance and emission can be further tuned at this stage by varying the acceptor.

**Synthesis of SiHD Dyes**

The synthesis of the SiHDs (**Scheme 2**) began with the treatment of 3-bromo aniline with ethyl iodide to yield 3-bromo-$N,N$-diethylaniline 2. Next, a Vilsmeier-Haack reaction was performed to obtain the para-formylated intermediate 3 in 48% yield. Protection of the aldehyde with ethylene glycol under acidic conditions afforded acetal 4 with 59% conversion. In parallel, the acetal-protected vinyl bromide building block 5 was prepared in 26% yield over 2-steps. With 4 and 5 in hand, we screened conditions to obtain dialdehyde 6. For instance, we activated one component (4 or 5) with $n$-butyllithium, followed by the sequential addition of dichlorodimethylsilane and the second component after lithium halogen exchange. Moreover, we explored activation of both compounds in one-pot at different ratios prior to the addition of the silane. We determined the most reproducible, scalable, and high yielding combination was the simultaneous activation of 4 and 5 (at a 1:1 molar ratio), which gave 6 in 34% upon acid-mediated deprotection.

**Scheme 1.** (top) Canonical route involving conjugate substitution and retro-Knoevenagel reactions to synthesize HD. (bottom) Retrosynthetic analysis of SiHDs.

**Scheme 2.** Synthesis of SiHD-1, SiHD-2, and SiHD-3.
After successfully preparing the proposed panel of SiHds, we proceeded to determine if any of these dyes were candidates for multimodal SWIR imaging. While with the $\lambda_{abs}$ for SiHD-1 (855 nm, 131 nm red-shift) and Si-HD-2 (850 nm, 110 nm red-shift) did not reach the SWIR window (Figure S1, S2), a direct comparison to the matching HD analogs provided crucial insight (Table S1). We found that installation of the SiMe$_2$ group onto this platform resulted in a $\lambda_{abs}$ shift that exceeds what is typically seen with other dyes (~90 nm). Moreover, we discovered that relative to SiHD-1, the benzo[cd] indolium modification (SiHD-3) further

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<th>Solvent</th>
<th>Molarity</th>
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Table 1. Conditions (base, additives, solvent, and molarity) explored to favor formation of vinylogous aldol condensation product 7.

In vitro Characterization and Stability Assays

Excitation of SWIR-HD at 988 nm resulted in SWIR emission with a $\lambda_{em}$ value of 1126 nm (QY = 0.01%, 138 nm Stokes shift) (Figure 1a). Of note, the entire emission spectrum of SWIR-HD fell within the SWIR window. Moreover, we observed a linear relationship between dye concentration and emission intensity up to at least 25 µM (Figures 1b, 1c). In the context of PA imaging, the best predictor of ultrasound intensity is an empirical term known as the PA Brightness Factor (PABF = molar extinction coefficient $\times$ (1 - fluorescent quantum yield)). Because the former term in this equation has a greater influence on the calculated PABF value, a measured $\epsilon$ value of $1.5 \times 10^4$ M$^{-1}$cm$^{-1}$ for SWIR-HD almost certainly ensures the PA readout will be sufficiently strong for in vivo applications. To determine the experimental PA properties of SWIR-HD, we obtained a PA emission spectrum by irradiating the dye from 650 to 1100 nm (Figure 1d, S3). Analysis of this spectrum reveal a $\lambda_{pa}$ of 1035 nm along with a blue-shifted shoulder at 945 nm. Next, we designed an experiment to predict whether SWIR could operate in a deep tissue context. This was accomplished by casting a tissue-mimicking phantom comprised of milk and agar that was three-fold thicker (3 cm) than phantoms routinely used to evaluate other photoacoustic dyes. Even with the substantial increase in phantom dimension, a strong PA signal could be readily observed at a dye concentration of 30 µM in both organic solvents and aqueous media (Figure 1e).

Beyond these excellent multimodal properties, it is essential for SWIR-HD to exhibit exceptional photo- and chemo-stability to serve as a viable dye for in vivo applications. First, because the body consists of various applications. To determine the experimental PA properties of SWIR-HD, we obtained a PA emission spectrum by irradiating the dye from 650 to 1100 nm (Figure 1d, S3). Analysis of this spectrum reveal a $\lambda_{pa}$ of 1035 nm along with a blue-shifted shoulder at 945 nm. Next, we designed an experiment to predict whether SWIR could operate in a deep tissue context. This was accomplished by casting a tissue-mimicking phantom comprised of milk and agar that was three-fold thicker (3 cm) than phantoms routinely used to evaluate other photoacoustic dyes. Even with the substantial increase in phantom dimension, a strong PA signal could be readily observed at a dye concentration of 30 µM in both organic solvents and aqueous media (Figure 1e).

Beyond these excellent multimodal properties, it is essential for SWIR-HD to exhibit exceptional photo- and chemo-stability to serve as a viable dye for in vivo applications. First, because the body consists of various
acids and alkaline gradients, we measured the absorbance across a pH range of 6.0 to 8.0 (Figure 2a). The intensity did not change substantially, indicating excellent tolerance to potential pH fluxes in the body. Next, we tested the likelihood of photobleaching by irradiating SWIR-HD at 988 nm using a pulsed laser set (1.25 MW). After 900 s, more than 95% of the dye remained (Figure 2b), indicating SWIR-HD would be sufficiently photostable during a typical imaging session. Lastly, we treated SWIR-HD with a panel of biologically relevant reactive oxygen and nitrogen species, as well as redox active metal ions (Figure 2c). Although the parent HD is prone to rapid oxidative decomposition, SWIR-HD was remarkably resistant against strong oxidants such as peroxynitrite even at levels 100,000-fold higher than the reported cellular concentration of 5-10 nM. Likewise, we observed no significant loss of SWIR-HD in the presence of redox-active metals (Fe and Cu) capable of generating ROS via Fenton and Fenton-like chemistry, respectively.

Figure 2. a) pH-absorbance profile of SWIR-HD (5 µM) measured at 965 nm. pH values tested = 6.00, 6.51, 7.00, 7.45, 8.01. b) Irradiation of SWIR-HD at 988 nm using a pulsed laser for 900 s (purple line). ICG (black line) shown for comparison. c) Percent SWIR-HD remaining after incubation with various ROS, RNS, and metal ions. d) Determination of singlet oxygen generation using DPBF after irradiation with a xenon arc lamp for 300 and 600 s. e) HT29, HCT116, and A549 cells were incubated with SWIR-HD (2, 4, or 6 µM) for 4 hrs. Cell viability was determined by comparing dye treatment to DMSO vehicle (n = 3).

Assessment of Biocompatibility

Prior to testing SWIR-HD in vivo, we performed two crucial experiments to assess biocompatibility. The substitution of oxygen with other group 16 elements (S and Se) or the installation of halides have been shown to facilitate intersystem crossing, which leads to singlet oxygen production upon exposure to light. This property would be a detriment to any multimodal contrast agent owing to unwanted phototoxicity. When SWIR-HD was irradiated in the presence of DPBF, a colormetric singlet oxygen sensor, the absorbance from 410-420 nm remained unchanged after 300 and 600 seconds (Figure 2d). This result indicates the inclusion of the SiMe₂ group does not result in singlet oxygen production, which is important to avoid ROS-mediated damage. In addition, we performed a standard MTT assay in HT29, HCT116, and A549 cancer cells to evaluate potential cytotoxicity when imaging with SWIR-HD. No loss of cell viability was observed when cells were incubated with SWIR-HD for 4 hours up to a concentration of 6 µM (Figure 2d).

Conclusion

To generate a robust FL or PA signal in vivo, a sufficient amount of incident light must be able to reach a given optical absorber. However, as photons pass through tissue, endogenous pigments (e.g., hemoglobin) can readily intercept and scatter light. A shift toward the SWIR imaging window where interference is minimized promises to enable access to deeper regions of the body. To facilitate this, we designed an efficient synthetic route to access SWIR-HD, in six-steps starting from readily accessible acetal precursors. Typically, Lewis acid catalysts are employed to shift the reactivity preference from the α-site to the γ-site. However, in our case we were able to access compound 7, which is a key intermediate in our synthesis through a non-templated vinylogous aldol reaction. Beyond strategically selecting the base, varying the solvent polarity, and controlling the reaction molarity, we discovered it was essential to include Burgess Reagent to facilitate a key intramolecular cyclization-dehydration sequence. The ability to substitute the endocyclic oxygen atom of the parent HD with an electron-deficient SiMe₂ group and vary the donor in the final step using this chemistry was instrumental in red shifting the λ_abs by 264 nm from 724 nm to 988 nm.

ASSOCIATED CONTENT

Supporting Information

Further experimental details, including synthetic procedures, spectral data, and supplemental in vitro and in vivo procedures and data are supplied in the Supporting Information document. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors.

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