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Tailored Near-Infrared Contrast Agents for Image Guided Surgery

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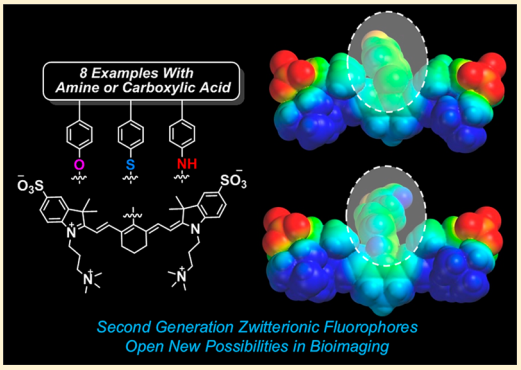
Supporting Information

ABSTRACT: The success of near-infrared (NIR) fluorescence to be employed for intraoperative imaging relies on the ability to develop a highly stable, NIR fluorescent, nontoxic, biocompatible, and highly excreted compound that retains a reactive functionality for conjugation to a cancer-recognizing peptide. Herein, systematic modifications to previously detailed fluorophore ZW800-1 are explored. Specific modifications, including the isosteric replacement of the O atom of ZW800-1, include nucleophilic amine and sulfur species attached to the heptamethine core. These novel compounds have shown similar satisfactory results in biodistribution and clearance while also expressing increased stability in serum. Most importantly, all of the synthesized and evaluated compounds display a reactive functionality (either a free amino group or carboxylic acid moiety) for further bioconjugation. The results obtained from the newly prepared derivatives demonstrate that the central substitution with the studied linking agents retains the ultralow background in vivo performance of the fluorophores regardless of the total net charge.



INTRODUCTION

Near-infrared (NIR) based molecular imaging has emerged as an effective technique for in vivo diagnostics. The numerous advantages of this technique compared to other traditional imaging techniques such as positron emission tomography (PET) or single photon emission computed tomography (SPECT) include the ability for real-time monitoring,[1−3] the use of nonionizing long wavelength deep tissue-penetrating light, the increased signal-to-background ratios, cost effectiveness, and tumor-targeting.[4,5]

Previously synthesized heptamethine NIR fluorophores, including indocyanine green **1** (ICG, Figure 1), are nonideal imaging agents because they exhibit elevated liver uptake, show background signal in the gastrointestinal (GI) tract, demonstrate inferior optical properties (low quantum yield/extinction coefficient), are unstable or nonsoluble in aqueous media, and are unable to conjugate covalently to targeting ligands.[6−8] Our previous efforts toward developing a NIR fluorophore for image guided surgery yielded a novel zwitterionic heptamethine dye **2** (ZW800-1, Figure 1) with enhanced photophysical characteristics (high extinction coefficient and quantum yield in serum) and physiological properties such as fast systemic circulation, reduced nonspecific binding, and rapid renal clearance.[6] Through the development of ZW800-1, we concluded that the balanced net zwitterionic charge directly influenced the improved in vivo performance. The use of phenylsulfonato (anionic) and alkylammonium (cationic) groups kept the surface charge balanced with the *meso*-carboxylate balancing the delocalized cation across the polymethine chain. Our previous hypothesis suggests that disturbing this zwitterionic charge-balanced system would sacrifice the optimum biological clearance which we define as high renal excretion to the urinary bladder within 4 h. To test the validity of this argument, we must alter the net charge of the molecule and observe the corresponding change in biodistribution properties. To fully elucidate the structural characteristics of fluorophores that are responsible for biodistribution and renal excretion, we modified the nonresonant structure attached to the *meso*-carbon on the fluorophore from the carboxylate seen in ZW800-1 (net zero charge) to a primary amine that would be protonated at physiological pH to yield an overall dicationic compound. The Stokes shifts of heptamethine cyanines are very dependent on the particular substituents that alter the stability of the excited state; similarly, added structural stability to overcome photobleaching of these compounds can be achieved using electron donating groups, especially on the central carbon of the polymethine chain. Increased Stokes shifts and structural stability in solution are very desirable, and through the incorporation of amino and sulfur moieties, a cyanine fluorophore can become increasingly stable even in solution when irradiated with light. Toward systematically probing this

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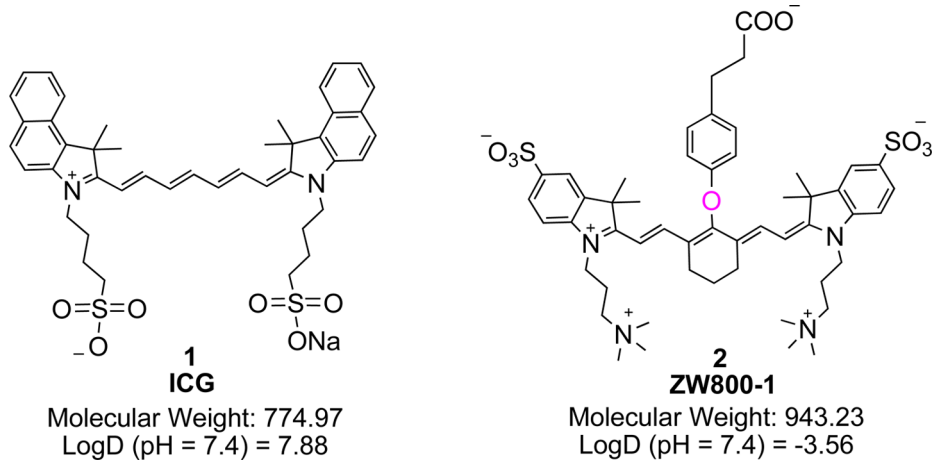
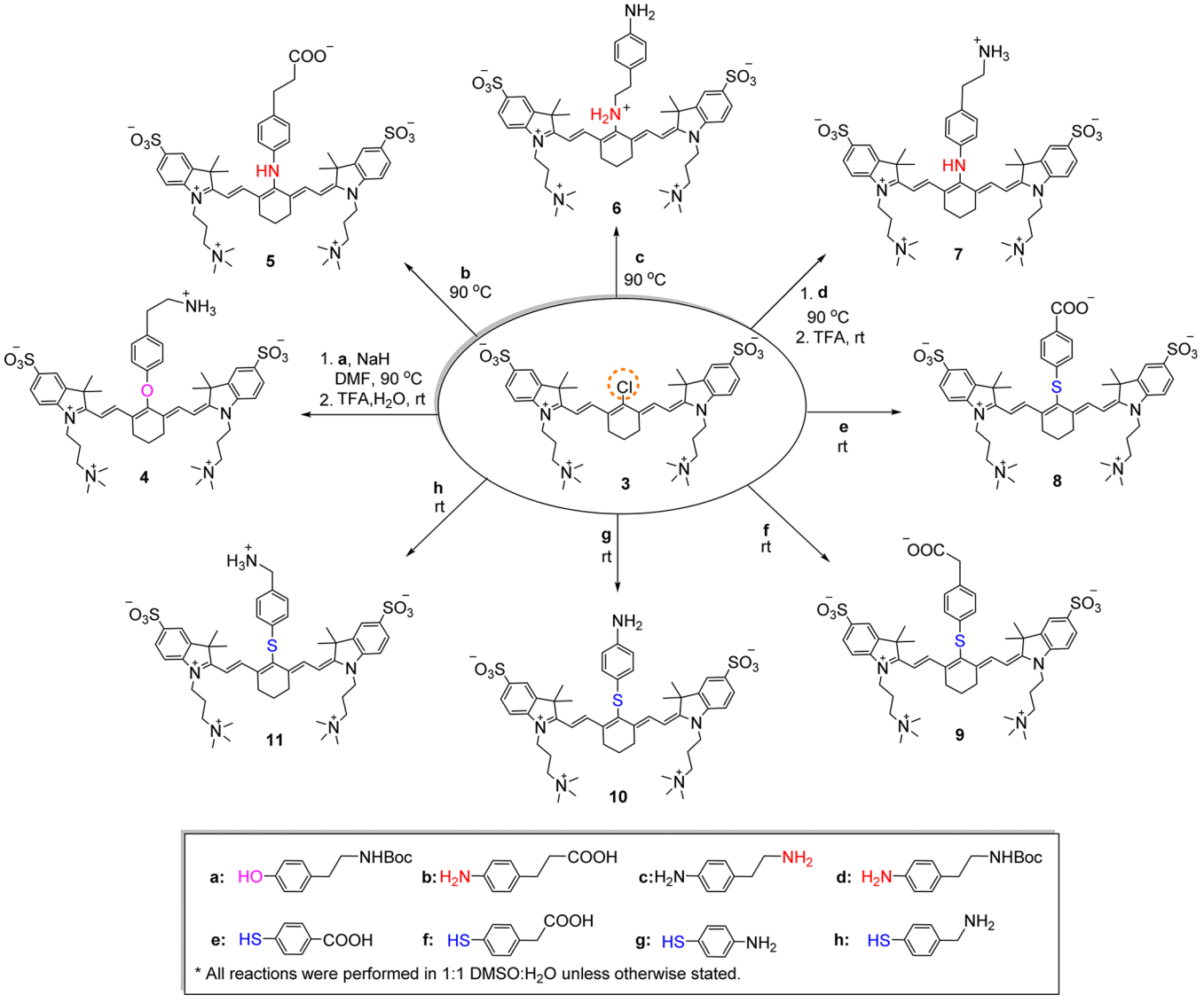


Figure 1. Chemical structures of ICG and ZW800-1.

Scheme 1. Synthetic Route to Novel Zwitterionic NIR Fluorophores with Various *meso*-Substitutions



conjecture of charge-dependent biodistribution, we varied the conjugating moiety attached to the *meso*-carbon for perfecting synthetic, photophysical, and biological efficacy. We have synthesized a number of complementary analogs (Scheme 1) for structurally modifying the conjugating moiety of ZW800-1 (**2**, Figure 1) and evaluated them to ascertain the biodistribution pattern with respect to the deviation from the net charge which was initially zero. Furthermore, by replacing

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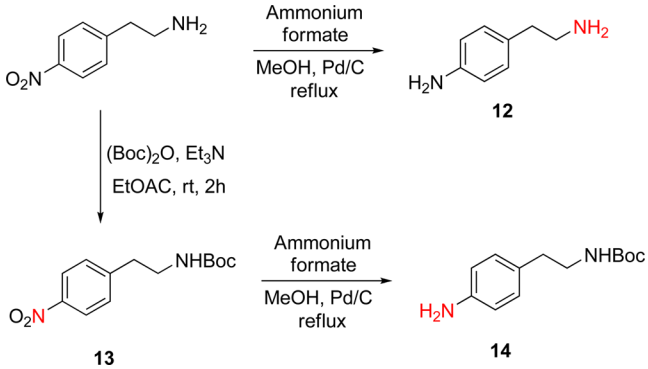
the carboxylic acid in ZW800-1 with an amine moiety, we now have the option to conjugate other biologically relevant molecules that possess a reactive carboxylic acid moiety in lieu of a primary amine (i.e., folic acid, etc.). The results of this study will provide more details about how to improve the optical properties and in vivo biodistribution for future implementation in the clinic.

RESULTS AND DISCUSSION

The chloro-substituted fluorophore **3** exhibits symmetric quaternary ammonium cations, and sulfonate substituents were prepared as already reported through salt condensation with Vilsmeier−Haack reagent to build the heptamethine carbocyanine core.[6] The desired dyes shown in Scheme 1 were synthesized by reacting the *meso* chlorine atom of the fluorophore **3** with appropriate nucleophiles, through the $S_{RN}1$ displacement pathway, in order to introduce a linking moiety (either carboxylic acid or amine) for subsequent bioconjugation.[9,10] Precursor **3** displays limited solubility in dimethyl sulfoxide (DMSO) or dimethylformamide (DMF), which has served as an ideal solvent for these reactions in the past;[6,11] however, because of the limited solubility of the fluorophore **3** in these solvents, water was used to improve the solubility of this fluorophore in DMSO, and this therefore led to improved yields for these $S_{RN}1$ reactions. Three nucleophilic atoms (O, N, and S) were utilized to incorporate new linking components containing either a terminal amine or carboxylic acid to our highly appealing zwitterionic framework. Aromatic amines and carboxylic acids are more difficult to couple via conventional amide-coupling strategies because of electron delocalization across the ring; therefore, we prepared both compounds with the direct aromatic attachment and also with carbon spacers between the aromatic system and the coupling component. As a direct comparison to ZW800-1, we began the synthesis by using boc-protected tyramine to link the phenolic moiety to the central carbon of the methine chain, the final ZW800-1 analog **4** displaying a free primary amine for bioconjugated amide bond formation. The selective reactivity at the oxygen atom was ensured by protection of the primary aliphatic amine group prior to the reaction with the chloro dye. The protonated oxygen exhibits limited nucleophilicity, and a base is required to generate the phenoxide ion which is more nucleophilic and is used in situ to react with the fluorophore **3** to provide the Boc protected product which is then converted to the free amine product by treatment with trifluoroacetic acid (TFA) in H_2O at room temperature. Purification by precipitation using DMSO in ethyl acetate followed by reverse phase column chromatography provided the pure product **4**.

Amino-substituted heptamethine cyanines were then prepared to observe a change in biological and photophysical properties; furthermore, we chose to study both an aliphatic and aromatic amine attached to the *meso*-carbon. We synthesized two substituted ZW800-1 analogs using the replacement of the *meso*-chlorine by the diamine compound shown in Scheme 2. The commercially available starting material, 2-(4-nitrophenyl)ethanamine is reduced to the diamino compound **12** using ammonium formate in Pd/C. The diamino precursor **12** displays two nucleophilic positions, and when the reaction is carried out with the chloro compound, we observed a large blue shift coming from the attachment of the aliphatic portion of the diamino precursor which afforded compound **6**. The selective nucleophilic reaction of the aliphatic amine in the presence of the aromatic amine is due

Scheme 2. Synthesis of Aliphatic Amine Linker 12 and Aromatic Amine Linker 14



to the increased nucleophilic nature of the alkylamine caused by the available lone pair of electrons as compared to the aromatic amine whose electrons are delocalized across the aromatic ring. It has been reported that a substantial blue shift is observed in the absorption wavelength when an aliphatic amine is attached to the central carbon; however, this phenomenon is not observed if the nucleophilic amine is aromatic. In order to achieve substitution with the arylamine, we proceeded using Boc protection of the aliphatic amine,[12] followed by reduction of the nitro group using ammonium formate in the presence of Pd/C at reflux to afford the Boc protected amine **14** as depicted in Scheme 2. This Boc protected amine **14** was used to replace the chlorine fluorophore **3** followed by deprotection in trifluoroacetic acid as elaborated in Scheme 1 to form compound **7**. The carboxylic acid derivative fluorophore **5** was prepared using similar protocol with commercially available linker 3-(4-aminophenyl)propanoic acid.

Four new dyes containing the thiol linker were synthesized by application of the $S_{RN}1$ reaction. As highlighted in Scheme 1, compounds **8** and **9** containing a carboxylic acid group were synthesized from the reaction between commercially available mercaptobenzoic acid and 2-(4-mercaptophenyl)acetic acid respectively with the dye **3**. These reactions were performed using DMSO and water as an effective cosolvent system. Purification of the crude was achieved by precipitation using DMSO in ethyl acetate and by reverse phase column chromatography using acetonitrile and water as the eluting solvent.

The dyes **10** and **11** containing the thiol linker but with a terminal amine group were obtained using the same procedure as described above for dyes **8** and **9**. After optimizing the reaction conditions, we achieved more efficient synthesis with 6 molar equivalents of the 4-aminobenzenethiol being allowed to react with 1 equiv of the dye **3** to provide compound **10**. In order to synthesize the final analog **11**, the novel linker **18** had to be prepared.

The synthesis of linker **18**, highlighted in Scheme 3, was initiated from the commercially available 4-hydroxybenzonitrile which was treated with dimethylcarbamothioic chloride in the presence of DABCO as base at 65 °C to provide *O*-(4-cyanophenyl) *N*,*N*-dimethylcarbamothioate **15**. Intermediate **15** was subjected to the Newman−Kwart rearrangement,[13,14] which prompted the observed intramolecular aryl migration of the *O*-thiocarbamate at elevated temperature to yield the corresponding *S*-(4-cyanophenyl) dimethylcarbamothioate **16** in quantitative yield which was converted to the free thiol **17** by treatment with potassium hydroxide in methanol.[15−17] The

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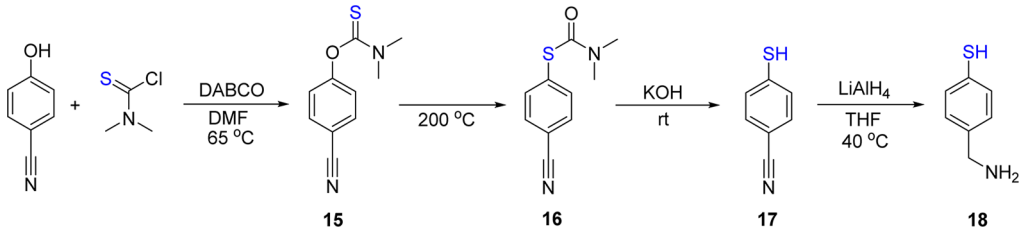
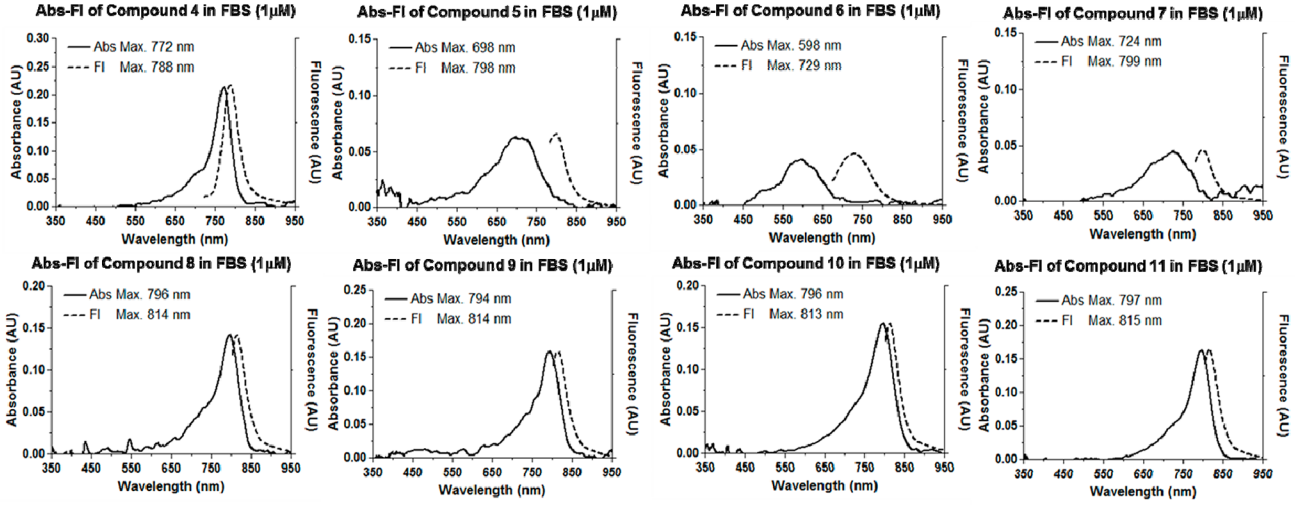
Scheme 3. Synthesis of Thiol Linker 18 Containing Aliphatic Amine





	4	5	6	7	8	9	10	11
Linker	-O-Ar	-NH-Ar	-NH-Alk	-NH-Ar	-S-Ar	-S-Ar	-S-Ar	-S-Ar
Functionality	Alk-NH_2	Alk-COOH	Ar-NH_2	Alk-NH_2	Ar-COOH	Alk-COOH	Ar-NH_2	Alk-NH_2
MW (Da)	915.23	942.24	914.25	914.25	931.23	945.26	903.25	917.27
Log D (pH = 7.4)	-2.98	-3.28	-1.97	-3.38	-3.3	-3.48	-0.43	-2.48
Linker pKa	N/A	1.70	8.44	1.95	N/A	N/A	N/A	N/A
Functionality pKa	9.81	4.49	4.41	9.93	3.58	3.72	2.47	9.31
Net Charge	+2	0	+2	+2	0	0	+1	+2
ε ($M^{-1}cm^{-1}$)	214,000	63,000	41,000	45,000	142,000	159,000	155,000	163,000
λ_{exc} (nm)	772	698	598	724	796	794	796	797
λ_{em} (nm)	788	798	729	799	814	814	813	815
Stokes shift (nm)	16	100	131	75	18	20	17	18
Φ (%)	22.3	3.1	6.9	3.8	11	10.8	4.5	11.4
Stability (%)	36	52	89	53	72	79	83	83

Figure 2. Absorbance and fluorescence spectra of the novel ZW800 analogs **4**–**11** recorded in 100% FBS, pH 7.4, at a 1 μM concentration (top). Summary of the physicochemical and optical properties of the various novel synthesized NIR dyes in serum at pH 7.4 (bottom). Note: The peak sizes of fluorescence spectra are arranged by the peak values of absorbance spectra to show them together as a single pair for clear observation of the Stokes shift. Abbreviations used are the following: Ar designates that the moiety is attached to an aromatic system, while Alk indicates attachment to an alkyl group.

resulting 4-mercaptobenzonitrile **17** was conveniently reduced to 4-(aminomethyl)benzenethiol **18** by reaction with lithium aluminum hydride.[18] After preparation of the linker, it was attached to the chloro compound **3** using similar reaction conditions previously described. The difference between compound **11** and the other compounds described was that it took a shorter time to achieve complete conversion of the product. It is important to note that because of the very high nucleophilicity of the sulfur atom compared to the nitrogen atom, no protecting group was required for the complete and selective reaction of the sulfur with the nucleofugal carbon of chloro compound **3** to afford the final ZW800-1 analogs **10** and **11**.

Physicochemical Characteristics, Optical Properties, and Stability Measurements. Figure 2 summarizes the physicochemical and optical properties of the various heptamethine ZW800-1 NIR analogs synthesized. These measurements were either theoretically calculated using the JChem plugin of ChemAxon or experimentally determined in fetal bovine serum (FBS). It can be observed from Figure 2 that the molecular weight and log *D* values are quite similar; however, there are great differences concerning the pK_a of the

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linker units and the functional moieties. Indeed, the net charge of the compounds and the resulting biodistribution properties are dependent on the pK_a of each of these ionizable units and the synthesized compounds span the breadth from a net charge of 0 to +2 depending on the placement of carboxylic acids and alkyl- or arylamines. From the optical measurements enumerated in Figure 2, it can be observed that the molar extinction coefficient of compound **4** is the highest, and this is due to the fact that the oxygen atom linked to the polymethine system has less effect on the polymethine chain compared to the compounds possessing the S linker such as compounds **8**–**11**. Compounds **5**–**7** containing the amine linkers have the lowest molar extinction coefficient probably because of the competing resonance pathways leading to a broad absorption spectrum.

As expected, the dyes containing the amine linkers have the largest Stokes shift compared to the dyes possessing the oxygen and sulfur linkers, as the nitrogen atom participates in an intermolecular charge transfer which affords the large Stokes shift.[19] Also, as expected we observe from the table in Figure 2 that the quantum yield of compound **4** is the highest while the dyes containing the amine linkers are the lowest. The stability studies, highlighted in Figure 3, were also performed in serum

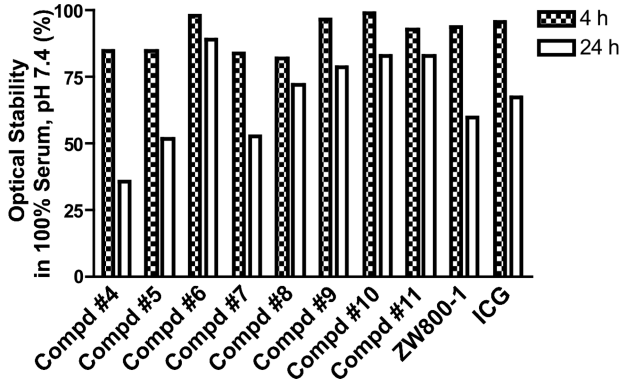

Figure 3. Optical stability of ZW800-1 derivatives in 100% FBS, pH 7.4, at 37 °C for 24 h.

at 37 °C for 24 h, and the results revealed that the least stable was compound **4** because of the ether linker which can easily be degraded, while the linkers containing sulfur atoms are larger and more highly polarizable which leads to increased stability without much influence on the polymethine resonance which is confirmed in the optical spectra. Also, as expected, the final compounds containing aliphatic amines expressed a better stability than the aromatic amines which can be explained by the better leaving group character in the aromatic anilide leaving group supplied by the aromatic character versus the primary amine.

Biological Studies and Comparison to ZW800-1. In order to confirm the in vivo performances of the various zwitterionic NIR fluorophores, the ZW800-1 analogs were administered intravenously into CD-1 mice, and their biodistribution and clearance were investigated in real time over 4 h. As shown in Figure 4, all of ZW800-1 analogs were eliminated from the body into urine by renal clearance with almost no significant nonspecific uptake in other organs and tissues, the same as the in vivo performance of ZW800-1, although compounds **8** and **9** showed weak uptake in liver. To compare the excretion amount of injected fluorophores in mice, the urines from bladder were collected after 4 h of imaging and the volumes and optical absorbance were measured. In Figure 5, compounds **4**–**7** containing the oxygen and amine linkers showed higher excretion amounts over 80–90% ID during 4 h postinjection, while compounds **8**–**11** containing sulfur linker showed similar excretion amounts with ZW800-1 over 70–80% ID. All of ZW800-1 analogs exhibited rapid and excellent renal excretion for 4 h compared to ICG showing only 3% ID in urine. In terms of the clinical use for safety and toxicity, we demonstrated that the zwitterionic NIR fluorophores excreted rapidly through renal filtration in certain time and showed ultralow nonspecific backgrounds. Therefore, ZW800-1 analogs may be good candidates for future in vivo applications such as ligand–receptor specific targeting; accordingly, we plan to exploit **9** and **11** with their increased stability in conjugated imaging using various targeting ligands.

The increased stability is very important during surgical resection of diseased tissue because the fluorophores must be retained and demonstrate optimum imaging capabilities for long periods in the body. These new sulfur-based fluorophores have the potential to greatly assist the medical community in these long-duration surgical resections, and we will begin assessing their efficacy postconjugation, which will be discussed in a separate manuscript.

CONCLUSIONS

We have demonstrated that the systematic modification of zwitterionic heptamethine fluorophore ZW800-1 with various charged linking functionalities does not have a significant impact on the renal excretion yet is facilitated by only the strong surface charge of the core. We can now utilize the amine functionalized zwitterionic compounds for bioconjugation without jeopardizing the effective clearance properties. The sulfur atom bound to the cyanine core has increased the overall stability of the fluorophore while allowing excellent optical and clearance properties.

MATERIALS AND METHODS

All chemicals and solvents were of American Chemical Society grade or HPLC purity and were used as received. HPLC grade acetonitrile (CH_3CN) and water were purchased from VWR International (West Chester, PA) and American Bioanalytic (Natick, MA), respectively. All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA), Sigma-Aldrich (Saint Louis, MO), and Acros Organics. The reactions were followed using silica gel 60 F_{254} thin layer chromatography plates (Merck EMD Millipore, Darmstadt, Germany). Open column chromatography was utilized for the purification of all hydrophobic final compounds using 60–200 μm, 60A classic column silica gel (Dynamic Adsorbents, Norcross, GA). Highly charged final products were isolated using revered phase C18 column chromatography (Fluka). The ^{1}H NMR and ^{13}C NMR spectra were obtained using high quality Kontes NMR tubes (Kimble Chase, Vineland, NJ) rated to 500 MHz and were recorded on a Bruker Avance (400 MHz) spectrometer using D_2O, DMSO-d_6, or MeOD-d_4 containing tetramethylsilane (TMS) as an internal calibration standard set to 0.0 ppm. NMR abbreviations used throughout the experimental section are as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, dd = doublet doublets, and bs = broad singlet. UV–vis/NIR absorption spectra were recorded on a Varian Cary 50 spectrophotometer. High-resolution accurate mass spectra (HRMS) were obtained either at the Georgia State University Mass Spectrometry Facility using a Waters Q-TOF micro (ESI-Q-TOF) mass spectrometer or utilizing a Waters Micromass LCT TOF ES+ Premier mass spectrometer. The purity of each compound tested was determined by using LC–MS instrument possessing a Waters 2487

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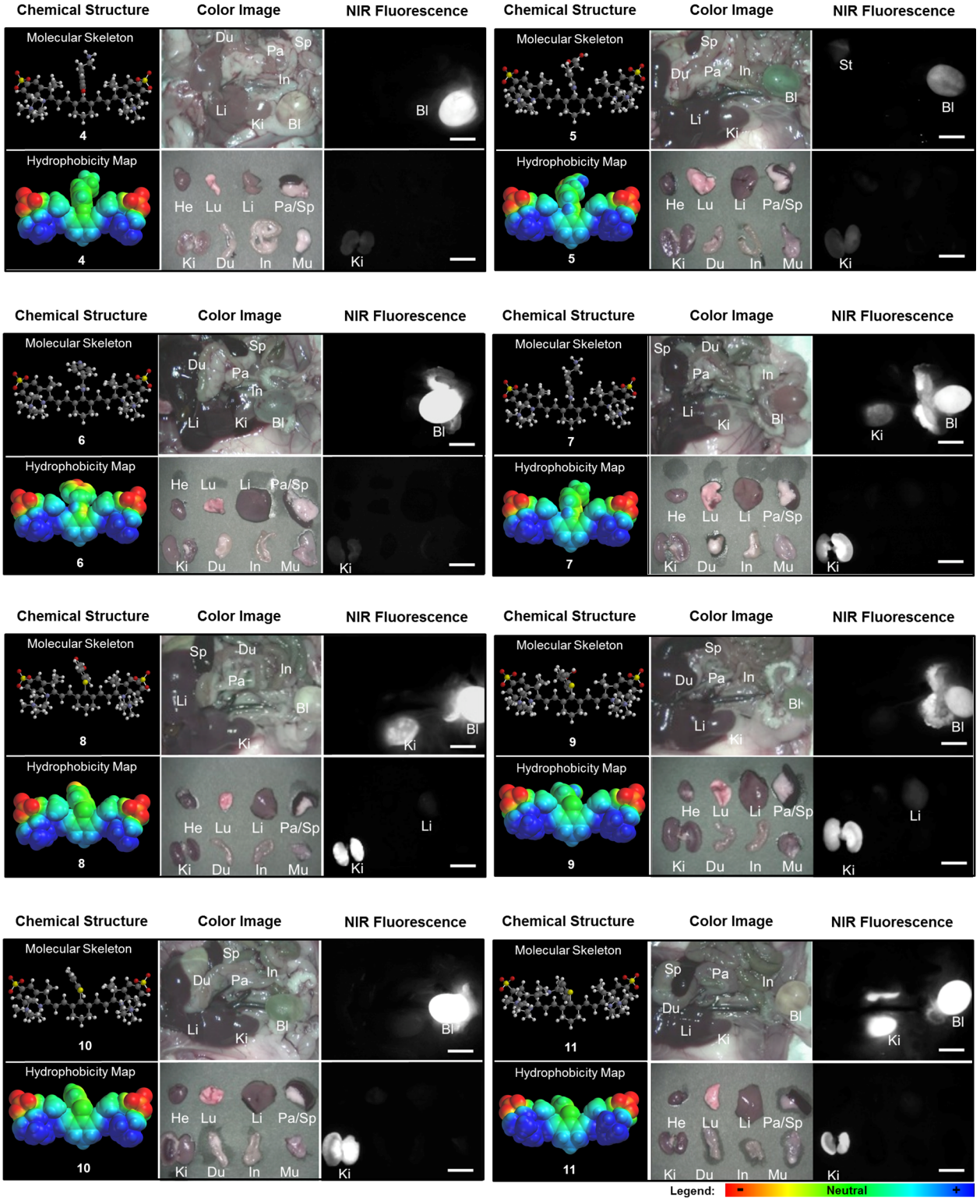

Figure 4. In vivo biodistribution and clearance of ZW800-1 derivatives. In silico calculations of minimized structures are calculated using density functional theory at the B3LYP level: red, negative charge; blue, positive charge; green, hydrophobicity. 10 nmol of each NIR fluorophore was injected intravenously into CD-1 mice 4 h prior to imaging. NIR fluorescence images for in vivo biodistribution (top) and resected organs (bottom) have identical exposure and normalizations. Abbreviations used are the following: Bl, bladder; Du, duodenum; He, heart; In, intestine; K_i, kidneys; Li, liver; Lu, lungs; Pa, pancreas; Sp, spleen; St, stomach. Scale bars are 1 cm.

single wavelength absorption detector with wavelengths set between 640 and 700 nm depending on the particular photophysical properties. The column used in LC was a Waters Delta-Pak 5 μm 100A 3.9 mm × 150 mm reversed phase C_{18} column, with a flow rate of 1 mL/min employing a 5−100% acetonitrile/water/0.1 formic acid gradient. A SEDEX 75 evaporative light scattering detection (ELSD) was also utilized in tandem with liquid chromatography to confirm purity. All compounds tested were >95% pure.

Synthesis. *Mono(2-((E)-2-((E)-2-(4-(2-aminoethyl)phenoxy)-3-((E)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)-*

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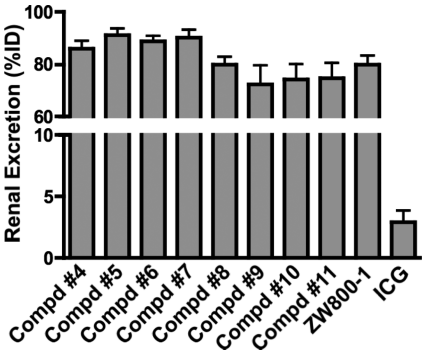

Figure 5. Renal excretion (% ID) of ZW800-1 derivatives. ZW800-1 and ICG are used as controls (N = 5).

*indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate) Tribromide (**4**).* To a solution of tyramine (1.25 g, 9.112 mmol) in DMF (20 mL) under N_2, was added triethylamine (1.38 g, 13.67 mmol) followed by Boc anhydride (2.98 g, 13.67 mmol), and the reaction mixture was stirred at rt for 2 h. To the above solution under N_2 was then added sodium hydride (0.22 g, 9.112 mmol), and the mixture was stirred at rt for 1 h. To the above was then added the chloro dye **3** (0.66 g, 0.6 mmol) under N_2, and the reaction mixture was stirred at rt for 17 h while monitoring by vis/NIR spectrophotometry. To the above solution was then added a mixture of TFA and water (10 mL/10 mL), and the reaction mixture was stirred at rt for 2 h. The solvents were then evaporated from the reaction mixture on rotavapor at 60 °C, and the obtained residue was stirred with ethyl acetate (200 mL), filtered, and dried under vacuum. The obtained crude product was stirred in methanol (100 mL), filtered, and dried under vacuum to afford the pure dye as a green solid (0.44 g, 80%). Mp: 230−232 °C. Vis/NIR abs, λ_{max}: 770 nm. ^{1}H NMR (D_2O): 7.85 (d, J = 14.0 Hz, 2H), 7.72 (d, J = 8.4 Hz, 2H), 7.69 (s, 2H), 7.26 (d, J = 8.0 Hz, 2H), 7.18 (d, J = 8.4 Hz, 2H), 7.06 (d, J = 8.4 Hz, 2H), 6.07 (d, J = 14.0 Hz, 2H), 4.10 (br s, 4H), 3.41 (br s, 4H), 3.04 (s, 18H), 2.88 (br s, 2H), 2.55 (br s, 4H), 2.22 (br s, 4H), 1.84 (br s, 2H), 1.23 (s, 12H). ^{13}C NMR (D_2O): 172.58, 164.51, 158.77, 143.56, 142.61, 141.17, 139.81, 131.31, 130.87, 126.83, 124.33, 119.72, 115.10, 110.72, 100.90, 63.09, 53.03, 48.81, 40.62, 32.13, 27.18, 23.78, 20.73. HRMS (TOF MS ES+) calcd for $C_{50}H_{69}N_5O_7S_2$: m/z 915.4638 ($[M + H]^+$). Found: m/z 457.7321 $[M + H/2]^+$.

*Mono(2-((E)-2-((E)-2-((4-(2-carboxyethyl)phenyl)amino)-3-((E)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate) Tribromide (**5**).* To a solution of the chloro dye **3** (1.0 g, 0.91 mmol) in a mixture of DMSO and water (15 mL/15 mL) under N_2, was added 3-(4-aminophenyl)propionic acid (0.45 g, 2.73 mmol), and the reaction mixture was stirred at 90 °C for 2h while monitoring by vis/NIR spectrophotometry. Water was then evaporated from the reaction mixture. The residue was cooled to room temperature and poured into ethyl acetate (300 mL) while stirring. The precipitated product was filtered, washed with ethyl acetate, and dried under vacuum. The crude material was dissolved in DMSO (70 mL) and methanol (20 mL) and poured into ethyl acetate (400 mL). The precipitated product was filtered and dried under vacuum. This precipitation procedure was repeated once more and the obtained pure product was dried under vacuum (0.67 g, 78%). Mp: 263−265 °C. Vis/NIR abs, λ_{max}: 700 nm. ^{1}H NMR (D_2O): 8.14 (d, J = 12.8 Hz, 2H), 7.94 (d, J = 8.0 Hz, 2H), 7.84 (s, 2H), 7.35−7.25 (m, 4H), 7.19 (d, J = 7.6 Hz, 2H), 6.12 (d, J = 13.6 Hz, 2H), 4.17 (br s, 4), 3.61 (br s, 4H), 3.29 (s, 18H), 2.91 (br s, 2H), 2.75−2.65 (br s, 6H), 2.41 (br s, 4H), 1.99 (br s, 2H), 1.29 (s, 12H). ^{13}C NMR (DMSO-d_6): 173.98, 170.45, 160.78, 144.96, 142.67, 140.19, 133.98, 130.05, 126.49, 124.87, 120.06, 118.52, 109.69, 98.59, 63.12, 53.00, 48.44, 35.69, 30.07, 28.21, 24.83, 21.90, 21.02. HRMS (TOF MS ES+) calcd for $C_{51}H_{69}N_5O_8S_2$: m/z 943.4588 ($[M + H]^+$). Found: m/z 471.7291 $[M + H/2]^+$.

*Mono(2-((E)-2-((E)-2-((4-aminophenethyl)amino)-3-((E)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate) Tribromide (**6**).* To a solution of the chloro dye **3** (0.3 g, 0.273 mmol) under N_2, in a mixture of DMSO and water (5 mL/5 mL) was added 4- aminophenethylamine (0.11 g, 0.82 mmol), and the reaction mixture was stirred at 90 °C for 1 h while monitoring by vis/NIR spectrophotometry. Water was evaporated from the reaction mixture on rotavapor. The residue was cooled to rt and then poured into ethyl acetate (200 mL) while stirring. The precipitated product was filtered and dried under vacuum. The obtained crude product was then purified by reverse phase column chromatography to obtain the pure target dye (0.21 g, 85%). Mp: 243−245 °C. Vis/NIR abs, λ_{max}: 580 nm. ^{1}H NMR (D_2O): 7.96 (d, J = 6.8 Hz, 2H), 7.91 (s, 2H), 7.57 (d, J = 11.6 Hz, 2H), 7.19 (br s, 4H), 6.99 (d, J = 8.0 Hz, 2H), 5.85 (d, J = 13.2 Hz, 2H), 4.23 (br s, 2H), 4.03 (br s, 4H), 3.59 (br s, 4H), 3.29 (s, 18H), 3.12 (br s, 2H), 2.56 (br s, 4H), 2.39 (br s, 4H), 1.82 (br s, 2H), 1.67 (s, 12H). ^{13}C NMR (D_2O): 166.52, 145.04, 140.18, 137.66, 136.82, 130.03, 126.86, 122.99, 119.88, 108.49, 95.13, 63.89, 53.34, 47.08, 39.57, 28.25, 25.28, 20.40. HRMS (TOF MS ES+) calcd for $C_{50}H_{70}N_6O_6S_2$: m/z 914.4798 ($[M + H]^+$). Found: m/z 457.2389 $[M + H/2]^+$.

Precursor to Dye 7. The pure Boc protected dye mono(2-((*E*)-2-((*E*)-2-((4-(2-((*tert*-butoxycarbonyl)amino)ethyl)phenyl)amino)-3-((*E*)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)-indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3*H*-indol-1-ium-5-sulfonate) tribromide was then subjected to deprotection using TFA and water (10 mL:10 mL) while stirring at rt and monitoring the reaction by vis/NIR spectrophotometry. At the end of 2 h, solvents were evaporated from the reaction mixture and the obtained solid product was dried under vacuum to afford the target dye (1.0 g, 83%). Mp: 260−263 °C. Vis/NIR abs, λ_{max}: 705 nm. ^{1}H NMR (D_2O): 8.21 (br s, 2H), 7.98 (d, J = 6.4 Hz, 2H), 7.92 (s, 2H), 7.45 (d, J = 7.2 Hz, 2H), 7.39 (br s, 2H), 7.34 (br s, 2H), 6.19 (d, J = 8.0 Hz, 2H), 4.31 (br s, 4H), 3.64 (br s, 4H), 3.31 (s, 18H), 3.18 (br s, 2H), 2.72 (br s, 4H), 2.48 (br s, 4H), 2.02 (br s, 2H), 1.53 (s, 12H), 1.52 (s, 2H). ^{13}C NMR (DMSO-d_6): 170.66, 160.26, 145.72, 145.08, 142.86, 142.58, 140.22, 130.48, 129.97, 126.54, 124.99, 120.07, 118.37, 109.72, 98.81, 63.17, 53.00, 48.45, 32.85, 28.16, 24.71, 21.83, 20.98. HRMS (TOF MS ES+) calcd for $C_{50}H_{70}N_6O_6S_2$: m/z 914.4798 ($[M + H]^+$). Found: m/z 457.2393 $[M + H/2]^+$.

*Mono(2-((E)-2-((E)-2-((4-(2-aminoethyl)phenyl)amino)-3-((E)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate) Tribromide (**7**).* To a solution of 4-nitrophenethylamine hydrochloride (1.0 g, 4.94 mmol) in ethyl acetate (15 mL) under N_2 was added triethylamine (0.75 g, 7.41 mmol) followed by Boc anhydride (1.18 g, 5.43 mmol), and the reaction mixture was stirred at rt for 17 h. The reaction mixture was then diluted with ethyl acetate (25 mL) and was washed with water (2 × 30 mL), dried over Na_2SO_4 and concentrated to afford 1.3 g of *tert*-butyl 4-nitrophenethylcarbamate as a pale yellow solid (quantitative). To a solution of the above *tert*-butyl 4-nitrophenethylcarbamate (1.3 g, 4.94 mmol) in methanol (25 mL) was added ammonium formate (1.56 g, 24.7 mmol) followed by 10% palladium on carbon (130 mg), and the reaction mixture was refluxed for 24 h. The reaction mixture was filtered through Celite and washed with methanol. The filtrate and washings were combined, and the solvent was then evaporated on rotavapor, diluted with ethyl acetate (50 mL), and washed with water (3 × 40 mL). The organic layer was then dried over Na_2SO_4 and concentrated to afford 1.04 g of *tert*-butyl 4-aminophenethylcarbamate (89%) which was used in the next step without further purification. To a solution of the above, *tert*-butyl 4-aminophenethylcarbamate (1.0 g, 4.24 mmol) in a mixture of DMSO and water (20 mL:20 mL) under N_2 was added the chloro dye **3** (1.55 g, 1.41 mmol), and the reaction mixture was stirred at 90 °C for 2 h while monitoring by vis/NIR spectrophotometry. Water was evaporated from the reaction mixture on rotavapor. The residue was cooled to rt and poured into ethyl acetate (300 mL) while stirring. The

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precipitated crude product was then filtered and dried under vacuum. Final purification was achieved by reverse phase column chromatography to obtain the Boc protected dye (1.14 g, 1.128 mmol, 80%). Mp: 250−252 °C. Vis/NIR abs, λ_{max}: 705 nm. ^{1}H NMR (D_2O): 8.14 (d, J = 12.8 Hz, 2H), 7.95 (d, J = 7.6 Hz, 2H), 7.84 (br s, 2H), 7.34 (d, J = 8.0 Hz, 2H), 7.29 (br s, 2H), 7.22 (br s, 2H), 6.15 (d, J = 13.2 Hz, 2H), 4.23 (br s, 4H), 3.65 (br s, 4H), 3.31 (s, 18H), 2.79 (br s, 2H), 2.68 (br s, 4H), 2.45 (br s, 6H), 1.96 (br s, 2H), 1.41 (s, 12H), 1.36 (s, 9H). ^{13}C NMR (DMSO-d_6): 170.43, 160.84, 145.07, 144.97, 142.73, 142.64, 140.16, 132.56, 130.36, 126.50, 124.73, 120.07, 118.68, 109.64, 98.53, 78.01, 63.14, 53.00, 48.42, 28.73, 28.23, 24.83, 21.91, 21.01.

*Mono(2-((E)-2-((E)-2-((4-carboxyphenyl)thio)-3-((E)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate) Tribromide (**8**).* To a solution of the chloro dye **3** (0.5 g, 0.613 mmol) under N_2 in a mixture of DMSO and water (10 mL/10 mL) was added 4-mercaptobenzoic acid (0.24 g, 1.55 mmol), and the reaction was stirred at rt for 30 min while monitoring by vis/NIR spectrophotometry. After complete conversion of the starting material, water was evaporated from the reaction mixture on rotavapor and the residue was cooled to rt and then poured into ethyl acetate (200 mL) while stirring. The precipitated product was filtered and dried under vacuum to obtain the pure target dye as a green solid (0.52 g, 91%). Mp: 250−255 °C. Vis/NIR abs, λ_{max}: 791 nm. ^{1}H NMR (D_2O): 8.62 (d, J = 13.6 Hz, 2H), 7.96 (d, J = 7.2 Hz, 4H), 7.79 (s, 2H), 7.51(d, J = 8.4 Hz, 2H), 7.38 (d, J = 8.4 Hz, 2H), 6.46 (d, J = 14 Hz, 2H), 4.37 (s, 4H), 3.71 (br s, 4H), 3.30 (s, 18 H), 2.77 (br s, 4H), 2.45 (br s, 4H), 1.93 (s, 2H), 1.43 (s, 12H). ^{13}C NMR (DMSO): 172.87, 167.12, 148.61, 146.11, 143.12, 142.29, 140.97, 134.49, 130.95, 128.58, 126.65, 126.21, 120.27, 111.07, 102.89, 62.83, 52.90, 49.37, 40.89, 27.59, 26.46, 21.34. LCMS ESI TOF calcd for $C_{49}H_{63}N_4O_8S_3^+$: m/z 932.2483 ([M + H]$^+$). Found: m/z 466.1337 [M + H/2]$^+$.

*Mono(2-((E)-2-((E)-2-((4-(carboxymethyl)phenyl)thio)-3-((E)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate) Tribromide (**9**).* To a solution of the chloro dye **3** (0.5 g, 0.61 mmol) under N_2 in a mixture of DMSO and water (10 mL/10 mL) was added 2-(4-mercaptophenyl)acetic acid (0.26g, 1.52 mmol), and the reaction was stirred at rt for 1 h while monitoring by vis/NIR spectrophotometry. After complete conversion of the starting material, water was evaporated from the reaction mixture on rotavapor and the residue was cooled to rt and then poured into ethyl acetate (300 mL) while stirring. The precipitated product was filtered and dried under vacuum. The obtained crude product was then purified by reverse phase column chromatography using water and methanol as the mobile phase to obtain the pure target dye as a green solid (0.42 g, 71%). Mp: 248−250 °C. Vis/NIR abs, λ_{max}: 791 nm. ^{1}H NMR (DMSO): 8.64 (d, J = 14 Hz, 2H), 7.70 (s, 4H), 7.59 (d, J = 8.4, 2H), 7.41(d, J = 8.4 Hz, 2H), 7.22 (s, 4H), 6.37 (d, J = 14 Hz, 2H), 4.21 (s, 4H), 3.51 (br s, 4H), 3.08 (s, 18 H), 2.79 (br s, 4H), 2.14 (br s, 4H), 1.92 (s, 2H), 1.38 (s, 12H)). ^{13}C NMR (DMSO): 172.88, 172.65, 150.64, 146.18, 145.80, 142.30, 140.98, 135.27, 134.62, 133.49, 131.19, 126.69, 126.55, 120.19, 111.02, 102.76, 63.03, 53.06, 49.35, 41.63, 27.81, 26.56, 21.39. LCMS ESI TOF calcd for $C_{50}H_{65}N_4O_8S_3^+$: m/z 946.4031([M + H]$^+$). Found: m/z 473.1717 [M + H/2]$^+$.

*Mono(2-((E)-2-((E)-2-((4-aminophenyl)thio)-3-((E)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate) Tribromide (**10**).* To a solution of the chloro dye **3** (1.0 g, 0.91 mmol) in a mixture of DMSO and water (15 mL/15 mL) under N_2, was added 4-aminothiophenol (0.68 g, 5.45 mmol), and the reaction mixture was stirred at rt for 17h while monitoring by vis/NIR spectrophotometry. Water was evaporated from the reaction mixture on rotavapor, the residue was cooled to rt and was then poured into ethyl acetate (200 mL) while stirring. The precipitated product was filtered and dried under vacuum. The obtained crude product was then dissolved in water (30 mL) and was washed with dichloromethane (3 × 40 mL) followed by ethyl acetate (3 × 40 mL) and the aqueous layer was evaporated to dryness to obtain the pure target dye as a green solid (0.63 g, 77%). Mp: 210−212 °C. Vis/NIR abs, λ_{max}: 790 nm. ^{1}H NMR (D_2O, 50 °C): 8.80 (d, J = 14.0 Hz, 2H), 8.00 (d, J = 8.0 Hz, 2H), 7.95 (s, 2H), 7.55−7.45 (m, 4H), 7.31 (d, J = 7.2 Hz, 2H), 6.47 (d, J = 14.4 Hz, 2H), 4.42 (br s, 4H), 3.69 (br s, 4H), 3.30 (s, 18H), 2.84 (br s, 4H), 2.50 (br s, 4H), 2.05 (br s, 2H), 1.60 (s, 12H). ^{13}C NMR (DMSO-d_6, 50 °C): 172.86, 146.16, 145.80, 142.41, 142.33, 140.96, 134.71, 128.13, 126.71, 121.97, 120.18, 111.04, 102.77, 63.01, 53.06, 52.91, 49.34, 41.58, 27.83, 27.73, 26.57, 21.39, 21.00. HRMS (TOF MS ES+) calcd for $C_{48}H_{65}N_5O_6S_3$: m/z 903.4097 ([M + H]$^+$). Found: m/z 451.7050 [M + H/2]$^+$.

*Mono(2-((E)-2-((E)-2-((4-(aminomethyl)phenyl)thio)-3-((E)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate) Tribromide (**11**).* To a solution of the chloro dye **3** (1.2 g, 1.47 mmol) under N_2 in a mixture of DMSO and water (10 mL/10 mL) was added 4-(aminomethyl)benzenethiol (1.23 g, 8.83 mmol), and the reaction was stirred at rt for 3 h while monitored by vis/NIR spectrophotometry. After complete conversion of the starting material, water was evaporated from the reaction mixture on rotavapor and the residue was cooled to rt and then poured into ethyl acetate (300 mL) while stirring. The precipitated product was filtered and dried under vacuum. The obtained crude product was then purified by reverse phase column chromatography using water and acetonitrile as the mobile phase to obtain the pure target dye as a green solid. (0.7 g, 52%). Mp: 240−245 °C. Vis/NIR abs, λ_{max}: 790 nm. ^{1}H NMR (DMSO): 8.63 (d, J = 13.6 Hz, 2H), 8.36 (s, 2H), 7.72 (d, 2H), 7.62 (d, J = 8.0 Hz, 2H), 7.47 (m, J = 8.0 Hz, 4H), 7.32 (d, J = 8.0 Hz, 2H), 6.41(d, J = 14.0 Hz, 2H), 4.24 (s, 4H), 3.92 (br s, 2H), 3.58 (t, 4 H), 3.10 (s, 18H), 2.82 (br s, 4H), 2.15 (br s, 4H), 1.92 (s, 2H), 1.44 (s, 12H)). ^{13}C NMR (DMSO): 172.79, 149.88, 145.96, 145.66, 142.33, 140.95, 137.51, 134.62, 132.20, 130.89, 126.60, 120.25, 111.07, 102.87, 62.83, 52.90, 49.33, 41.98, 40.88, 27.74, 26.49, 21.34. LCMS ESI TOF calcd for $C_{49}H_{66}N_5O_6S_3^+$: m/z 917.4242 ([M + H]$^+$). Found: m/z 458.1678 [M + H/2]$^+$.

Synthesis of Linkers. *O-(4-Cyanophenyl) Dimethylcarbamothioate (**15**).* A 250 mL flask equipped with a magnetic stirring bar was charged with 4-hydroxybenzonitrile (5.0 g, 41.9 mmol), DABCO (9.41 g, 83.8 mmol), and 10 mL of DMF. To this well stirred mixture was added *N*,*N*-dimethylthiocarbamoyl chloride (6.21 g, 50.28 mmol). The resulting mixture was heated to 65 °C and monitored by TLC until complete disappearance of the starting material. The reaction mixture was poured into a beaker containing ice, and the pH of the solution is reduced to 2 by adding HCl. The crystals formed are filtered, and further purification is accomplished by recrystallization in ethanol to afford the pure compound **15** as a cream white solid (8.0 g, 93%). ^{1}H NMR ($CDCl_3$): 7.67 (d, J = 8.0 Hz, 2H), 7.18 (d, J = 8.0 Hz, 2H), 3.42 (s, 3H), 3.33 (s, 3H). ^{13}C NMR ($CDCl_3$): 186.30, 157.10, 133.36, 124.22, 118.38, 109.62, 43.38, 39.01.

*S-(4-Cyanophenyl) Dimethylcarbamothioate (**16**).* To a 250 mL round-bottom flask equipped with a magnetic stir bar and a reflux condenser was charged with *O*-(4-cyanophenyl) dimethylcarbamothioate (4.80 g, 23.27 mmol). The contents of the flask were maintained under N_2 and immersed in a preheated oil bath maintained at 200 °C while stirring. The reaction was monitored by TLC, and complete disappearance of the starting material was observed after 4 h of reaction. After completion of the reaction, the content of the flask solidified when cooled to rt. This provided the pure product **16** which was used in the next step without further purification ((4.50 g, 94%). ^{1}H NMR ($CDCl_3$): 7.64−7.58 (m, J = 8.0 Hz, 4H), 3.08−3.03 (d, 6H). ^{13}C NMR ($CDCl_3$): 164.83, 136.36, 133.24, 118.40, 112.45, 37.05

*4-Mercaptobenzonitrile (**17**).* To a 250 mL round-bottom flask equipped with a magnetic stir bar was added *S*-(4-cyanophenyl) dimethylcarbamothioate **13** (4.0 g, 19.4 mmol) and dissolved in THF. To this solution was added KOH (2.40 g, 16.27 mmol) dissolved in MeOH, and this was stirred at rt for 3 h while monitoring with TLC. After completion of the hydrolysis, the mixture was poured into crushed ice, acidified with HCl to attain a pH value of 2, and stirred

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well until the oil solidified. The precipitate formed was collected, washed with cold water, and dried to yield the desired product as a light brown solid (2.20 g, 84%). ^{1}H NMR ($CDCl_3$): 7.49 (d, J = 8.0 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 3.72 (s, 1H). ^{13}C NMR ($CDCl_3$): 139.30, 132.52, 128.70, 118.66, 108.73.

*4-(Aminomethyl)benzenethiol (**18**).* 4-Mercaptobenzonitrile (2.0 g, 14.8 mmol) was dissolved in anhydrous THF. The solution was added dropwise to a stirred suspension of lithium aluminum hydride (0.67 g, 17.7 mmol) in THF under N_2 atmosphere. This suspension was slowly heated to reflux. After 4 h, the excess hydride was removed by adding ethyl acetate followed by methanol and filtered. The filtrate was poured into crushed ice and the precipitate filtered, collected, and dried to yield the desired product as a white solid (1.82 g, 89%). Mp: 215−218 °C. ^{1}H NMR ($CDCl_3$): 8.75 (br s, 2H), 7.56−7.51 (m, J = 8.0 Hz, 4H), 3.95 (s, 2H), 3.47 (s, 1H). ^{13}C NMR ($CDCl_3$): 136.10, 134.11, 130.73, 127.47, 41.95.

Determination of Optical Properties and Prediction of Physicochemical Descriptors. All optical measurements were performed at 37 °C in 100% fetal bovine serum (FBS) buffered with 50 mM HEPES, pH 7.4. Absorbance and fluorescence emission spectra of the series of NIR fluorophores were measured using fiber optic HR2000 absorbance (200−1100 nm) and USB2000FL fluorescence (350−1000 nm) spectrometers (Ocean Optics, Dunedin, FL). NIR excitation was provided by 8 mW of either 655 or 765 nm NIR laser diode light source (Electro Optical Components, Santa Rosa, CA) (depending on the exact chemical structure) coupled through a 300 μm core diameter, NA 0.22 fiber (Fiberguide Industries, Stirling, NJ). For fluorescence quantum yield (QY) measurements, oxazine 725 in ethylene glycol (QY = 19%) and ICG in DMSO (QY = 13%) were used as a calibration standards, under conditions of matched absorbance at 655 and 765 nm. In silico calculations of the partition coefficient (log D at pH 7.4) and pK_a were done using Marvin and JChem calculator plugins (ChemAxon, Budapest, Hungary). Molecular skeleton (2D) and hydrophobicity (3D) structures were minimized and calculated using Spartan density function theory calculations at the B3LYP level using the 6-311++G(2df,2pd) basis set.

Biological Fluorescence Imaging. Animals were housed in an AAALAC-certified facility and were studied under the supervision of BIDMC IACUC in accordance with approved institutional protocol (no. 101-2011 for rodents). Male CD-1 mice weighing ∼25 g (Charles River Laboratories, Wilmington, MA) were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally (Webster Veterinary, Fort Devens, MA). For in vivo fluorescence imaging in real-time, the FLARE imaging system has been described in detail previously.[20−22] In this study, 11 mW/cm^2 of 760 nm excitation light was used with white light (400−650 nm) at 40 000 lx. Color and NIR fluorescence images were acquired simultaneously with custom software at rates up to 15 Hz over a 15 cm diameter field of view. The imaging system was positioned at a distance of 18 in. from the surgical field. For each experiment, camera exposure time and image normalization were held constant.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H NMR and ^{13}C NMR spectra along with LC−MS data for the final synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

NIR, near-infrared; SPECT, single photon emission computed tomography; ICG, indocyanine green; ZW800-1, zwitterionic heptamethine dye; $S_{RN}1$, unimolecular radical nucleophilic substitution; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; TFA, trifluoroacetic acid; DABCO, 1,4-diazabicyclo-[2.2.2]octane; FBS, fetal bovine serum; % ID, percent injected dose

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